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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

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Insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs) play an important role in cell growth and differentiation. Clinical and epidemiological studies have indicated that measuring IGFs and IGFBPs in blood has potential implications in assessing growth-related abnormalities and risks of certain types of cancer. To facilitate the application, we reported a large collection of reference ranges of IGFs and IGFBPs in normal population and evaluations of these molecules in serum and plasma as well as the impact of freeze-thaw cycles on the measurement. IGF-I, IGFBP-3 and ALS showed a similar pattern of change associated with age. Levels of these molecules were low at birth and increased with age through puberty. After puberty the levels declined slowly with age. Overall, IGF-I, IGFBP-3 and ALS were slightly higher in females than in males. Free IGF-I accounted for about 1% of the total IGF-I and its variation with age was similar to total IGF-I. IGF-II levels were also increased with age from birth to puberty, but became stable after puberty. There was little difference in IGF-II levels between genders. IGFBP-2 levels declined with age from birth to puberty. Levels of IGFBP-6 in contrast were increased with age. These IGF binding proteins were higher in males than in females. IGFs, IGFBP-3 and ALS were 5-10% higher in serum than in plasma. IGFBP-2 and IGFBP-6 differed substantially between serum and plasma. Freeze-thaw treatment up to five cycles had little impact on plasma levels of IGFs and IGFBP-3. Our observations suggest that levels of IGFs and their binding proteins are varied with age, gender, and types of specimen and that these variations need to be taken into consideration when IGFs and their binding proteins are utilized in clinic and research. J. Clin. Lab. Anal. 13:166–172, 1999.

Key words: IGFs; free IGF-I, IGFBP-2, IGFBP-3; IGFBP-6; ALS

INTRODUCTION

Insulin-like growth factors (IGFs) including IGF-I and IGF-II are 70 and 67 amino-acid single chain polypeptides with molecular weights of 7.65 and 7.47 kDa, respectively (1). These growth factors, working in concert with growth hormone, insulin, sex steroids, and other growth factors exert acute anabolic effects on protein and carbohydrate metabolisms, and have a long-term impact on cell proliferation, differentiation, and apoptosis (2). In addition, IGFs are potent mitogens for a variety of cancer cells (3). The actions of IGFs are mediated through a specific cell membrane receptor, insulin-like growth factor-I receptor (IGF-IR), which has tyrosine kinase activity. Binding of IGFs to this receptor activates the enzyme that subsequently initiates a signal transduction pathway (4). The interaction between IGFs and IGF-IR is modulated by a group of IGF binding proteins (IGFBPs). Six IGF binding proteins (IGFBP-1 to -6) with high affinity to IGFs have been characterized (5). In most situations, IGFBPs inhibit the actions of IGFs via blocking the binding of IGFs to IGF-IR, but under certain circumstances IGFBPs

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enhance the IGF action through protecting IGFs from degradation, as a result, increasing the bioavailability of IGFs in target tissues (2).

Both IGFs and IGFBPs are present in blood, and most circulating IGFs are bound to IGFBP-3 which binds further to another large protein named acid labile subunit (ALS) (6,7). The three molecules form a 150-kDa ternary complex that is considered the major form of IGFs in circulation. Liver is believed to be the main source of IGFs, IGFBP-3, and ALS in blood. Levels of IGFs and IGFBP-3 decline when the liver function is impaired (8,9). Other conditions that affect the production and degradation of IGFs or IGFBPs may also have impact on the levels of these molecules in circulation. Growth hormone stimulates the production of IGF-I and IGFBP-3. Children suffering from growth hormone deficiency tend to have declined levels of IGF-I and IGFBP-3 in serum (10,11). In contrast, individuals with acromegaly have substantially increased serum IGF-I and IGFBP-3 (12,13). In addition to the above observations, recent epidemiologic studies suggest that premenopausal women and men with high levels of IGF-I in plasma tend to have increased risks for breast cancer and prostate cancer respectively (14–17). A case-control study also shows high IGF-I to be associated with an increased risk of lung cancer (18).

Given the potential that measuring IGFs and their binding proteins in circulation may have significant implications in assessing growth abnormalities and cancer risk, we describe in this report concentrations of IGF-I, free IGF-I, IGF-II, IGFBP-2, IGFBP-3, IGFBP-6, and total ALS in the serum and plasma of normal individuals. We also evaluated levels of IGF-I, IGF-II, IGFBP-2, IGFBP-3, IGFBP-6, and ALS between serum and plasma specimens as well as the impact of freeze-thaw cycles on the measurement of IGF-I, IGF-II, and IGFBP-3.

