

# Small patients, big challenges: navigating the complex world of pediatric reference intervals

Măriuca Mănescu<sup>1\*</sup>, Minodora Dobreanu<sup>2,3</sup>

1. Department of Pediatrics, Emergency County Clinical Hospital of Targu Mures, Romania

2. Department of Laboratory Medicine, George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures, Romania

3. Clinical Laboratory, Emergency County Clinical Hospital of Targu Mures, Romania

## ABSTRACT

With about 60%-70% of medical decisions based on laboratory investigations, modern medicine is increasingly more reliant on laboratory medicine. Pediatric healthcare particularly depends on laboratory testing, with reference intervals (RIs) serving as critical tools for accurate result interpretation. However, establishing pediatric RIs poses distinctive challenges due to developmental, growth, nutritional, and disease-related variations across age groups, along with technical, ethical, and logistical challenges. Despite these limitations, there are several ongoing initiatives for developing pediatric RIs globally, underscoring the importance of developing accurate pediatric RIs. This review outlines the particularities of pediatric laboratory medicine and the specific challenges in establishing pediatric RIs, highlighting the recent initiatives in this area. It also addresses biological and analytical variability issues and explores future perspectives aimed at enhancing diagnostic accuracy and elevating patient care standards.

**Keywords:** biological variation, pediatric reference range initiatives, pediatrics, reference change values, reference intervals

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## INTRODUCTION

It is widely recognized that modern medicine is increasingly more dependent on laboratory investigations, which are becoming more diverse, complex, and accessible. The US Centers for Disease Control and Prevention estimate that 14 billion tests are requested each year in the United States, making them the most utilized medical tool [1]. In the majority of evidence-based clinical guidelines, laboratory testing is essential for the accurate diagnosis and effective management of various pathologies. Approximately 60%-70% of medical decisions are based on laboratory test results, both in hospital and ambulatory settings, highlighting their significant impact on medical practice and patient care [1].

Assessment of health and disease in pediatric patients is greatly reliant on laboratory testing as well, especially considering the challenges of obtaining a complete medical history and conducting a thorough physical examination in this age group. Various tests can be used in the laboratory to evaluate children, and efforts have been made to improve the process of collecting blood samples, which has historically been a challenging step in pediatrics. However, there are still significant gaps in

accurately interpreting results due to the unique aspects of this age group, such as development, nutrition, and growth.

A test result by itself has very little utility unless it is accompanied by a precise and pertinent clinical interpretation. Reference intervals (RIs) and clinical decision limits (CDLs) are the main parameters used to interpret laboratory findings, by answering two essential questions: 1) Is the patient healthy or not? 2) Is the patient at risk for a disease, currently diseased, or experiencing a worsening condition?

RIs answer the first question by describing the typical distribution of results seen in a healthy reference population. According to Horn and Pesce, „the reference interval is the most widely used medical decision-making tool” [2]. It can be defined as the interval between the two reference limits derived from the distribution of results obtained from a sample of the reference population [3]. For example, the 95% confidence interval, represented by the range between the 2.5th and 97.5th percentiles, is the most commonly used. Individuals with values outside the reference range are statistically more likely to have a particular condition. Notably, RIs are closely related to biological variation (BV), particularly between-subject BV,

\* Corresponding author: Măriuca Mănescu, Department of Pediatrics, Emergency County Clinical Hospital of Targu Mures, Romania. E-mail: manescu.mariuca@gmail.com

and are influenced by a wide range of factors. These factors are presented in Figure 1 alongside other elements that can influence laboratory test results.

On the other hand, clinical decision limits (CDLs) are predetermined cut-off values established by clinical studies and/or expert consensus, which are linked to a specific clinical risk or outcome. They answer the second question by providing a threshold value above or below which the results can be associated with a diagnosis of a specific disease or a significantly higher risk of adverse clinical outcomes [4, 5]. An example of clinical decision limits includes serum glucose levels used to diagnose diabetes mellitus or hypoglycemia. Critical or panic values are also a special type of clinical decision limit. Critical values are laboratory results that fall outside the typical range to an extent that could pose an urgent health threat to the individual and require prompt action/ immediate intervention by the clinician (for example, a blood potassium level higher than 6.0 mmol/L) [4, 6].

Today, more than 50 years after the introduction of these concepts in laboratory medicine, their applicability remains incomplete and, to some extent, unclear. Medicine has greatly evolved in recent years, but as we work to comprehend the structural and functional dynamics at play in individuals, we must also dedicate time and effort to understanding what "normal/ healthy" means and what the foundations of our comparisons are. Despite living in an era of evidence-based medicine and witnessing significant medical advancements, in pediatrics we often lack a solid basis for making accurate comparisons, which is essential for providing the best interpretations.

