Serum Levels of Insulin-like Growth Factor-I and Insulin-like Growth Factor-I Binding Protein-3: Quality Control for Studies of Stored Serum

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Abstract

The insulin-like growth factor (IGF) axis, particularly IGF-I and IGF binding protein-3 (IGFBP-3), has been the subject of much attention because of its role in juvenile growth and their association with cancers at several sites. However, epidemiologic studies of IGF-I and IGFBP-3 have had mixed results and several authors have speculated that quality control (QC), sample storage history, and other methodologic concerns could play a role in this heterogeneity. This article documents the results of storage history and QC efforts for a study of IGF-I and IGFBP-3 in 6,226 serum samples from the National Health and Nutrition Examination Survey III (NHANES III). The study was carried out on site at Diagnostic Systems Laboratories in Webster, Texas, using the IGF-I ELISA (DSL 10-5600) and the IGFBP-3 immunoradiometric assay (DSL 6600). A run-in study of assay performance suggested that plates, days, and weeks significantly affected the variance of both assays. Analysis of samples with different storage histories also indicated strong effects of storage history. Serum samples disbursed to laboratories for measurement of diverse analytes and then returned for storage showed reductions in serum IGF-I level averaging 43% and reductions in IGFBP-3 of 25% compared with samples shipped immediately to the repository for long-term storage at −80°C. Therefore, the main study was carried out using samples that had been shipped directly to the National Center for Health Statistics/NHANES collection center for storage. Laboratory analyses of NHANES III and QC samples were carried out over ~10 months. QC was monitored through repeated testing of blood samples from six individuals, with two individuals tested twice on each plate. Assay performance was stable over the entire study and coefficients of variation averaged 2% to 3% within plates and ~14% for IGF-I and ~11.5% for IGFBP-3 over the entire study. Coefficients of variation varied significantly among individual QC subjects, ranging from 12.3% to 17.6% for IGF-I and 8.9% to 12.8% for IGFBP-3. Based on Levy-Jennings plots, ~5% of the plates used for IGF-I in the main study were out of compliance. Finally, location on a plate had small but significant effects on IGF-I level. Together, these results highlight the need for care in large studies of putative biomarkers for cancer risk and illustrate some probable sources of heterogeneity in past epidemiologic studies of the IGF axis and cancer. (Cancer Epidemiol Biomarkers Prev 2007;16(5):1017–22)

Introduction

Insulin-like growth factors (IGF) are related to growth and development and also have metabolic activities including mitogenic actions regulating cellular proliferation and differentiation (1). Two IGFs (IGF-I and IGF-II) and six specific IGF binding proteins (IGFBP-1 to IGFBP-6) have been identified, but recent epidemiologic research has focused on IGF-I and its main binding protein (IGFBP-3; ref. 1). Both basic and epidemiologic studies of IGFs and risk of cancer have been motivated by their effects on cell proliferation and apoptosis and their place in insulin signaling (2). Results from the epidemiologic studies suggest that high levels of IGF-I are related to increased risk for colorectal (2-4), prostate (5), and premenopausal breast cancers (2, 6), although the data are not always consistent (2, 6). There is also considerable interest in associations between IGF levels and cancer risk factors (1), including breast density (7), alcohol consumption (8, 9), diet (10-12), and other lifestyle factors (10, 11). The IGF system seems to be related to risk of other diseases, notably ischemic heart disease (13) and congestive heart failure (14). In these studies, lower levels of IGF-I have been associated with increased risk.

Clinical tests for IGFs show that current assays are sufficient for diagnosing growth-related deficiencies (15). However, epidemiologic studies examine associations of IGF across the entire range from low to normal to high values, and therefore require a higher degree of accuracy and precision in the assay performance than IGF assays used for clinical purposes. The success of these epidemiologic studies is dependent on high-quality, sensitive laboratory assays that can be done on thousands of samples over weeks or months and proper storage and sample handling. Some of the variability in results of studies of cancer and IGFs could be due to variability in assay performance or because of differences in assays from different vendors (16). Indeed, concerns have been raised about the reliability of the current IGF and IGFBP assays (2, 4, 16-19).