MATERIALS AND METHODS

Specimens

Serum and plasma samples used for the normal reference range were residual specimens collected for various research purposes. All blood samples were drawn through venipuncture and were centrifuged at around 3,000*g* for 15 to 30 minutes. Serum or plasma samples were collected after centrifugation and were stored at –20°C or lower temperature. Samples used for free IGF-I and ALS were kept frozen until the analysis, but samples used for IGF-I, IGF-II, IGFBP-2, IGFBP-3, and IGFBP-6 had been thawed a few times before.

Specimens used for measuring IGF-I, IGF-II, and IGFBP-3 were gathered from four different sources, which included: (1) 58% sera or EDTA plasma from healthy individuals aged 6–31 years who were enrolled in two epidemiologic studies on hypertension conducted in 1987 and 1988 in The Netherlands (19,20); (2) 15% sera from healthy blood donors aged 29–67 years who provided their blood to the Red Cross Blood Bank at Utrecht (The Netherlands), 1991 through 1993; (3) 5% EDTA plasma from healthy volunteers aged 25–45 years working in the Department of Endocrinology at Wilhelmina Children's Hospital; and (4) 21% EDTA plasma provided by doctors who collected the specimens from patients with minor throat, nose, or ear conditions as well as from patients' healthy brothers or sisters in several hospitals across The Netherlands between 1991 and 1994. Thus, for IGF-I, IGF-II, and IGFBP-3, the specimens were mixed samples of serum and EDTA plasma. However, for IGFBP-2, only EDTA plasma was used.

Specimens used for measuring free IGF-I, IGFBP-6, and ALS were all serum samples from healthy Danish children and adults. The samples were residual specimens from several epidemiologic studies conducted in Denmark. Serum specimens from healthy children were collected from five public schools in Copenhagen area between 1990 and 1991, and adult specimens were from a large population-based study on risk factors of cardiovascular disease in Copenhagen (21). Presented in a different format, the free IGF-I and ALS data has been published previously (21,22).

Specimens used for comparing levels of IGFs and IGFBPs between serum and plasma were collected from 14 male and 12 female healthy volunteers, aged 20–60 years (median age 35). Each individual provided three types of blood specimens including serum, EDTA plasma, and heparin plasma. Ten heparin plasma specimens were used to determine levels of IGFs and IGFBP-3 after each of five freeze-thaw cycles.

Immunoassays

We measured concentrations of IGF-I, free IGF-I, IGF-II, IGFBP-2, IGFBP-3, IGFBP-6, and total ALS in serum and plasma using immunoassay kits from Diagnostic Systems Laboratories, Inc. (Webster, Texas). The methods for IGF-I, free IGF-I, IGF-II, and IGFBP-3 were immunoradiometric assay (IRMA) which employed in each kit two specific antibodies to form the sandwich complex. The ranges of calibrators were 4.5–640 ng/mL for IGF-I, 0.15–20 ng/mL for free IGF-I, 50–2,000 ng/mL for IGF-II, and 2.5–100 ng/mL for IGFBP-3. Samples analyzed for IGFBP-3 were diluted 1:100-fold in a standard solution before measurement. For IGF-I and IGF-II, the specimens were pre-treated (1:1,000) with an acid-ethanol extraction buffer to separate IGFs from their binding proteins. The efficiency of the extraction procedure was shown to be similar to that of acid-column chromatography (23). The intra- and interassay precision was between 1.5–3.4 and 1.5–8.2% of coefficient of variation (CV) respectively for IGF-I, 3.3–10.3 and 3.6–10.7% CV for free IGF-I, 4.2–7.2 and 6.3–10.4% CV for IGF-II, and 0.5–1.9 and 1.8–3.9% CV for IGFBP-3.

Measurements of IGFBP-2 and -6 were performed with specific competitive radioimmunoassays (RIA) using polyclonal antibodies. The calibrators ranged from 2.5 to 100

ng/mL for IGFBP-2 and from 3.0 to 220 ng/mL for IGFBP-6. Samples were diluted in a standard solution 1:50 for IGFBP-2 and 1:20 for IGFBP-6. The intra- and interassay precision was between 4.7–8.5 and 4.5–7.4% CV respectively for IGFBP-2, and 6.4–10.7 and 6.1–9.5% CV for IGFBP-6. The method for total ALS was an enzyme linked immunosorbent assay (ELISA) using two specific antibodies against ALS (24). The calibrators ranged from 0.6 to 60 μg/mL and the samples were diluted 1:100-fold in a sample pretreatment buffer before measurement. The intra-assay precision was 3.8–7.5% CV and the interassay precision was 2.8–8.9% CV.