To this end, this review focuses on pediatric reference intervals (RIs) by highlighting the particularities and challenges of pediatric laboratory medicine, the current status of pediatric RI studies, limitations and variability issues, and future perspectives for improving pediatric RIs to enhance diagnostic accuracy and patient care.

## PEDIATRIC PATIENTS: A DIFFERENT MEDICAL LANDSCAPE

„Children are not small adults” is not just a cliché. Pediatric laboratory testing is uniquely challenging, therefore applying adult RIs to pediatric populations could lead to unnecessary follow-up testing and misinformed clinical decision-making.

It is important to note that major organ systems undergo growth and maturation from birth until 18 years of age. By 4-6 months of age, children's weight will have doubled, and by 12 months, it will have tripled, while their body length will have increased by 50% during the first year of life [7, 8]. Various physiological changes occur during growth and development in children, which necessitate pediatric RIs that take into account the variability in physiological changes with age, body mass index (BMI), gender, Tanner stage (sexual development), and ethnicity.

Current laboratory systems are unable to accurately determine the age of premature infants or establish age-specific references based on gestational age. Premature delivery can result in a variety of conditions arising from

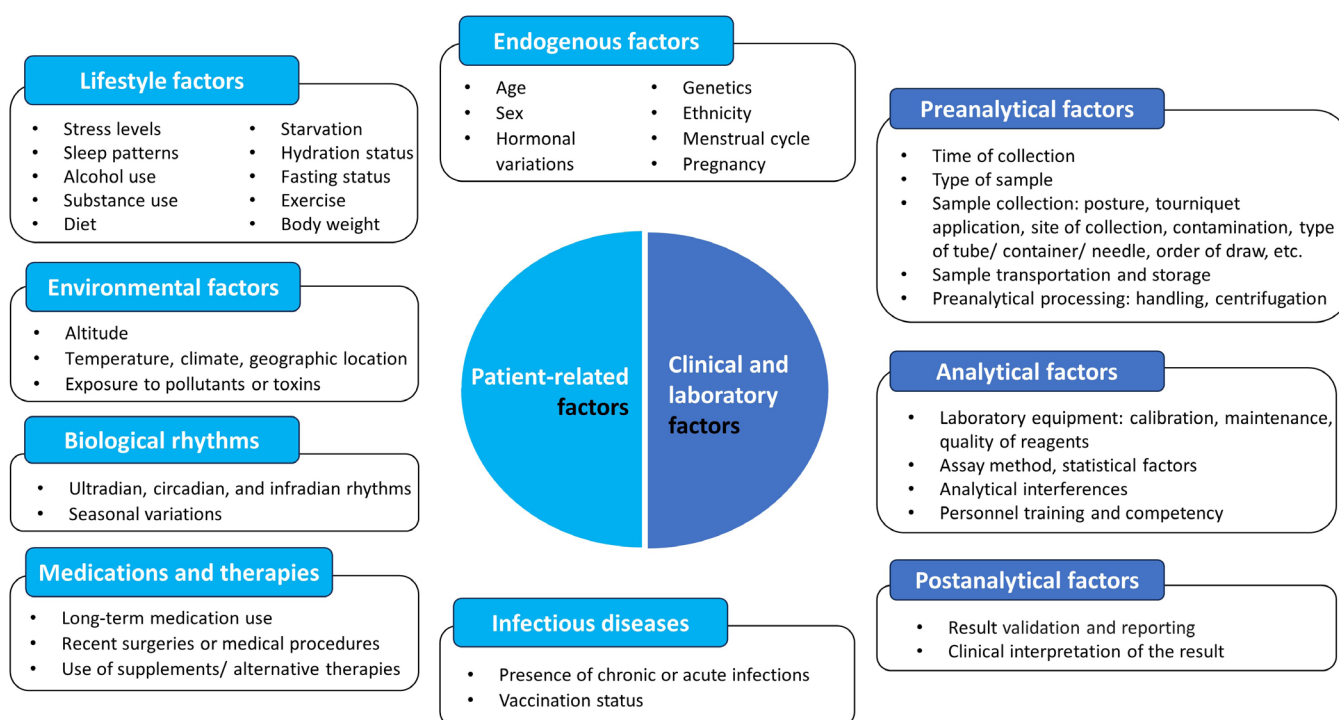


Figure 1. Factors influencing laboratory test results. Several of these factors also impact reference intervals.

underdeveloped organ systems, particularly impacting the lungs and the central nervous system, and leading to significant effects on test outcomes and diagnostic precision [9, 13].

During the newborn period, alterations in normal metabolism and pathological metabolic decompensation can happen abruptly due to active metabolism, lack of energy reserves such as glycogen and storage lipids, immature enzymatic systems, and regulation of hemostasis. Thus, alterations in the concentrations of various analytes can occur very rapidly, as seen with hourly changes in blood levels of bilirubin in neonates due to physiological hemolysis, liver immaturity, and reabsorption of bilirubin from the gut [14].

