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National and international initiatives and approaches for the establishment of reference intervals in pediatric laboratory medicine

Nationale und internationale Initiativen und Ansätze für die Festlegung von Referenzintervallen in der pädiatrischen Laboratoriumsmedizin

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Abstract: Thoroughly validated laboratory medicine reference intervals for children of all ages groups have been published increasingly during the last years. The aim of this review is to present a number of these studies and the various approaches to the generation of reference intervals. Population-based data obtained in large cohorts of healthy children in Germany, Northern Europe, North America, and selected other countries as well as patient-derived data collected in many areas of the world are discussed. Additionally, special research aspects such as reference intervals for preterm neonates, preanalytical issues, intraindividual variation of analytes, or follow-up studies that are covered in many of these studies are presented.

Keywords: age dependence; newborn; pediatric laboratory medicine; preterm neonates; reference intervals.

Zusammenfassung: In den vergangenen Jahren sind viele sorgfältig validierte Referenzintervalle für laboratoriumsmedizinische Kenngrößen bei Kindern aller Altersgruppen publiziert worden. Das Ziel dieser Übersicht ist es, wichtige Beispiele für derartige Studien vorzustellen und die verschiedenen Ansätze zur Ermittlung der Referenzintervalle aufzuzeigen. Bevölkerungsbezogene Ergebnisse, die in großen Kollektiven gesunder Kinder in Deutschland, Nordeuropa, Nordamerika und einzelnen anderen Ländern ermittelt wurden, werden ebenso beschrieben wie patientenbezogene Daten, die ebenfalls in vielen Ländern der Welt gesammelt wurden. Darüber hinaus werden besondere Aspekte diskutiert, die in vielen dieser Studien über die Ermittlung von Referenzintervallen hinaus untersucht wurden, so etwa Referenzintervalle für Frühgeborene, präanalytische Besonderheiten, intraindividuelle Schwankungen laboratoriumsmedizinischer Kenngrößen oder aus bevölkerungsbasierten Studien resultierende Folgeprojekte.

Schlüsselwörter: Altersabhängigkeit; Frühgeburten; Neugeborene; pädiatrische Laboratoriumsmedizin; Referenzintervalle.

Introduction

For a valid judgment on a child's health status, age-specific reference intervals for results of clinical laboratory investigations are an essential prerequisite [1] because the different phases of physiological development of the healthy child from or even before birth to adolescence are reflected in rather different concentrations of a variety of clinical laboratory analytes in blood. Validated and reliable statistical methods are needed to establish these reference intervals [2]. To achieve this goal, two principally different approaches can be followed, either population-based reference intervals or reference intervals derived from patients' data stored in laboratory information systems (LIS). In recent years, a variety of activities for the establishment of reference intervals has been performed in several regions of the world, studying a substantial number of healthy children in each age group and using well-characterized or traceable methods.

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Studies to be mentioned here are the NORICHILD initiative [3] and subsequent studies of the Scandinavian Societies of Clinical Chemistry or the Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER) carried out in Canada [4]. In the USA, the National Children's Study (NCS), a long-term project, had been started a few years ago and was intended to yield clinical laboratory results on more than 100,000 children [5]. In Germany, the German Health Interview and Examination Survey for Children and Adolescents ("KiGGS") [6], has recently provided a large set of reference intervals for many parameters used in routine laboratory investigations, based on a large number of children with well characterized health status [7]. In several other countries, similar studies have been performed; furthermore, data resulting from multinational activities focusing on special aspects of pediatric laboratory medicine have been published recently.

The IFCC Task Force on Pediatric Laboratory Medicine (TF-PLM) has recently formed an international "reference interval consortium" comprising scientists from Canada, Switzerland, Singapore, Australia, Germany, and the USA.

This review summarizes some of the recently published results contributing to the establishment of a broad and solid set of reference intervals in pediatric laboratory medicine worldwide. Results of population-based as well as patient-derived reference intervals are discussed, and further results of the studies underlying these reference intervals focusing on related aspects of children's health are also described.

Population-based reference intervals

Ideally, samples from healthy subjects are studied to obtain a valid database of laboratory test results. This may be achieved using a population-based approach asking volunteers to give blood or other body fluids for investigation. Recently, in a number of European, North American, and other countries from different continents, many important studies have been performed with this kind of approach.

Germany

In Germany, a very large nationwide survey on the health status of children (KiGGS) has been conducted only a few years ago. The subjects for the KiGGS study had been randomly selected from the official registers of local residents, and 167 study locations (sample points) had been chosen all over Germany [6]. The health status of the children was thoroughly evaluated using questionnaires filled in by parents and (using parallel questionnaires) by the children aged 11 years and older. Physical examinations and other tests as well as computer-assisted personal interviews were carried out by a study team of physicians and health workers. As the study had been designed to comprise a number of 1000 children per age group spanning 1 year, about 18,000 children from age 0 to 18 years participated in the study. As a result, ca. 14,000 EDTA blood samples, ca. 14,000 serum samples, and ca. 14,000 urine samples were collected from children older than 1 year. In younger children, the design of the study did not permit collection of blood and urine samples. Following a strict preanalytical protocol, the laboratory investigations were carried out at central laboratories using well-defined standardized methods (e.g. IFCC reference methods) when available [7], using equipment by Roche Diagnostics (Mannheim, Germany; Hitachi 917 analyzer for general clinical chemistry analytes and Elecsys 2010 analyzer for immunoassays) or Abbott Laboratories (Abbott Park, IL, USA; Cell-Dyn 3500 for hematology analytes).

Laboratory parameters investigated in the KiGGS study were focused on general health indices (such as clinical chemistry analytes, red blood count, and urine status), markers of the nutritional state, iron metabolism as well as that of the thyroid, indices for atopic sensitization (allergy-specific IgE), and markers of past infections or the immunization status. After the initial evaluation of the data, a comprehensive overview of results for ca. 25 analytes of the study has been published in German [8]. It contains age-dependent percentiles (from 3^{rd} to 97^{th}) in table as well as graphic form, which were obtained using elaborate statistical methods such as the LMS (λ-μ-σ) approach in order to deal with nonparametric distributions. Figure 1 shows an example of continuous age-dependent percentile curves for alkaline phosphatase (ALP) concentrations, the parameter with one of the most significant effects of age.

These data may already serve as a basis for age-specific reference intervals, although it may still be necessary to examine the clinical data of the children studied and maybe rule out some of the results. Meanwhile, as a variety of clinical information is available for these children, it will be possible to study the influence of parameters like body weight or other biometric and physiological data on some of the laboratory parameters investigated.

