# Age- and Sex-Specific Reference Intervals Across Life Span for Insulin-Like Growth Factor Binding Protein 3 (IGFBP-3) and the IGF-I to IGFBP-3 Ratio Measured by New Automated Chemiluminescence Assays

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**Context:** Measurement of IGF-binding protein-3 (IGFBP-3) can aid the diagnosis of GH-related diseases. Furthermore, epidemiological studies suggest that IGFBP-3 and the molar IGF-I to IGFBP-3 ratio are associated with clinical end points like cancer or cardiovascular disease. However, their clinical use is limited by the lack of validated reference intervals.

**Objective:** The objective of the study was the establishment of age- and sex-specific reference intervals for IGFBP-3 and the molar IGF-I to IGFBP-3 ratio by newly developed automated immunoassays.

**Setting:** This was a multicenter study with samples from 11 cohorts from the United States, Canada, and Europe.

**Participants:** A total of 14 970 healthy subjects covering all ages from birth to senescence participated in the study.

**Main Outcome Measures:** Concentrations of IGFBP-3 and the IGF-I to IGFBP-3 ratio as determined by the IDS iSYS IGF-I and IGFBP-3 assays were measured.

**Results:** Both the concentration of IGFBP-3 and the IGF-I to IGFBP-3 ratio are mainly determined by age. IGFBP-3 concentrations increase until the age of 22 years, with a plateau being visible between 15 and 25 years. Determined by the high peripubertal peak in IGF-I, the peak in the IGF-I to IGFBP-3 ratio occurs already around the age of 15 years, with a slightly earlier and higher peak in females. Beyond the age of 60 years, IGFBP-3 concentrations remain higher in females, whereas IGF-I as well as the IGF-I to IGFBP-3 ratio remains significantly higher in males.

**Conclusions:** We present an extensive set of assay-specific age- and sex-adjusted normative data for concentrations of IGFBP-3 and the molar IGF-I to IGFBP-3 ratio and demonstrate distinct sex specific differences across the life span. **(*J Clin Endocrinol Metab* 99: 1675–1686, 2014)**

The IGFs represent a family of proteohormones with high sequence homology to proinsulin. The IGFs are involved in a wide spectrum of physiological processes including cell proliferation, apoptosis and protein synthesis, carbohydrate homeostasis, lipid metabolism, and bone metabolism (1). Their biological effects are mediated and modified through a complex system consisting of three IGF receptors (IGF receptors I and II; insulin receptor) and a superfamily of six high-affinity IGF-binding proteins (IGFBPs) (1, 2). It is widely accepted that binding proteins like the IGFBPs regulate their specific ligands by prolonging IGF half-life in circulation. Furthermore, locally produced IGFBPs are likely to act as autocrine/paracrine regulators of IGF action in various tissues. In vivo as

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Abbreviations: IGFBP, IGF-binding protein; LMS method, skewness [L], mean [M], coefficient of variation [S]; SHIP, Study of Health in Pomerania.

well as in vitro studies suggest that the IGFBPs also have IGF-independent effects (3). For example, in contrast to IGF-I but similar to GH, IGFBP-3 increases circulating glucose and insulin levels (4, 5).

IGF-I is predominantly synthesized in the liver in response to pituitary GH and is the main mediator of the peripheral GH effects. In the circulation, most IGF-I is bound to IGFBP-3 and acid labile subunit. In the resulting 150-kDa ternary complex, IGF-I is unable to cross the vascular endothelium (1). Therefore, the molar ratio between IGF-I and IGFBP-3 has been suggested to reflect an estimate of tissue-available IGF-I (6). In accordance with this view, the molar ratio of IGF-I to IGFBP-3 appears to correlate with free as well as with bioactive IGF-I (7). Whereas GH secretion follows a pulsatile pattern, the concentrations of circulating IGF-I and IGFBP-3 are largely stable during the course of a day as well as during longer periods and are therefore considered to reflect the long-term status of GH secretion.

A recent meta-analysis (8) of 12 studies including a total of 14 906 subjects demonstrated an U-shaped association between IGF-I concentrations and all-cause mortality as well as cancer- and cardiovascular-related mortality. That study, however, contained no information on IGFBP-3 or the IGF-I to IGFBP-3 ratio. On the other hand, we recently, analyzed 1988 men and 2069 women in the Study of Health in Pomerania (SHIP) and demonstrated that low levels of IGFBP-3 were associated with higher all-cause mortality (9). Findings from other large epidemiological studies also support the notion that IGFBP-3 as well as the IGF-I to IGFBP-3 ratio is associated with important clinical end points like cancer (10) or cardiovascular disease (11).

Although epidemiological data suggest an important role for IGFBP-3 as an additional biomarker of GH secretion and as a modulator of IGF effects, in clinical routine the diagnosis of GH-related diseases as well as monitoring of therapy is mainly based on measurements of IGF-I (12, 13). One reason for the less frequent use of IGFBP-3 measurements and the neglect of the IGF-I to IGFBP-3 ratio in the clinical context may relate to the fact that solid data on reference intervals for IGFBP-3 and the IGF-I to IGFBP-3 ratio obtained from large cohorts of well-characterized healthy subjects are scarce. To our knowledge, all larger studies are restricted to children and adolescents only (14–16). The availability of such data, however, represents a prerequisite for using a biomarker as a diagnostic tool. In the context of IGF-I, this has been enforced by the recent consensus statement (17). Based on these facts, we aimed to establish reference intervals for IGFBP-3 and the IGF-I to IGFBP-3 ratio from birth to senescence for a newly developed set of automated assays (IGF-I, IGFBP-3). We validated the assays and conducted a large multicenter study involving well-characterized subjects of all ages from several cohorts in different countries to provide robust reference intervals for IGF-I, IGFBP-3, and the IGF-I to IGFBP-3 ratio. The IGF-I assay and the construction of the respective reference intervals are presented in the accompanying manuscript by Bidlingmaier et al (59); here we present the respective studies for IGFBP-3 and the IGF-I to IGFBP-3 ratio.

## Subjects and Methods

### *Development of the iSYS IGFBP-3 assay*

The new iSYS IGFBP-3 assay involves two mouse monoclonal antibodies and follows a one-step protocol with a predilution step (6 $\mu$L of sample). Diluted samples and antibodies are incubated at 37°C followed by the addition of streptavidin-coated magnetic particles. The resultant signal is generated by flash chemiluminescence initiated by the addition of trigger solutions. Initially we used the reference material code 93/560 from the National Institute for Biological Standards (Hertfordshire, United Kingdom) for calibration, but the final version of the assay uses recombinant glycosylated human IGFBP-3 as the working standard. Methodological details as well as the results of the development, validation, and characterization of the assay are given in the Supplemental Text, Tables, and Figures.

