Specimen processing time and measurement of total insulin-like growth factor-I (IGF-I), free IGF-I, and IGF binding protein-3 (IGFBP-3)

Tiffany G. Harris [a,*], Howard D. Strickler [a], Herbert Yu [b], Michael N. Pollak [c], E. Scott Monrad [a], Mark I. Travin [a], Xiaonan Xue [a], Thomas E. Rohan [a], Robert C. Kaplan [a]

[a] *Albert Einstein College of Medicine, Bronx, NY, USA*
[b] *Yale University School of Medicine, New Haven, CT, USA*
[c] *McGill University, Montreal, Que., Canada*

Abstract

Background: An increasing number of epidemiologic studies are investigating the relationship between serum levels of insulin-like growth factor-I (IGF-I) and IGF binding proteins (IGFBPs) and risk of cancer, cardiovascular disease, and other diseases. However, little is known regarding the effects of blood specimen processing time on measured levels of total and free IGF-I, and on IGFBP-3, the major binding protein.

Design: Two serum separation tubes were collected from each of 12 subjects. One tube was centrifuged as soon as possible following blood collection (a mean of 47 min; range = 30–80 min), and serum aliquots were placed into −70 °C storage either shortly after centrifugation, or following 2, 4, 10, or 24 h at ambient temperature (measured from the time of blood draw). The second serum separation tube was maintained at ambient temperature for 24 h before centrifugation and freezing. Total IGF-I, free IGF-I, and IGFBP-3 levels were determined using commercial enzyme linked immunosorbent assays (ELISAs) commonly employed in epidemiologic studies. The effects of time until centrifugation and freezing on seroassay results were evaluated using generalized estimating equation (GEE) linear regression models and Spearman correlation.

Results: Total IGF-I and IGFBP-3 levels did not vary significantly with the amount of time at ambient temperature following centrifugation, even up to 24 h, in blood specimens that were centrifuged soon after collection (all $p_{\chi^2} > 0.10$). However, free IGF-I levels increased significantly with increasing time intervals between centrifugation and freezing in these same specimens ($p_{\text{trend}} < 0.001$). Total IGF-I/IGFBP-3 molar ratio, a crude measure of free IGF-I levels, showed no clear association. In blood specimens that were not centrifuged for 24 h, total IGF-I, free IGF-I, and IGFBP-3 were each significantly elevated (each $p_{\chi^2} < 0.001$) compared with results in blood specimens that were centrifuged and frozen soon after collection, whereas the total IGF-I/IGFBP-3 molar ratio was decreased ($p_{\chi^2} < 0.001$). Nonetheless, all total IGF-I, free IGF-I, IGFBP-3, and total IGF-I/IGBFBP-3 molar ratio values altered by delays in processing were highly correlated with the values in specimens processed as soon as possible (all Spearman rank correlation coefficients $\geqslant 0.84$).

Conclusions: Total IGF-I and IGFBP-3 can be fairly stably measured in serum with commonly used commercial assays regardless of the interval between blood collection and freezing, up to at least 24 h, as long as centrifugation and serum aliquoting take place shortly after blood collection. Free IGF-I levels, however, increase steadily with the time interval until freezing, even if serum

* Corresponding author.
E-mail address: tharris@aecom.yu.edu (T.G. Harris).

separation has been completed soon after blood collection. Because the altered serum values are highly correlated with the referent values, analysis of total IGF-I, free IGF-I, IGFBP-3, and total IGF-I/IGFBP-3 molar ratio data by quartile might help mitigate concerns regarding the effects of delays in processing time.

Keywords: Blood preservation; Centrifugation; Enzyme-linked immunosorbent assay; Insulin-like growth factor I; Insulin-like growth factor binding protein 3; Serum; Time factors

