Biomarkers of inflammation for population research: Stability of C-reactive protein and alpha$_1$-acid glycoprotein in dried blood spots.

Running title: CRP and AGP in dried blood spots

Authors and institutions:

Eleanor Brindle[1]
Masako Fujita[1,2]
Bettina Shell-Duncan[1,2]

[1] Center for Studies in Demography and Ecology, University of Washington, Seattle, WA 98195
[2] Department of Anthropology, University of Washington, Seattle, WA 98195

Corresponding author:
Eleanor Brindle
Center for Studies in Demography and Ecology
University of Washington
Seattle, WA 98195-3100
Phone: (206) 616-9369
Fax: (206) 543-3285

Abbreviations:
CRP, AGP, ELISA, DBS

BACKGROUND

Biomarkers of acute phase inflammation are associated with risk of morbidity and mortality from cardiovascular disease, stroke, diabetes, and other conditions [1-3]. Inflammatory markers are indispensable in accurately assessing micronutrient status, as levels of iron, vitamin A, and several other micronutrients are altered by inflammation. C-reactive protein (CRP) is a widely-used marker of both chronic and acute inflammation [4], and high-sensitivity assays have revealed its importance as an indicator of morbidity and mortality risk [5, 6], particularly for cardiovascular disease (i.e. [1, 7, 8] and others). CRP climbs sharply and quickly following injury or infection, and declines almost as quickly [9]. Alpha-1-acid glycoprotein (AGP) is another important biomarker of inflammation with a more gradual pattern in the acute phase response. AGP begins to increase more than 24 hours after the onset of inflammation, and remains elevated up to one week following infection or injury. CRP and AGP can thus be complementary biomarkers of acute phase inflammatory response.

Because CRP and AGP are valuable biomarkers for population-level health research, there is a demand for less expensive and more efficient methods appropriate for large-scale, non-clinical population research with limited budgets and facilities. Methods for measuring CRP and AGP in dried blood spot (DBS) specimens, made from capillary blood collected from a finger prick, have greatly simplified the process of incorporating these measures into non-clinical studies. Serum and plasma specimens can be prohibitively difficult to collect, process, and transport in non-clinical research settings. DBS collection is less invasive than venipuncture, and DBS specimens eliminate many of the obstacles to collecting blood specimens, yet can provide comparable measures of many biomarkers. However, there is limited and conflicting evidence regarding the stability of acute-phase proteins in DBS specimens. For example, McDade et al. [10] find no effect of storage on CRP in DBS after 3 days at very warm ambient temperatures, 14 days at room temperature or under refrigeration, or after repeated freeze-thaw cycles, while Erhardt et al. [11] found that CRP degraded rapidly in very warm field conditions simulating desert and tropical environments, while AGP remained relatively stable in simulated desert conditions, but degraded under tropical field conditions.

METHODS

Assays

Several methods for measuring CRP are available [12, 13], including those designed for clinical diagnostic use and commercial assay kits designed for non-clinical research. However, these can be prohibitively expensive for large-scale research, may be limited in the sample types demonstrated to yield valid results, and may not offer the flexibility needed to optimize assay efficiency for high-throughput. An assay described by Wu and colleagues [14] for use in serum, and later described by McDade et al. [10] for use in dried blood spots (DBS), filled the demand for appropriate methods very effectively, but the polyclonal antibodies needed have recently become unavailable. We developed an enzyme immunoassay using readily available monoclonal antibodies to measure CRP in serum, plasma, or DBS. The new CRP ELISA performance was evaluated by standard assay validation methods, and by comparing CRP concentrations measured in the new assay with those from the previously described assay that is no longer available.

Like for CRP, many clinical diagnostic assays and assay kits are available to measure AGP, but often these are not well-suited to population-level research. We chose an AGP ELISA commercially available as an assay kit in a format well suited to population-level work, with only major reagents, assay performance data, and instructions provided by the manufacturer (Genway). The kit is less expensive than most ELISA kits, and allows more flexibility for large-scale work

than traditional kits. We tested this assay for use with DBS specimens by comparing results from paired serum and DBS specimens.

Specimen collection and storage

CRP and AGP were assessed after collection and storage in both laboratory and field settings using paired serum and DBS specimens. In the laboratory, paired serum and DBS specimens from nine healthy US adults were collected simultaneously as part of another study. Participants provided written informed consent, and procedures were approved by the Institutional Review Board of the University of Washington. For the field study, matched DBS and serum specimens from venous blood samples of 23 individuals randomly selected from a larger study among 241 lactating Ariaal women of Marsabit District, northern Kenya. DBS were prepared by pipetting drops of blood collected by venous blood draw, and stored in a sealed plastic bag with desiccant at ambient field temperatures (12-28C) for 13-42d. Remaining blood specimens were kept away from light, allowed to clot and serum separated after field centrifugation. Sera were frozen within 3 hrs of venipuncture and stored in liquid nitrogen for 13-42d and then transported to KEMRI laboratory in Nairobi. All samples were then shipped via air express on dry ice to UW and stored at -20C until analysis. Research procedures were approved by the Institutional Review Boards of the University of Washington and the Kenya Medical Research Institute (KEMRI). Whatman 903 filter paper cards were used for all DBS specimens in both the laboratory and field collections.

