Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 7, Issue, 08, pp.19067-19070, August, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

IMPORTANCE OF HIGH SENSITIVITY C-REACTIVE PROTEIN (Hs-CRP)IN STORED SAMPLES OF HUMAN SERUM

***Dr. K. Saradamba and Dr. M. Lakshmi Rajeswari**

Department of Biochemistry, Andhra Medical College, King George Hospital, Visakhapatnam, Andhra state, India

ARTICLE INFO

Article History:
Received 21st May, 2015
Received in revised form
18th June, 2015
Accepted 07th July, 2015
Published online 21st August, 2015

Key words:

C-reactive protein,
Archived samples,
Long-term storage,
Stability,
Biomedical research.

ABSTRACT

Background: The stability of biomarkers in stored biomedical samples is crucial, especially when storage is for extended periods of time. High-sensitivity CRP (Hs-CRP) is a biomarker of low grade inflammation that is extensively used to identify and study card in vascular and/or
inflammatory processes in clinical care and large epidemiologic studies. (Ayo *et al*., 2014) Therefore, assessing Hs-CRP stability in archived samples at a given temperature is important to insure precision of measurements over time and the validity of studies using archived samples.

Methods: We evaluated the stability of Hs-CRP in 30 randomly selected human serum samples by measuring Hs-CRP concentrations in freshly collected sample (Hs-CRP (0)) and in the same set of samples after 7–11 years of storage at −80°C (Hs-CRP (LT)).

Results: Hs-CRP did not significantly change up to 11 years of storage at −80°C as shown by a negligible median difference between Hs-CRP (0) and Hs-CRP (LT), delta(Hs-CRP (0)- Hs-CRP (LT)=−0.01, p=0.45. There was a good concordance and agreement between Hs-CRP (0) and Hs-CRP (LT) as measured respectively by Lin's coefficient of correlation (ρC= 0.98) and Bland-Altman analysis (mean difference = −0.02, 95% CI (−0.04–0.0045) p=0.107). In addition, the data also suggest that the time elapsed between collection and Hs-CRP measurement does not affect Hs-CRP stability over time when samples are kept under the appropriate conditions.

Conclusions: Long-term storage at −80°C for up to 11 years did not significantly affect the stability of serum Hs-CRP. Given the cost and time for collecting fresh samples, this observation represents an important finding for biomedical research and clinical care.

***Citation*:** K. Saradamba and M. Lakshmi Rajeswari, 2015. "Importance of high sensitivity c-reactive protein (Hs-CRP)in stored samples of human serum", *International Journal of Current Research*, 7, (8), 19067-19070.

INTRODUCTION

Studies often use archived samples to retrospectively measure the concentrations of biomarkers in plasma or serum (Bays *et al*., 2002; Bea *et al*., 2011; Townsend *et al*., 2013). This practice is particularly common among large research consortia that combine biomarkers data assayed from samples stored in freezers over varying time intervals, sometimes for several years after initial sample collection (Dehghan *et al*., 2011). The increasing use of large biobanks to procure samples for research studies also means that more investigators than ever are using samples stored at low temperatures for periods ranging from months to years, even up to decades (Floegel *et al*., 2013; Mercuri *et al*., 2013). These practices in biomedical research raise the problem of stability of serum biomarkers over time as well as the validity of the results of such studies.

***Corresponding author: Dr. K. Saradamba,**
Department of Biochemistry, Andhra Medical College, King George Hospital, Visakhapatnam, Andhra state, India.

C-reactive protein (CRP) is an important acute phase reactant protein in many epidemiologic studies designed to investigate the relationship between inflammation and several chronic conditions including heart disease and metabolic diseases. High-sensitivity CRP (Hs-CRP) is routinely used in conjunction with lipid panels to access cardiovascular risk in clinics and population studies (Ridker, 2003; Ridker, 2004). Like many other biomarkers, Hs-CRP concentrations may be affected by preclinical handling and storage conditions. While manufacturers of the reagents used to measure such biomarkers given some information about biomarkers stability in biological fluids in the accompanying laboratory specifications, most of them do not have data on stability that exceed periods of few months at −20°C (Banfi *et al*., 2002; Cobas Integra 2009). This leaves a tremendous gap between what is known about the stability period of samples stored for few months and samples stored for few years or decades. For example, issues surrounding the long-term stability of serum Hs-CRP remain unresolved. One study reported that Hs-CRP was stable in samples stored for a couple of years at −20°C (Brindle *et al*., 2010) while others have reported contradictory results from