In this article, we characterize the long-term performance of two commonly used assays, one for IGF-I and one for IGFBP-3, and we report a number of other quality control (QC) results relevant to large studies of IGFs from stored serum samples. These studies were carried out as part of a cross-sectional study of ~6,000 samples from the Third National Health and Nutrition Examination Survey (NHANES III) and included analysis of ~700 QC samples. We first describe a run-in study of the test plates and kits used in our analysis. This study used

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samples from the same subjects used for QC in the main study. The run-in was used to determine coefficients of variation (CV) between replicate samples from a vial, between vials obtained from single subjects, among plates, between days, and between 2 weeks. Second, we report the effects of storage history and sample handling on IGF-I and IGFBP-3 levels in ~100 NHANES III samples that were handled in two different ways after collection. Lastly, we examine the performance of the two assays over 6 months during an analysis of the 6,000 samples, which included ~700 control samples obtained from six QC subjects. This extensive QC data set allowed us to document individual and temporal variation in assay performance. These results should be of considerable interest for the design of future studies of IGF-I and IGFBP-3 in large cohorts and surveys and should aid in the interpretation of past epidemiologic studies of the IGF axis.

Materials and Methods

This study characterizes the performance of the Diagnostic System Laboratories (DSL) Inc. (Webster, TX) IGF-I ELISA (DSL 10-5600) and IGFBP-3 immunoradiometric assay (IRMA; DSL 6600). The IGF-I ELISA is an enzymatically amplified ''one-step'' sandwich-type immunoassay. The assay includes a simple extraction step in which IGF-I is separated from its binding protein in serum. The IGFBP-3 IRMA is a noncompetitive assay in which the analyte to be measured is ''sandwiched'' between two antibodies (20). The first antibody is immobilized to the inside walls of the tubes and the other antibody is radiolabeled for detection. The analyte present is bound by both of the antibodies to form a ''sandwich'' complex.

All assays described were done by a single technician at the DSL facility in Webster, Texas. For the IGF-I ELISA, a single batch of reagents sufficient for the entire experiment was frozen at the study onset. The IGFBP-3 IRMA required fresh batches of radioactive tracer every 3 weeks; all other reagents were from a single lot. Throughout the study, we reanalyzed samples if the CV for replicate samples from a single vial was >15%. This was rare, as only 10 samples for IGF-I and 2 samples for IGFBP-3 had CVs >15% between replicates. The 63 out-of-range IGF-I samples were diluted 50:50 and reanalyzed. Out-of-range samples had values of IGF-I greater than the highest DSL laboratory standard included on each plate.

The QC efforts associated with this study had three components. First, we did a run-in study, which characterized assay performance using recently collected QC samples. The QC samples consisted of serum samples from six volunteers who visited the NIH Department of Transfusion Medicine donor center. Three of the volunteers were men of ages 23, 38, and 58 years; three were women of ages 31, 37, and 59 years. Serum was frozen at −80°C 1 to 3 h after collection. After 1 to 3 weeks, the serum from each subject was thawed and aliquoted into 360 0.5-mL vials and then refrozen at −80°C. These samples were used in the pilot study and as QC samples during the analysis of NHANES III subject samples. A factorial design was used for the run-in study. For each of the six QC subjects, we analyzed duplicate samples from two different vials on two plates per day for 3 days in the first week and 2 days in the following week. This resulted in 240 measurements from 120 vials for IGF-I and IGFBP-3.

