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The effects of freeze–thaw on β-trace protein and β2-microglobulin assays after long-term sample storage

Stephen P. Juraschek[a,b], **Josef Coresh**[a,b,c], **Lesley A. Inker**[d], **Gregory P. Rynders**[e], **John H. Eckfeldt**[e], and **Elizabeth Selvin**[a,b,*]

[a]Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health and the Welch Center for Prevention, Epidemiology and Clinical Research, Johns Hopkins Medical Institutions, Baltimore, MD, USA

[b]Department of Medicine, Johns Hopkins Hospital, Baltimore, MD, USA

[c]Department of Biostatistics, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

[d]Division of Nephrology, Tufts Medical Center, Boston, MA, USA

[e]Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN, USA

Abstract

Objectives—To evaluate the effect of a freeze–thaw cycle on β-trace protein (βTP) and β2-microglobulin (β2M).

Design and methods—We compared βTP and β2M concentrations before and after a single freeze–thaw cycle in long-term stored samples from 172 participants of the Third National Health and Nutrition Examination Survey (NHANES III).

Results—Measurements of βTP and β2M before and after freeze–thaw were highly correlated with Spearman's coefficients of 0.90 and 0.99, respectively. Serum concentrations of βTP were slightly lower after freeze–thaw (−0.05 mg/L, P=0.006). Measurements of β2M did not differ before and after freeze–thaw (P=0.35).

Conclusions—βTP and β2M measurements were robust to a single freeze–thaw cycle, although β2M appeared more stable than βTP. These results have implications for future studies of these biomarkers.

Keywords

β-trace protein (βTP); β2-microglobulin; National Health and Nutrition Examination Survey; Freeze–thaw; Kidney filtration marker

Introduction

β-trace protein (βTP) and β2-microglobulin (β2M) are low molecular weight serum proteins freely filtered by the glomerulus and are increasingly being viewed as useful markers of glomerular filtration rate. βTP, a 168 amino acid glycoprotein weighing 23–29 kDa [1],

*Corresponding author at: Johns Hopkins University, 2024 E. Monument, Suite 2-600, Baltimore, MD 21205, USA. Fax: +1 410 955 0476. lselvin@jhsph.edu (E. Selvin).

originates from the choroid plexus of the central nervous system, while β2M, a subunit of the major histocompatibility (MHC) class I molecule weighing 12 kDa, is produced by all nucleated cells [2,3]. In clinical and epidemiological studies, researchers often rely on stored serum samples for the measurement of numerous biomarkers; however, previous studies have not described the potential effects of freeze–thaw cycles on βTP or β2M. Freeze–thaw cycles have been shown to denature some serum proteins and cause dissociation into inactive dimers [4–6] although many are robust to the freeze–thaw process [7]. The purpose of this study was to determine the influence of a single freeze–thaw cycle on concentrations of βTP and β2M measured in stored serum samples.

Materials and methods

We selected a random sample of 200 specimens obtained from persons 60 years or older who participated in the Third National Health and Nutrition Examination Survey (NHANES III), a large cross-sectional survey of the civilian non-institutionalized population conducted in 1988–1994 [8]. This freeze–thaw study was conducted as part of a larger project to measure markers of kidney function in stored serum samples in NHANES III [9,10]. Informed consent was obtained from all participants. Protocols for the conduct and implementation of this study were approved by the Institutional Review Boards of both the National Center for Health Statistics and the Johns Hopkins Bloomberg School of Public Health.

In 2009 the specimens, which had been frozen immediately after collection, were thawed and βTP and β2M were measured. These samples were refrozen and subsequently thawed for second βTP and β2M measurements 4 to 13 months later. Samples were stored at −70 °C until the time of βTP and β2M measurement. βTP and β2M were measured at the University of Minnesota using particle-enhanced immunonephelometric assays (N Latex β-trace protein assay and N Latex β2-microglobulin assay, Siemens Diagnostics, IL). The inter-assay coefficients of variation for the βTP and β2M assays were 5.7% (mean 0.594 mg/L) and 2.7% (mean 1.757 mg/L), respectively. Assays were performed from September 2009 through July 2010 and entailed 2 reagent lots and 2 calibrators for βTP, and 3 reagent lots and 2 calibrators for β2M. To monitor reagent performance, monthly means were calculated for both βTP and β2M, and controls were assayed twice daily during all assay runs. There were no progressive shifts in assay means for βTP or β2M.

For the two biomarkers, we excluded extreme outliers (difference>3 standard deviations from the mean; equivalent to 0.3% of observations from a normal distribution). We compared measurements before and after freeze–thaw using paired t-tests, Pearson's and Spearman's correlation coefficients, and the intra-class correlation coefficient using one-way analysis of variance (ANOVA). We also calculated the coefficient of variation of the difference between the two measurements (CV_d), using the following equation:

i. CV_d= standard deviation/mean

ii. $$\text{Standard deviation}=\text{sqrt}\left(\sum_i^n (x_i-y_i)^2/2n\right)$$

iii. $$\text{Mean}=\left(\sum_i^n x_i/n+\sum_i^n Y_i/n\right)/2$$

where x is the value after freeze–thaw, y is the value before freeze–thaw, and n is the total number of pairs.

