Age- and Sex-Specific Dynamics in 22 Hematologic and Biochemical Analytes from Birth to Adolescence

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BACKGROUND: Pediatric laboratory test results must be interpreted in the context of interindividual variation and age- and sex-dependent dynamics. Reference intervals as presently defined for separate age groups can only approximate the age-related dynamics encountered in pediatrics. Continuous reference intervals from birth to adulthood are not available for most laboratory analytes because of the ethical and practical constraints of defining reference intervals using a population of healthy community children. We applied an indirect method to generate continuous reference intervals for 22 hematologic and biochemical analytes by analyzing clinical laboratory data from blood samples taken during clinical care of patients.

METHODS: We included samples from 32 000 different inpatients and outpatients (167 000 samples per analyte) from a German pediatric tertiary care center. Measurements were performed on a Sysmex-XE 2100 and a Cobas Integra 800 during clinical care over a 6-year period. The distribution of samples considered normal was estimated with an established indirect statistical approach and used for the calculation of reference intervals.

RESULTS: We provide continuous reference intervals from birth to adulthood for 9 hematology analytes (hemoglobin, hematocrit, red cell indices, red cell count, red cell distribution width, white cell count, and platelet count) and 13 biochemical analytes (sodium, chloride, potassium, calcium, magnesium, phosphate, creatinine, aspartate transaminase, alanine transaminase, γ-glutamyltransferase, alkaline phosphatase, lactate dehydrogenase, and total protein).

CONCLUSIONS: Continuous reference intervals capture the population changes in laboratory analytes during pediatric development more accurately than age groups. After local validation, the reference intervals provided should allow a more precise consideration of these dynamics in clinical decision making.

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The interpretation of laboratory test results in pediatrics is performed in the context of age- and sex-dependent dynamics, as physiological development leads to changes in many of the analytes measured, particularly in the first years of life and during puberty. To reflect inter- and intraindividual variation in laboratory tests, clinical decision making is generally guided by reference intervals, which are defined as the 2.5th and 97.5th percentiles of a healthy population’s distribution (1–4).

Partition into discrete age groups for both males and females is commonly performed to represent the age and sex dependence of laboratory analytes when reference intervals are used. Age groups are selected using visual inspection and statistical tests to approximate change in analyte concentration with age (5, 6). However, discrete age groups do not adequately reflect the continuous changes of biological development and thus cannot always represent the exact extent and onset of age-dependent dynamics. In analogy with other developmental quantities routinely specified in relation to age (e.g., weight- and height-for-age charts), a continuous description would seem to be a more appropriate approach for laboratory analytes (7, 8).

Such an approach is restricted by the requirement of a large number of samples from healthy children (9). According to the generally accepted recommendation of an IFCC expert group, approximately 120 samples are needed to establish reliable reference intervals for a homogenous population (1, 2); creation of continuous age-dependent reference intervals with these procedures would require many more samples to account for variation in analyte concentration with age. Because access to blood samples from healthy children is limited by ethical

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and practical constraints, this procedure is infeasible in many settings (3, 9). Newborn and infant children are most affected by these restrictions, although precise age-adjusted reference intervals are especially important in these age groups because of the significant age-related contribution to pediatric morbidity and pronounced physiological development with consecutive changes in laboratory analytes. Great efforts have been undertaken to address these generally recognized issues (10, 11). The Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER)4 provides sex-specific reference intervals from birth to adulthood for >80 biochemical analytes derived from a population of >8600 healthy children (6, 12–15). The continuous dynamics for many of the examined analytes are acknowledged and carefully considered in the selection of age partitions. Separation into age groups with significant differences, however, invariably leads to a discontinuous representation of change with age. In the German Health Interview and Examination Survey for Children and Adolescents (KiGGS), continuous reference intervals are reported for 28 analytes on the basis of a representative German cohort of 14 255 children (16, 17). However, the critical age group of newborns and infants was excluded from blood sampling in the KiGGS survey for the ethical and practical reasons mentioned above.