In comparing levels of IGFs and IGFBP-3 between serum and plasma and evaluating the impact of freeze-thaw cycles on values of IGFs and IGFBP-3, we used three ELISA methods, each of which employed two specific antibodies. The intra- and interassay precision were 4.5–8.6% CV and 3.3–6.8% CV respectively for IGF-I, 3.4–6.7% CV and 5.9–7.9% for IGF-II, and 7.3–9.6% CV and 8.2–11.4% CV for IGFBP-3. These analytical features were similar to those of the IRMA methods mentioned earlier.

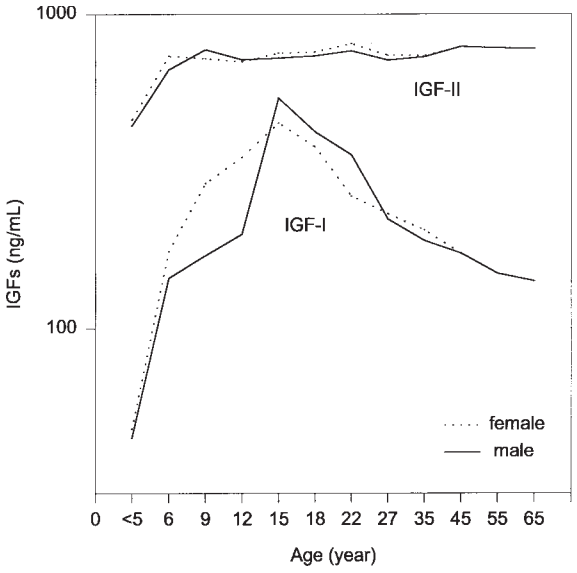

Fig. 1. Concentrations of IGF-I (ng/ml) and IGF-II (ng/ml) by age and sex.

Statistical analysis

Mean concentrations of IGFs and IGFBPs were presented in age- and sex-specific categories. Based on the number of specimens available for each category as well as the knowledge of these growth factors in relation to growth, twelve age groups were created with different age intervals. Log transformed means were used in the figures for IGF-I, free IGF-I, IGFBP-3, IGFBP-2, and IGFBP-6 as these molecules had a positively skewed distribution. To evaluate the differences in values of IGFs or IGFBPs between serum and plasma specimens, we used the paired student *t*-test to compare the original or log-transformed values between the matched specimens. Correlations of IGFs or IGFBPs between serum and plasma were assessed using either the Pearson or Spearman correlation coefficient where appropriate. Two-way analysis of variance (ANOVA) was used to compare levels (original or log transformed) of IGFs and IGFBP-3 at different freeze-thaw cycles. All statistical analyses were performed using the computer software SAS (SAS Institute, Cary, NC).

RESULTS

IGF-I

IGF-I concentration was measured in 738 serum and plasma specimens, and the frequency distribution was positively skewed. Log transformation of the original values helped to normalize the distribution (data not shown). Figure 1 shows the age- and sex-specific log-transformed means. Overall, IGF-I levels were slightly higher (8%) in females than in males, and the concentrations changed with age. IGF-I levels were low at birth and increased with age through puberty. The increase started earlier in girls than in boys. The trend for increase leveled off in both genders after puberty and then declined slowly with age.

Free IGF-I

Free IGF-I was measured in 1,140 serum specimens. Approximately 1% of total IGF-I in circulation was free from any IGF binding proteins and the percentage seemed to be relatively constant across the age groups. The frequency distribution of free IGF-I was similar to that of total IGF-I: positively skewed. Changes with age and differences between genders in free IGF-I were same as to those of total IGF-I, e.g., higher levels in females than in males and increase in concentrations with age until puberty (Fig. 2).

IGF-II

Eight hundred and twenty-nine serum and plasma samples were analyzed for IGF-II and a normal symmetric (Gaussian) distribution was observed for the values of these samples. The concentrations were low at birth and increased with age until puberty (Fig. 1). After puberty IGF-II levels remained unchanged throughout the rest of life. Gender difference was minimal: only 2%.