samples stored over long periods of time i.e. up to 10 years (Ishikawa *et al*., 2007; Nilsson *et al*., 2005). Found that Hs-CRP concentrations increased with time whereas Nilsson *et al.* found that Hs-CRP concentrations were barely affected by long-term storage at low temperature (Doumatey *et al*., 2012). The two studies used different study designs, outcome measurements, and also had different overall objectives, which could explain the observed inconsistency. Therefore, the issue of Hs-CRP stability in human samples stored over several years remains an open question. In contrast to most previous studies that have assessed Hs-CRP stability, the focus of our study is to determine the effect of long-term storage on Hs-CRP stability in one type of sample, namely serum. Evaluating the effects of sample type (plasma, serum) or anticoagulant (citrate, EDTA) on Hs-CRP stability is outside the scope of this study. Evaluating the expected stability of Hs-CRP after years of storage is of potential utility to epidemiologic studies and biobanks that often conduct assays on serum samples that have been banked for years to decades.

MATERIALS AND METHODS

Subjects

The participants of this study were drawn from the patients of King George Hospital & Andhra Medical College. The individuals are healthy volunteers who were not ascertained on any phenotype and were recruited between 2001 and 2008 (Doumatey *et al*., 2012). All subjects included in this study provided written informed consent. The study was approved by the ethics committee of the Hospital. The thirty (30) participants included in this analysis were randomly selected from a subset of HUFS (N=1174) that consists of individuals that have baseline Hs-CRP measured and have more than one serum aliquots archived.

Measurement of baseline Hs-CRP

Blood samples were obtained from participants in plain vacutainer tubes and allowed to clot at room temperature for 1 hour and then spun to separate serum. Serum samples were stored in externally threaded 2-ml cryovials. Each cryovial was filled with about 1.8 ml of serum leaving about 0.2 ml of space to allow for liquid expansion during freezing. Hs-CRP was measured on Cobas Integra 400 Plus using a latex particle-enhanced immunoturbidimetric assay following the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). The measuring range for this assay is 0.01–20 mg/dL. The baseline Hs-CRP was obtained the day of collection when possible or samples are stored following manufacturer's recommendations (stored at 4°C for 8 days or at −20°C for up to 3 years) (Banfi *et al*., 2002) and batch- assayed thereafter. The average storage time at −20°C before the baseline Hs-CRP (Hs-CRP (0)) measurement was 3 months. After the baseline measurements, all samples were stored at −80°C.

Measurement of Hs-CRP on archived samples

The 30 samples randomly selected for this study were slowly thawed at 4°C and then thoroughly mixed before long-term Hs-CRP (Hs-CRP (LT)) was measured using the same method as described above for the baseline measurement (Roche Diagnostics, Indianapolis, IN). While the methodology remained the same throughout the study, the reagents were slightly modified by the manufacturer to improve the assay performance. The accuracy remains similar with a measuring range of 0.03–35mg/dL.

Statistical methods

All statistical analyses were run in SPSS version 19 and SAS package. Agreement between Hs-CRP (0) and Hs-CRP (LT) was assessed using: Bland –Altman limits of agreement analysis, Lin's concordance coefficient (Bland *et al*., 1999; Brindle *et al*., 2010) and the Wilcoxon signed rank test (a nonparametric test for related samples test). Non-parametric correlation between the two endpoints was also determined by Kendall's tau-b coefficient. To visualize the difference between Hs-CRP (0) and Hs-CRP (LT) for each sample and across all samples, line plots and Bland-Altman plots (Brindle *et al*., 2010) were generated. To determine if the relationship between Hs-CRP (0) and Hs-CRP (LT) is affected by the length of time elapsed between samples collection and Hs-CRP measurement, the data was split into 3 groups:

1) Samples assayed between 0–8 days after collection.
2) Samples assayed between 9–80 days.
3) Samples assayed more than 80 days after collection.

The Wilcoxon signed rank test was used to determine if the differences between the medians are statistically significant.