In a recent pilot study, we have shown an alternative method for the determination of continuous reference intervals that avoids these obstacles (18). Reference intervals are calculated from a laboratory database containing a mixture of healthy and pathologic samples; the distribution of supposedly healthy samples is estimated from the whole data set with a statistical approach and used to calculate continuous reference intervals. Comparison with reference intervals determined with conventional methods from the KiGGS survey showed a high concordance of reference limits and their age-dependent dynamics, thereby proving the feasibility of this indirect method and making it a viable alternative to conventional approaches when these are limited.

The purpose of the present report is to apply the indirect approach to a broader panel of hematologic and biochemical analytes (Table 1). The resulting continuous reference intervals should demonstrate the gradual influences of age and sex and therefore allow more precise clinical decision making on the basis of laboratory results. The effect on the classification of laboratory samples as healthy or pathologic is analyzed by comparing the original categorization (performed with conventional reference intervals) to categorization performed with the newly established continuous reference intervals.

**Methods**

We analyzed measurements of 22 analytes performed during clinical care of patients in the Department of Pediatrics and Adolescent Medicine, University Hospital Erlangen, a tertiary care center covering the entire spectrum of pediatrics.

**STUDY POPULATION**

The population in Germany—and our hospital’s patient population—is composed predominantly of white individuals; stratification according to ethnicity was not performed.

**SELECTION OF ANALYTES**

Analytes were selected according to clinical relevance and methodologic requirements. We considered analytes for which a large number of measurements would allow application of the indirect approach used and where the availability of sex-stratified continuous reference intervals from birth to adulthood might benefit clinical decision making. This resulted in the identification of 13 biochemical analytes [sodium, chloride, potassium, calcium, magnesium, phosphate, creatinine (enzymatic), aspartate transaminase (AST), alanine transaminase (ALT), γ-glutamyl transferase (γ-GT), alkaline phosphatase, lactate dehydrogenase (LDH), and total protein] and 9 hematology analytes [hemoglobin, hematocrit, mean red cell hemoglobin (MCH), mean red cell hemoglobin concentration (MCHC), mean red cell volume (MCV), red cell count, red cell distribution width, white cell count, and platelet count] (see Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol61/issue7). All the measurements of these analytes performed for inpatients and outpatients aged ≤18 years, including patients from intensive care units and specialty units, were retrieved from the laboratory’s database. The time period examined spanned January 2004 to April 2013 (biochemical analytes except for AST, ALT, γ-GT) and April 2008 to April 2013 (hematologic analytes, AST, ALT, and γ-GT) to provide a maximum number of samples measured with the same methods. Analysis of γ-GT activity was performed for children aged 6 months to 18 years, as the number of available measurements allowed valid application of the algorithm in this interval only. For each analyte, 45 978 to 210 239 measurements (from 11 162 to 34 807 distinct individuals) were available for analysis (Table 1).

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*Nonstandard abbreviations: CALIPER, Canadian Laboratory Initiative on Pediatric Reference Intervals; KiGGS, German Health Interview and Examination Survey for Children and Adolescents; AST, aspartate transaminase; ALT, alanine transaminase; γ-GT, γ-glutamyl transferase; LDH, lactate dehydrogenase; MCH, mean red cell hemoglobin; MCHC, mean red cell hemoglobin concentration; MCV, mean red cell volume.*
ANALYTICAL PROCEDURES
Measurements of biochemical analytes were performed on a Cobas Integra 800 (Roche Diagnostics), and blood counts were measured on a Sysmex XE-2100 (Sysmex Europe). Method details are available in online Supplemental Table 1, and precision data are presented in online Supplemental Table 2. The stability of each analyte over time during the study period is demonstrated by stable monthly median values (see online Supplemental Tables 3 and 4).

CALCULATION OF CONTINUOUS REFERENCE INTERVALS
We calculated continuous reference intervals with an indirect method described and validated previously (18); method details are available in the online Supplement. The approach is based on the assumption that the input data set consists of a mixture of parametrically distributed samples from healthy individuals and random pathologic samples (i.e., the proportion of nonpathologic samples is modeled with a statistical distribution, whereas the pathologic samples are assumed to be scattered randomly). When applied to a sufficiently large data set, these 2 sample sets can be reliably distinguished by a statistical algorithm. We estimate the underlying parametric distribution of healthy samples, and its 2.5th and 97.5th percentiles define the reference interval. The algorithm comprises 3 basic steps for each analyte: (a) partition of input data into age groups; (b) generation of discrete reference intervals for these groups; and (c) conversion of these discrete reference intervals into continuous reference intervals.