Following the run-in study, we conducted a site visit to the DSL facility in Webster. A review of the assays led to two small tests aimed at determining whether sample dilution or radioactive tracer age (I-125, half life = 60.1 days) contributed to variance in the initial test results. Although these factors did not conclusively seem to influence our results, we chose to replace the radioactive tracers used for the IRMA every 3 weeks, 1 week earlier than the recommended time limit for the commercially available kit. We also tested new batches of radioactive tracer with our QC samples. None of these tests suggested aberrant assay performance. The run-in study was analyzed using a general linear model that allowed us to quantify variance components at the vial, plate, day, and week levels.

Second, we tested the effects of sample preparation and storage on 108 NHANES III surplus serum samples; recall that these samples were collected between 1988 and 1994 (21). These samples were stored using two different procedures. Surplus serum samples were frozen on collection, shipped directly to testing labs, thawed for analysis of various serum factors, refrozen in −80°C freezers, and made available for further study. During their time at the testing labs, the surplus serum samples may have been frozen and thawed one or more times for reanalysis and experienced variable periods at refrigerator and room temperature. The exact treatment these samples received is unknown. A second group of samples, the liquid nitrogen samples, were frozen on collection, shipped to Atlanta, defrosted on an ice table, and aliquoted into four 0.5-mL vials. Liquid nitrogen samples were then stored in liquid nitrogen until released for our study.

Third, in the main study of ~6,000 subjects from the fasting subsample of NHANES III, we analyzed IGF-I and IGFBP-3 using the liquid nitrogen samples. Both the liquid nitrogen study samples and our QC samples experienced two freeze-thaw cycles before this study. IGF-I was analyzed on 96-well plates. Samples for 36 NHANES III subjects and two samples from each of two of the QC subjects were analyzed in replicates on each of the plates (72 wells of subject serum, 8 wells of QC serum, and 16 wells of internal standards). QC subjects were placed on plates at random, but replicate samples from a single vial were adjacent to one another. We recorded location of samples on each plate. The same pairs of QC samples (designated A-F for the six individuals) were used throughout the study (A and B, C and D, and E and F).

Samples were analyzed for IGFBP-3 in batches of coated tubes on the same days as the IGF-I samples using the same scheme of replication for the NHANES samples and QC subjects (i.e., groups of 36 samples and two samples from each of two QC subjects were analyzed in replicate). One to four racks and batches of IGFBP-3 were analyzed on weekdays over the course of ~10 months (June 2004-February 2005). Sample sizes for each QC subject are not exactly equal because of uneven numbers of samples in batches of samples shipped to DSL for analysis and because of erroneous QC sample placement in a small number of batches that resulted in extra assays of certain pairs of QC subjects. For example, there were 114 replicates for QC subjects F and E and 140 replicates for subjects A and B.

Standardized deviates for the QC subjects were computed from individual values by subtracting the subject's overall average and dividing by the subject's SD as computed across all available plates. For IGF-I, the subject-specific means and SDs were computed from log-transformed values. After analyzing all samples, we used a modified Levy-Jennings approach to identify outliers (see below and ref. 22). This approach identified 15 plates for reanalysis. For 12 of these plates, both IGF-I and IGFBP-3 were reanalyzed. In the remaining three plates, only IGF-I was reanalyzed because only one IGF-I QC sample was aberrant (i.e., had a SD >3.0 in absolute value). Plates were selected for this reanalysis if the average of the two SDs for each QC subject was >1.96 in absolute value for IGF-I or IGFBP-3.

A new data set was created with 188 plates after the initial Levy-Jennings analysis and further QC studies were done, including construction of additional Levy-Jennings plots to identify plates that were potentially out of compliance. For these additional Levy-Jennings plots, a plate would be classified as noncompliant only if the SDs for a given QC subject were either both below or both above some appropriately chosen bounds ($\pm c$), where c is chosen to give the appropriate experiment-wise error rate. We wanted to ensure that the