Of particular note, the data obtained during the KiGGS study are available from the Robert Koch-Institute as public use files upon submittal of a request containing the intended use for scientists with a documented interest in the field of pediatric medicine. Taken together, the

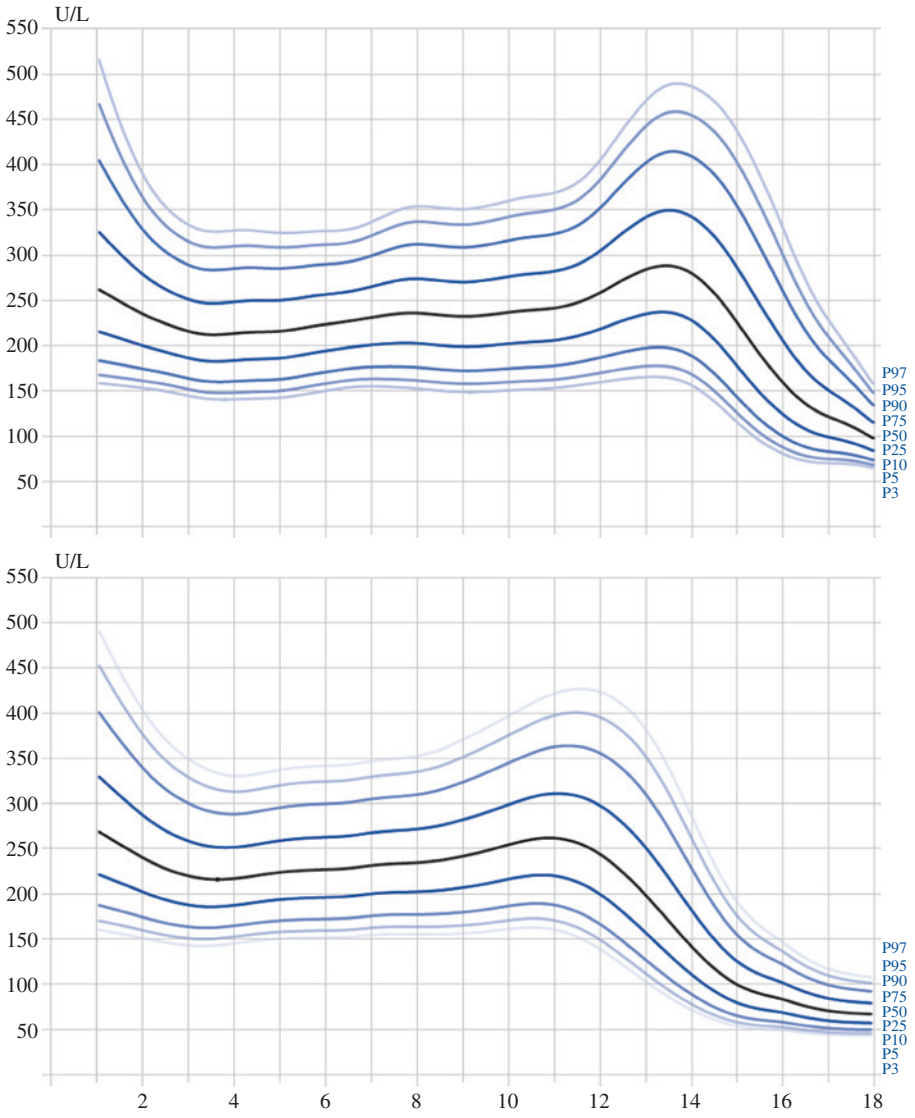

Figure 1: Smoothed percentiles (3rd, 5th, 10th, 25th, 50th, 75th, 90th, 95th, and 97th) of ALP activities (y-axis) as a function of the exact age (x-axis, given in years) in healthy boys (n=7299; top) and girls (n=6956; bottom) obtained in the KiGGS study.
(Robert Koch-Institut, editor. Bevölkerungsbezogene Verteilungswerte ausgewählter Laborparameter aus der Studie zur Gesundheit von Kindern und Jugendlichen in Deutschland (KiGGS). Beiträge zur Gesundheitsberichterstattung des Bundes. Berlin: RKI, 2009:93; [8] reproduced with permission by the Robert Koch Institute, Berlin, Germany.)

results of the KiGG study serve as an invaluable tool for researchers trying to solve specific problems related to children's health.

Scandinavian countries

The prototype of pediatric reference interval studies has been undertaken already in the early 1990s, with the NORICHILD project [3]. Later on, this was followed by studies in Sweden and Denmark [9] in which healthy children of almost all ages volunteered to participate.

In a recently published review by Ridefelt et al. [10], three prospective community-based projects utilizing blood samples from healthy children in Sweden and Denmark and Canada are named to have "substantially improved the situation" for common clinical chemistry and hematology analyses. One of the most recently performed study included 694 apparently healthy children [11, 12], evenly distributed from 6 months to 18 years of age. They were recruited as volunteers at child care units and schools, and information on their health status was obtained using a questionnaire filled out by parents and older children. Analytes determined in this study

comprised alanine aminotransferase (ALT), albumin, aspartate aminotransferase (AST), bilirubin, conjugated bilirubin, C-reactive protein (CRP), creatine kinase (CK), γ-glutamyltransferase, HbA_{1c}, lactate dehydrogenase (LD), myoglobin, and pancreatic amylase, which were analyzed on the Abbott Architect ci8200, and for HbA_{1c}, on the Tosoh G7 (Tosoh Clinical Diagnostics, Tokyo, Japan) and a mono S-system. The authors found age- and gender-related reference intervals (2.5^{th} and 97.5^{th} percentiles) to be substantially different to comparable studies for some analytes. In the same study, hematology and anemia analytes were determined on the Siemens Advia 2120 platform (Siemens Healthcare Laboratory Diagnostics, Erlangen, Germany; hemoglobin [Hb], erythrocytes, reticulocytes, leukocytes, lymphocytes, monocytes, neutrophils, eosinophils, basophils, platelets, iron, transferrin, transferrin saturation) [13]. In the third study, thyroid-stimulating hormone (TSH), free thyroxine (fT4), free triiodothyronine (fT3), total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, and prolactin were again analyzed on Abbott Architect ci8200 [14]. The thyroid hormone levels were similar to previously reported data for the Abbott Architect platform, but exhibited differences from studies performed with other methods. Prolactin displayed wide reference ranges, but relatively small age-related changes, and a marginal difference between sexes during adolescence.

Levels of LDL and total cholesterol were higher than those reported for children in Canada, but lower than those reported for children in China. The authors concluded that age- and gender-specific pediatric reference intervals presented in their studies differ from previously recommended reference intervals, which may be due to the fact that in many earlier investigations, retrospective hospital-based data had been used, which may include various subgroups.

Canada

Within the last 10 years, a most remarkable activity on the establishment of pediatric reference intervals has taken place in Canada. The CALIPER [15] has led to an impressive collection of invaluable data on routine and special laboratory investigation results in large cohorts of children. In a review by investigators of the CALIPER project, the challenges specific to establishing pediatric reference intervals and recent initiatives aimed at closing existing gaps in current knowledge are highlighted [16].