Partition into age groups: We split the samples in overlapping age groups to achieve a sample count of 500–2500 per group, depending on the analyte (Table 1) (18). Samples in each age group were selected from different

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<th>Table 1. Number of samples and distinct patients available for each analyte and number of groups after partition into age groups. a</th>
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a Group size denotes the number of samples in each age group.
Because the number of available samples is variable and depends on the age period examined, each age group encompasses a time interval of a different length. Separation into overlapping age groups resulted in 38–197 groups (Table 1).

**Generation of discrete reference intervals.** We applied an indirect method for reference interval determination—an expanded version of an algorithm developed by Arzideh and colleagues (19–21)—to each age group. Briefly, a smoothed kernel density function is estimated for the distribution of the data. The “central” part of this distribution is assumed to represent the main part of the healthy population and is defined by truncation points in the Box–Cox transformed data set with an optimization method. A gaussian distribution of the central part is estimated, and its 2.5th and 97.5th percentiles are calculated to obtain the reference intervals.

**Conversion into continuous reference intervals.** For each analyte, we generated parametric curves for the 2.5th, 50th, and 97.5th percentiles. We used the R software’s smooth.spline function to convert the reference limits into a continuous interval (22) and chose the number of degrees of freedom by visual inspection of the curves generated. Two different curves were created for most analytes to adequately represent the different dynamics of the neonatal period and infancy on the one hand and toddlerhood to adolescence on the other hand.

**COMPARISON OF REFERENCE INTERVALS**

A comparison of the generated reference intervals to results from KiGGS (17) and CALIPER (6) is shown in online Supplemental Figs. 1 and 2. In KiGGS, hematology analyses were performed on a Cell-Dyn 3500 (Abbott) and biochemical analyses were performed on a...
Hitachi 917 (Roche Diagnostics); in CALIPER, analyses were performed on an Architect c8000 (Abbott). The same methods were used in general, except for phosphate, magnesium, and calcium (phosphomolybdate, Arsenazo, and Arsenazo III, respectively, in CALIPER).

The hypothetical effect of the newly established continuous reference intervals on classification of samples in the examined data set as healthy or pathologic is quantified in Table 2. We compared classification with the current categorization method (using conventional reference intervals from published literature or the manufacturer) to categorization performed with the newly established continuous reference intervals. Patients with multiple measurements of a certain analyte were classified as healthy if all their measurements for that analyte were also classified as healthy.

Results

We calculated continuous age- and sex-specific reference intervals for 13 biochemical analytes and 9 hematology analytes. Graphical representations of the reference intervals are provided in Fig. 1A (creatinine), Fig. 2 (biochemical analytes without creatinine), and Fig. 3 (hematologic analytes). Data tables containing age-specific upper and lower reference limits to enable integration of the calculated reference intervals into laboratory information systems are available in the online Supplemental Database.

The majority of the analytes showed substantial age-specific dynamics, especially in the first months and years of life and after the onset of puberty. These age-dependent changes could only be approximated by reference intervals for distinct age groups, as cutoff points that would allow partition into separate age groups are nonexistent. The continuous change in creatinine reference intervals with age and the progressive divergence of male and female reference intervals are highlighted in Fig. 1A. The upper and lower reference limits underlying the reported continuous reference intervals for creatinine concentration are presented in Fig. 1B to demonstrate that a separation into age groups would be arbitrary and not due to biological changes in analyte concentration. Fig. 1C shows a comparison of continuous reference intervals for creatinine concentration to age-grouped reference intervals from CALIPER and highlights the problem of representing the age dependence of creatinine concentration with distinct age groups. Deficiencies become especially apparent at age group margins in infancy, e.g., when creatinine reference limits change by a factor of 3 [from 0.32–0.92 mg/dL (0–14 days) to 0.10–0.36 mg/dL (15 days to <2 years)]. Detailed consideration of sex-specific differences shows that these can be observed after the onset of puberty, but not before, in many analytes (alkaline phosphatase, ALT, AST, \( \gamma \)-GT, LDH, hematocrit, hemoglobin, red cell count). Examples of this correlation are highlighted in Fig. 4, which shows sex-specific differences in alkaline phosphatase activity and hemoglobin and creatinine concentration in relation to Tanner stage PH2 (i.e., the appearance of pubic hair as a physical indicator of puberty onset).