RESULTS

CRP in DBS and serum stored at -20C in the laboratory were highly correlated when assayed 4 months after collection (Pearson correlation .997, $p<.01$), and absolute values were similar (DBS values averaged 110% ± 11% of serum). The specimens remained stable for 15 months, with concordance between DBS and serum values remaining high (.965, $p<.01$, DBS 103% ± 11% of serum), and DBS and serum results were 90% ± 11% and 85% ± 13%, respectively, of previous values. CRP serum and DBS specimens stored in rural Kenya field conditions, then stored for almost 12 months at -20C in the laboratory, however, did not show similar concordance. Pearson correlations were still significant (.913, $p<.01$) but DBS values averaged only 61% ± 20% of serum values.

The AGP assay was not available until 15 months after the specimens were first collected in the laboratory, but AGP in DBS and serum showed excellent concordance after storage 15 months in lab conditions (Pearson correlation .913, $p<.01$, DBS averaged 99% ± 10% of serum values). As with CRP, AGP results from DBS and serum samples for specimens collected in remote field conditions suggest degradation has occurred, with DBS values averaging 56% ± 15% of serum values, and Pearson correlation of .560, $p<.05$.

CONCLUSIONS

Overall, results showed that CRP and AGP in DBS stored in the laboratory at -20C with humidity controlled were stable for more than one year, while DBS stored under field conditions showed evidence of degradation (Figure 1).

LITERATURE CITED

1. **Pearson, T.A., et al., *Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association.* Circulation, 2003. 107(3): p. 499-511.**

2. **Christiansen, M.S., et al., *Orosomucoid in urine is a powerful predictor of cardiovascular mortality in normoalbuminuric patients with type 2 diabetes at five years of follow-up.* Diabetologia, 2005. 48(2): p. 386-93.**
3. **Crimmins, E.M. and C.E. Finch, *Infection, inflammation, height, and longevity.* Proc Natl Acad Sci U S A, 2006. 103(2): p. 498-503.**
4. **Pepys, M.B. and G.M. Hirschfield, *C-reactive protein: a critical update.* J Clin Invest, 2003. 111(12): p. 1805-12.**
5. **Finch, C.E. and E.M. Crimmins, *Inflammatory exposure and historical changes in human life-spans.* Science, 2004. 305(5691): p. 1736-9.**
6. **Ford, E.S., et al., *C-reactive protein and body mass index in children: findings from the Third National Health and Nutrition Examination Survey, 1988-1994.* J Pediatr, 2001. 138(4): p. 486-92.**
7. **Ridker, P.M., P.W. Wilson, and S.M. Grundy, *Should C-reactive protein be added to metabolic syndrome and to assessment of global cardiovascular risk?* Circulation, 2004. 109(23): p. 2818-25.**
8. **Danesh, J., et al., *Low grade inflammation and coronary heart disease: prospective study and updated meta-analyses.* Bmj, 2000. 321(7255): p. 199-204.**
9. **Gabay, C. and I. Kushner, *Acute-phase proteins and other systemic responses to inflammation.* N Engl J Med, 1999. 340(6): p. 448-54.**
10. **McDade, T.W., J. Burhop, and J. Dohnal, *High-sensitivity enzyme immunoassay for C-reactive protein in dried blood spots.* Clin Chem, 2004. 50(3): p. 652-4.**
11. **Erhardt, J., et al., *Recovery of proteins in dried blood spots used for the measurement of the vitamin A, iron, and infectious status-test of different environmental conditions*, in *Micronutrient Forum*. 2007: Istanbul, Turkey.**
12. **Roberts, W.L., et al., *Evaluation of nine automated high-sensitivity C-reactive protein methods: implications for clinical and epidemiological applications. Part 2.* Clin Chem, 2001. 47(3): p. 418-25.**
13. **Clarke, J.L., et al., *Comparison of differing C-reactive protein assay methods and their impact on cardiovascular risk assessment.* Am J Cardiol, 2005. 95(1): p. 155-8.**
14. **Wu, T.L., et al., *Development of ELISA on microplate for serum C-reactive protein and establishment of age-dependent normal reference range.* Clin Chim Acta, 2002. 322(1-2): p. 163-8.**

Figure 1. CRP and AGP in serum vs. DBS stored lab and field storage conditions.