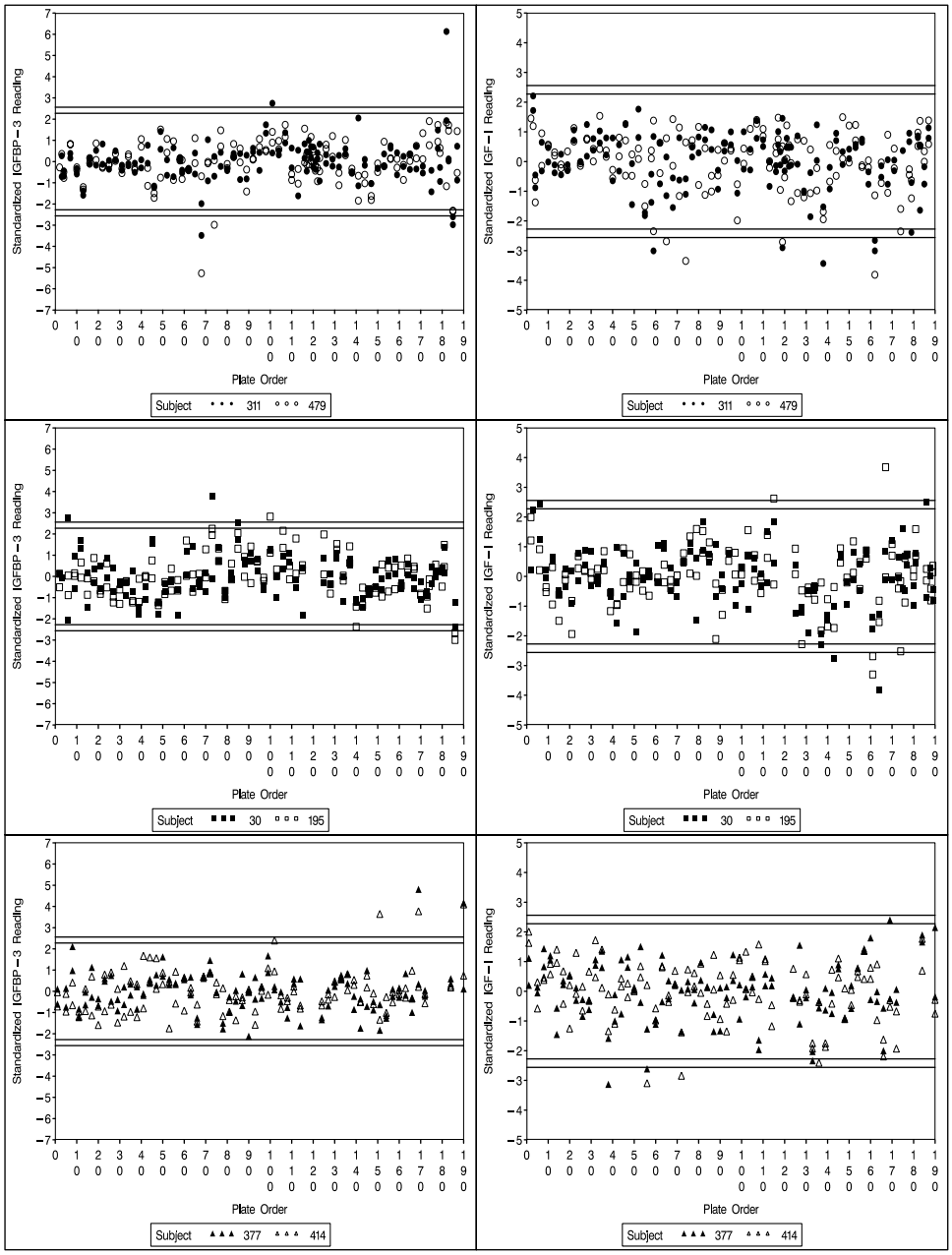

Figure 1. Modified Levy-Jennings plots for six QC subjects over 188 plates/batches for IGF-I and IGFBP-3 assays.