### Assay validation and characterization

According to the Clinical and Laboratory Standards Institute recommendations (18, 19), the limits of detection and limits of quantification, imprecision, linearity, and recovery were determined. Comparability of results obtained on different instruments was also investigated.

### Cross-reactivity and Interference

Specificity was tested by analyzing assay buffer and pooled serum samples spiked with increasing amounts of insulin up to 18 000 mU/L (66/304; National Institute for Biological Standards), IGF-I to 2000 ng/mL, IGF-II to 6000 ng/mL, and IGFBP-1, -2, -4, -5, and -6 to 4000 ng/mL (GroPep). The poten-

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tial impact of high amounts of hemoglobin (Lampire), bilirubin (Merck Millipore), and triglycerides (Sigma-Aldrich) was also tested.

### Sample type and stability

To investigate the potential impact of different collection tube types of blood for IGFBP-3, parallel measurements were performed in plain serum, serum in separator tubes, and in different types of plasma (Na citrate-, Li heparin-, Na heparin-, and K-EDTA). Stability of IGFBP-3 levels was tested by measuring five human serum and plasma samples after storage at 4°C and 25°C for approximately 4 days (96 h) and after four freeze-thaw cycles in six human serum samples.

### Comparison with existing method

The new IDS-iSYS IGFBP-3 assay was compared with the Immulite 2500 (Siemens) by parallel assessment of serum samples from the SHIP cohort (n = 4121). Historical data from the same samples measured by the Nichols Advantage IGFBP-3 assay (Nichols Institute Diagnostics) were additionally compared with the iSYS IGFBP-3 assay. All assays were performed according to the respective manufacturers' instructions.

### Subjects included in the reference interval study

To establish method-specific reference intervals for IGFBP-3 and the IGF-I to IGFBP-3 ratio, both parameters were measured using the newly developed, automated IDS-iSYS assays in samples obtained from 11 different cohorts: one newborn study (cord blood), five pediatric cohorts (Canada, Denmark, Germany, Sweden, United States), and four adult cohorts (Germany). Detailed information about the different cohorts is provided in the accompanying manuscript by Bidlingmaier (59) et al and also summarized in Table 1. All studies were approved by the respective local institutional review board, and informed consent was obtained from participants or parents when appropriate. Collection procedures, sample type, and details of the respective cohorts are given elsewhere (20–23). All samples were stored at −20°C or −80°C before analysis.

### Measurements

Samples were analyzed on IDS-iSYS instruments in seven laboratories in five countries (Table 1) according to a standardized protocol. Due to limitations in sample volume, IGF-I was measured in 15 014 samples, whereas IGFBP-3 was measured only in 14 970 samples. All analyses took place between 2010 and 2011. The same batch of reagents was used in all laboratories, with the exception of the measurement of the samples from KORA (Cooperative Health Research in the Region Augsburg) F4, in which another batch was intentionally used to investigate independence of the results from the reagent batch. Variability between laboratories was assessed using the same set of three quality control samples in all assay runs and between instrument agreement was formally assessed in a separate study (see Supplemental Tables 2 and 3).

Detailed information about the IGF-I assay used in this study is provided in the accompanying manuscript by Bidlingmaier et al (59).

### Statistical analysis

EP Evaluator Software (version 8.0.0114; Data Innovations Europe) has been used for the analysis of the data obtained during the assay validation. For correlation of results obtained by the new IGFBP-3 IDS-iSYS assay to those obtained by widely used existing or previous IGFBP-3 assays (Siemens Immulite 2500 and Nichols Advantage), Passing-Bablok analysis was used to obtain the slope and intercept and Bland-Altman plots are displayed. Statistical analyses were performed with SAS version 9.1 (SAS Institute Inc).

Acquired results from the multicentric reference interval study were explored by several statistical models. A modification of the LMS method (skewness [L], mean [M], and coefficient of variation [S]) (24, 25) allowed the best fit and therefore was used to construct reference ranges according to age and sex. In a subset of samples from children and adolescents, information about Tanner stages was available. In this subset, reference intervals for both sexes according to Tanner stage were developed using the Harrell-Davis estimate of quantiles.

To further investigate the robustness of the reference intervals obtained, three adult cohorts (KORA F4, KORA Age, and SHIP)

**Table 1.** Details of the Cohorts and Laboratories Taking Part in the Reference Interval Study

| Study Group | Laboratory Measured | Samples, n | | Sample Matrix | Age Range, y |
|---|---|---|---|---|---|
| | | IGFBP-3 | IGF-I to IGFBP-3 Ratio | | |
| Munich, Germany | Munich | 144 | 144 | Cord blood | 0 |
| CALIPER, Canada | San Clemente | 1359 | 1357 | Serum | 0–19 |
| CALIPER new | Liege | 602 | 588 | Serum | 0–20 |
| Randers, Denmark | Aarhus | 854 | 854 | Serum | 5–20 |
| Gothenburg, Sweden | Gothenburg | 319 | 319 | Serum | 0.9–1.1 |
| Leipzig, Germany | Munich | 190 | 190 | Serum | 7–18 |
| Athens, Georgia | San Clemente | 716 | 682 | Serum | 3–19 |
| MESY-BEPO, Germany | Berlin | 2619 | 2616 | Serum | 18–87 |
| SHIP, Germany | Greifswald | 4109 | 4109 | Serum | 20–81 |
| KORA F4, Germany | Munich | 3018 | 2988 | Plasma | 32–81 |
| KORA Age, Germany | Munich | 1040 | 1040 | Serum | 65–94 |
| Total | | 14 970 | 14 887 | | |

Abbreviations: CALIPER, Canadian Laboratory Initiative on Pediatric Reference Interval Database; MESY-BEPO, Metabolisches Syndrom Berlin Potsdam study; KORA, Cooperative Health Research in the Region Augsburg. Detailed information about the exact number of subjects of each sex falling into each age group is given in Supplemental Tables 12–15.