1. Introduction

Mounting evidence suggests that high serum insulin-like growth factor-I (IGF-I) levels are associated with increased risks of colorectal, breast, and several other common cancers [1,2]. IGF-I may also be associated with risk of cardiovascular disease, albeit the nature of this association, if any, remains unclear [3–5]. To better understand these relationships, an increasing number of molecular epidemiologic studies are measuring IGF-I and IGF binding proteins (IGFBPs) in serum. In field studies, such as these, there may often be considerable time delays between the collection and centrifugation and freezing of blood specimens. Two common scenarios in epidemiologic studies are that: (i) blood specimens are centrifuged onsite to separate serum soon after blood collection, but the separated serum aliquots remain at ambient temperature for some period of time before reaching a storage facility where they are then frozen and (ii) blood specimens are held or shipped overnight before being centrifuged and frozen at a storage facility. Little is known, however, regarding the effects of delays in blood specimen processing on the measured serum levels of IGF-I and IGFBP-3 (the protein which binds most of the IGF-I in circulation [1]). To address this issue, we varied the time intervals between the collection of blood, centrifugation, and freezing, and assessed the impact of this on levels of total IGF-I, unbound or ''free'' IGF-I, which is commonly thought to be the main bioactive component of IGF-I [6–8], and IGFBP-3, as measured using a brand of commercial seroassays that has been commonly employed in epidemiologic studies [9–13].

2. Materials and methods

2.1. Subjects and specimens

Sequential patients ($n = 11$) who presented for diagnostic coronary angiography, during November and December, 2002 at a hospital-based referral center in the Bronx, NY were recruited as subjects. Eligibility was restricted to men and women 30–79 years of age. Patients were excluded if they had New York Heart Association Class III or IV heart failure, renal failure, hepatic disease, or cancer (other than skin cancer). One healthy 46 year-old female volunteer was also enrolled to assess whether similar results were observed in an entirely healthy individual.

Sociodemographic data and medical history were obtained using a structured interview conducted by a trained research nurse and by medical record review. Prior to angiography, two venous blood samples were collected in serum separation tubes (SST^{TM} tubes; BD Vacutainer Systems, Franklin Lakes, NJ). One of the two SST^{TM} tubes from each subject was centrifuged as soon as possible following blood collection (a mean of 47 min; range = 30–80 min), and the resulting serum was then pipetted into 0.5 mL aliquots. One of the serum aliquots was placed into −70 °C storage shortly after centrifugation, delayed only by the time required to aliquot and label specimens (a mean of 28 min following centrifugation; each less than 35 min except one of 65 min), while the other serum aliquots were maintained at ambient room temperature before being placed into −70 °C storage at 2, 4, 10, and 24 h following blood draw. The second SST^{TM} blood specimen was maintained at ambient room temperature for 24 h, at which point the specimen was centrifuged, and 0.5 mL aliquots from the resulting serum specimen were placed into storage at −70 °C. The frozen specimens were later shipped overnight on dry ice to the testing laboratory (HY). This study was approved by the institutional review boards at Albert Einstein College of Medicine and Montefiore Medical Center.

2.2. IGF assays

Concentrations of total IGF-I, free IGF-I, and IGFBP-3 in serum were determined using commercially available enzyme linked immunosorbent assays (ELISAs) from Diagnostic Systems Laboratories, Inc. (DSL; Webster, TX) [9,13]. The total IGF-I (DSL kit #10-2800), free IGF-I (DSL kit #10-9400), and IGFBP-3 (DSL kit #10-6600) assays were conducted in specific accordance with the manufacturer's recommendations. All specimens were tested as part of a single batch, using three separate ELISA kits from a single manufacturer's production lot for each biomarker. Specimens were tested in replicate with each pair of replicates tested on the same assay plate in sequential wells to avoid between-plate variation. The mean value for each pair of replicates was used as the result in each

assay. Any samples with disparate values, defined as a coefficient of variation (CV) greater than 10%, were repeated and any plates with quality control samples that had values outside the expected range were also repeated. Previous testing by our laboratory of 45 batches of samples that included masked duplicate aliquots found that the CVs between and within batches, respectively, were 3.605% and 4.900% for total IGF-I, 9.849% and 16.956% for free IGF-I, and 4.182% and 5.545% for IGFBP-3 (data not shown).