probability (α) of incorrectly classifying one of the 188 plates as noncompliant was small. Let p denote the probability that both replicates (denoted r_1 and r_2) for a QC subject fall outside the same (lower or upper) bound strictly by chance:

$$\begin{aligned} p &= \Pr[(r_1 > c \text{ and } r_2 > c) \text{ or } (r_1 < -c \text{ and } r_2 < -c)] \\ &= \Pr(r_1 > c \text{ and } r_2 > c) + \Pr(r_1 < -c \text{ and } r_2 < -c) \\ &= [\Pr(r_1 > c)]^2 + [\Pr(r_1 < -c)]^2 \\ &= 2[\Pr(r_1 > c)]^2 \end{aligned}$$

The last equality follows from the assumed symmetry of the distributions of SDs, and the next-to-last equality follows from the assumption that the SDs are statistically independent from one another. The value of p in the above equation is called the comparison-wise error rate because it applies only to one plate. Because we made 188 comparisons per analyte, each with probability p of indicating noncompliance strictly by chance, we used a Bonferroni-type adjustment to maintain the so-called experiment-wise error rate α. To ensure $\alpha \leq 0.05$, we set p equal to $0.05/188 = 2.66 \times 10^{-4}$, which implies $\Pr(r_1 > c)$ is equal to the square root of 1.33×10^{-4} (= 0.0115). Finally, we chose c so that the area under the standard normal curve to the right of c is 0.115 to arrive at $c = 2.275$. The inner pair of bounds shown on the Levy-Jennings plots of Fig. 1 corresponds to the aforementioned choice of c that holds $\alpha < 0.05$, whereas the outer pair of bounds correspond to a choice of $c = 2.56$, which holds $\alpha < 0.01$.

We illustrate the results of our QC efforts in three ways. First, we characterize overall levels of variation in the QC subjects. Second, we graphically illustrate the behavior of QC samples using Levy-Jennings plots. Third, we report on the effects of position in a plate on estimated serum levels of IGF-I using ANOVA.

Results

Run-in Study. Variance components for the run-in and main study are summarized in Table 1. Note a large between-week

Table 1. Variance components for assays of IGF-I and IGFBP-3 from run-in and main study

(A) Variance components

Source*	IGFBP-3		Ln IGF-1	
	Run-in	Main study	Run-in	Main study
Between-subject	891,683	834,353	0.0861	0.0884
Between-week	259,217	0	0.0011	0.0006
Between-day	24,617	7,627	0.0012	0.0040
Between-plate	89,176	50,683	0.0035	0.0054
Within-subject	122,571	74,849	0.0150	0.0133

(B) Percent of variance associated with different factors

Source*	IGFBP-3		Ln IGF-1	
	Run-in	Main study	Run-in	Main study
Between-subject	64.3	86.2	80.5	82.7
Between-week	18.7	0	1.0	0.6
Between-day	1.8	0.8	1.1	3.7
Between-plate	6.4	5.2	3.3	5.0
Within-subject	8.8	14.0	14.0	12.4

NOTE: Day is nested in week and plate is nested in day and week.
*Variance estimates obtained with restricted maximum likelihood approach.

effect for IGFBP-3 in the run-in study. This was due to a large difference in average values for IGFBP-3 between weeks 1 and 2 of the run-in. Following a site visit and a change in reagent replacement schedule (in subsequent analyses, we replaced the radioactive tracer used in the IGFBP-3 assay every 3 weeks), the between-week effect was no longer present. We also considered the possibility that sample dilution would improve assay performance by improving antibody binding efficiency. We found no evidence for this in comparisons of analyses with and without a 50% dilution of QC samples (results not shown).