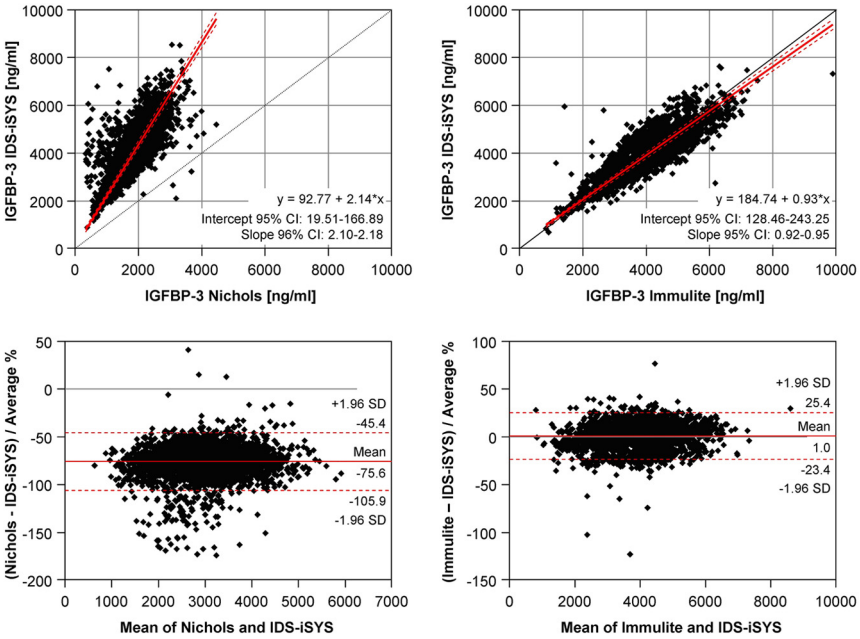


**Figure 1.** Comparison of the IGFBP-3 levels measured by Nichols Advantage and IDS-iSYS assays (based on the SHIP-0 cohort) on the left side and Immulite 2500 and IDS-iSYS assays (based on the SHIP-1 cohort) on the right side. Passing-Bablok regression plots are displayed in the top: the thick solid red line represents the regression line, and the dashed red lines represents the 95% confidence interval for the fit. Bland-Altman plots are displayed in the bottom.

were used to analyze the influence of different exclusion criteria (renal function, diabetes, etc) by calculating references intervals based on quantile regression (26) with restricted cubic splines with three predefined knots (27).

Further statistical analysis was done with Statview version 5.0 (1998; SAS Institute Inc). A nonparametric Mann-Whitney *U* test or a Kruskal-Wallis one-way ANOVA was used for between-group comparisons as appropriate.

Concentrations of IGFBP-3 are given in nanograms per milliliter throughout the manuscript, but the calculation of the IGF-I to IGFBP-3 ratio was performed after conversion into nanomoles per liter (IGF-I: nanograms per milliliter × 0.1307 = nmol/L; IGFBP-3: nanograms per milliliter × 0.03478 = nanomoles per liter). To better illustrate the relative abundance of IGF-I and IGFBP-3 molecules in blood, the resulting molar ratio was expressed as a percentage [ratio = IGF-I (nanomoles per liter) to IGFBP-3 (nanomoles per liter)*100].

## Results

### Assay characteristics

Validation of the assay in different laboratories confirmed a broad measurement range up to supraphysiological concentrations (10 000 ng/mL; Supplemental Table 4). The limit of quantification was below 50 ng/mL (Supplemental Table 1 and Figure 1). Excellent reproducibility and recovery of the measurements could be demonstrated between instruments, laboratories, and reagent batches (Supplemental Tables 2, 3, and 5). No cross-reactivity or interference was seen for any of the tested compounds, including the other IGFBPs (Supplemental Tables 6, A and B and 7). We show the linear dilution of the glycosylated recombinant human IGFBP-3 used for the calibration and parallel dilution with samples containing high IGFBP-3 concentrations (Supplemental Table 4, A and B). Furthermore, measured IGFBP-3 concentrations in normal samples did not change after overnight incubation with third-trimester samples at 37°C, expected to exhibit high proteolytic activity (Supplemental Table 16). We also extensively tested the impact of preanalytical conditions and repeated freezing and thawing, but IGFBP-3 concentrations remained stable under all conditions tested (Supplemental Tables 8–10). Measured concentrations were comparable in most matrices but 10%–20% lower when citrate or oxalate plasma was used (Supplemental Table 11).

### Comparison with other IGFBP-3 assays

Samples from the baseline examination in SHIP had originally been measured using the Nichols Advantage IGFBP-3 assay. We now reassayed these samples using the IDS-iSYS assay. The results from both assay correlated [R = 0.78 (95% confidence interval 0.77–0.79)], but IGFBP-3 levels measured by the Nichols Advantage assay were significantly lower than those reported by the new IDS-iSYS assay (iSYS = 92.8 + 2.14 × Nichols; Figure 1). In contrast, in samples recently collected during the first 5-year follow-up examination in SHIP, which were analyzed in parallel using the Siemens Immulite 2500 and the IDS-iSYS assay, a strong correlation with a much closer agreement was observed (slope 0.93; 95% confidence interval 0.92-0.95). The mean difference between the methods was only about 1% (52.7 ng/mL).

### Generation of reference intervals for IGFBP-3 and the IGF-I to IGFBP-3 ratio

In the present study, data from 14 970 and 14 887 subjects were available for the calculation of reference intervals for IGFBP-3 and the IGF-I to IGFBP-3 ratio, respectively (Table 1). Summarized sex-specific L, M, and S values and the fitted percentiles for IGFBP-3 and the IGF-I to IGFBP-3 ratio are given for males (Table 2) and females (Table 3), and individual data points are displayed in Figure 2, A and B. An extended version of the tables showing