Some analytes showed similar patterns of age-dependent change. A decline in concentration during the
first months and years of life and a subsequent rise, with higher concentrations in males than in females after puberty, was observed in hemoglobin concentration, hematocrit, red cell count, and creatinine concentration (Figs. 3 and 1A). A fast decline in activity of AST, ALT, and LDH during infancy was followed by a slower decline until 18 years of age, with a lower activity in females after puberty (Fig. 2). Red cell indices (MCH, MCHC, and MCV), red cell distribution width, and white cell count decreased continuously in infancy and stabilized afterward without substantial sex-specific differences. Plasma electrolytes and total protein concentration likewise exhibited age-specific changes in concentration but no substantial sex-specific differences. Platelet count rose during the first months of life and decreased afterward, and a higher platelet count was observed in females than in males in infancy and adolescence. Alkaline phosphatase showed the most complex pattern of change in analyte concentration over time: a decline in the first 4 years of life was followed by a rise with sex-specific onset, extent, peak, and subsequent decline. The increase in activity observed in females had an earlier onset, peak, and decline but was less pronounced than the rise in activity in males.

Comparison of the reference intervals provided with results from CALIPER and KiGGS showed consistent upper and lower reference limits and onset of age-dependent changes (see online Supplemental Figs. 1 and 2). Major differences, however, were observed between magnesium reference intervals in the CALIPER trial and those reported herein; comparison of our results to findings from the KiGGS survey and other sources (23, 24) showed no such differences and confirmed the reported reference intervals.

Use of the continuous reference intervals in comparison to the currently used reference intervals...
(mainly supplied by manufacturers or from published literature) resulted in a substantial reduction in the number of samples considered pathologic. The decrease in samples classified as pathologic was most pronounced for blood count analytes (except red cell distribution width) and less pronounced for most biochemical analytes. The proportion of samples classified as pathologic increased for AST, ALT, potassium, and LDH. Differences in classification of test results as healthy or pathologic with either the continuous reference intervals or the currently used reference intervals are summarized in Table 2.

Discussion

We report continuous reference intervals for 22 important hematologic and biochemical analytes from birth to adulthood. Unlike most reference intervals currently used, which are valid for discrete age intervals stratified according to sex, we present a continuous classification strategy, allowing a precise representation of age- and sex-dependent change during development. This is possible because the analysis of a comprehensive clinical laboratory database with an indirect approach has made a large number of measurements available for evaluation.
The number of samples analyzed (45,978 to 210,239, depending on the analyte) eliminates the need to approximate age-dependent change in analyte concentration with separate age groups. Accordingly, our data show gradual changes in analyte concentration over time rather than abrupt changes at well-defined time points (Figs. 1A, 2, and 3). Likewise, analysis of the discrete reference limits underlying the reported continuous reference intervals shows the absence of physiologically sensible cut-off points (Fig. 1B). This reflects the notion that separation into age groups is a technical limitation that can be overcome when a sufficient number of samples are available (7, 8).

The physiological developments leading to variation with age include the effects of increased creatinine production with muscle growth and maturation of renal function on creatinine concentrations (Fig. 1A). Transition from fetal to adult erythropoiesis and the physiologic anemia of infancy are accompanied by gradual changes in hemoglobin concentration, red cell count, red cell indices (MCV, MCH), and red cell distribution width (Fig. 3). Additionally, sex-dependent changes during and after puberty can be observed in many analytes (Figs. 2 and 4). The continuous nature of these changes is well established and is generally incorporated into clinical decision making on the basis of laboratory test results (3, 10, 25, 26). However, the availability of continuous reference intervals allows more precise consideration of these dynamics and better differentiation of change due to physiological development and change due to disease. Direct comparison of continuous reference intervals to age-grouped reference intervals highlights these advantages (Fig. 1C; online Supplemental Fig. 2). The potential benefits of continuous reference intervals could be limited in practice by current approaches to laboratory test result reporting that might in fact complicate interpretation. Therefore, improved forms of result reporting are necessary. Suitable representations include graphical result display (e.g., as in growth charts) or reporting of z-scores/percentiles instead of absolute values. Although the technical basis exists for such representations, they are rarely incorporated into current clinical practice. The continuous reference intervals provided can therefore serve as an incentive for laboratory software manufacturers to implement new strategies for result display.