Confidence intervals for the CV_d were obtained by bootstrapping 1000 replications with a correction for bias. We used scatterplots and Bland–Altman plots to visually compare the

βTP and β2M measurements before and after freeze–thaw. We fit linear regression models using Deming regression to correct for measurement error.

Results

After excluding 6 outliers, there were 172 samples with sufficient volume and valid measurements of βTP and β2M after the first and after the second freeze–thaw. The mean age of individuals in the study sample was 70 years (SD, 7). Forty eight percent were male, 58% were non-Hispanic white, 24% were non-Hispanic black, 16% were Mexican American, and 3% were of other race/ethnicity.

Summary statistics are shown in Table 1. Values of βTP before and after the freeze–thaw cycle were highly correlated with a Pearson's correlation of 0.94. However, the values of βTP were slightly lower on average after the single freeze–thaw cycle (mean difference: −0.05 mg/L, 95% CI: −0.06, −0.04; P=0.006). The CV_d was 13.2% (95% CI: 11.8, 14.6). Consistent with the slightly lower values of βTP after freeze–thaw, the slope from the Deming regression model was significantly less than 1.0 (slope=0.91; 95% CI: 0.85, 0.97) (Fig. 1A). The Bland–Altman plot shows that the difference for βTP was relatively consistent across the range of the data (Fig. 1C).

Values of β2M were highly correlated with a Pearson's correlation of 0.99 but there was no significant difference in values before and after the freeze–thaw cycle (mean difference: 0.01, 95% CI: −0.01, 0.02; P=0.35) (Table 1). Variability between the measurements was lower than for βTP, with a CV_d of 3.85% (95% CI: 3.25, 4.44). The slope of the Deming regression line was slightly greater than the identity line (Fig. 1B) and was almost significantly different from 1.0 (slope=1.03; 95% CI: 1.00, 1.06). However, this positive slope was accompanied by a negative constant of −0.06, and linear regression with no constant negated most of the observed increase in slope (slope=1.0). The Bland–Altman plot shows that the differences appeared to be randomly distributed across the range of β2M values.

Discussion

In this study we evaluated the effects of a single freeze–thaw cycle on serum concentrations of βTP and β2M measured in long-term stored samples. We found that both of markers were robust to freeze–thaw with high correlations between the two measurements. We observed some evidence of a minor systematic difference in βTP after freeze–thaw, suggesting possible degradation. This small (6.7%) reduction in βTP after the additional freeze–thaw was slightly greater than the expected range of error for the assay (inter-assay CV of 5.7%). However, the correlation between βTP values before and after freeze–thaw was extremely high, such that the relative rankings of individuals would be unchanged. This would result in minimal to no effects on relative measures of association in epidemiologic studies such as relative risks or odds ratios.

For all tests of reliability, β2M performed better than βTP in this study. β2M had a lower CV_d, higher correlation coefficients, and no significant difference in values before and after the freeze–thaw cycle. Our results suggest that β2M is more robust to freeze–thaw cycles compared to βTP. This difference may also reflect higher precision of the β2M assay (inter-assay CV of 2.7% for β2M vs 5.7% for βTP).

This study has several limitations that should be considered in the interpretation of the results. First, serum specimens were originally collected from NHANES participants in 1988–1994. Samples were stored for approximately 20 years prior to our first measurement. Thus, the results from this study may not be generalizable to fresh serum samples. Second,

we only examined the impact of a single freeze–thaw cycle and there were 4–13 months between the two measurements.

Ultimately, our results suggest that measurements of βTP and β2M in long-term stored serum samples are relatively unaffected by a single freeze–thaw cycle. Our findings are consistent with prior research, suggesting limited effects of freeze–thaw cycles on a large number of different serum proteins stored at −70 °C [7]. These findings have important implications for clinical and epidemiologic studies that utilize previously thawed samples for the measurement of βTP or β2M.

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Abbreviations

βTP	β-trace protein
β2M	β2-microglobulin
NHANES III	Third National Health and Nutrition Examination Survey
ANOVA	one-way analysis of variance
CV	coefficient of variation