**Table 2.** LMS Charts for Males for Each Age Group

| | IGFBP-3, ng/mL | | | | | IGF-I to IGFBP-3 Ratio[a] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LMS Parameter (λ = 0.7190) | | Percentiles | | | LMS Parameter (λ = 0.4072) | | Percentiles | | |
| | $\mu$ | $\sigma$ | 2.5% | 50% | 97.5% | $\mu$ | $\sigma$ | 2.5% | 50% | 97.5% |
| Age, y | | | | | | | | | | |
| 0 | 2072.6077 | 0.2558 | 1113 | 2073 | 3180 | 9.7254 | 0.4194 | 3.57 | 9.73 | 19.76 |
| 1 | 2378.7990 | 0.2528 | 1289 | 2379 | 3634 | 10.2974 | 0.4115 | 3.87 | 10.30 | 20.68 |
| 2 | 2678.0241 | 0.2498 | 1465 | 2678 | 4074 | 10.9794 | 0.4037 | 4.22 | 10.98 | 21.80 |
| 3 | 2965.7883 | 0.2468 | 1637 | 2966 | 4492 | 11.7516 | 0.3959 | 4.62 | 11.75 | 23.07 |
| 4 | 3234.9229 | 0.2438 | 1801 | 3235 | 4878 | 12.5967 | 0.3882 | 5.07 | 12.60 | 24.44 |
| 5 | 3458.4976 | 0.2409 | 1942 | 3458 | 5193 | 13.5140 | 0.3806 | 5.55 | 13.51 | 25.92 |
| 6 | 3600.7249 | 0.2381 | 2039 | 3601 | 5384 | 14.5105 | 0.3732 | 6.09 | 14.51 | 27.53 |
| 7 | 3670.4182 | 0.2353 | 2096 | 3670 | 5466 | 15.6094 | 0.3660 | 6.68 | 15.61 | 29.29 |
| 8 | 3741.0823 | 0.2326 | 2153 | 3741 | 5550 | 16.8557 | 0.3589 | 7.36 | 16.86 | 31.29 |
| 9 | 3830.0772 | 0.2301 | 2221 | 3830 | 5660 | 18.2445 | 0.3520 | 8.12 | 18.24 | 33.51 |
| 10 | 3939.5526 | 0.2277 | 2300 | 3940 | 5801 | 19.7240 | 0.3451 | 8.94 | 19.72 | 35.85 |
| 11 | 4058.3802 | 0.2254 | 2385 | 4058 | 5956 | 21.2109 | 0.3382 | 9.80 | 21.21 | 38.14 |
| 12 | 4165.1026 | 0.2232 | 2463 | 4165 | 6093 | 22.5791 | 0.3312 | 10.62 | 22.58 | 40.17 |
| 13 | 4249.6017 | 0.2212 | 2528 | 4250 | 6198 | 23.6721 | 0.3242 | 11.35 | 23.67 | 41.65 |
| 14 | 4312.7807 | 0.2192 | 2580 | 4313 | 6272 | 24.3608 | 0.3171 | 11.90 | 24.36 | 42.39 |
| 15 | 4348.5313 | 0.2174 | 2614 | 4349 | 6306 | 24.5955 | 0.3101 | 12.23 | 24.60 | 42.34 |
| 16 | 4366.2753 | 0.2157 | 2638 | 4366 | 6316 | 24.4011 | 0.3033 | 12.35 | 24.40 | 41.55 |
| 17 | 4378.8828 | 0.2141 | 2657 | 4379 | 6319 | 23.8399 | 0.2968 | 12.27 | 23.84 | 40.18 |
| 18 | 4394.2924 | 0.2126 | 2678 | 4394 | 6327 | 22.9879 | 0.2907 | 12.02 | 22.99 | 38.37 |
| 19 | 4413.4744 | 0.2113 | 2700 | 4413 | 6341 | 21.9323 | 0.2850 | 11.64 | 21.93 | 36.28 |
| 20 | 4435.5676 | 0.2100 | 2723 | 4436 | 6361 | 20.7612 | 0.2799 | 11.16 | 20.76 | 34.06 |
| 21–25 | 4453.2249 | 0.2074 | 2753 | 4453 | 6361 | 17.8642 | 0.2692 | 9.86 | 17.86 | 28.81 |
| 26–30 | 4306.8442 | 0.2047 | 2683 | 4307 | 6127 | 14.2328 | 0.2572 | 8.09 | 14.23 | 22.51 |
| 31–35 | 4197.6191 | 0.2053 | 2610 | 4198 | 5977 | 12.7923 | 0.2544 | 7.33 | 12.79 | 20.14 |
| 36–40 | 4177.3891 | 0.2090 | 2571 | 4177 | 5982 | 12.2280 | 0.2566 | 6.96 | 12.23 | 19.32 |
| 41–45 | 4161.0794 | 0.2155 | 2515 | 4161 | 6018 | 11.2808 | 0.2620 | 6.34 | 11.28 | 17.98 |
| 46–50 | 4022.4845 | 0.2239 | 2374 | 4022 | 5891 | 11.1402 | 0.2701 | 6.14 | 11.14 | 17.99 |
| 51–55 | 3913.7597 | 0.2328 | 2251 | 3914 | 5808 | 10.9564 | 0.2781 | 5.92 | 10.96 | 17.92 |
| 56–60 | 3801.3203 | 0.2413 | 2133 | 3801 | 5711 | 10.9734 | 0.2815 | 5.87 | 10.97 | 18.05 |
| 61–65 | 3693.6761 | 0.2487 | 2027 | 3694 | 5610 | 10.8437 | 0.2791 | 5.84 | 10.84 | 17.77 |
| 66–70 | 3579.3183 | 0.2552 | 1926 | 3579 | 5487 | 11.1745 | 0.2752 | 6.08 | 11.17 | 18.20 |
| 71–75 | 3364.5920 | 0.2609 | 1779 | 3365 | 5201 | 11.1568 | 0.2712 | 6.13 | 11.16 | 18.05 |
| 76–80 | 3219.0924 | 0.2663 | 1673 | 3219 | 5015 | 11.2884 | 0.2651 | 6.30 | 11.29 | 18.09 |
| 81–85 | 3194.2581 | 0.2717 | 1632 | 3194 | 5014 | 11.5752 | 0.2560 | 6.60 | 11.58 | 18.27 |
| 86–90 | 3312.7544 | 0.2770 | 1665 | 3313 | 5239 | 11.6855 | 0.2449 | 6.85 | 11.69 | 18.12 |

The variables $\mu$ and $\sigma$ as well as the estimated percentiles (2.5%, 50%, and 97.5%) are provided. Note that an extended version of this table providing the data split by smaller increments in age and also providing the exact number of subjects of each sex falling into each age group is given in Supplemental Tables 12–15.

[a] Ratio = IGF-I (nanomoles per liter) to IGFBP-3 (nanomoles per liter) * 100.

percentiles and variables for smaller increments (<1 y up to age 30 y, 1 y thereafter) is given in Supplemental Tables 12–15). These tables also show the number of subjects from the reference population falling into each age group.

As expected, concentrations of IGFBP-3 showed a strongly age-related pattern in both sexes (Figure 2A). The increase during childhood continues until the age of 22 years, and thus, peak concentrations are reached later than peak IGF-I concentrations. Peak values for the percentiles for IGFBP-3 were higher in females (50th percentile, 4649 ng/mL) than in males (4454 ng/mL). In contrast to the very pronounced peripubertal peak in IGF-I concentrations, which is followed by a rapid decline, IGFBP-3 concentrations exhibit a plateau in concentrations between the ages of 15 and 25 years. At age 30 years, IGFBP-3 concentrations are very similar in both sexes [50th percentile, 4241 ng/mL (M) and 4265 ng/mL (F)] followed by a more pronounced decline in males than in females, with lower concentrations at age 60 years (50th percentile, M vs F: 3751 vs 3904 ng/mL) and 80 (M vs F: 3190 vs 3634 ng/mL). Thus, during 50 years of life, the 50th percentile of IGFBP-3 concentrations declines by about 25% in males as opposed to only 15% in females.