Effect of Storage Conditions. Following the site visit, samples subject to two different storage conditions were analyzed (Fig. 2). Paired t tests indicate significantly lower values for IGF-I and IGFBP-3 in samples sent to laboratories for the original NHANES III laboratory assays (surplus serum samples) than those sent directly to Atlanta for storage (liquid nitrogen samples). The liquid nitrogen samples were frozen, shipped directly to National Center for Health Statistics collection center, thawed, aliquoted, and then stored in liquid nitrogen for 10 to 16 years until the current study. The surplus serum samples were stored at −80°C but could have experienced multiple freeze-thaw cycles and unknown amounts of time at refrigerator or room temperature at the laboratories where the standard NHANES III serum analyses were done. The mean difference for IGF-I was 129 ng/mL (95% confidence interval, 105-154; $t = 10.47$, $P < 0.001$, $n = 96$) and for IGFBP-3 was 1,050 ng/mL (95% confidence interval, 841-1,260; $t = 9.9$, $P < 0.001$, $n = 102$). Compared with the mean IGF-I and IGFBP-3 values in the liquid nitrogen stored specimens, these correspond to 43% and 25% reductions, respectively. Degradation was not associated with age or race/ethnicity in either analyte, but degradation of IGFBP-3 was somewhat ($P = 0.035$) higher in women (mean difference, 1,257; SE, 142; $n = 55$) than in men (mean difference, 813; SE, 152; $n = 48$). Subsequent analyses were done entirely on the liquid nitrogen specimens.

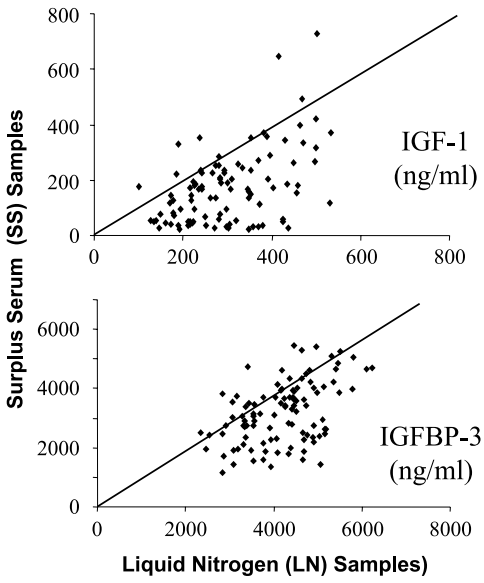

Figure 2. Effects of storage and handling history on IGF-I and IGFBP-3 levels from samples of the NHANES 1988-1994 surplus serum.

Samples included in the 12 plates selected for reanalysis for both IGF-I and IGFBP-3 indicated that an additional freeze-thaw cycle did not influence levels of either analyte, confirming previous studies of these assays (19) and suggesting that extended time spent at room temperature may be more important for IGF degradation than an additional freeze thaw cycle.

Assay Performance. Log transformation resulted in a close to normal distribution of SDs for IGF-I. Transformation did not improve the fit of IGFBP-3 SDs. The distributions of SDs for each analyte had a mean of zero and a variance of 1, and were fairly symmetrical (Fig. 3). Results for the six QC subjects throughout the entire 188 plates analyzed during the main study period are summarized in Table 2 and illustrated in Fig. 1. Note that these results are based on analyses of means of two replicates for each QC subjects. Coefficients of variance between replicate samples from a single vial were very low [mean CV IGF-I, 2.2% (95% confidence interval, 2.17-2.26; $N = 7{,}530$); mean CV IGFBP-3, 1.91 (95% confidence interval, 1.87-1.95; $N = 7{,}415$)]. Average values of these replicates were used in all subsequent analyses. Variances within subjects differed significantly (Bartlett's test) for both IGF-I ($P < 0.001$) and IGFBP-3 ($P < 0.001$) in this analysis and CVs ranged from 12.3% to 17.6% for IGF-I and from 8.9% to 12.8% for IGFBP-3 (Table 2). Between-subject effects accounted for 82.7% and 86.2% of variance for IGF-I and IGFBP-3, respectively (Table 2). We observed significant effects of day (2-4% of total variance) and plate (~5% of total variance) on assay performance (Table 1). Thus, samples should be analyzed on the same plate and on the same day to the extent possible. Plate effects were somewhat larger than day effects, especially for IGFBP-3.