2.3. Statistical analysis

Total IGF-I, free IGF-I, and IGFBP-3 test results were plotted separately for each subject, according to the time intervals between blood collection, centrifugation, and freezing. To more formally assess the effects of time until centrifugation and freezing on seroassay results, we used generalized estimating equation (GEE) linear regression models [14], with the seroassay results modeled as continuous outcomes. The time interval until freezing was modeled as a categorical variable and the reference period set as freezing shortly following centrifugation. We additionally modeled the time until freezing as an ordered variable to assess the overall linear trend in assay results with increasing time until freezing. To adjust the standard errors for repeated observations per subject, each GEE model employed an independent correlation structure and we used standard errors which were robust to the correlation structure imposed [15]. Furthermore, each GEE model was repeated using assay values that were "standardized" by subtracting the mean assay value and then dividing by the standard deviation (SD) in order to make the test data analytically similar across the assays. Interestingly, the total IGF-I/IGBP-3 molar ratio has been used in some epidemiologic studies as an estimate of the level of free (bioactive) IGF-I, since IGFBP-3 binds approximately 80% of IGF-I in circulation. Although this molar ratio is a crude and indirect measure of free IGF-I levels, because it does not account for several other IGFBPs, we used GEE to evaluate the effects of delays in specimen processing on its values, given the usage of total IGF-I/IGFBP-3 molar ratio in the literature. Correlations between serum values were assessed using the Spearman rank correlation coefficient. All analyses were performed using Stata Statistical Software (Release 8.2).

3. Results

The 12 subjects had an average age of 63 years (range 41–79). Six subjects were female, four were Caucasian, six were African American, and two were Hispanic. Four subjects had diabetes. The healthy female volunteer had a similar time pattern to her assay results as those observed in the angiography patients, and therefore her data were included in our analysis.

3.1. Total IGF-I

The reference serum specimens (i.e., blood centrifuged soon after collection and frozen shortly thereafter) had a mean total IGF-I level of 334.4 ng/mL (SD = 105.0). There was no association between total IGF-I levels and time until freezing in serum aliquots that, like the reference serum samples, were from specimens centrifuged soon after blood collection (all $p_{\chi^2} > 0.20$; Table 1 and Fig. 1A). However, in aliquots that were not centrifuged for 24 h, the mean total IGF-I level was higher by 23.6 ng/mL, or 0.21 SDs, compared with the reference aliquots ($p_{\chi^2} < 0.001$) (Table 1).

3.2. Free IGF-I

The reference serum samples had a mean free IGF-I level of 0.25 ng/mL (SD = 0.15). Free IGF-I levels increased with the time until freezing in serum from specimens centrifuged soon after blood collection ($p_{\text{trend}} < 0.001$; Table 1 and Fig. 1B). Free IGF-I levels were higher by 0.69 SDs after 10 h at ambient temperature, and by 1.47 SDs after 24 h at ambient temperature. Compared with the reference serum samples, serum from blood specimens that were not centrifuged for 24 h also had significantly elevated levels of free IGF-I by 1.27 SDs ($p_{\chi^2} < 0.001$) (Table 1).

3.3. IGFBP-3

The reference serum samples had a mean IGFBP-3 of 3750.9 ng/mL (SD = 1075.8). There was no overall association ($p_{\text{trend}} = 0.20$) between IGFBP-3 levels and time until freezing in serum from specimens centrifuged soon after blood collection (Table 1 and Fig. 1C); albeit, IGFBP-3 levels were slightly, non-significantly higher (all increases were less than 6.7%) with delayed freezing following centrifugation. Serum from blood specimens that were not centrifuged for 24 h had a significantly higher total IGFBP-3 level (by 0.78 SDs) than the reference serum ($p_{\chi^2} < 0.001$) (Table 1).

3.4. Total IGF-I/IGFBP-3 molar ratio

The reference serum samples had a mean total IGF-I/IGFBP-3 molar ratio of 0.093 (SD = 0.028). Similar to the findings for total IGF-I and IGFBP-3 individually (above and Table 1), the molar ratio had no significant overall association with time to freezing in serum from specimens that were centrifuged soon after blood collection ($p_{\text{trend}} = 0.16$) (Table 1). We note, however,

Table 1
Linear regression results for the effects of delaying serum separation and freezing on total IGF-I, free IGF-I, and IGFBP-3