The corresponding reference intervals for the IGF-I to IGFBP-3 ratio also exhibit a strong increase during childhood. Peak concentrations for the ratio are mainly deter-

**Table 3.** LMS Charts for Females for Each Age Group

| | IGFBP-3, ng/mL | | | | | IGF-I to IGFBP-3 Ratio[a] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LMS Parameter ($\lambda$ = 0.8399) | | Percentiles | | | LMS Parameter ($\lambda$ = 0.3208) | | Percentiles | | |
| | $\mu$ | $\sigma$ | 2.5% | 50% | 97.5% | $\mu$ | $\sigma$ | 2.5% | 50% | 97.5% |
| Age, y | | | | | | | | | | |
| 0 | 2113.7322 | 0.2692 | 1053 | 2114 | 3271 | 10.4133 | 0.3789 | 4.46 | 10.41 | 20.27 |
| 1 | 2417.1846 | 0.2652 | 1221 | 2417 | 3721 | 10.5573 | 0.3718 | 4.60 | 10.56 | 20.32 |
| 2 | 2711.3549 | 0.2613 | 1388 | 2711 | 4151 | 10.8268 | 0.3649 | 4.80 | 10.83 | 20.61 |
| 3 | 2992.6286 | 0.2573 | 1553 | 2993 | 4557 | 11.2273 | 0.3581 | 5.07 | 11.23 | 21.14 |
| 4 | 3257.3873 | 0.2534 | 1713 | 3257 | 4933 | 11.7641 | 0.3515 | 5.40 | 11.76 | 21.92 |
| 5 | 3479.9041 | 0.2496 | 1854 | 3480 | 5242 | 12.4383 | 0.3451 | 5.80 | 12.44 | 22.94 |
| 6 | 3605.4271 | 0.2458 | 1945 | 3605 | 5403 | 13.2495 | 0.3391 | 6.27 | 13.25 | 24.20 |
| 7 | 3699.0712 | 0.2421 | 2019 | 3699 | 5515 | 14.2414 | 0.3334 | 6.84 | 14.24 | 25.78 |
| 8 | 3794.7955 | 0.2385 | 2096 | 3795 | 5629 | 15.4551 | 0.3281 | 7.52 | 15.46 | 27.73 |
| 9 | 3903.2395 | 0.2350 | 2180 | 3903 | 5762 | 16.9106 | 0.3231 | 8.33 | 16.91 | 30.10 |
| 10 | 4021.3541 | 0.2317 | 2270 | 4021 | 5908 | 18.5648 | 0.3184 | 9.25 | 18.56 | 32.79 |
| 11 | 4140.0713 | 0.2285 | 2360 | 4140 | 6055 | 20.2738 | 0.3138 | 10.22 | 20.27 | 35.54 |
| 12 | 4246.4293 | 0.2255 | 2444 | 4246 | 6184 | 21.8330 | 0.3094 | 11.12 | 21.83 | 38.00 |
| 13 | 4334.6486 | 0.2226 | 2517 | 4335 | 6286 | 23.0439 | 0.3052 | 11.86 | 23.04 | 39.83 |
| 14 | 4406.2559 | 0.2199 | 2580 | 4406 | 6365 | 23.7757 | 0.3011 | 12.36 | 23.78 | 40.82 |
| 15 | 4466.1923 | 0.2173 | 2636 | 4466 | 6428 | 23.9725 | 0.2974 | 12.57 | 23.97 | 40.90 |
| 16 | 4510.8040 | 0.2150 | 2682 | 4511 | 6470 | 23.6591 | 0.2941 | 12.51 | 23.66 | 40.15 |
| 17 | 4542.3031 | 0.2128 | 2718 | 4542 | 6495 | 22.9153 | 0.2913 | 12.20 | 22.92 | 38.71 |
| 18 | 4566.3094 | 0.2108 | 2749 | 4566 | 6510 | 21.8598 | 0.2890 | 11.70 | 21.86 | 36.79 |
| 19 | 4590.5104 | 0.2090 | 2779 | 4591 | 6527 | 20.6236 | 0.2873 | 11.08 | 20.62 | 34.60 |
| 20 | 4617.5735 | 0.2073 | 2809 | 4618 | 6550 | 19.3455 | 0.2860 | 10.43 | 19.35 | 32.39 |
| 21–25 | 4646.5472 | 0.2040 | 2855 | 4647 | 6559 | 16.4255 | 0.2846 | 8.88 | 16.43 | 27.44 |
| 26–30 | 4430.4156 | 0.2002 | 2752 | 4430 | 6219 | 13.4803 | 0.2868 | 7.25 | 13.48 | 22.60 |
| 31–35 | 4137.1585 | 0.1998 | 2573 | 4137 | 5804 | 12.8042 | 0.2905 | 6.83 | 12.80 | 21.60 |
| 36–40 | 4055.1332 | 0.2022 | 2504 | 4055 | 5709 | 12.4502 | 0.2920 | 6.62 | 12.45 | 21.05 |
| 41–45 | 3956.5718 | 0.2070 | 2409 | 3957 | 5610 | 11.6841 | 0.2928 | 6.20 | 11.68 | 19.79 |
| 46–50 | 3921.5147 | 0.2134 | 2343 | 3922 | 5612 | 11.1640 | 0.2954 | 5.88 | 11.16 | 18.99 |
| 51–55 | 3944.5084 | 0.2205 | 2306 | 3945 | 5703 | 10.4867 | 0.3006 | 5.46 | 10.49 | 17.99 |
| 56–60 | 3914.0402 | 0.2276 | 2238 | 3914 | 5717 | 9.6143 | 0.3063 | 4.93 | 9.61 | 16.65 |
| 61–65 | 3859.9509 | 0.2342 | 2161 | 3860 | 5691 | 9.1422 | 0.3082 | 4.67 | 9.14 | 15.88 |
| 66–70 | 3747.4925 | 0.2401 | 2059 | 3747 | 5572 | 9.1030 | 0.3053 | 4.68 | 9.10 | 15.74 |
| 71–75 | 3707.0794 | 0.2449 | 2005 | 3707 | 5549 | 9.0263 | 0.2976 | 4.73 | 9.03 | 15.41 |
| 76–80 | 3648.8049 | 0.2486 | 1950 | 3649 | 5490 | 9.0088 | 0.2880 | 4.83 | 9.01 | 15.13 |
| 81–85 | 3639.4316 | 0.2516 | 1925 | 3639 | 5498 | 9.5279 | 0.2788 | 5.22 | 9.53 | 15.76 |
| 86–90 | 3740.5262 | 0.2543 | 1961 | 3741 | 5672 | 9.6706 | 0.2701 | 5.41 | 9.67 | 15.77 |

The variables $\mu$ and $\sigma$ as well as the estimated percentiles (2.5%, 50%, and 97.5%) are provided. Note that an extended version of this table providing the data split by smaller increments in age and also providing the exact number of subjects of each sex falling into each age group is given in Supplemental Tables 12–15.