Newborns, with their limited prior exposure to infectious diseases and immature immune systems, are particularly susceptible to specific infections [9]. These infections often require targeted testing, such as TORCH screening for congenital infections or evaluations for neonatal sepsis caused by pathogens like Group B *Streptococcus* and *Listeria monocytogenes* [10]. Metabolic disorders and genetic diseases such as cystic fibrosis and sickle cell disease are typically identified during childhood. While neonatal screening programs are expanding, there is a lack of accurately established cut-off values for new and emerging pediatric biomarkers [15].

Furthermore, serology test results can be influenced by the transfer of maternal IgG through the placenta and by the immune responses of infants and children [10]. For instance, diagnosing syphilis in newborns using serologic tests is complicated by the transplacental transfer of maternal IgG antibodies, which can result in a false positive test result [11]. Serological testing for Human Immunodeficiency Virus (HIV) is not appropriate for infants under 18 months because it cannot distinguish between maternal antibodies transmitted during pregnancy and antibodies generated by an HIV-infected infant [12]. Elevated antibody levels following routine childhood vaccinations may also affect serological tests.

Mainstream coagulation tests are designed for monitoring coagulation times and are not suitable for diagnosing genetic diseases such as hemophilia in children [16]. Assessing hormone levels in pediatric patients requires careful consideration of age and sex [17]. Sex hormones undergo distinct surges during development: one in infancy, critical for postnatal gonadal development via the transient activation of the hypothalamic–pituitary–gonadal axis, and another during puberty, essential for sexual maturation [70]. Growth hormone levels fluctuate significantly with age, peaking during the pubertal growth spurt [71]. Alkaline phosphatase activity is significantly higher in childhood compared to adulthood and varies notably among pediatric patients due to factors

such as growth, changes in bone metabolism, and the maturation of the hepatobiliary system, with peaks observed during infancy and puberty [89].

Differences in metabolism between children and adults also impact therapeutic drug monitoring and therapy, with certain antibiotics specifically indicated for pediatric use [18, 19]. Lipid levels are considerably lower in children compared to adults due to growth and maturation and also depend on age, gender, and ethnicity [20].

Consensus guidelines established for adults do not directly apply to children's care due to significant physiological and metabolic differences between age groups, as seen in the management of hyperlipidemia [21].

All these aspects highlight the critical need for establishing comprehensive pediatric RIs to improve clinical diagnosis and patient care. However, carrying out studies to ensure multiple reference ranges categorized by different factors can be challenging both in adult medicine and even more so in pediatric care.

## CHALLENGES AND LIMITATIONS IN ESTABLISHING PEDIATRIC RIs

There are significant limitations in clinical laboratories when it comes to pediatric patients. Technical constraints, the high demand for resources, and various ethical restrictions (especially in premature infants and neonates) hinder research [22–25]. Furthermore, children's dynamic physiology and concerns about blood volume depletion complicate study designs [25–28]. Consequently, numerous studies aiming to establish RIs have involved a restricted population of pediatric individuals, with some studies even excluding certain categories, such as premature infants.

Currently, there is a concern over the availability of credible pediatric RIs as many of them are known to be out of date and were determined using outdated techniques and methodologies that may no longer meet the accuracy standards of contemporary laboratory practices [29, 30]. Moreover, changes in instrumentation and methodology often occur without corresponding adjustments to reference ranges, which can lead to discrepancies in clinical analysis [31, 32].

The lack of standardization or harmonization of laboratory assays is another issue, as methods may vary between manufacturers. Many laboratories rely on reference ranges provided by the manufacturer of the analyzer or test. This can cause confusion among patients and even among clinicians, as different methods may be used within the same hospital, leading to variations in RIs. Also, children visiting multiple medical centers because of the growing integration of healthcare networks

may encounter different assays, systems, or reported RIs, potentially leading to inconsistent patient management. This leads to inaccurate and erroneous interpretations, increasing the risk of misdiagnosis and inappropriate treatment, adding to the pressures felt by patients and their families. There is a need for standardization and the adoption of commonly accepted RIs [33-35].

## ESTABLISHING RIs: DIRECT AND INDIRECT METHODS

The document "Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory: Approved Guideline—Third Edition (EP28-A3c)", published by the Clinical & Laboratory Standards Institute (CLSI) and The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), is widely used as a source for establishing recommendations regarding RIs for interpreting laboratory results [3].

Professional guidelines often emphasize the need for laboratories to determine their own RIs and to periodically review these established limits, taking into account the specific population being tested. Age stratification is also necessary [3]. However, in reality, many laboratories do not meet these requirements due to limitations in recruiting appropriate individuals, sampling-related challenges, lack of time, and high costs. Due to these difficulties, instead of establishing their own RIs, they choose to verify the RIs provided by the manufacturer or another laboratory through a study involving 20 healthy subjects, despite the various transference issues [3, 36]. Transferring RIs from an older method to a newer, more modern method, is also a common practice, requiring the establishment of comparability between the two methods [37]. Therefore, many RIs presented in laboratory reports rely on data from the manufacturer, published literature, expert recommendations, or old studies. This approach may not accurately account for the unique characteristics of their patient population, leading to potential misdiagnosis and mistreatment. Laboratory professionals are encouraged to review their RIs in use, estimate new RIs, and share their results widely, fostering collaboration and innovation within the field.