IGFPB-3

IGFBP-3 was measured in 793 serum and plasma specimens and the concentration varied widely from 1,000 ng/mL to 7,000 ng/mL. The distribution was slightly skewed towards the lower end. Log transformation helped to normalize the distribution. Overall, IGFBP-3 levels were 8% higher in fe-

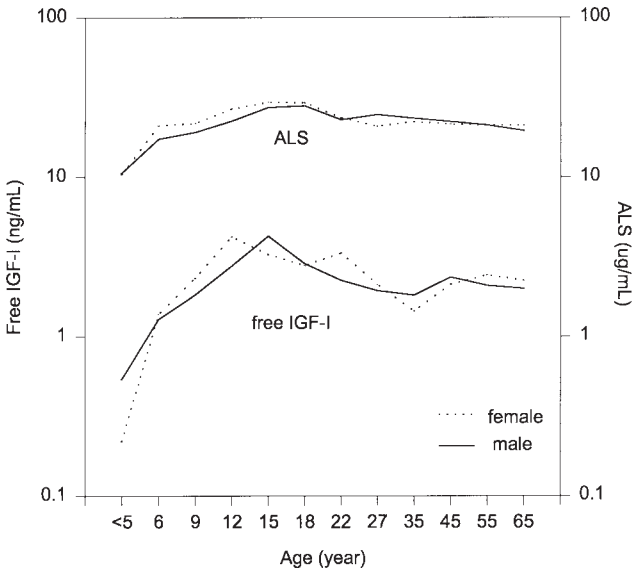

Fig. 2. Concentrations of free IGF-I (ng/ml) and ALS by age and sex.

males than in males and were elevated with age from birth to puberty. The concentrations reached the highest level at puberty and then started to decline gradually with age (Fig. 3).

IGFBP-2

IGFBP-2 level was determined in 628 EDTA plasma samples. The distribution of the values was positively skewed. Compared to IGFBP-3, the age-associated change in IGFBP-2 was completely different. The highest concentrations of IGFBP-2 were shown at birth. IGFBP-2 levels declined with age during early years of life, but had little change after puberty (Fig. 3). Males had slightly higher values of IGFBP-2 than females.

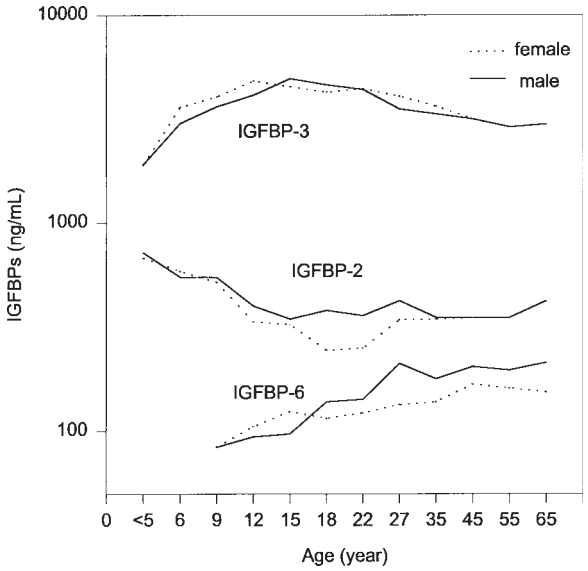

Fig. 3. Concentrations of IGFBP-3 (ng/ml), IGFBP-2 (ng/ml), and IGFBP-6 (ng/ml) by age and sex.

IGFBP-6

Sera from 409 individuals were analyzed for IGFBP-6 and the distribution of the values was positively skewed. IGFBP-6 levels increased significantly with age from birth to puberty, and after puberty the change with age became minimal (Fig. 3). In most age groups, especially after puberty, males had higher levels of IGFBP-6 than females.

ALS

ALS was measured in 1,187 serum specimens. The distribution of the data was symmetric. ALS levels were low at birth and were elevated with age until puberty. After that, the level declined slightly with age (Fig. 2). The age-associated change in ALS seemed to be similar to those in IGF-I and IGFBP-3. Overall, ALS levels were higher in women than in men.

Comparison of IGFs and IGFBPs Between Serum and Plasma

Table 1 shows the comparisons of IGF-I and IGF-II between serum and plasma specimens. Levels of both IGFs were about 10% higher in serum than in plasma, and the differences were statistically significant (all P values < 0.001). IGFs were not significantly different between the two plasma specimens ($P = 0.211$ for IGF-I and $P = 0.654$ for IGF-II, respectively). IGF levels correlated well between serum and plasma samples ($r \geq 0.95$, $P < 0.001$).