RESULTS

The mean age for the 30 individuals from which the samples were collected is 35.7 years. Mean± STD for Hs-CRP at baseline (Hs-CRP (0)) is 0.24 ±0.3 mg/dL with a range of 0.01–1.34 mg/dL and a median value of 0.13 mg/dl. Upon storage at −80°C for an average of 8.7 years (range: 7–11 years), we observed a slight increase in Hs-CRP concentrations (Mean ± STD= 0.26±0.33 mg/dl, range (0.03–1.35 mg/dl) and median=0.14 mg/dL). However, the increase was not statistically significant as shown by the Wilcoxon signed rank test statistics ($Z=-0.76$, $p= 0.44$). Furthermore, Bland-Altman analysis, a measure of agreement, showed no bias between the two measurements of Hs-CRP i.e. at baseline and after long-term storage (mean difference= −0.02, p= 0.10 and 95% limits of agreement (−0.15–0.11))

(Figure 1A) and the absence of difference between the two measurements is also confirmed by the two line plots representing Hs-CRP (0) and Hs-CRP (LT) respectively in the 30 samples (Figure 1B).

The correlation between Hs-CRP (0) and Hs-CRP (LT) is strong (Kendall's tau-b=0.87, $p<0.0001$). Since high correlation does not always mean agreement or concordance, we also estimated Lin's coefficient of correlation, ρc, which shows a high concordance between Hs-CRP (0) and Hs-CRP (LT) ($\rho c = 0.98$). Subset analysis based on the time elapsed between sample collection and Hs-CRP measurement showed that the duration between collection and testing did not significantly affect Hs-CRP concentrations.

DISCUSSION

This study shows that serum Hs-CRP concentrations remain stable for up to 11 years at −80°C. There was a slight increase (2%) in Hs-CRP level overtime that was not significantly meaningful. Similar to the findings of this study, Ishikawa S *et al*. observed an increase in Hs-CRP concentrations, but in contrast to this study's findings, the increase they reported was statistically significant and more pronounced especially for samples that have low baseline Hs-CRP (Nilsson *et al*., 2005). The discrepancy between these two studies could be due to several factors including the freeze/thaw cycle which can affect Hs-CRP stability by disrupting CRP pentameric structure resulting in monomers that can be detected and thus lead to overestimation of Hs-CRP concentrations. While the effects of long -term storage on Hs-CRP have been investigated by others (Ishikawa *et al*., 2007; Nilsson *et al*., 2005; Lin, 1989; Ledue and Rifai, 2003), our study design is unique. In fact, we used a sample randomization procedure to select our samples unlike other studies which either used randomly selected samples from groups stratified based on Hs-CRP concentrations or used different type of samples for the stability study (serum at baseline and plasma after long term storage).

Secondly, samples used in this study were fasting samples which is important for precision especially when the assay principle, turbidimetry, depends on optical clarity. Thirdly, in this study we only focused on the effect of long -term storage on a single type of sample, serum, to avoid the intrinsic variability that may be introduced as confounder due to the use of different type of samples. A change in analytical methods or change in reagent lots can influence the accuracy of measurements. To minimize the effect of such change on our results, we have used strict quality control processes such as retaining the same analytical methodology and Doumatey *et al*. (2012) Page 4 *Clin Biochem*. Author manuscript; available in PMC 2015 March 01. manufacturer for all assays, re-assaying samples previously assayed with earlier reagent lots using newly procured lots and accessing concordance between reagent lots. A limitation of this study is the relatively small sample size.

The increase in sample size in such experiment may further narrow the limits of agreement interval between the before and after storage Hs-CRP measurements and allow an enhanced generalization of our findings. In conclusion, our study shows that Hs-CRP concentration is remarkably stable after longterm storage in −80°C freezer for up to 11 years. These findings are significant for investigators involved in international collaborations in which samples are shipped between countries or studies involving the use of biobank samples.

REFERENCES

Ayo P Doumatey, Jie Zhou, Adebowale Adeyemo, and Charles Rotimi *Clin Biochem*., 2014 March; 47(0): 315–318. doi:10.1016/j.clinbiochem. 2013.12.014.

Banfi, G., Bauer, K., Brand, W., Buchberger, M., Deom, A., Ehret, W. *et al*. Use of Anticoagulants in Diagnostic Laboratory Investigations, 2002. World Health Organization; Geneva: 2002. p. 1-64.