Assay	Mean (SD)[a]	β coefficient using non-standardized values (95% CI)	β coefficient using standardized values (95% CI)[b]	p_{χ^2}	p_{trend}[c]
Total IGF-I					0.88
Reference period[d]	334.4 (105.0)	Ref.	Ref.	Ref.	
2 h	339.8 (118.6)	5.5 (−3.7, 14.7)	0.05 (−0.03, 0.13)	0.24	
4 h	337.3 (112.5)	2.9 (−5.0, 10.9)	0.03 (−0.04, 0.10)	0.47	
10 h	333.9 (108.3)	−0.4 (−7.9, 7.1)	0.00 (−0.07, 0.06)	0.92	
24 h	336.5 (118.0)	2.1 (−10.6, 14.8)	0.02 (−0.09, 0.13)	0.75	
Not centrifuged for 24 h	357.9 (118.5)	23.6 (12.8, 34.4)	0.21 (0.11, 0.30)	<0.001	
Free IGF-I					<0.001
Reference period[d]	0.25 (0.15)	Ref.	Ref.	Ref.	
2 h	0.26 (0.16)	0.01 (−0.03, 0.05)	0.05 (−0.15, 0.24)	0.63	
4 h	0.27 (0.14)	0.02 (−0.01, 0.05)	0.11 (−0.05, 0.27)	0.18	
10 h	0.39 (0.22)	0.14 (0.09, 0.20)	0.69 (0.41, 0.97)	<0.001	
24 h	0.55 (0.31)	0.30 (0.19, 0.42)	1.47 (0.91, 2.03)	<0.001	
Not centrifuged for 24 h	0.51 (0.31)	0.26 (0.14, 0.38)	1.27 (0.69, 1.85)	<0.001	
IGFBP-3					0.20
Reference period[d]	3750.9 (1075.8)	Ref.	Ref.	Ref.	
2 h	3833.0 (945.3)	82.1 (−176.0, 340.1)	0.09 (−0.19, 0.36)	0.53	
4 h	3894.8 (838.3)	143.8 (−48.0, 335.7)	0.15 (−0.05, 0.35)	0.14	
10 h	3872.4 (946.5)	121.5 (−53.1, 296.0)	0.13 (−0.06, 0.31)	0.17	
24 h	3998.9 (1001.6)	247.9 (−104.7, 600.6)	0.26 (−0.11, 0.63)	0.17	
Not centrifuged for 24 h	4496.2 (1184.5)	745.2 (388.2, 1102.3)	0.78 (0.41, 1.16)	<0.001	
Total IGF-I / IGFBP-3 molar ratio					0.16
Reference period[d]	0.093 (0.028)	Ref.	Ref.	Ref.	
2 h	0.091 (0.027)	−0.002 (−0.007, 0.003)	−0.09 (−0.28, 0.10)	0.36	
4 h	0.087 (0.023)	−0.006 (−0.013, 0.001)	−0.23 (−0.51, 0.06)	0.12	
10 h	0.088 (0.025)	−0.005 (−0.009, −0.0004)	−0.19 (−0.36, −0.02)	0.03	
24 h	0.087 (0.027)	−0.006 (−0.014, 0.001)	−0.25 (−0.54, 0.04)	0.09	
Not centrifuged for 24 h	0.083 (0.026)	−0.010 (−0.015, −0.005)	−0.40 (−0.61, −0.19)	<0.001	

[a] Units are ng/mL.
[b] Standardized values were created by subtracting the mean and then dividing by the standard deviation.
[c] Excludes the specimens that were not centrifuged for 24 h.
[d] The reference period serum specimens were from blood centrifuged soon after collection, and frozen shortly thereafter.

that compared with the reference value, the molar ratios were on average slightly reduced with delayed freezing (each reduction was less than 6.5%), and at 10 h (but only 10 h) the reduction (5.4%) was statistically significantly ($p_{\chi^2} = 0.03$). These reductions were mainly explained by the small non-significant variations in IGFBP-3 levels (above). Delaying centrifugation for 24 h resulted in a significant decrease in the molar ratio (by 0.40 SDs) compared to the reference serum ($p_{\chi^2} < 0.001$), a decrease that was mainly explained by a greater average increase in IGFBP-3 (19.9%) than total IGF-I (7.0%) levels with delayed centrifugation.