There are two primary methods for establishing reference intervals, the direct method and the indirect method. The direct method is the conventional approach, which involves recruiting a sufficiently large group of healthy people (minimum sample size of 120-400 subjects) while accounting for the relevant covariates, such as age, gender, ethnicity, etc. [3, 38]. Although using the direct method for establishing RIs is currently considered the gold standard, it does not necessarily reflect the actual health status of individuals (which can be difficult

to define) and has relatively low applicability [38, 39]. The recruitment process can be quite demanding due to logistical, practical, and financial constraints [38-41]. Defining an individual as "normal" or "healthy" is a challenging task, given the uncertainty surrounding potential subclinical issues. On the other hand, outlier exclusion is a vital step of direct approaches, which in turn can lead to RIs that are based on a subgroup of "super-healthy" individuals and may not accurately represent the overall population [38]. The selection of statistical methods and exclusion criteria can also have an impact on the RIs obtained, potentially leading to unnecessary flagging of normal results [41]. When it comes to children, additional factors can complicate matters, including ethical limitations and challenges related to blood collection, as mentioned before. These difficulties underscore the importance of accurately stratifying and establishing appropriate RIs, particularly for vulnerable pediatric populations. A series of articles have pointed out the intricacies of this method, presenting both its benefits and limitations [13, 24, 30, 31, 38-41].

The indirect method is an alternative approach to determining RIs by harnessing the power of stored patient data in laboratory databases. This method uses a database originally created for a different purpose, such as routine clinical care or screening tests [38, 40]. Assuming most of the test results are non-pathological, this data can be employed to establish RIs using different statistical approaches. One major advantage of the indirect method is that it requires minimal resources, mainly expertise, time, and access to a laboratory information system database [38, 40-44]. The challenge lies in the requirement for statistical methods to deconvolve a mixed population dataset containing both healthy individuals and patients into a distribution consisting solely of healthy individuals. This can be quite challenging using traditional techniques, but there have been significant developments in recent years [40, 41, 43, 44]. Some notable statistical approaches used for the indirect method are the Hoffmann method, the Bhattacharya analysis, the Price method, the Reference Limit Estimator (RLE), the Transformed Maximum Curve (TMC), as well as the innovative Kosmic and RefineR algorithms [40, 45].

The use of indirect methods to establish pediatric RIs has become widespread due to ethical challenges in this age group as well as practical advantages (less time-consuming, less costly) [38, 46, 47]. This method allows for establishing precise RIs for young age groups, particularly neonates and young infants, who are overrepresented in laboratory databases due to their significant contribution to pediatric morbidity. The extensive availability of samples enables more detailed age stratification, better capturing the continuous dynamics of many analytes during pediatric development.

Despite being practically more challenging, some studies have demonstrated the superiority of the direct method in pediatric settings, particularly when compared to classical approaches like the Hoffmann method. According to Shaw et al., RIs obtained from hospital data through the Hoffmann method were usually wider in comparison to those determined using the direct method, such as in the CALIPER study [48]). Interestingly, none of the RIs established with the Hoffmann method completely overlapped with the 90% confidence intervals calculated by CALIPER [48]. This underscores the need for ongoing research to find an optimal approach for leveraging hospital databases and using the indirect method in the development of RIs.

## ADVANCING PEDIATRIC HEALTH: EXPLORING PEDIATRIC RIs INITIATIVES

According to the IFCC Global Reference Interval Database, there are various national and international pediatric reference range initiatives around the world [49], demonstrating the feasibility of conducting this kind of study.

The program available in the United States, **The Children's Health Improvement through Laboratory Diagnostics (CHILDX)**, has collected samples from a cohort of 902 healthy children aged between 6 months and 17 years. A unique feature of this project is the collection of both blood and urine samples to establish RIs for various analytes, including enzymes, hormones, vitamins, bone markers, and coagulation tests [50-55].

The **Lifestyle of Our Kids (LOOK)** program, based in Australia, has focused on establishing RIs in pediatrics through a community-based longitudinal study. They enrolled 852 healthy 8-year-old children to investigate the effects of early physical activity on health, with follow-up assessments at ages 10 and 12 [56]. Blood samples were analyzed for 37 common analytes, further completing the study by providing RIs for NT-pro-BNP in relation to age and gender [57].