We also examined the possibility of variation in IGF-I assay results based on location on the ELISA plate. We found some evidence for a location effect. Samples in the outer rows of the plates had higher levels of IGF-I, adjusted for between-subject variation ($P = 0.0482$). However, the magnitude of this effect was small, as the least square mean IGF-I level in wells in the outer rows was 246.8 (SE, 1.7) but slightly lower in inner wells (242.7; SE, 1.13). These estimates were obtained from ANOVA with QC subject, position, and subject by position as independent variables and IGF-I level as the dependent variable.

Discussion

This study characterized the performance of commonly used assays for IGF-I and IGFBP-3 in the context of a large cross-sectional study of serum samples. Three salient points

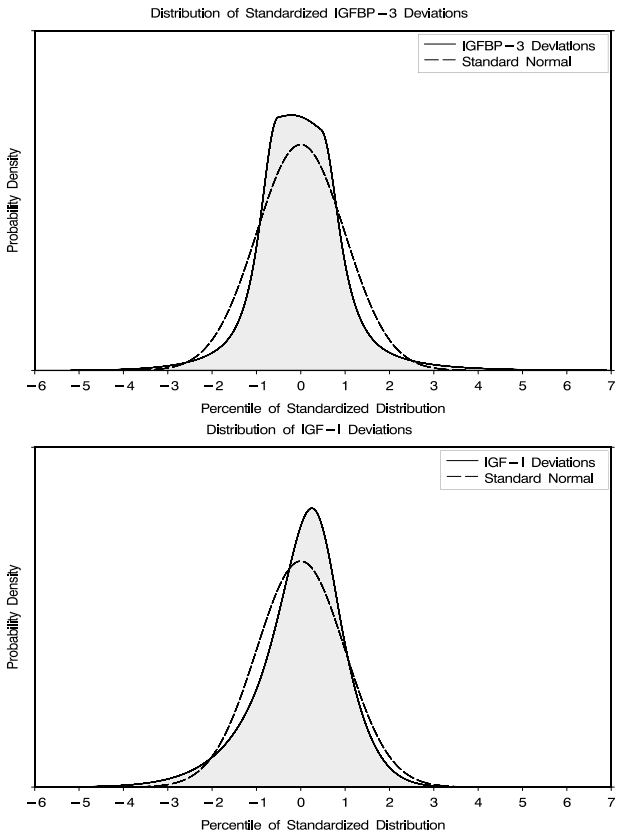

Figure 3. Distribution of SDs for IGF-I and IGFBP-3 QC samples.

emerged from this analysis. First, the performance of the assay can vary significantly by plate/batch, day, and week of the assay. Samples must be randomized over the time required for analysis. Importantly, studies of matched samples should analyze matches on the same plates. Second, sample degradation may occur due to handling issues such as freeze-thaw cycles, time spent at room temperature during sample aliquoting for other assays, or storage conditions. Careful efforts to quantify and minimize sample degradation are required. The present study and other recent studies of children and adults (e.g., refs. 23, 24) will provide reference values for IGF-I and IGFBP-3 and preliminary measurements can be used to determine whether sample degradation has occurred. Third, the DSL ELISA for IGF-I and IRMA for IGFBP-3 showed good temporal stability. This is important for large-cohort or surveillance studies that require months for complete analysis.