3.5. Correlations

We examined whether the total IGF-I, free IGF-I, and IGFBP-3 values in the serum samples that were not immediately centrifuged and frozen remained correlated with the reference serum sample values. For specimens not centrifuged for 24 h, the correlations with the reference serum sample values were 0.98 (95% CI = 0.93, 0.99) for total IGF-I; 0.85 (95% CI = 0.53, 0.96) for free IGF-I; 0.84 (95% CI = 0.51, 0.95) for IGFBP-3; and 0.92 (95% CI = 0.74, 0.98) for total IGF-I/IGFBP-3 molar ratio. For specimens centrifuged but not frozen for varied periods of time, free IGF-I values were shown (above) to vary from the reference values, but they were still highly correlated with reference values; the correlation of the reference values with those from specimens that remained unfrozen for 10 h was 0.90 (95% CI = 0.67, 0.97), and it was 0.87 (95% CI = 0.58, 0.96) for those that remained unfrozen for 24 h.

4. Discussion

In this study, we found that total serum IGF-I and IGFBP-3 levels, measured using commercial seroassays that are commonly employed in epidemiologic studies, were not significantly affected by the period of time that

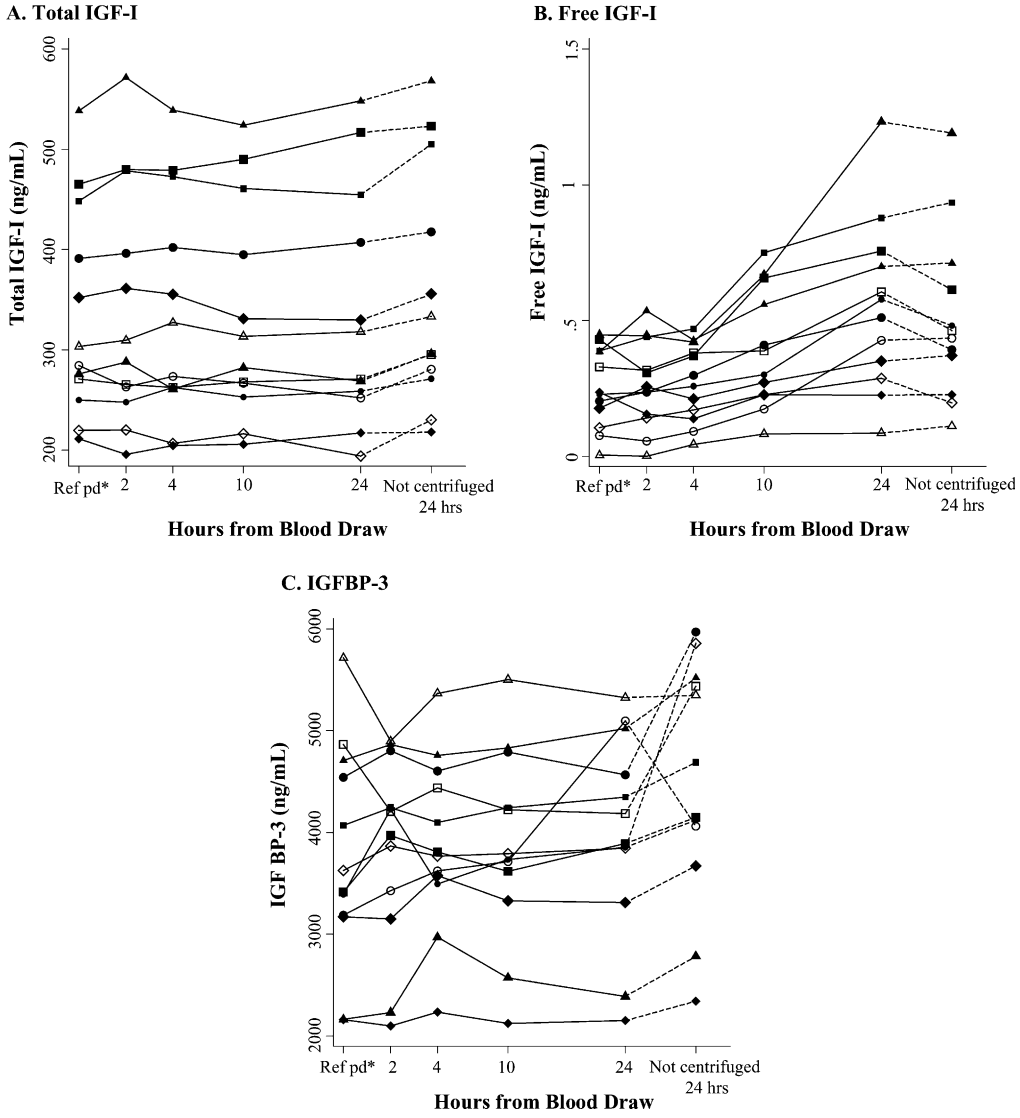

Fig. 1. Effect of delaying serum separation and freezing on total IGF-I (A), free IGF-I (B), and IGFBP-3 (C) results. *Ref. pd, reference period serum specimens were from blood centrifuged soon after collection, and frozen shortly thereafter.