[a] Ratio = IGF-I (nanomoles per liter) to IGFBP-3 (nanomoles per liter) * 100.

mined by the high peak in IGF-I-concentrations, and therefore, the ratio reached peak values before IGFBP-3 concentrations at approximately 15 years in both sexes (Figure 2B). The highest values for the IGF-I to IGFBP-3 ratio occur slightly earlier (females: 14.5–15.0 y; males: 15.0–15.5 y), and the 50th percentile is slightly lower in females as compared with males (23.9% vs 24.6%; Supplemental Tables 14 and 15). Beyond puberty, ratios decline rapidly and are approximately 13% at age 30 years in both sexes. Females maintain higher IGFBP-3 concentrations at older age, whereas IGF-I concentrations remain higher in males beyond the age of 50 years, and accordingly, the corresponding IGF-I to IGFBP-3 ratio is also higher in older males (50th percentile at 80 y: males vs females: 11.4% vs 9.3%).

To better reflect pubertal changes, we also constructed reference ranges for IGFBP-3 concentrations the IGF-I to IGFBP-3 ratio according to Tanner stages from a pediatric cohort [n = 854 (393 boys, 461 girls); Tables 4 and 5]. The highest IGFBP-3 concentrations are seen in Tanner stage IV/V. In both sexes, peak values for the 2.5th percentile for IGFBP-3 concentrations were significantly lower when calculated based on chronological age (Tables 2 and 3) as compared with the calculation based on Tanner stages (Table 4; males: 2753 vs 3867 ng/mL; females: 2855 vs 3934 ng/mL). This difference between the calculation

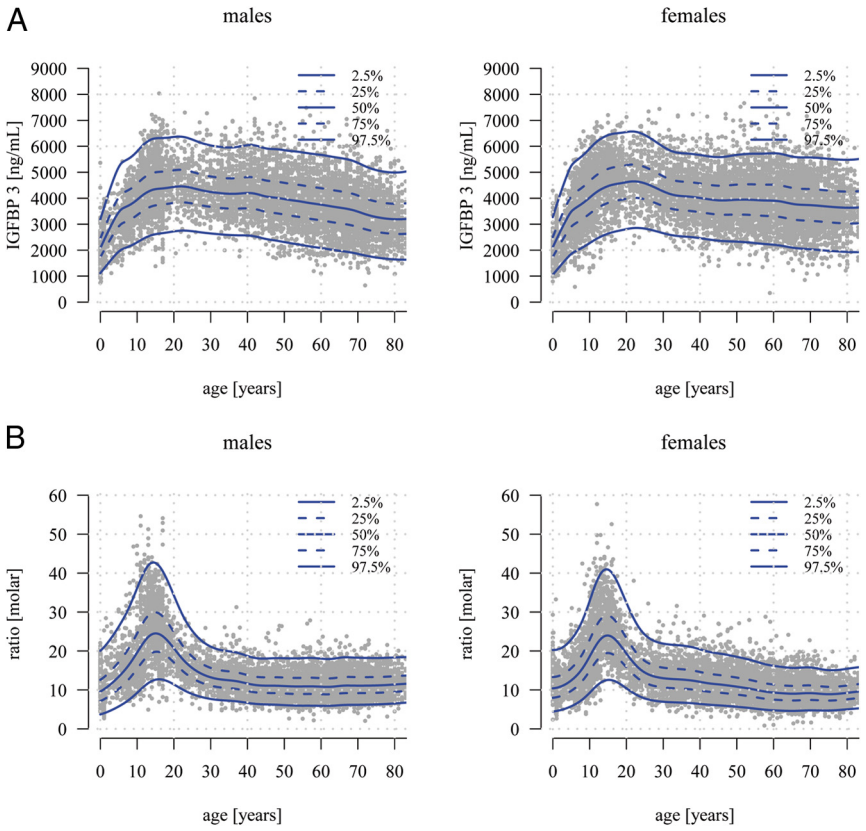


**Figure 2.** Serum IGFBP-3 (A) and IGF-I to IGFBP-3-ratio (B) values in approximately 15 000 subjects for males (left side) and females (right side). The lines represent the 2.5%, 25%, 50%, 75%, and 97.5% centiles calculated by quantile regression via vector generalized additive models. Ratio = IGF-I (nanomoles per liter) to IGFBP-3 (nanomoles per liter) * 100.

based on chronological age and Tanner stage was much less pronounced at the 50th percentile and almost absent at the upper end of the reference interval (97.5th percentile; males: 6361 vs 6651 ng/mL; females: 6570 vs 6559 ng/mL).

Based on three population-based adult studies (KORA F4, KORA Age, and SHIP), in which detailed information on comorbidities and anthropometric measures were available, we investigated the impact of the application of different exclusion criteria to define the reference population on the resulting reference intervals (Table 6). The overall impact of applying stricter exclusion criteria on the reference intervals constructed was small. It is very obvious that the minor differences between the resulting reference ranges are clinically irrelevant.

## Discussion

Here we report the development and validation of a new, automated chemiluminescence immunoassay for the measurement of IGFBP-3. We also report extensive method-specific age- and sex-adjusted reference intervals for IGFBP-3 derived from a large multicenter study including samples from about 15 000 newborns, children, adolescents, and adults of all ages from different geographic regions. Finally, because the same automated assay system also allows for the measurement of IGF-I, we are, for the first time, able to present reference intervals for the IGF-I to IGFBP-3 ratio in such a large population.