The publication of the CALIPER containing the largest data set was conducted with 2188 healthy children and adolescents, newborn to 18 years of age, recruited from a multiethnic population with informed parental consent [3]. The health status was assessed from completed questionnaires and according to defined exclusion criteria. Whole-blood samples were collected in which 40 serum biochemical markers were determined on the Abbott Architect c8000 analyzer. Reference intervals were generated according to the C28-A3 statistical guidelines of the Clinical Laboratory Standards Institute (CLSI). Caucasians, East Asians, and South Asian participants were evaluated with respect to the influence of ethnicity, and statistically significant differences were observed for seven specific biomarkers. Figure 2 shows examples of the data obtained, displaying individual results for common analytes such as creatinine or urea. As part of CALIPER, a new comprehensive database of pediatric reference intervals was established, which is available at the online version of the publication in *Clinical Chemistry* (for an example, cf. Table 1). This database, which will be filled continuously, should assist laboratorians and pediatricians in interpreting test results more accurately and thereby lead to improved diagnosis of childhood diseases. It will also be of global benefit once reference intervals are validated in transference studies with other analytical platforms and local populations, as recommended by the CLSI.

Although the above-mentioned study was focused on standard biochemical tests, a subset of CALIPER investigated fertility hormones [17]. Especially in this field, accurate reference intervals established on the basis of a healthy, nonhospitalized pediatric population and reflecting age-, gender-, and pubertal stage-specific changes are essential for test result interpretation. Healthy children and adolescents (n=1234) were recruited from a multiethnic population as part of the CALIPER study. After written informed parental consent was obtained, participants filled out a questionnaire including demographic and pubertal development information (assessed by self-reported Tanner stage). Concentrations of seven fertility hormones including estradiol, testosterone, progesterone, sex hormone-binding globulin, prolactin, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were determined by use of the Abbott Architect i2000 analyzer for which age-, gender-, and Tanner stage-specific reference intervals were calculated according to CLSI C28-A3 guidelines. Not surprisingly, a complex pattern of change in each analyte concentration from the neonatal period to adolescence was observed. Consequently, many age and sex partitions were required to cover the changes in most fertility hormones over this period.

Following the CALIPER approach, other areas of laboratory medicine, such as endocrinology parameters, were

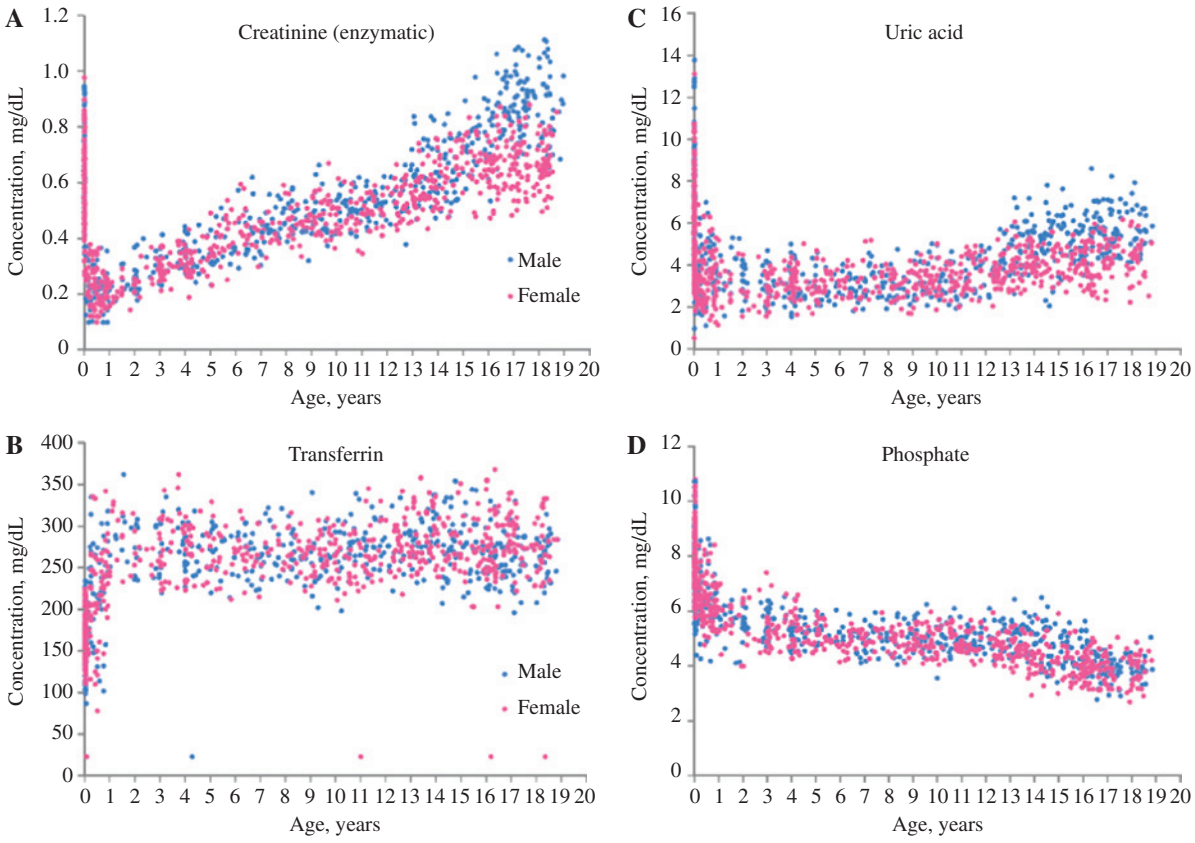

Figure 2: Examples of individual results of routine clinical chemistry analytes obtained in the CALIPER study.
(Colantonio DA, Kyriakopoulou L, Chan MK, Daly CH, Brinc D, Venner AA, et al. Closing the gaps in pediatric laboratory reference intervals: a CALIPER database of 40 biochemical markers in a healthy and multiethnic population of children. Clin Chem 2012;58:854–68; [4] reproduced with permission from the AACC.)

Table 1: Excerpt of original data file of the CALIPER study.

Participants Gender	Participants Age, years	Alkaline Phosphatase, U/L	ALT, U/L	Amylase, U/L	AST, U/L	Bilirubin – total, mg/dL
Male	0.005			3	94	
Female	0.005			10		30.6
Female	0.008			2.9	129	1.7
Female	0.008	117	11			
Female	0.008		14			
Female	0.008		14		90	
Female	0.008	91	21	10	95	
Female	0.008	164	33	2.9	162	
Female	0.008		12			
Female	0.008	150	9			
Male	0.008			4	71	40.1
Female	0.008	177	18		97	
Female	0.008	155	20		112	38.2
Female	0.008	175	19		99	

Colantonio DA, Kyriakopoulou L, Chan MK, Daly CH, Brinc D, Venner AA, et al. Closing the gaps in pediatric laboratory reference intervals: a CALIPER database of 40 biochemical markers in a healthy and multiethnic population of children. Clin Chem 2012;58:854–68. [4] The complete Excel file may be downloaded at http://www.clinchem.org/content/suppl/2012/02/03/clinchem.2011.177741.DC1/clinchem.2011.177741-7.xls.

investigated in a subsequent study: α-fetoprotein, cobalamin, folate, homocysteine, ferritin, cortisol, troponin I, 25(hydroxy)-vitamin D [25(OH)-vitamin D], intact parathyroid hormone (iPTH), TSH, total thyroxine (TT4), total triiodothyronine (TT3), fT4, and free triiodothyronine (fT3) were determined on the Abbott Architect i2000 in a total of 1482 samples collected from ethnically diverse healthy children ages 2 days to 18 years [18]. Nonparametric and robust methods were used to establish the 2.5th and 97.5th percentiles for the reference intervals. In this study, statistically significant differences were found between ethnic groups for fT4, TT3, TT4, cobalamin, ferritin, iPTH, and 25(OH)-vitamin D.