the serum samples were held at ambient temperature prior to freezing, up to at least 24 h, as long as the blood specimens were centrifuged and the serum aliquoted soon after blood collection. In contrast, in these same serum samples, free IGF-I levels showed a statistically significant increase with the period of time that the samples were held at ambient temperature prior to freezing. One possible explanation for why free IGF-I was significantly affected by the amount of time at ambient temperature, and total IGF-I and IGFBP-3 were not, is that a delay in freezing might lead to increased dissociation of IGF-I from its binding proteins. If so, delays in processing and freezing blood specimens would be likely to cause overestimation of the true free IGF-I levels in serum regardless of the seroassay system utilized. Total IGF-I/IGFBP-3 molar ratio, which is a crude and indirect measure of free IGF-I levels that was associated with cancer in some previous studies [13,16–18], was either unchanged or only slightly (<6.5%) reduced by delays in freezing following centrifugation. We also observed that serum from blood specimens that were not centrifuged for 24 h had significantly higher levels of total IGF-I, free IGF-I, IGFBP-3, but lower total IGF-I/IGFBP-3 molar ratio values than the reference serum aliquots. The reduction in total IGF-I/IGFBP-3 molar ratio was explained by a greater average increase in IGFBP-3 than total IGF-I values. Based on these results, further studies are needed to better determine how soon specimens must be centrifuged following collection before there is an effect on total IGF-I, free IGF-I, and IGFBP-3 levels, and to measure the size of these effects per unit time.

Despite the observed time-dependent changes in total IGF-I, free IGF-I, IGFBP-3, and total IGF-I/IGFBP-3

molar ratio values, their measured values always remained highly correlated with the reference serum values. When categorized by quartile (or tertile, etc.), therefore, most patients would be correctly stratified. Moreover, by collapsing IGF-I and IGFBP-3 results and their molar ratio into quartiles the effects of inconsistent specimen processing time will be reduced. As long as the delay in centrifugation and freezing is roughly similar for all subjects, epidemiologic studies can mitigate the potential effects of variations in specimen processing time on their findings by analyzing serum IGF-I and IGFBP-3 by quartile. Thus, it is critical that there be a standard protocol that includes a time table for the processing of all blood specimens. Fortunately, this is already common in the field, even though the specific concerns above were not, to our knowledge, previously taken into consideration. On the other hand, when studying weak associations (e.g., low relative risks) the small amount of residual misclassification could still be a problem, and great care will need to be taken to process serum specimens in a fixed time frame.

There are only limited prior data addressing the effects of delayed centrifugation and freezing of blood specimens on IGF-I and IGFBP-3 seroassay results. A study by Kristal et al. [19] that tested plasma samples using the same commercial ELISAs as in our study found that delays in centrifugation and freezing of refrigerated specimens up to 32, 72, and 144 h resulted in small significant decreases in total IGF-I and a delay of up to 144 h also resulted in a small significant decrease in IGFBP-3. However, as in our analysis, the results from the delayed specimens were highly correlated with those from specimens processed immediately. In two other studies that also tested plasma, Hankinson et al. [12] (using the same commercial ELISAs) and Jane Ellis et al. [20] (using a radioimmunoassay), found that delays in centrifugation and freezing up to 36 h [12] and 24 h [12,20], respectively, had no effect on the measured total IGF-I and IGFBP-3 levels. Thus, there have been conflicting prior findings regarding the effects of specimen processing delays of 24 h or more. Our study is the first, to our knowledge, to examine the effects of delays in specimen processing of less than 24 h.