Experimental application of the continuous reference intervals, in contrast to the reference intervals used previously, leads to substantial shifts in classification of samples as pathologic or not (Table 2). These data have to be interpreted with caution, as they are highly specific to our hospital population and the analytical methods used. Furthermore, the same data set that has been used to generate reference intervals has also been examined for shifts in classification; a decrease in samples classified as pathologic is therefore to be expected. However, 2 observations are noteworthy. First, the extent of change in classification is substantial, ranging from a 38% decrease to a 9% increase in the number of samples considered pathologic and a 23% decrease to a 14% increase in patients having at least 1 measurement considered pathologic. Second, the shifts in classification are more distinct in analytes exhibiting pronounced variation with age (e.g., hemoglobin, red cell indices, creatinine concentration) than in those analytes that do not (e.g., plasma electrolytes). These findings highlight the clinically relevant limitations of current reference intervals in representing the dynamics encountered in pediatric laboratory medicine.

Reference intervals in our study were determined with an indirect approach; the population analyzed consisted of inpatients and outpatients from a pediatric tertiary care center. The use of indirect methods for reference interval estimation is controversial, and the capability of these methods to correctly identify the subset of nonpathologic samples has been challenged (3, 7, 27–29). Therefore, validation of our results in other patient cohorts and with other analytical instruments is strongly warranted. However, concerns related to indirect methods are partly due to algorithm-specific restrictions, which include the assumption of an underlying normal gaussian distribution (i.e., a symmetrical distribution) of nonpathologic samples (28, 30). The algorithm we used does not assume a normal gaussian distribution of healthy samples but a Box–Cox distribution, which is not symmetrical and is often used in statistics to represent skewed data (31). Furthermore, we have validated the method in a previous study and shown that it generates reference intervals comparable to those of direct approaches, independent of the proportion of pathologic samples in the setting of a comprehensive hospital population (18). This is confirmed by comparison of our results to findings from CALIPER and KiGGS, showing a high concordance of reference limits and their age-dependent dynamics (see online Supplemental Figs. 1 and 2). The single analyte displaying major differences—magnesium reference intervals from the CALIPER trial—has been shown to be nontransferable in a CLSI-based transference study (32), explaining the observed disagreement. Furthermore, indirect approaches have been applied for reference interval calculation in the context of predominantly conventional methods: in the CALIPER trial, samples for children <1 year old were collected from the maternity ward and outpatient clinics from “apparently healthy children” rather than from healthy children recruited from the community (6). On the other hand, the KiGGS survey covered aspects of pediatric health from birth to adolescence, yet blood samples were obtained only from children >1 year old (17). The unique practical and ethical challenges of pediatric laboratory medicine therefore require the application of
indirect and innovative approaches for reference interval calculation.

In the present study, we examined a data set of 22 frequently measured analytes from a single laboratory. The reference intervals were established in a German population of mainly white origin on a Sysmex XE-2100 (blood count) and a Cobas Integra 8000 (clinical chemistry). The reported values are directly applicable only for this population and these analytical platforms, in which case the data tables published online enable integration into laboratory information systems to allow automatic classification of test results (see online Supplemental Database). However, transferability of the published reference intervals according to guidelines is possible and allows their use in global populations and platforms (1). Moreover, the indirect approach exemplified here can be applied universally given a sufficiently large number of samples, which will allow validation of our results and testing of the presented approach in other patient cohorts. Furthermore, analysis of rare analytes or special patient subgroups can be performed with aggregate data from multiple centers. This will enable us to meet the particular challenges in the establishment of pediatric reference intervals, which are limiting the interpretation of laboratory test results in children and adolescents.

Conclusions

We report the sex- and age-dependent dynamics of 22 common hematologic and biochemical analytes from birth to adulthood and provide reference intervals for their interpretation. The complex dynamics in many analytes cannot be adequately represented by separation into age groups; the continuous description provided can therefore improve clinical decision making when interpreting pediatric laboratory test results.

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