The differences in IGFBPs and ALS between serum and plasma are shown in Table 2. IGFBP-3 levels were not different between serum and heparin plasma ($P = 0.75$), but were

TABLE 1. Comparison of IGFs Between Serum and in Plasma

Specimen	Mean (SD)	Median	Range
IGF-I (ng/mL)			
Serum	157.5 (45.6)	153.5	77–265
EDTA plasma	137.8 (42.5)	134.5	64–237
Heparin plasma	141.3 (39.5)	144.5	66–236
Paired *t*-test:	serum and EDTA plasma, $P < 0.001$		
	serum and heparin plasma, $P < 0.001$		
	EDTA and heparin plasma, $P = 0.211$		
IGF-II (ng/mL)			
Serum	520.3 (91.0)	497	390–799
EDTA plasma	474.3 (84.8)	460	336–757
Heparin plasma	478.0 (95.1)	461	327–782
Paired *t*-test:	serum and EDTA plasma, $P < 0.001$		
	serum and heparin plasma, $P < 0.001$		
	EDTA and heparin plasma, $P = 0.654$		

TABLE 2. Comparison of IGFBPs and ALS Between Serum and in Plasma

Specimen	Mean (SD)	Median	Range
IGFBP-3 (ng/mL)			
Serum	3460 (500)	3375	2470–4910
EDTA plasma	3288 (532)	3235	2220–4420
Heparin plasma	3446 (523)	3365	2530–4610
Paired *t*-test:	serum and EDTA plasma, $P < 0.001$		
	serum and heparin plasma, $P = 0.752$		
	EDTA and heparin plasma, $P < 0.001$		
IGFBP-2 (ng/mL)			
Serum	310.8 (111.4)	226.1	183.3–650.6
EDTA plasma	359.0 (105.3)	328.3	219.7–689.9
Heparin plasma	196.6 (75.2)	186.7	70.5–367.6
Paired *t*-test:	serum and EDTA plasma, $P < 0.001$		
	serum and heparin plasma, $P < 0.001$		
	EDTA and heparin plasma, $P < 0.001$		
IGFBP-6 (ng/mL)			
Serum	175.4 (56.7)	165.4	91.8–274.8
EDTA plasma	157.2 (53.7)	146.2	76.6–265.0
Heparin plasma	141.8 (56.8)	129.8	70.0–268.8
Paired *t*-test:	serum and EDTA plasma, $P = 0.010$		
	serum and heparin plasma, $P < 0.001$		
	EDTA and heparin plasma, $P = 0.115$		
ALS (μ/mL)			
Serum	131.4 (6.2)	30.2	20.7–43.0
EDTA plasma	28.5 (6.0)	27.4	17.0–41.4
Heparin plasma	29.9 (5.6)	28.8	18.4–42.6
Paired *t*-test:	serum and EDTA plasma, $P < 0.001$		
	serum and heparin plasma, $P = 0.025$		
	EDTA and heparin plasma, $P = 0.009$		

significantly higher in these two specimens than in EDTA plasma ($P < 0.001$). IGFBP-2 levels were significantly different among the three types of specimens ($P < 0.001$). Heparin specimens contained the lowest levels, whereas EDTA samples had the highest concentrations. The difference between the two was substantial: 83% higher in EDTA than in heparin plasma. IGFBP-6 levels were significantly higher in serum than in plasma ($P \leq 0.01$), but were not different between the plasma samples ($P = 0.115$). The variations of ALS levels among the specimens were similar to those of IGFBP-3, i.e., little difference between serum and heparin plasma ($P = 0.03$) and significant difference between these specimens and EDTA plasma ($P < 0.01$). Despite different levels in serum and plasma, values of these IGF binding proteins were correlated well in these specimens.

Impact of Freeze-Thaw Cycle

Levels of IGF-I, IGF-II, and IGFBP-3 were measured in ten heparin plasma samples after each of five freeze-thaw cycles. There was no indication that concentrations of IGFs and IGFBP-3 would decline after up to five times of repeated freeze-thaw treatment (Fig. 4).