Steroids commonly used as biomarkers for the diagnosis and monitoring of endocrine diseases such as congenital adrenal hyperplasia were addressed in another separate CALIPER study in which the authors used a new liquid chromatography tandem mass spectrometry (LC/MS/MS) method for the determination of serum cortisol, corticosterone, 11-deoxycortisol, androstenedione, 21-hydroxyprogesterone, testosterone, 17-hydroxyprogesterone, and progesterone [19]. Here, a total of 337 serum samples from children between the ages of 0 and 18 years were analyzed. Not surprisingly, reference intervals for all hormones required significant age-dependent stratification, whereas testosterone and progesterone required additional sex-dependent stratification.

For a monitoring of deficiencies due to malabsorption secondary to gastrointestinal disorders in the pediatric population, concentrations of fat-soluble micronutrients such as vitamins A and E are most commonly determined. Thus, in another subset of CALIPER, a total of 342 blood samples were collected from healthy children 1 day to 19 years of age recruited from the community [20]. Retinol and α-tocopherol were extracted from serum using hexane before concentrations were measured with HPLC. According to these results, vitamin A demonstrated increasing levels with age necessitating four distinct age stratifications, while vitamin E levels peaked within the first year of life, requiring only two age partitions. Ratios of vitamin E to cholesterol and triglyceride were also calculated and correlated well to vitamin E levels. Sex-specific differences were not observed.

Transference of CALIPER results to different instrument platforms

Despite its importance for the establishment of reference intervals, the data obtained in the CALIPER study suffer from an inherent disadvantage because its original database was only directly applicable for Abbott Architect assays. Therefore, in a subsequent study [21], the authors sought to expand the scope of this database to biochemical assays from other major manufacturers, allowing for a much wider application of the CALIPER database. Based on CLSI C28-A3 and EP9-A2 guidelines, CALIPER reference intervals were transferred (using specific statistical criteria) to assays performed on four other commonly used clinical chemistry platforms including Beckman Coulter DxC800 (Beckmann Coulter Diagnostics, Brea, CA, USA), Ortho Vitros 5600 (Ortho Clinical Diagnostics, Rochester, NY, USA), Roche Cobas 6000, and Siemens Vista 1500. The resulting reference intervals were subjected to a thorough validation using 100 reference specimens (healthy community children and adolescents) from the CALIPER bio-bank. In general, the transferred pediatric reference intervals were similar to those established in the authors previous study. However, assay-specific differences in reference limits were observed for many analytes, and in some instances were considerable. The results of the External Quality Assessment (EQA) evaluation generally mimicked the similarities and differences in reference limits among the five manufacturers' assays. In addition, the majority of transferred reference intervals were validated through the analysis of CALIPER reference samples.

USA

The establishment of pediatric reference intervals for laboratory parameters was expected to constitute part of the outcome of the NCS in the USA, a planned large-scale, long-term National Institutes of Health (NIH) study of US children and their parents designed to study environmental influences on child health and development [5], which would have followed 100,000 children from before birth to age 21 years. However, in late 2014, the NIH director decided to close the NCS, but it was announced that the agency will make the collected data and specimens of the pilot study (already comprising 5000 children in 40 locations across the USA) available to researchers, and the American Association of Clinical Chemistry's (AACC) Pediatric Reference Range Initiative, a project of the Pediatric-Fetal/Maternal-Division, is currently planning the optimal use of this material.

Other initiatives to obtain pediatric reference intervals in the USA had previously been started by the Pediatric and Maternal-Fetal Division of the AACC as well as by ARUP Laboratories, a large commercial reference laboratory ("ChildX").

Other areas of the world

Ethiopia

In an approach to establish reference intervals for concentrations of electrolytes in Ethiopian children, cord blood from 60 newborns and venous blood samples from 57 infants were collected and analyzed by direct potentiometry on the AVL 9181 electrolyte analyzer (AVL, now Roche Diagnostics, Mannheim, Germany) [22]. Although the levels of Na and K showed differences between newborns and infants, combined reference intervals were suggested by the Haris and Boyd rule, 126–143 mmol/L for sodium and 4.0–7.9 mmol/L for potassium. Differences in chloride concentrations between newborns and infants could not be observed, and thus, a combined reference interval was determined (100–111 mmol/L). According to this study, maternal, neonatal, and infantile factors did not affect the concentrations of the electrolytes. Thus, combined reference intervals were suggested for the interpretation of electrolyte values in Ethiopian newborns and infants without taking the effect of maternal, neonatal, and infantile factors into account. However, the authors concluded that as these reference intervals for electrolytes were significantly different from previously reported values, it appears to be appropriate to apply them for the interpretation of electrolyte values in the Ethiopian pediatric population from now on.

Sub-Saharan Africa

Troy et al. [23] recently published a study using 542 blood samples from 269 HIV-uninfected, black Zimbabwean infants at 3, 5, and 9 months of age in which hematological and immunological parameters were analyzed using the Sysmex KX-21N hematology analyzer (Sysmex Corporation, Kobe, Japan) and a Partec "Cyflow" flow cytometer (Partec, Muenster, Germany) to calculated reference intervals based on ethnicity and geographic location in Zimbabwe. These authors found, however, that substantial proportions of the platelet counts (44%), Hb concentrations (19%), and mean erythrocyte corpuscular volumes (41%) were outside published "normal ranges". They also observed that 65% of these children's Hb concentrations qualified as "adverse events" as defined by the US NIH Division of AIDS (NIH DAIDS), which are commonly used in clinical trials, and the majority (71%) of relative CD4 cell counts indicated immunodeficiency by World Health Organization criteria. Thus, hematological and immunological reference intervals that are intended to be used to evaluate toxicities in pediatric trials in sub-Saharan Africa need to be reevaluated to account for differences in ethnicity, geographic location, nutrition, and socioeconomic status.

Other authors reported similar results, which were obtained in samples from 655 HIV-seronegative, healthy children from 1 month to 18 years of age from the Kilimanjaro Region of Tanzania [24]. These investigators used the Beckman Coulter "AcT 5 Diff" hematology analyzer and a Becton Dickinson "FACSCalibur" flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Although median Hb concentrations for all age groups were higher than previously established East African reference intervals, reference ranges encompassed lower values for Hb, mean corpuscular volume, and platelets as compared to the corresponding US reference intervals. Again, it was found that by applying the NIH DAIDS adverse event grading criteria to the reference range participants, 21% of the children would be classified as having an adverse event related to the Hb concentration. In this study, a significant decline of absolute counts of CD4-positive T-lymphocytes was observed with increasing age. Most importantly, percentages of CD4-positive T-lymphocyte observed in Tanzanian children younger than 5 years were significantly lower than those established in their counterparts living in developed countries. From these studies, it may also be concluded that the criteria of health used for children in the developed world may not be applicable in countries like Zimbabwe.