More recently, the **HAPPI Kids (The Harmonising Age Pathology Parameters in Kids)** Australian program included children and adolescents from 0-18 years of age undergoing minor day surgical procedures. They aimed to establish continuous RIs for variate analytes (general biochemistry, hematology, immunology markers) using 5 different analyzer platforms, therefore providing a more accurate evaluation of laboratory results throughout a child's development [58]. The HAPPI Kids project was groundbreaking as it was the first study to conduct comparison tests across analyzers, adding significant scientific evidence supporting the standardization of assay methodologies. They recently established pediatric RIs for cystatin C [59].

Expanding the scope of research, **AHRIP (Australian Harmonised Reference Intervals for Paediatric)** is an ambitious project aimed at establishing RIs for common biochemical parameters across laboratories in Australia and New Zealand. Through a comprehensive data mining method, the AHRIP team analyzed over 1.8 million laboratory results from 15 pediatric facilities to create standardized reference ranges [60].

One of the most renowned and extensive projects is **CALIPER (The Canadian Laboratory Initiative on Pediatric Reference Intervals)**, which has recruited over 14,000 participants from the diverse Canadian community, ranging from newborns to 18-year-olds [61]. This effort has built a comprehensive database for establishing RIs in children and adolescents. The CALIPER team has conducted or is currently working on studies to determine RIs for a wide range of analytes, including chemistry and immunological markers on new analytical systems (e.g. Abbott Alinity, Siemens Attelica), hematological markers on multiple platforms (Sysmex, Beckman, Mindray), markers of inflammatory disease (cytokines, calprotectin, autoimmune markers), markers of nutritional deficiency (essential trace elements), markers of environmental toxicity (heavy metals), and cancer biomarkers. [61-73]. Additionally, they are examining critical care markers on point-of-care testing platforms [74]. Several sub-studies are also underway to address specific gaps, such as pregnancy and infancy, lipid metabolism in adolescents with insulin resistance and obesity, and a seroprevalence study for monitoring antibody levels following COVID-19 vaccination [61].

The **PRINCE study (Pediatric Reference Intervals in China)**, is a large program funded by the Chinese government that addresses the critical gap in pediatric RIs from this geographic area. The project aimed to develop a platform for pediatric evidence-based research in China, recruiting a total of 15,150 healthy children and adolescents aged from 0 to 19 years [75-78]. They established RIs for hematological markers and common biochemical analytes, conducting validation studies across 21 hospitals to ensure the accuracy of the established intervals [76-78].

In the European countries, three notable initiatives stand out: Nordic Reference Interval Project (NORIP) / Scandinavian Initiative for the Establishment of Pediatric Reference Intervals (NORICHILD), German Health Interview and Examination Survey for Children and Adolescents (KiGGS), and Pediatric Reference Intervals Initiative (PEDREF).

NORIP launched the Scandinavian Initiative for the **NORICHILD** in 2012 to establish direct pediatric RIs for the Nordic population. The project collected blood samples from 1,429 healthy Danish children and adolescents, which were used to determine RIs for 21 biochemical parameters [79, 80].

**KiGGS** is a national health monitoring program that was started in 2003 to collect detailed health data on children aged from 0 to 17 years old. A two-phase sampling methodology was employed in the study to choose a representative sample of subjects from 167 localities around Germany [81]. The KiGGS cohort has been followed up twice: from 2009 to 2012, a computer-assisted telephone interview survey was used for the first follow-up (KiGGS Wave 1) [82], and from 2014 to 2017, a health examination and interview survey were used for the second follow-up (KiGGS Wave 2) [83]. With data on health habits, healthcare consumption (encompassing medical visits, hospitalizations, medication use, preventive services, therapies, medical tests and procedures), personal, familial, environmental, and socioeconomic determinants, as well as physical and mental health markers, the KiGGS cohort seeks to give a thorough picture of health development over the life cycle [84-86].

Finally, **PEDREF** is a multicenter initiative that seeks to develop a new generation of pediatric RIs using the indirect method on laboratory data from 12 German care centers and laboratory service providers [47]. A collaborative database comprising over 15 million pediatric samples aged 0 to 18 years has been established by the study and age- and sex-specific RIs for a range of biochemical and hematological analytes have been developed [87-89].

## CUSTOMIZING RIs FOR POPULATIONAL DIVERSITY

It is vital to understand that establishing RIs is a critical task that requires a nuanced approach, extending beyond simple statistics or science. It requires a deep understanding of the intricacies of human physiology, the nuances of clinical practice, and the complexities of population variability, demanding expert supervision from professionals in laboratory medicine, physiology, and pathology. Hence, relying on RIs derived from other projects/ pediatric reference interval initiatives is erroneous, as they may not reflect the population served by the laboratory. Individuals from different populations may have unique physiological characteristics, requiring a personalized approach rather than a one-size-fits-all solution.

Beyond age stratification, it's also crucial to consider other factors such as weight, sex, ethnicity, and geographic location, as well as the distinctive features of the patient population being served. Only by combining rigorous statistical analysis with expert input and consideration of population diversity can we ensure that RIs are accurate, reliable, and clinically relevant.