The strong influence of age on circulating IGFBP-3 concentrations seen in our study confirms observations from previous studies reporting reference intervals in smaller cohorts. Some of these studies focused on the generation of normative data in children and adolescents and included between 468 and 1692 individuals (15, 16, 28–33). Reference intervals for IGFBP-3 concentrations have also been reported from studies in adults (30, 34, 35), which included from 1584 to 2499 individuals. There are only three studies in children (15, 16, 32) and one study in adults (30) describing reference intervals for the IGF-I to IGFBP-3 ratio. Some of these studies reported ratios of mass concentrations (15, 30), whereas others (16, 32) reported the ratio of the molar concentrations. We followed the latter approach to accurately reflect the considerable difference in size of the two molecules. Although the study by Alberti et al (16) included only children and adolescents between ages 6 and 20 years and was conducted using different assays (manual RIAs), the values reported for the ratio during early childhood are in a similar range: the 50th percentile was approximately 15% in both studies, and at the peripubertal peak, the ratio was approximately 30% at age 15 years (16, 32) and during Tanner IV in our study. Based on our data, at the pubertal peak, the molar IGF-I concentrations constituted on average about one third of the molar IGFBP-3 concentration. In contrast, after birth and at old age, the molar IGF-I concentrations are only approximately 10% of the corresponding molar IGFBP-3 concentrations.

Many different manual or automated assays involving polyclonal antisera or monoclonal antibodies of different specificity have been used to establish reference intervals for IGFBP-3 concentrations. The cohorts used differed in size, age ranges, and geographical origin. Furthermore, various statistical methods have been used for calculation of reference intervals. These factors might contribute to

**Table 4.** Percentiles for IGFBP-3 According to Tanner Stages Based on the Danish Cohort (n = 854)

| | IGFBP-3, ng/mL | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sex** | **Tanner** | **Age Range** | **2.5%** | **25%** | **50%** | **75%** | **97.5%** |
| Males | I | 6.1–12.9 | 9.36 | 13.02 | 15.03 | 17.05 | 23.12 |
| | II | 8.1–14.8 | 10.55 | 18.34 | 22.39 | 26.42 | 33.00 |
| | III | 10.9–16.0 | 20.96 | 27.40 | 28.99 | 32.40 | 39.58 |
| | IV | 12.4–17.1 | 19.33 | 27.05 | 31.07 | 35.15 | 41.82 |
| | V | 13.5–20.0 | 18.43 | 23.47 | 26.98 | 29.74 | 35.21 |
| Females | I | 5.8–12.1 | 10.30 | 14.55 | 17.27 | 20.39 | 26.32 |
| | II | 9.3–14.1 | 12.93 | 17.21 | 20.92 | 25.84 | 32.44 |
| | III | 9.3–15.1 | 20.41 | 26.13 | 29.51 | 32.14 | 38.95 |
| | IV | 11.8–16.6 | 15.25 | 24.88 | 29.18 | 33.55 | 41.62 |
| | V | 12.5–19.9 | 14.25 | 20.00 | 25.69 | 29.02 | 36.14 |

Estimated percentiles (2.5%, 25%, 50%, 75%, and 97.5%) derived by Harrell-Davis estimate of quartiles are provided. Ratio = IGF-I (nanomoles per liter) to IGFBP-3 (nanomoles per liter) * 100.

the considerable differences in the reported absolute concentrations of IGFBP-3 between studies, eg, the upper limit of normal at the pubertal peak varies from 4800 (28) to 13 800 ng/mL (29), although most studies (15, 16, 30, 31, 33) report peak values at approximately 7000 ng/mL. These dramatic variations cannot be explained by biological variation alone. Analytical heterogeneity between immunoassays has been studied and discussed for several years, albeit mainly for GH and IGF-I assays, for which a recent consensus statement demanded the use of uniform standards to minimize between-method differences (17). In this respect, until today much less attention was given to IGFBP-3 assays, although potential factors leading to heterogeneity in IGFBP-3 assay have been described: it is important to keep in mind that IGFBP-3 circulates in different forms, resulting from glycosylation (36). The assay presented here is calibrated against human glycosylated IGFBP-3 expressed in mammalian cells, and our validation experiments have shown full recovery of the glycosylated standard in human samples as well as parallel dilution of the calibrator and samples containing high endogenous IGFBP-3 concentrations. Furthermore, IGFBP-3 can be proteolysed (37), particularly under specific clinical conditions like pregnancy (38). We could demonstrate that in the new IGFBP-3 assay measured concentrations in normal samples are not changed after incubation with third-trimester samples at 37°C, indicating the assay is not significantly affected by proteolysis.

Comparison of the IGFBP-3 concentrations obtained by the new IDS-iSYS assay with those obtained by two other automated assays, which were or are widely used, confirmed that also in the case of IGFBP-3, considerable differences between methods exist. Results obtained by the Nichols Advantage IGFBP-3 assay correlated to those obtained by the IDS-iSYS but were dramatically lower. In contrast, the IDS-iSYS results were highly correlated and agreed with those obtained by the Immulite 2500 IGFBP-3 assay. It remains a limitation of our study that due to the discontinuation of the Nichols assay long before we started to develop the iSYS assay, we could not specifically investigate the reasons for the remarkable difference between the results. More specifically, it was not possible to directly compare the (potentially) different preparations used for calibration. Limited stability of IGFBP-3 in sam-

**Table 5.** Percentiles for IGF-I to IGFBP-3 Ratio According to Tanner Stages Based on the Danish Cohort (n = 854)

| | IGF-I to IGFBP-3 Ratio, % | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sex** | **Tanner** | **Age Range** | **2.5%** | **25%** | **50%** | **75%** | **97.5%** |
| Males | I | 6.1–12.9 | 9.36 | 13.02 | 15.03 | 17.05 | 23.12 |
| | II | 8.1–14.8 | 10.55 | 18.34 | 22.39 | 26.42 | 33.00 |
| | III | 10.9–16.0 | 20.96 | 27.40 | 28.99 | 32.40 | 39.58 |
| | IV | 12.4–17.1 | 19.33 | 27.05 | 31.07 | 35.15 | 41.82 |
| | V | 13.5–20.0 | 18.43 | 23.47 | 26.98 | 29.74 | 35.21 |
| Females | I | 5.8–12.1 | 10.30 | 14.55 | 17.27 | 20.39 | 26.32 |
| | II | 9.3–14.1 | 12.93 | 17.21 | 20.92 | 25.84 | 32.44 |
| | III | 9.3–15.1 | 20.41 | 26.13 | 29.51 | 32.14 | 38.95 |
| | IV | 11.8–16.6 | 15.25 | 24.88 | 29.18 | 33.55 | 41.62 |
| | V | 12.5–19.9 | 14.25 | 20.00 | 25.69 | 29.02 | 36.14 |

Estimated percentiles (2.5%, 25%, 50%, 75%, and 97.5%) derived by Harrell-Davis estimate of quartiles are provided. Ratio = IGF-I (nanomoles per liter) to IGFBP-3 (nanomoles per liter) * 100.