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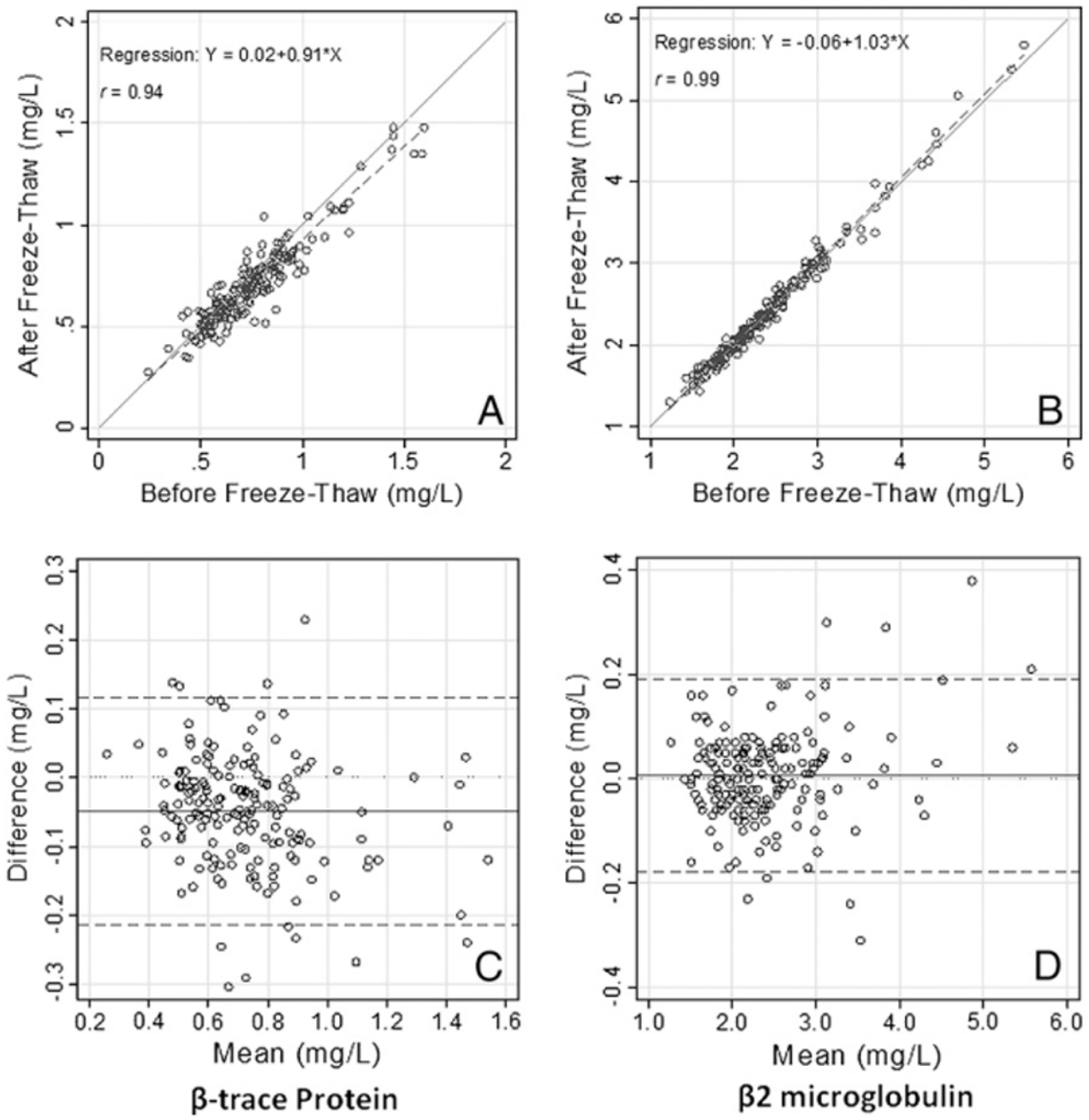

Fig. 1.

(A and B) Scatterplots comparing concentrations of βTP or β2M before and after a single freeze–thaw cycle. The solid line is the 45° line of identity (y=x). The dashed line is the Deming regression line. (C and D) Bland–Altman plots of the differences in concentrations of βTP or β2M before and after freeze–thaw versus the mean. The solid line is the mean difference, the dashed lines represent ±2 standard deviations.

Table 1

Summary statistics for serum measurements of β-trace protein and β2 microglobulin before and after a single freeze–thaw cycle, N=172 pairs.

	β-Trace protein (mg/L)	β2 Microglobulin (mg/L)
Before freeze–thaw, reference range[a]	0.40 to 1.36	1.50 to 4.56
After freeze–thaw, reference range[a]	0.43 to 1.45	1.51 to 4.43
Before freeze–thaw, mean (SD)	0.75 (0.23)	2.42 (0.72)
After freeze–thaw, mean (SD)	0.70 (0.22)	2.42 (0.74)
Mean difference (before–after) (95% CI)	−0.05 (−0.06 to −0.04)	0.01 (−0.01 to 0.02)
P-value[b]	0.006	0.35
$CV_{Difference}$, (95% CI)[c]	13.2 (11.8 to 14.6)	3.85 (3.25 to 4.44)
Pearson's correlation, *r*	0.94	0.99
Intra-class correlation coefficient (95% CI)	0.91 (0.86 to 0.97)	0.98 (0.98 to 0.99)
Deming regression coefficient, β_1 (SE)	0.91 (0.03)	1.03 (0.01)
Deming regression constant, β_o (SE)	0.02 (0.02)	−0.06 (0.03)
Linear regression with no constant, β (SE)	0.93 (0.01)	1.00 (0.003)

[a]The reference range represents the 2.5th to 97.5th percentiles.

[b]Paired *t*-test of the hypothesis that the means are equal.

[c]Confidence intervals generated from 1000 bootstrap replications.