It is a very well-known fact that pediatric obesity continues to escalate as a significant public health issue [90],

and therefore understanding how analyte levels change with Body Mass Index (BMI) is crucial. As average BMI levels increase in populations, RIs for analytes affected by BMI may also change [91-92]. It is important to identify which parameters are specifically impacted by BMI and to understand the importance of these changes in a medical context, enabling the differentiation between physiological variations devoid of clinical significance and the potential presence of hidden disease.

Research conducted on the CALIPER cohort revealed that elevated adiposity significantly impacts the levels of various markers in children and adolescents, encompassing liver enzymes, lipids/lipoproteins, inflammatory markers, and uric acid [93]. Other studies have analyzed the impact of obesity on laboratory results; however, many of them focus on adults [94] or have examined a limited number of analytes [95]. As this issue escalates, there is a growing demand for research studies to accurately assess the effects of obesity on laboratory findings and their clinical relevance.

Furthermore, the impact of geographical location and ethnicity on laboratory tests have been extensively documented in numerous publications, highlighting the significance of these factors in influencing test results and, consequently, medical decision-making.

A study published in *Clinical Chemistry* by the CALIPER team found statistically significant differences between ethnic groups for several biomarkers, including 25(OH)-vitamin D [25(OH)D], intact parathyroid hormone (iPTH), total thyroxine (TT4), total triiodothyronine (TT3), free thyroxine (FT4), cobalamin, and ferritin [68]. Another study conducted in Canada analyzed the concentrations of 52 biomarkers in a multiethnic population of healthy children and found that 18 biomarkers demonstrated statistically significant ethnic differences [96].

Previous studies in African children have consistently demonstrated that red blood cell parameters, including hemoglobin, hematocrit, and mean corpuscular volume, are generally lower compared to those of Caucasian children [97, 98]. Notably, however, platelet counts tend to be higher in African children, whereas eosinophil counts are often elevated, likely due to the prevalence of helminth infestations [99].

The European geographical area is characterized by a remarkable diversity of ethnic groups, living in regions with varying climatic conditions and lifestyles. This heterogeneity has a profound impact on laboratory test results and disease prevalence. In Europe, vitamin D RIs can vary significantly depending on ethnicity and geographical zone [100, 101]. The prevalence of vitamin D deficiency is notably lower in northern latitude countries such as Norway, Iceland, and Finland, compared to mid-latitude countries like the United Kingdom, Ireland, the

Netherlands, and Germany. This trend persists even after accounting for ethnicity. While skin color plays a role in vitamin D status, it is only part of the equation. Other factors, including dietary habits, cultural practices, and biological differences, significantly influence the prevalence of vitamin D deficiency across different ethnic groups [101].

Thyroid dysfunction stands as a prominent endocrine disorder, prevalent across European countries. Research conducted in formerly iodine-deficient regions like Poland reveals significant differences in the established reference ranges for serum thyroid function tests compared to iodine-sufficient areas [102]. This underscores the profound impact of geographical location on health dynamics.

The higher occurrence of certain diseases, such as thalassemia, in certain populational groups has created the need for adjusting reference ranges of hemoglobin fractions to account for the genetic and physiological differences observed in these populations [103]. The distribution of  $\beta$ -thalassemia is highly skewed towards certain ethnicities, including the Mediterranean area, but the prevalence has also been rising in Eastern and Northern Europe due to migration [104].

There is a higher prevalence of celiac disease in people of European descent, especially in regions like Ireland and Scandinavia [105, 106]. Screening for celiac disease is more common in these populations due to genetic predisposition.

Populational migration can significantly affect RIs for laboratory tests due to several factors related to the genetic, environmental, and cultural diversity introduced into new regions. Without accurate and culturally relevant RIs, healthcare providers may struggle to accurately diagnose and treat patients from diverse backgrounds, leading to potential misdiagnosis and mistreatment.

In an increasingly interconnected world, defining ethnicity has become more complex due to widespread population movements. Although we recognize the differences in certain analytes among various ethnic groups, available data are often limited and incomplete. As a result, the precise impact of ethnicity on clinical variability remains uncertain. Employing genetic profiling and a personalized approach could provide a more accurate method for identifying relevant subgroups, thus improving the precision and effectiveness of medical treatments.