Bays HE, Stein EA, Shah AK, Maccubbin DL, Mitchel YB, Mercuri M. Effects of simvastatin on C-reactive protein in mixed hyperlipidemic and hypertriglyceridemic patients. *Am J Cardiol*., 2002; 90(9):942–6. (PubMed: 12398959)

Bea JW, Wright NC, Thompson P, Hu C, Guerra S, Chen Z. Performance evaluation of a multiplex assay for future use in biomarker discovery efforts to predict body composition. *Clin Chem Lab Med*., 2011; 49(5):817–24. (PubMed: 21361852)

Bland JM, Altman DG. Measuring agreement in method comparison studies. *Stat Methods Med Res*., 1999; 8(2):135–60. (PubMed: 10501650)

Brindle E, Fujita M, Shofer J, O'Connor KA. Serum, plasma, and dried blood spot highsensitivity C-reactive protein enzyme immunoassay for population research. *J Immunol Methods*, 2010; 362(1–2):112–20. (PubMed: 20850446)

Dehghan A, Dupuis J, Barbalic M, Bis JC, Eiriksdottir G, Lu C, *et al*. Meta-analysis of genomewide association studies in N80 000 subjects identifies multiple loci for C-reactive protein levels. *Circulation*, 2011; 123(7):731–8. (PubMed: 21300955)

Diagnostics, R. Cobas Integra 400/8002009. Roche Diagnostics; 2009. Cardiac C-Reactive Protein (Latex) High Sensitive; p. 1-5.

Doumatey AP, Bentley AR, Zhou J, Huang H, Adeyemo A, Rotimi CN. Paradoxical Hyperadiponectinemia is Associated With the Metabolically Healthy Obese (MHO) Phenotype in African Americans. *J Endocrinol Metab*., 2012; 2(2):51–65. (PubMed: 23293696)

Floegel A, von Ruesten A, Drogan D, Schulze MB, Prehn C, Adamski J, *et al*. Variation of serum metabolites related to habitual diet: a targeted metabolomic approach in EPIC-Potsdam. *Eur J Clin Nutr*., 2013; 67(10):1100–8. (PubMed: 23942179)

Ishikawa S, Kayaba K, Gotoh T, Nakamura Y, Kario K, Ito Y, *et al*. Comparison of Creactive protein levels between serum and plasma samples on long-term frozen storage after a 13.8 year interval: the JMS Cohort Study. *J Epidemiol*., 2007; 17(4):120–4. (PubMed: 17641447)

Ledue TB, Rifai N. Preanalytic and analytic sources of variations in C-reactive protein measurement: implications for cardiovascular disease risk assessment. *Clin Chem.,* 2003; 49(8):1258–71. (PubMed: 12881440)

Lin LI. A concordance correlation coefficient to evaluate reproducibility. *Biometrics*, 1989; 45(1):255–68. (PubMed: 2720055)

Macy EM, Hayes TE, Tracy RP. Variability in the measurement of C-reactive protein in healthy subjects: implications for reference intervals and epidemiological applications. *Clin Chem*., 1997; 43(1): 52–8. (PubMed: 8990222)

Mercuri A, Turchi S, Borghini A, Chiesa MR, Lazzerini G, Musacchio L, *et al*. Nitrogen biobank for cardiovascular research. *Curr Cardiol Rev*., 2013; 9(3):253–9. (PubMed: 23909635)

Nilsson TK, Boman K, Jansson JH, Thogersen AM, Berggren M, Broberg A, *et al*. Comparison of soluble thrombomodulin, von Willebrand factor, tPA/PAI-1 complex, and high-sensitivity CRP concentrations in serum, EDTA plasma, citrated plasma, and acidified citrated

plasma (Stabilyte) stored at −70 degrees C for 8–11 years. *Thromb Res*., 2005; 116(3):249–54. (PubMed:15935834)

Ridker PM. High-sensitivity C-reactive protein and cardiovascular risk: rationale for screening and primary prevention. *Am J Cardiol*., 2003; 92(4B):17K–22K.

Ridker PM. High-sensitivity C-reactive protein, inflammation, and cardiovascular risk: from concept to clinical practice to clinical benefit. *Am Heart J*., 2004; 148(1 Suppl.):S19–26.(PubMed: 15211329)

Townsend MK, Clish CB, Kraft P, Wu C, Souza AL, Deik AA, *et al*. Reproducibility of Metabolomic Profiles among Men and Women in 2 Large Cohort Studies. *Clin Chem*., 2013;59(11):1657–67. (PubMed: 23897902)

****