CVs for IGF-I and IGFBP-3 were comparable to other recent studies of IGF-I and IGFBP-3 using DSL kits. For example, Rinaldi et al. (16) report intrabatch and interbatch CVs of 6.3% and 8.3% for IGF-I and 7.2% and 13.9% for IGFBP-3 with DSL ELISAs, and Hankinson et al. (25) report interbatch and intrabatch CVs of 8.7% and 15.6% for IGF-I and 9.3% and 19.4% for IGFBP-3, respectively. Variation in IGF-I in this study was a little higher than past studies but variation in IGFBP-3 was lower. We cannot determine whether this is due to differences in sample characteristics or assay performance. Somewhat lower CVs have been reported in some studies (26); again, it is difficult to determine whether this was due to assay performance, laboratory differences, or sample characteristics. Fewer studies have used the DSL IGFBP-3 IRMA, but one article reports intra-assay and interassay CVs of 3.9% and 1.9%, respectively (27). Larger studies and those with longer duration seem to have higher CVs. These relatively high CVs and evidence for heterogeneity within individuals clearly suggest that improvements in laboratory practice, careful sample handling procedures, and improved assays would be welcome for both analytes. Finally, note that CVs between replicates within a plate were very low (~2%) for both IGF-I and IGFBP-3.

Overall, these assays showed moderately high CVs for both analytes. Comparison with past studies is not always easy because very few details are given about QC efforts and analysis in many epidemiologic studies involving IGFs or other serum components. A little more detail about QC and sample storage conditions in such studies could help improve comparisons among studies and assessment of study quality. Variation in assay performance has been suggested as a cause of heterogeneity in epidemiologic studies of associations between cancer and the IGF system (2, 28), and a recent study suggests that the performance of a commonly used DSL ELISA for IGFBP-3 may have changed over time (16). Better methods of measuring biologically relevant aspects of the IGF system in serum and in specific tissues are needed to improve our understanding of the epidemiology of cancer and the IGF system.

Sample degradation can be a problem for studies of serum analytes with long, complex storage histories. Time at room temperature, storage temperature, and number of freeze-thaw cycles have been suggested as potential factors influencing stability of IGF-I and IGFBP-3 (18, 26, 29). For example, delays in shipping before freezing samples reduced IGFBP-3 levels by 3% (18), but IGF-I and IGFBP-3 seemed to be stable in response to five freeze-thaw cycles in a study (26) using IRMA from DSL. We are unaware of studies examining the interactive effects of freeze-thaw cycles, time at room temperature, and duration of storage. In any case, careful attention should be paid to storage history, and our experiences clearly indicate that a subset of representative samples should be tested before embarking on a large study. Detailed records of sample history would facilitate assessment of potential causes of sample degradation. Lastly, availability of reference values from diverse studies should help in the evaluation of sample integrity.

This article highlights the need for careful consideration of sample storage and handling history, and it documents the performance of commonly used assays for IGF-I and IGFBP-3 in a large cross-sectional study that included subjects

Table 2. Descriptive statistics for the six QC subjects analyzed in this study

Subject	*N*	IGF-I (ng/mL)					IGFBP-3 (ng/mL)				
		Mean	SD	Min	Max	CV	Mean	SD	Min	Max	CV
A	140	322.3	40.9	201.0	430.5	12.7	2,908.5	363.9	1,641.0	5,139.0	12.5
B	140	324.5	49.8	169.8	413.7	15.4	4,726.5	447.6	2,369.5	5,579.5	9.5
C	125	219.6	38.6	121.9	409.8	17.6	2,838.5	315.0	1,895.5	3,726.5	11.1
D	126	188.0	27.9	103.9	271.8	14.9	4,398.0	393.1	3,463.5	5,881.5	8.9
E	114	250.8	30.9	167.5	321.7	12.3	2,556.2	328.2	1,983.5	3,900.5	12.8
F	114	153.1	22.4	95.0	216.6	14.6	3,005.9	296.6	2,378.5	4,432.0	9.9

representing a wide range of age, socio-demographic, and health status characteristics. Assay performance was stable over time but CVs of ~10% for both analytes will reduce study power. On the other hand, both analytes have substantial between-person variability, potentially allowing assessment of associations between the IGF axis and the demographic, behavioral, and health-related variables. These results should help guide the design and analysis of future studies of IGF-I and IGFBP-3, candidate biomarkers of cancer risk, and potential targets for chemopreventive interventions at several sites.

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