**Table 6.** Impact of Different Exclusion Criteria on the Reference Limits Derived From Three Large Adult Cohorts

| | Reference Limits (2.5%–97.5%) for IGFBP-3, ng/mL | | | | Reference Limits (2.5%–97.5%) for IGF-I to IGFBP-3 ratio[a] | | | |
|---|---|---|---|---|---|---|---|---|
| | Men | | Women | | Men | | Women | |
| Exclusion Criteria | 30 y | 70 y | 30 y | 70 y | 30 y | 70 y | 30 y | 70 y |
| No exclusion | 3194–6414 | 1867–5449 | 3235–6359 | 2277–5697 | 7.19–20.71 | 5.78–16.82 | 6.83–20.63 | 4.63–14.25 |
| Exclusion of subjects with diabetes, history of cancer, creatinine clearance <60 ml/min per 1.73 $m^2$, liver disease, disease of the pituitary gland | 3205–6438 | 2019–5340 | 3222–6377 | 2265–5622 | 7.42–20.86 | 5.99–16.47 | 6.67–20.59 | 4.72–14.19 |
| Exclusion of subjects with diabetes, history of cancer, creatinine clearance <60 ml/min per 1.73 $m^2$, liver disease, disease of the pituitary gland, BMI < 18 or > 30 kg/$m^2$ | 3152–6361 | 2126–5261 | 3244–6417 | 2332–5627 | 7.52–20.51 | 6.32–16.39 | 7.01–21.02 | 4.81–14.22 |
| Exclusion of subjects with diabetes, history of cancer, creatinine clearance <30 ml/min per 1.73 $m^2$, liver disease, disease of the pituitary gland | 3205–6434 | 2018–5370 | 3242–6350 | 2300–5692 | 7.32–20.75 | 5.95–16.53 | 6.79–20.61 | 4.91–14.21 |
| Exclusion of subjects with diabetes, history of cancer, creatinine clearance <30 ml/min/1.73 $m^2$, liver disease, disease of the pituitary gland, BMI < 18 or > 30 kg/$m^2$ | 3147–6368 | 2121–5273 | 3244–6408 | 2338–5585 | 7.50–20.23 | 6.26–16.55 | 7.14–21.00 | 4.97–14.16 |

Abbreviation: BMI, body mass index. The analyses are based on the KORA F4, KORA Age, and SHIP cohorts.

[a] Ratio = IGF-I (nanomoles per liter) to IGFBP-3 (nanomoles per liter) * 100.

ples stored over periods of several years has also been discussed as a potential explanation. However, the fact that the age-adjusted IGFBP-3 concentrations in samples from the SHIP cohort collected several years ago and the very new KORA Age study were not different when measured by the new IGFBP-3 assay might provide indirect evidence against a larger degradation of the measure and during long-term storage. Regardless of what the reason is, the differences between the methods underline the need for assay-specific reference intervals also in case of IGFBP-3.

The subjects included in our study came from Europe, Canada, and the United States, and most of them were of Caucasian origin, and therefore, applicability of our reference intervals to other ethnic groups has not been proven. A recent study had shown that common genetic variants influence circulating levels of IGFBP-3 similarly in five ethnic groups (39). However, earlier studies had reported that although ethnicity has limited impact on circulating concentrations of IGF-I, it accounts significantly for the variation seen in IGFBP-3 concentrations (40). Therefore, comparative studies using the new IGFBP-3 assay in samples from different ethnic groups are warranted.

Our study collected data from a very large sample of subjects of all ages and both sexes, allowing a detailed study of changes in concentrations across the life span. Although from a clinical point of view, the differences in concentrations of IGFBP-3 between sexes were minor, we provide the reference intervals for each sex separately based on at least 170 subjects of each sex per decade. Despite overall similar concentrations, our analysis could show that during puberty, but especially beyond the age of 60 years, females have higher IGFBP-3 concentrations. In contrast, at older ages, males have higher concentrations of IGF-I. Consequently, the IGF-I to IGFBP-3 ratio is higher in older males than in older females, an observation that also was made in several larger epidemiological studies in older adults (41, 42). Unraveling the mechanism causing this sex difference was beyond the scope of the present analysis, and the interrelationship between the GH/IGF axis and sex hormones are complex (43, 44). However, the occurrence of the dimorphism after menopause suggests a role of sex steroids (45).

Potential clinical implications of the analysis of changes in concentrations of IGFBP-3 and also the IGF-I/IGFBP-3 ratio are also suggested by epidemiological data showing significant associations between IGFBP-3 or the IGF-I to IGFBP-3 ratio and various diseases ranging from cancer, coronary events, hepatic steatosis, and lung function to mortality (9, 46–52). In addition, studies on the molecular level suggest a role of IGFBP-3 in the development of the diseases: IGFBP-3 possesses IGF-independent actions, which mainly are related to apoptosis and cell growth, and IGFBP-3 concentrations seem to modify glucose uptake (53). In vitro, IGFBP-3 inhibits the insulin-stimulated glu-

cose uptake in adipocytes (54, 55), and in vivo overexpression of IGFBP-3 leads to hyperglycemia, impaired glucose tolerance, and insulin resistance independent of IGF-I (56). In patients with non-insulin-dependent diabetes, a higher IGFBP-3 proteolytic activity was found compared with healthy controls, suggesting a role for IGFBP-3 in metabolic diseases (57). In this context, it is interesting that an investigation of the Third National Health and Nutrition Examination Survey reported a 3-fold higher odds of the metabolic syndrome in subjects with a low IGF-I to IGFBP-3 ratio (58).

With our present study, we are able to provide method-specific age- and sex-adjusted reference intervals across all age groups from birth to senescence for serum concentrations of IGFBP-3 and for the molar IGF-I to IGFBP-3 ratio. We identified differences between sexes in the age-related changes in the IGF-I to IGFBP-3 ratio, which is significantly higher in aged males. The huge data set obtained from the analysis of samples from well-characterized cohorts and analyzed by the new combination of automated assays for IGF-I and IGFBP-3 might facilitate the application of these parameters in clinical studies, research, and potentially also in clinical practice.

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