In this investigation, all IGF assays were performed in serum, and therefore we were unable to address whether the impact of specimen processing on assay results may differ between serum and plasma. Indeed, several studies [21–24] have reported differences in absolute IGF-I (and IGFBP) levels measured in paired serum and plasma specimens and in paired plasma specimens with different types of anti-coagulant, although the levels are highly correlated between each of the specimen types [21,22]. The biologic basis for the systematic differences in IGF-I and IGFBP results obtained using serum versus plasma are not known, but could involve differences in their protein content, as well as the characteristics of the anti-coagulants used in the collection of plasma. For example, certain IGFBPs may bind heparin, a common anti-coagulant [25,26]. Khosravi et al. [24] found that free IGF-I levels following storage overnight at room temperature were increased 2.6-fold in serum and 1.8-fold in heparinized plasma compared to the levels in fresh specimens. Of note, the investigators also found that there was an increase in free IGFBP-3, supporting our hypothesis that the increase in free IGF-I levels with delayed specimen processing may have reflected an increase in its disassociation from IGFBP-3 when left at ambient temperature. In the same study, free IGF-I levels measured in EDTA plasma were not appreciably different in the fresh and overnight stored specimens. Another limitation is that our study did not examine whether high versus low biomarker levels were more affected by processing time. If high values are less affected by processing time than low values, then the high values will be more precisely measured and less misclassified (or vice versa if low values are more precisely measured). An analysis of this type would require a much greater sample size than obtained in our investigation.

In conclusion, the results of our study indicate that if serum separation tubes are centrifuged soon after blood collection, total IGF-I and IGFBP-3 can be fairly stably measured using a commonly employed brand of commercial assays, regardless of the interval between blood collection and freezing, up to at least 24 h. However, free IGF-I levels increase progressively with the time interval between blood collection and freezing, even in blood specimens that are centrifuged soon after blood collection. Waiting for 24 h until centrifugation and freezing leads to increased levels of all three biomarkers. These effects need to be taken into account when planning the processing of blood specimens for epidemiologic studies of IGFs, but analysis of total IGF-I, free IGF-I, and IGFBP-3 data by quartile can mitigate some concerns regarding the effects of delays in processing time, as long as the delay is similar for all patients. More generally, it is essential that the effects of delays in processing and freezing on the biomarkers of interest be evaluated prior to embarking upon epidemiologic and clinical investigations.

Acknowledgements

Support for this work was provided by Public Health Service grants R01CA93881 from the National Cancer Institute and R01HL075516 from the National Heart, Lung, and Blood Institute. Additional support was provided by the Albert Einstein College of Medicine Diabetes Research Center.

References

[1] H. Yu, T. Rohan, Role of the insulin-like growth factor family in cancer development and progression, J. Natl. Cancer Inst. 92 (2000) 1472–1489.

[2] S.J. Moschos, C.S. Mantzoros, The role of the IGF system in cancer: from basic to clinical studies and clinical applications, Oncology 63 (2002) 317–332.

[3] A. Juul, T. Scheike, M. Davidsen, J. Gyllenborg, T. Jorgensen, Low serum insulin-like growth factor I is associated with increased risk of ischemic heart disease: a population-based case-control study, Circulation 106 (2002) 939–944.

[4] S. Kawachi, N. Takeda, A. Sasaki, et al., Circulating insulin-like growth factor-1 and insulin-like growth factor binding protein-3 are associated with early carotid atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 617–621.

[5] R.C. Kaplan, H.D. Strickler, T.E. Rohan, R. Muzumdar, D.L. Brown, Insulin-like growth factors and coronary heart disease, Cardiol. Rev. 13 (2005) 35–39.

[6] J. Frystyk, Free insulin-like growth factors – measurements and relationships to growth hormone secretion and glucose homeostasis, Growth Horm. IGF Res. 14 (2004) 337–375.

[7] J.W. Chen, K. Hojlund, H. Beck-Nielsen, J. Sandahl Christiansen, H. Orskov, J. Frystyk, Free rather than total circulating insulin-like growth factor-I determines the feedback on growth hormone release in normal subjects, J. Clin. Endocrinol. Metab. 90 (2005) 366–371.

[8] I.M. Chapman, M.L. Hartman, K.S. Pieper, et al., Recovery of growth hormone release from suppression by exogenous insulin-like growth factor I (IGF-I): evidence for a suppressive action of free rather than bound IGF-I, J. Clin. Endocrinol. Metab. 83 (1998) 2836–2842.

[9] H. Yu, M.R. Spitz, J. Mistry, J. Gu, W.K. Hong, X. Wu, Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis, J. Natl. Cancer Inst. 91 (1999) 151–156.