Saudi Arabia

In a cross-sectional study, conducted among Saudi schoolchildren, fasting blood samples were collected from 2149 children (53% boys and 47% girls), aged 6–18 years old [25]. Using these samples, concentrations of glucose, cholesterol, triglycerides, and HDL and LDL cholesterol were analyzed on the Architect c8000 Chemistry System, and reference intervals were established by nonparametric methods between the 2.5th and the 97.5th percentiles. According to this study, significant differences were observed between boys and girls for cholesterol and triglycerides concentrations in all age groups, but only at age 6–7 years, and in adolescents, HDL and LDL levels were found to be significantly different between boys and girls. No significant differences were seen in glucose levels except at age 12–13 years. The authors concluded that Saudi children have comparable serum cholesterol levels than their Western counterparts, which probably reflects changing dietary habits and increasing affluence in Saudi Arabia. Increased lipid screening is anticipated, and these reference intervals will aid in the early

assessment of cardiovascular and diabetes risk in Saudi pediatric populations.

Melanesia

Another area of the world where pediatric reference intervals derived from studies in Western countries may not be applicable is the Pacific, especially Melanesia. Therefore, in a study with plasma samples from 327 healthy Melanesian children living in the Madang Province of Papua New Guinea, specific reference intervals for common biochemical and hematological analytes were established [26]. Concentrations of clinical chemistry analytes were determined on a Cobas Integra800 platform (Roche Diagnostics) with a few exceptions such as vitamin B12 (Roche Elecsys 201) or vitamin D (Dia Sorin Liaison, Dia Sorin, Saluggia, Italy). The authors found substantial differences in the concentrations of Hb, soluble transferrin receptor, ferritin, calcium, phosphate, and CRP as compared with reference intervals from children from Western countries and/or African children. Furthermore, differences in the upper limits of reference intervals for bilirubin and ALT were also observed.

Multinational studies

For selected analytes, especially those relevant to an evaluation of the endocrine status of children, multinational studies have been performed to obtain a sufficiently large number of samples. A recent publication by Bidlingmaier et al. [27] covers such an approach for insulin-like growth factor 1 (IGF1), which is a cornerstone in diagnosis and monitoring of growth hormone (GH)-related diseases. The authors conducted a multicenter study with samples from several cohorts from the USA, Canada, Denmark, Sweden, Germany, and Austria including also 4106 children. As considerable discrepancies between analytical methods exist especially in this case, all concentrations of IGF1 were determined using only one specific assay (IDS iSYS) (Immunodiagnostics Systems, Boldon, UK), which was calibrated against the recommended standard (02/254) and found to be insensitive to the six high-affinity IGF-binding proteins (IGFBPs). Age- and sex-adjusted reference intervals derived from this large cohort using criteria defined by a recent consensus conference reflect the age-related pattern of IGF1 secretion showing a decline immediately after birth followed by an increase until a pubertal peak. Later in life, values decrease continuously. The impact of gender is small, although across the lifespan, women have lower mean IGF1 concentrations. Remarkably, geographical region, sampling setting (community or hospital based), and rigor of exclusion criteria did not affect the reference intervals.

This multinational study was subsequently extended toward determination of IGFBP-3, not at least because epidemiological studies suggest that IGFBP-3 and the IGF1/IGFBP-3 molar ratio are associated with clinical end points like cancer or cardiovascular disease [28]. Again, concentrations of IGFBP-3 and the IGF1/IGFBP-3 ratio were determined using only one assay system. Both the concentration of IGFBP-3 and the IGF1/IGFBP-3 ratio are mainly determined by age (Figure 3). Resulting from the high peripubertal peak in the concentrations of IGF1 (see above), the peak in the IGF1/IGFBP-3 ratio occurs already around the age of 15 years, with a slightly earlier and higher peak in girls. In their publication, the authors present an extensive set of pubertal stage-, age-, and sex-adjusted normative data for concentrations of IGFBP-3 (for an example, see Table 2) and the IGF1/IGFBP-3 molar concentration ratio.

Patient-based reference intervals

Although considerable difficulties are associated with obtaining blood samples of healthy children based on

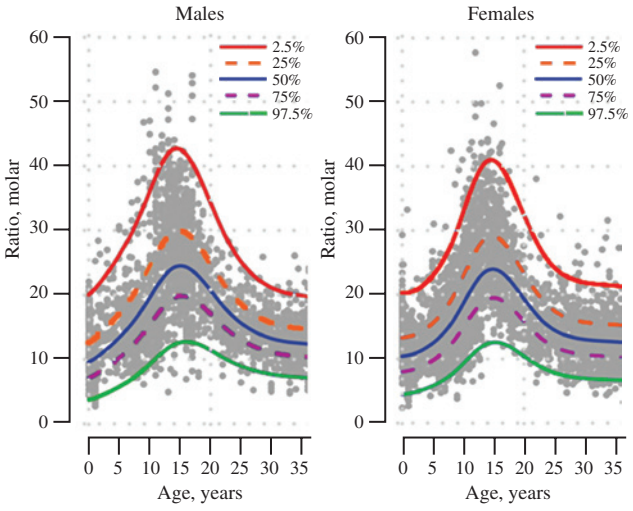

Figure 3: Ratio of IGF-I to IGFBP-3 molar concentration values (multiplied by a factor of 100) in serum of male (left side) and female (right side) subjects from early childhood to young adult age. The lines represent the 2.5th, 25th, 50th, 75th, and 97.5th percentiles calculated by quantile regression via vector generalized additive models. (Modified after Friedrich N, Wolthers OD, Arafat AM, Emeny RT, Spranger J, Roswall J, et al. 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. J Clin Endocrinol Metab 2014;99:1675–86. [28]).

Table 2: Reference intervals (2.5th and 97.5th percentiles) for concentrations of IGFBP-3 (μg/L) derived from the study of 854 healthy Danish children, separated by Tanner stage.

Gender	Tanner stage	Age range, years	2.5%	97.5%
Males	I	6.1–12.9	9.4	23.1
	II	8.1–14.8	10.6	33.0
	III	10.9–16.0	21.0	39.6
	IV	12.4–17.1	19.3	41.8
	V	13.5–20.0	18.4	35.2
Females	I	5.8–12.1	10.3	26.3
	II	9.3–14.1	12.9	32.4
	III	9.3–15.1	20.4	38.9
	IV	11.8–16.6	15.2	41.6
	V	12.5–19.9	14.2	36.1

Friedrich N, Wolthers OD, Arafat AM, Emeny RT, Spranger J, Roswall J, et al. 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. J Clin Endocrinol Metab 2014;99:1675–86. [28].

a volunteer participation, there is an enormous wealth of laboratory test results for children's samples resting in the LIS worldwide [29, 30]. Using several statistical approaches [31, 32], these data were used to derive age-specific reference intervals in several studies. The rationale behind the approach in these studies was usually to use outpatient children's samples and introducing corrections for obviously pathological results.