## ADDRESSING WITHIN-SUBJECT VARIATION IN LABORATORY TESTING

As discussed previously, the stratification of RIs based on age is indispensable in laboratory medicine, given the dynamic changes occurring across the lifespan for

diverse laboratory parameters. However, we must also consider other types of variations. Certain analytes exhibit predictable cyclic variations over specific time-frames, influenced by ultradian, circadian, infradian, or seasonal biorhythms [107, 108]. Ultradian rhythms are repetitive fluctuations within shorter intervals throughout the day, such as episodic hormone secretions that regulate many endocrine functions [107]. Cortisol and melatonin follow daily circadian rhythms, fluctuating predictably throughout the day [107]. Hormones like estrogen and progesterone fluctuate across menstrual cycles, reflecting infradian rhythms [107]. Additionally, analytes such as vitamin D and serotonin vary seasonally, influenced by changes in sunlight and environmental conditions [107]. This presents a nuanced challenge in establishing precise reference values for each point along these cycles, underscoring the necessity of identifying the optimal timing for investigation. Furthermore, the absence of these patterns may serve as a compelling indicator of underlying pathology.

Beyond these anticipated fluctuations, individuals manifest distinct variability in laboratory outcomes across various testing instances. In healthy individuals, this variability oscillates randomly around a homeostatic set point [109]. Such fluctuations are attributed to pre-analytical variations (e.g. patient preparation, sample collection, sample transportation/storage), analytical variations (e.g. test imprecision during replicated analysis), and within-subject BV [108, 109]. All these sources of variation contribute to a statistical distribution of potential analyte concentrations around an individual's own average of values (homeostatic point), effectively establishing an "individual" reference interval (RI) (Figure 2). Consequently, deviations that exceed the expected variation (falling outside the individual's RI) can signal underlying health conditions, even if they remain within the population RI. This highlights the crucial need for recognizing and comprehending these fluctuations within clinical contexts. For the same reason, RIs are not particularly helpful when interpreting consecutive results for an individual. While stratifying by factors like sex, age, and other demographic variables can enhance the accuracy of RIs, it is important to recognize the inherent limitations of population-based RIs [110]. These ranges, taken from a large group of people, may not precisely represent the unique differences and individual variability observed in serial results from one person. The physiological baseline and natural fluctuations of each person may vary greatly from the population average. Consequently, population-based RIs may not detect important changes in an individual's test results over time [110-112].

The RI is typically determined as the mean  $\pm$  2 standard deviations (SD), encompassing central 95% of the

values from the studied population. Hence, 5% of individuals will consistently fall outside the RI (2.5% below the lower limit and 2.5% above the higher limit of RI). Laboratories routinely flag such results, which may result in unnecessary follow-up actions, such as additional testing [112]. Moreover, each person's range of values typically covers only a segment of the overall RI (Figure 2). Variations within this range may not attract significant attention from laboratory personnel and clinicians, as they are often considered unimportant. Therefore, more objective methods for comparing serial test results in individuals are needed.

For example, the RI for creatinine in newborns is 0.4-1.0 mg/dL. However, if a particular newborn's creatinine levels regularly fluctuate between 0.4-0.6 mg/dL, an increase to 0.9 mg/dL would be abnormal for that newborn. Despite this, such a change might not be recognized as a medical problem because it still falls within the broader RI. This underscores the need for more precise and individualized approaches to interpreting serial test results.

In order to address this limitation, it is essential to consider the concept of reference change values (RCVs). RCVs are precise thresholds that account for both ana-

lytical and biological variability, enabling a better understanding of consecutive results for an individual [112, 113]. They establish a customized standard for determining the significance of variations in test results from both statistical and clinical viewpoints. The difference is considered significant when it exceeds the RCV, regardless of whether the most recently measured value falls within the reference range or not. By utilizing RCVs, healthcare providers are able to better detect important changes that could indicate the onset or progression of a disease, even if these changes fall within the normal reference ranges. Therefore, even though population-based RIs are important in laboratory medicine, it is crucial to incorporate RCVs for a more accurate and personalized assessment of consecutive test results (Figure 2).

Calculating RCVs requires understanding that variation between consecutive tests in the same subject can arise from the laboratory assay (analytical variation), from the within-subject variation of the analyte itself (biological variation), or from both [112, 113]. Fortunately, laboratories are generally aware of the imprecision of their assays, and the BVs of most analytes are known and constantly updated by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) BV database [114].