[10] B.D. Li, M.J. Khosravi, H.J. Berkel, et al., Free insulin-like growth factor-I and breast cancer risk, Int. J. Cancer 91 (2001) 736–739.

[11] J.M. Chan, M.J. Stampfer, E. Giovannucci, et al., Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study, Science 279 (1998) 563–566.

[12] S.E. Hankinson, W.C. Willett, G.A. Colditz, et al., Circulating concentrations of insulin-like growth factor-I and risk of breast cancer, Lancet 351 (1998) 1393–1396.

[13] X. Wu, H. Yu, C.I. Amos, W.K. Hong, M.R. Spitz, Joint effect of insulin-like growth factors and mutagen sensitivity in lung cancer risk, J. Natl. Cancer Inst. 92 (2000) 737–743.

[14] S.L. Zeger, K.Y. Liang, Longitudinal data analysis for discrete and continuous outcomes, Biometrics 42 (1986) 121–130.

[15] S.L. Zeger, K.Y. Liang, P.S. Albert, Models for longitudinal data: a generalized estimating equation approach, Biometrics 44 (1988) 1049–1060.

[16] E.K. Wei, J. Ma, M.N. Pollak, et al., A prospective study of C-peptide, insulin-like growth factor-I, insulin-like growth factor binding protein-1, and the risk of colorectal cancer in women, Cancer Epidemiol. Biomarkers Prev. 14 (2005) 850–855.

[17] S. Rinaldi, R. Kaaks, A. Zeleniuch-Jacquotte, et al., Insulin-like growth factor-I, IGF binding protein-3, and breast cancer in young women: a comparison of risk estimates using different peptide assays, Cancer Epidemiol. Biomarkers Prev. 14 (2005) 48–52.

[18] A.P. Chokkalingam, M. Pollak, C.M. Fillmore, et al., Insulin-like growth factors and prostate cancer: a population-based case-control study in China, Cancer Epidemiol. Biomarkers Prev. 10 (2001) 421–427.

[19] A.R. Kristal, I.B. King, D. Albanes, et al., Centralized blood processing for the selenium and vitamin E cancer prevention trial: effects of delayed processing on carotenoids, tocopherols, insulin-like growth factor-I, insulin-like growth factor binding protein 3, steroid hormones, and lymphocyte viability, Cancer Epidemiol. Biomarkers Prev. 14 (2005) 727–730.

[20] M. Jane Ellis, J.H. Livesey, M.J. Evans, Hormone stability in human whole blood, Clin. Biochem. 36 (2003) 109–112.

[21] H. Yu, J. Mistry, M.J. Nicar, et al., Insulin-like growth factors (IGF-I, free IGF-I and IGF-II) and insulin-like growth factor binding proteins (IGFBP-2, IGFBP-3, IGFBP-6, and ALS) in blood circulation, J. Clin. Lab. Anal. 13 (1999) 166–172.

[22] A.G. Renehan, J. Jones, S.T. O'Dwyer, S.M. Shalet, Determination of IGF-I, IGF-II, IGFBP-2, and IGFBP-3 levels in serum and plasma: comparisons using the Bland-Altman method, Growth Horm. IGF Res. 13 (2003) 341–346.

[23] M.J. Evans, J.H. Livesey, M.J. Ellis, T.G. Yandle, Effect of anticoagulants and storage temperatures on stability of plasma and serum hormones, Clin. Biochem. 34 (2001) 107–112.

[24] M.J. Khosravi, A. Diamandis, J. Mistry, Factors influencing immunoassay levels of free insulin-like growth factors (IGFs) as determined by new ELISAs. Presented at The Endocrine Society Annual Meeting, June 24–26, 1998, New Orleans, LA, USA.

[25] J. Beattie, K. Phillips, J.H. Shand, M. Szymanowska, D.J. Flint, G.J. Allan, Molecular recognition characteristics in the insulin-like growth factor (IGF)-insulin-like growth factor binding protein -3/5 (IGFBP-3/5) heparin axis, J. Mol. Endocrinol. 34 (2005) 163–175.

[26] S.M. Firth, R.C. Baxter, Cellular actions of the insulin-like growth factor binding proteins, Endocr. Rev. 23 (2002) 824–854.