In a large study performed in Singapore and Australia [33], results of 16 common clinical biochemistry tests of ambulatory pediatric patients aged 0–19 years, requested by primary care physicians over a period of 12 months, were retrospectively evaluated and used to construct smoothed centile charts using a penalized maximum likelihood method [34]. The authors observed increased concentrations of sodium, bicarbonate, creatinine, urate, total protein, and albumin with increasing age of the children. In contrast, the concentrations of potassium, chloride, anion gap, calcium, phosphate, and LD decreased with increasing age of the children. Changes in the concentrations of urea, ALP, glucose, and total cholesterol varied by age. Generally, boys and girls shared similar trend patterns until 10–15 years of age, when variations in the age of onset of puberty and development caused the trends of some analytes to differ.

In a similar study performed by Loh et al. [35], the authors used the test results resting in their LIS to obtain data on within-individual biological variation (CVi) in children because it is a particular challenge to derive these values by direct sampling. Laboratory results of 22 basic clinical chemistry assays performed on 9356 children who visited primary care physicians more than once over a year were obtained from a large laboratory network in Australia. Smoothed 50th centile (median) CVi charts were derived using the LMS method [36]. In general, the median CVi trends for this pediatric cohort remained relatively stable with increasing age. Only for AST, γ-globulin, phosphate, urea, and creatinine, differences of more than 30% between the highest and the lowest median CVi were observed. Furthermore, the differences between child and adult CVi were relatively small. Nearly all the analytes had child/adult CVi ratios of 1.0±0.5. The authors concluded that median CVi derived from patients with only two repeat biochemistry measurements may be considered reasonable estimates of CVi values among children seeking treatment at primary care settings. The LMS approach allowed a visualization of the continuous trends of CVi with age and extended the pediatric CVi estimation to an age of younger than 4 years.

Søeby et al. (29) used concentrations of creatinine as a model analyte to explore the utility of hospital laboratory data as a source of information. Creatinine concentrations (determined using an enzymatic method) in plasma of 9700 children aged 0–18 years were obtained from LIS databases of two large pediatric hospitals and partitioned into high-resolution gender and age groups. Normal probability plots were used to deduce the parameters of the distributions and thus reference intervals for creatinine values in the mixed hospital data sets. Furthermore, for an examination of developmental patterns in periods of changing creatinine levels, temporal trajectories were generated from repeated creatinine determinations. As observed previously, concentrations of creatinine showed a great age dependence from birth throughout childhood. These pronounced transitions in creatinine levels at different time points after birth and around the early teens again underscore the need for an establishment and the usefulness of age-specific reference intervals. The authors showed the reference interval generated from their LIS data to be well comparable to those determined in studies in the healthy population and that, at least in the case of creatinine, hospital laboratory data may be used to obtain valid reference intervals.

Another approach to derive continuous age-dependent reference intervals from clinical laboratory databases containing datasets of both healthy and pathological samples was used by Zierk et al. [37] for hematology parameters determined using the Sysmex XE-2100 hematological analyzer in ca. 60,000 individual samples of children from birth to adulthood. Results were separated according

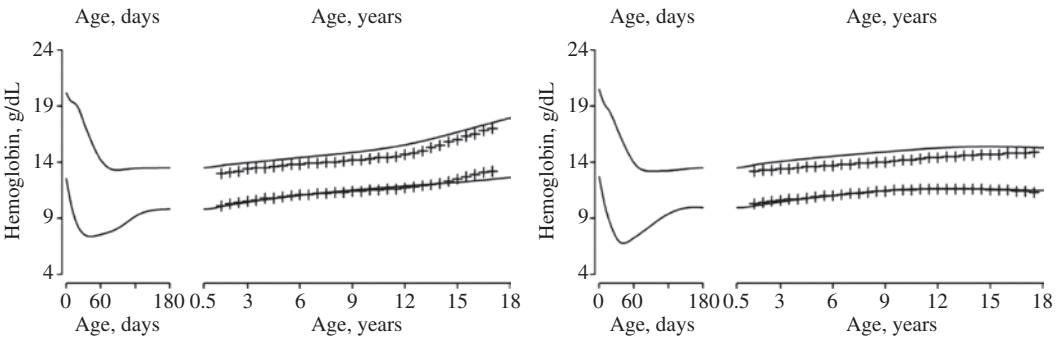

Figure 4: Graphical representation of Hb reference intervals (RIs) calculated using patient-derived data.
Calculated Hb RIs (continuous line) vs. RIs determined in the KiGGS study (crosses). (Left) Boys. (right) Girls. (Zierk J, Arzideh F, Haeckel R, Rascher W, Rauh M, Metzler M. Indirect determination of pediatric blood count reference intervals. Clin Chem Lab Med 2013;51:863–72. [37] With permission from De Gruyter Publishers.)

to age, and a density function of the proportion of samples from healthy children was estimated for each age group. The resulting reference intervals were merged to obtain continuous reference intervals from birth to adulthood, which were compared to those generated by identical laboratory instruments, and to population study-generated data such as those obtained in the KiGGS study. This comparison showed a high concordance as exemplified in Figure 4. Although the authors concluded that the indirect approach are well-suited to create continuous, intralaboratory reference intervals from LIS databases and that they are comparable to those created using population derived methods, a thorough examination of the results suggests a small but not neglectable bias.

Using outpatients' blood samples obtained at both Children's National Medical Center and Georgetown University (Washington, DC, USA) over a period of more than 4 years, Soldin et al. [38] determined concentrations of the steroid hormones aldosterone, 17α-hydroxyprogesterone, dehydroepiandrosterone, testosterone, and 25(OH)-vitamin D_3 using isotope dilution LC/MS/MS. From the respective results, reference intervals for children from birth to 18 years of age were established. These authors found that all the analytes exhibited at least some age dependence, whereas gender differences between early and late childhood and adolescence were found for 17α-hydroxyprogesterone and testosterone. Not surprisingly, seasonal differences were apparent for 25(OH)-vitamin D_3.

Other studies describing the generation of reference intervals from patient-derived laboratory results comprise a cohort of 2474 patients, aged 2–16 years, who underwent a short-stature workup but were diagnosed as normal in Korea [39] in which concentrations of serum calcium, inorganic phosphorus, blood urea nitrogen, creatinine, uric acid, glucose, total cholesterol, total protein, albumin, ALP, aspartic aminotransferase, ALT, and total bilirubin were determined, and a study by Cangemi et al. [40] who used their LIS data to establish reference intervals for total adiponectin determined in more than 4000 samples. These authors found no correlation between age and adiponectin concentrations in obese children, but significant relations were observed in the total group of patients and in healthy control subjects.