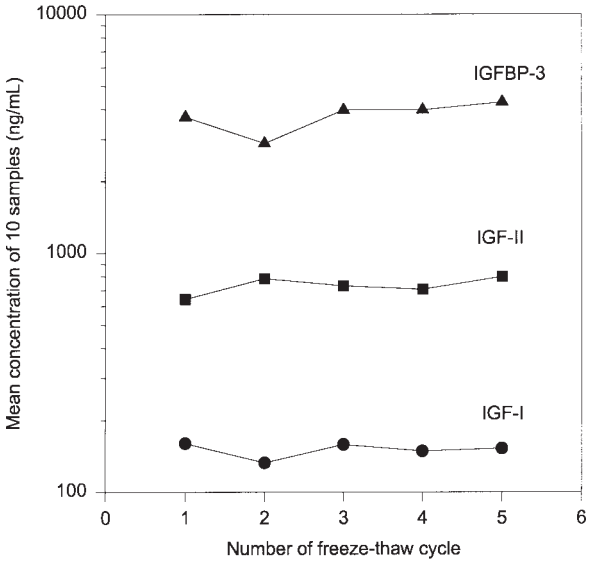

Fig. 4. Concentrations of IGF-I (ng/ml), IGF-II (ng/ml), and IGFBP-3 (ng/ml) in plasma samples measured after each of the 5 cycles of freeze and thaw.

DISCUSSION

In a comparison of the age-specific concentrations among members of the IGF family studied, IGF-I, free IGF-I, IGFBP-3, and ALS seemed to share a similar pattern of change in association with age. A similar pattern of change may suggest a closer relationship among these molecules. IGF-II levels increased slightly after birth, but the level maintained relatively constant after puberty. Different patterns of change related to age were observed for IGFBP-2 and IGFBP-6. Levels of IGFBP-6 were increased with age, whereas IGFBP-2 levels declined with age. This difference may suggest that these binding proteins play a different role in regulating the actions of IGFs and/or have different functions of their own.

Overall, there was little difference between gender in values of these IGF molecules. Most of the gender differences appeared during puberty. This would be another indication that the growth factors are involved in pubertal growth. Our observations of the age- and gender-associated variation in levels of IGFs and IGFBP-3 were consistent with the findings in the literature (21,25–31). However, there were few publications describing the normal reference ranges of IGFBP-2, IGFBP-6, and ALS (32–34).

Although the actual differences were relatively small, there were statistically significant discrepancies in values of IGFs and IGFBPs between serum and plasma when the specimens collected from the same individuals were compared. For IGF-I and IGF-II, the differences were only about 10%. For IGFBP-3, the difference was even smaller, less than 5%. Since the reference ranges for IGF-I, IGF-II, and IGFBP-3 were generated from mixed plasma and serum specimens, our data tended

to have 5 to 10% greater variation compared to the data generated from serum or plasma alone. The slightly wide variation does not obscure our observations on the variations of these molecules in relation to age and gender. However, one should take the specimen type into consideration when the concentrations are monitored for changes over time or before and after medical treatment or intervention.

IGFBP-2 levels were shown to have substantial differences in the three types of blood specimens, and the largest difference existed between EDTA and heparin plasma: more than 80% higher in EDTA than in heparin. IGFBP-6 concentrations were also different among the specimens, 12 to 24% higher in serum than in EDTA or heparin plasma, despite no statistically significant difference between the two plasma specimens. The reasons for these differences remain largely unknown. Two studies demonstrated that IGFBP-2 was able to bind to heparin (35,36). It would be interesting to know if IGFBP-2 could bind to heparin in heparinized plasma resulting in low measurement of IGFBP-2 in this type of specimen. Another possible reason for these differences would be the suppression of the IGF binding protein proteases in EDTA plasma. Slow degradation may raise IGFBP-2 levels in the EDTA samples.

We assessed the impact of freeze-thaw cycle on levels of IGFs and IGFBP-3 in heparin plasma and found no significant change in concentration after the samples underwent five cycles of freeze-thaw treatment, suggesting that IGF-I, IGF-II, and IGFBP-3 are relatively stable in plasma.

In conclusion, levels of IGFs and their binding proteins in circulation varied with age and gender. The age- and gender-associated variations were different among members of the IGF family. Most of the differences were associated with the growth at puberty. Levels of IGFs, IGFBP-3, and ALS tended to be slightly higher (5–10%) in serum than in plasma. Differences between serum and plasma were substantial for IGFBP-2 and IGFBP-6. Limited freeze-thaw treatment did not show significant impact on values of IGFs and IGFBP-3 in plasma. Findings of our study indicate that concentrations of IGFs and IGFBPs in blood are varied with age, gender, and type of specimen. These variations need to be adjusted when applications of IGFs and IGFBPs are considered in disease diagnosis, patient management, risk assessment, and disease prevention intervention.

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