By the investigators of the CALIPER project, a critical study on the validity of patient-derived reference intervals has recently been published [41]. They analyzed LIS-based data for 13 analytes (calcium, phosphate, iron, ALP, cholesterol, triglycerides, creatinine, direct bilirubin, total bilirubin, ALT, AST, albumin, and magnesium), determined using the thin-film slide technique ("Vitros 5600"; Ortho Clinical Diagnostics), which had been collected over a period of 4 years. The data for each analyte were partitioned by age and gender in the same way as in the CALIPER study. After removal of outliers, reference intervals were calculated by extrapolating to identify the 2.5^{th} and 97.5^{th} centiles in each partition. Using the 90% confidence intervals for the reference intervals defined by CALIPER, the LIS-based reference intervals were compared to those reported previously by CALIPER. The LIS-calculated reference intervals were found to be generally wider than those calculated by CALIPER, and none fell completely within the 90% confidence intervals calculated by CALIPER. These authors concluded that calculating pediatric reference intervals from hospital-based data may be useful as a guide in some cases but will likely not replace the need to establish reference intervals in healthy pediatric populations.

Table 3 lists a synopsis of selected studies for the establishment of pediatric reference intervals for both

Table 3: Synopsis of selected recent studies for the establishment of pediatric laboratory medicine reference intervals.

Country	Number of children	Age range covered	Analytes or areas covered (main emphasis)	References
(A) Population-based results				
Germany	14,000	1–18 years	General clinical chemistry (enzymes, electrolytes, metabolites, proteins), blood count, iron metabolism (iron, ferritin, soluble transferrin receptor), vitamins (D and B12/folate)	[7, 8]
Denmark	1429	5–19 years	General clinical chemistry (enzymes, electrolytes, metabolites, proteins)	[9]
Sweden	694	6 months–18 years	Electrolytes, metabolites, enzymes, iron metabolism, thyroid hormones, lipids	[11–14]
Canada	2188	0–18 years	General clinical chemistry (enzymes, electrolytes, metabolites, proteins)	[3]
Canada	1234	0–18 years	Fertility hormones (estradiol, testosterone, progesterone, SHBG, prolactin, FSH, LH)	[17]
Canada	1482	0–18 years	Hormones (cortisol, vitamin D, PTH, TSH, T4, fT4, T3, fT3), ferritin, vitamin B12, folate, homocysteine, troponin I	[18]
Ethiopia	60 57	Neonates Infants	Electrolytes (Na, K, Cl)	[22]
Zimbabwe	269	3–9 months	Blood count, CD4 lymphocytes	[23]
Tanzania	655	1 months–18 years	Blood count, CD4 lymphocytes	[24]
Saudi Arabia	2149	6–18 years	Glucose, cholesterol, HDL and LDL cholesterol, triacylglycerol	[25]
Melanesia	327	1–10 years	Blood count, iron metabolism (ferritin, soluble transferrin receptor), electrolytes, proteins	[26]
(B) Patient-based results				
Australia	56,700	0–19 years	General clinical chemistry	[33]
Denmark	9700	0–18 years	Creatinine	[29]
Germany	60,000 (number of samples)	0–19	Blood count	[37]
Canada	1200	0–18 years	General clinical chemistry (selected electrolytes, enzymes, proteins)	[41]

the population-based approach and the patients' data approach.

Preterm neonates: the most highly challenging group

Although the collection of blood samples from volunteering healthy children may be relatively easy to realize, this approach is virtually impossible for preterm neonates. However, reference intervals for laboratory results are needed for this patient group even more badly than for older children. Consequently, the use of data obtained during clinical care and stored in the LIS are currently the only source of reference intervals for preterm neonates, which has been shown in a few recent publications.

As the clinical interpretation of gonadotropins is important in the context of ambiguous genitalia, Greaves et al. [42] performed a study to develop reference intervals for LH and FSH in infants born between 24 and 29 weeks' gestation. Samples were collected at 0–43 days after birth from 82 premature infants, and concentrations of LH and FSH were determined on the Siemens "Advia Centaur" analyzer. Forty-three male infants demonstrated a range of LH levels from 0.1 to 13.4 IU/L and of FSH levels from 0.3 to 4.6 IU/L. Thirty-nine female infants demonstrated a range of LH levels from 0.2 to 54.4 IU/L and of FSH levels from 1.2 to 167 IU/L. The ratio of LH/FSH levels differed with males, ranging from 0.3 to 9.4, and females, at <0.5. These data may provide guidance for the interpretation of LH and FSH levels for the first 6 weeks of life in extremely premature infants born between 24 and 29 weeks' gestation.

For the same authors, the immaturity of the endocrine system and its potential impact on morbidity served as motivation for another study to obtain age appropriate hormone reference intervals for preterm neonates using the Siemens "Immulite2000" analyzer [43]. Serum samples were collected from babies born 23–29 weeks' gestation at 1, 4, 7, 14, 21, 28, and 42 days after birth. Using the results

from the 107 infants who survived beyond 40 weeks' corrected gestational age, reference intervals for cortisol, dehydroepiandrosterone sulfate, growth hormone, and progesterone concentrations were calculated and found to be highest during the first 7 days, whereas fT4 levels were as low as <2.6 pmol/L for the first 28 days with the nadir at 7 days. Concentrations of estradiol showed a broad range from <73 to 1626 pmol/L over the 6 weeks of the study, whereas concentrations of IGF-1 were below the analyzer's sensitivity. There were no differences in reference intervals between male and female infants.

Zhu et al. [44] investigated the concentrations of thyroid hormones (T3, fT3, T4, fT4, and thyrotropin) as determined using an electrochemiluminescence assay in samples of 247 hospitalized preterm infants from 28 to 36 weeks' gestation at 8–15 postnatal days. They observed no differences in concentrations of TSH between the different age groups using Kruskal-Wallis H tests, which consequently led to a single reference interval; significant differences in T3, fT3, T4, and fT4 concentrations between the age groups were detected by analysis of variance (ANOVA), which resulted in gestational age-related reference intervals.

Cystatin C is increasingly regarded as superior alternative to creatinine for assessing the renal function. Hahn and Bae [45] recently published the results of a study with on a total of 883 blood samples collected from 246 neonates including very-low-birth-weight infants during the first 30 days of life for the concurrent determination of serum cystatin C and creatinine concentrations as well as the calculation of the ratio of these analytes. For their data analyses, they excluded infants with symptoms or signs of acute kidney injury, systemic illness, congenital anomaly, or renal pathology. These authors found that the concentration ratio increased with the postconceptional age, except in the first three postnatal days, and it correlated positively with gestational age at birth, birth weight, postnatal age, and postconceptional age. They calculated reference intervals for the concentration ratio according to postnatal and postconceptional age. Concentrations of cystatin C were also the focus of two other investigations published by Elmas et al. [46] and Bariciak et al. [47] who studied 52 and 128 preterm and term neonates, respectively. The first group observed only a trend toward higher cystatin C concentrations in the infants with the lowest gestational age but the differences were not statistically significant. Concentrations of cystatin C were also independent of birth weight and gender. The second team calculated reference intervals, categorized by age, for their group of 128 neonates and observed a decline in concentrations of cystatin C from day 1 to day 3 after birth reflecting he maturation of renal function after birth.

Long-term observational studies

By definition, assessing the health status from laboratory results obtained in cross-sectional studies lacks the element of individuality, which may, however, be taken into account using a concept of an intraindividual reference range, at least for analytes in which it has been shown that the intraindividual variation is much smaller than the interindividual variation. For this approach, it is necessary to have access to results of long-term observational studies. Southcott et al. [48] studied a cohort of 852 healthy 8-year-old Australian children who were enrolled in a community-based multidisciplinary longitudinal study investigating how early physical activity contributes to health. The same children came back for reassessment at ages 10 and 12 years. Each time, blood samples were analyzed for a total of 37 different clinical chemistry analytes. Reference intervals and intraindividual variation were derived for all the analytes for males and females separately.

In a subset of this study, Koerbin et al. [49] determined concentrations of cardiac troponin I (cTnI), calculated the 99th percentile values, and made estimates of the long-term biological variation. They found that concentrations of cTnI were above the limit of detection in the vast majority of the children and that the 99th percentiles were lower compared to a healthy adult population in both girls and boys at all ages studied. Reflecting the element of individuality, the calculated 99th percentile varied markedly depending upon whether the lowest or highest cTnI measurement for an individual child was included in the calculation. The biological variation of the concentrations of cTnI varied markedly between 0% and 136%, and the reference change value was an increase of 147% or a decrease of 59%. As different children showed concentrations of cTnI above the 99th percentile at the 3 periods of study assessment, the authors of the study concluded that this 99th percentile may not be a reliable index of silent cardiac disease in children, but rather be indicating a low-grade intercurrent illness.

Preanalytical considerations

In many of the studies performed to obtain pediatric reference intervals, preanalytical conditions could not always be strictly optimized. However, this may not always lead to the loss of validity of its results, but the effects have to be known. In a study to quantify the impact of food consumption and the time of day [50], blood samples were

drawn from 27 healthy children and adolescents (aged 4–18 years) with informed consent at four time points: after overnight fast, mid-morning after breakfast, within 2 h after lunch, and late afternoon. Fasting significantly affected the concentrations of 22 of the 38 analytes determined, as evaluated by paired, two-tailed Student's *t*-tests, with HDL cholesterol being the most highly affected. Values tended to decrease postprandially, except for five analytes, including triglycerides, which increased. The analysis of the data by paired, repeated-measures ANOVA showed that 28 analytes significantly differed across times of day tested. Thus, it is necessary to use fasting samples for the determination of certain analytes in children, and pediatricians should consider diurnal factors in the ordering of laboratory investigations.

Determination of clinical chemistry analytes in population-based studies is commonly performed using batches of serum or plasma samples stored at low temperatures. In a study aimed to determine the stability of selected analytes under these conditions [51], serum samples collected from children of 0 to 18 years of age attending outpatient clinics were pooled into a single pool or into age group-specific pools. Following baseline measurement, each pool was aliquoted and kept frozen at −80 °C until analysis. Concentrations of 57 analytes were determined at monthly intervals over a 10- to 13-month period. The comparison of results obtained at monthly intervals to baseline measurements revealed that concentrations of the majority of analytes determined in this study showed no significant time-dependent change relative to baseline or any trend over time after up to 13 months of storage. However, concentrations of PTH declined by up to –27.2% after 10 months of storage with most of the decline becoming evident already after 2 months. The variability that was observed for the concentrations of most analytes over time probably reflects assay variability rather than changes in analyte stability. Thus, samples do not require immediate testing for reference interval determination for the selected analytes with possible exception of PTH.

Special aspects and follow-up studies of reference interval investigations

Obviously, the influence of the pubertal stage can be expected to have an additional impact beyond that of age on reference intervals for hormones related to growth and sexual development. However, information about the pubertal stage present in healthy children participating in population-derived studies is rarely available. A few data are nevertheless available and illustrate the need to take this variable into account. One example is the analysis of IGF1 and IGFBP-3 concentrations in a Danish cohort with classification according to Tanner stages by Friedrich and colleagues [27] (see Table 2), which clearly shows that the Tanner stage of a child in puberty should be known for the correct evaluation of an individual IGF1 or IGFBP-3 concentration.

Sometimes, studies conducted for the establishment of pediatric reference intervals may reveal additional information. Ridefelt et al. [52] used data from a Swedish project to detect transient hyperphosphatasemia, an often unnoticed benign entity primarily affecting children younger than 5 years of age with an unknown prevalence. Of the 97 subjectively healthy subjects aged 6–22 months studied, 6 children (4 girls and 2 boys), but none of the older children, showed concentrations of ALP of >1000 U/L. Thus, the prevalence in the age group from 6 months to 2 years can be calculated as 6.2%. Although the study did not include the follow-up of these apparently healthy children, conditions others than transient hyperphosphatasemia explaining the elevated ALP could not be excluded. However, concentrations of liver enzymes, calcium, intact PTH, and vitamin D were essentially normal in these children supporting the diagnosis of transient hyperphosphatasemia.

Data collected during studies conducted with healthy children are often used not only for establishing reference intervals but also for investigations on other areas that are in many cases the main focus of the study. Thus, in the years following their collection, the data obtained during the German KiGGS study have been further evaluated in numerous projects [53–63], e.g. investigations on the interrelationships of endocrinological parameters such as thyroid hormone concentrations and iodine status, or assessment of seropositivity rates for several viral infective agents. Furthermore, correlations of the data obtained in the laboratory with clinical parameters have been investigated, such as vitamin D concentrations and endurance performance or thyroid hormone concentrations and blood pressure.

Conclusions

In contrast to the situation encountered a decade ago, clinical laboratory scientists can today rely on valid reference intervals for many common analytes covering all ages of children from birth to adolescence as basis for

their clinical decisions. Although it is still sometimes tedious to find the appropriate data, modern information technology has facilitated this task substantially. It is hoped that by the ongoing activity of the IFCC TF-PLM, a growing database accessible to all health-care providers for children will be continuously filled with increasing numbers of analytes in all areas of clinical laboratory medicine including novel biomarkers, eventually creating an international "reference interval pool". Obviously, this will only be possible with the help of many contributors in all areas of the world submitting the results of their studies or observations.

There are many more analytes used in today's laboratories than those for which pediatric reference intervals have been published up to now, especially in the field of endocrinology, where the lack of standardization and the multitude of different immunoassays often inhibit large studies. Furthermore, as laboratory medicine progresses, e.g. into areas such as more detailed metabolic analyses or proteomics, which are evidently of great importance for the health care for children of all ages, a need of establishing reference intervals also for these analytes will concomitantly arise. Thoroughly validated normative data regarding these areas, however, will probably be even harder to obtain than the analytes investigated up to now. Again, this will require the cooperation of all scientists and physicians who are active in pediatric laboratory medicine worldwide.

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