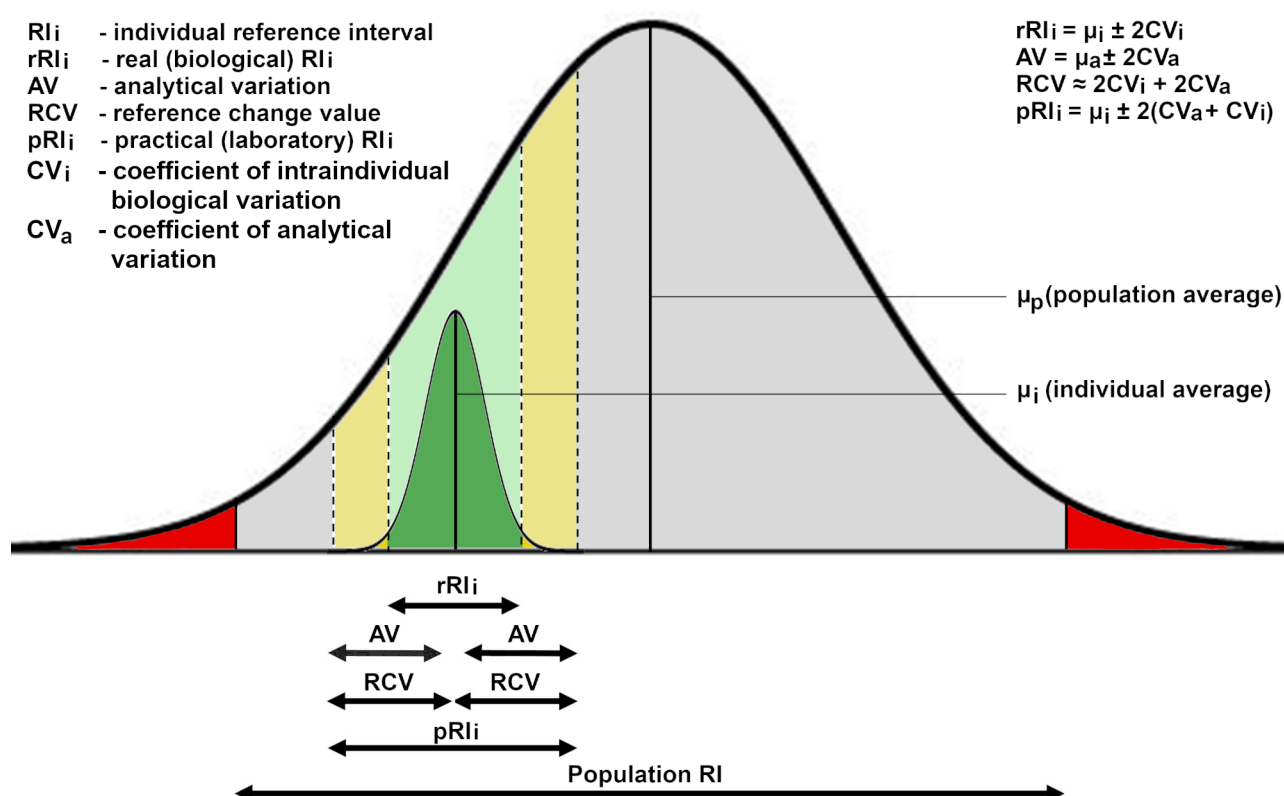


**Figure 2.** Individual vs population reference intervals and RCV. The green area represents the individual's biological reference interval (rRI<sub>i</sub>). Analytical variation can expand this interval (yellow area), resulting in a *de facto* individual reference interval (pRI<sub>i</sub>). Values within the gray area, although still within the population reference interval, fall outside the individual's reference interval. Note: the established formula for RCV is  $2^{1/2} \times Z \times (CV_a^2 + CV_i^2)^{1/2}$  where Z is the number of standard deviations for a given probability. For Z=1.96, corresponding to a 95% confidence interval, RCV can be approximated by the simpler equation  $2CV_a + 2CV_i$ .

RCV can be successfully used for delta check. Delta check (checking the difference) is an important quality control tool developed to evaluate the differences between consecutive test results for the same patient [115, 116]. Laboratory personnel are notified by the delta check program when significant changes or unusual trends are identified, which may signal either an alteration of the patient's condition or sample issues, such as misidentification, contamination, hemolysis, etc.

Delta-check offers the advantage of being evidence-based, serving as a statistical tool in clinical practice. Setting alert limits for delta-check algorithms often involves employing various approaches, such as utilizing the standard deviation of prior results, assessing the percentage change from the previous result, and reviewing historical data trends [115]. Using RCVs to establish delta-check alert limits has the advantage of incorporating not only BV but also analytical variation [117]. Even if clinicians were aware of BV (which is unlikely, though there are databases they could consult, such as the EFLM Biological Variation Database [114]), RCVs still offer a significant benefit. It is impractical for clinicians to stay updated on the analytical variation of the laboratory, which can differ for each analyte measured. Therefore, the automatic calculation of RCVs and their adoption into delta-check algorithms is advantageous as it automates the process. Exceeding these limits then triggers an alert (flagged sample), which can be investigated through collaboration between the clinician and the laboratory.

It should be noted that, even in the absence of statistical tools, clinicians frequently discern patterns or identify potential deviations that, while still within the RI, show significant changes in biological parameters. Thus, they often employ delta-check reasoning in their daily medical decision-making process. However, it is crucial for clinicians and laboratory personnel to collaborate closely. This ensures that the intuitive judgments, primarily based on the clinician's experience, are validated by the more statistics-oriented laboratory, and followed by well-supported medical decisions. Efforts should be made in order to improve the collaboration between clinicians and laboratory personnel, promoting a collaborative relationship based on teamwork rather than operating as separate entities.

## ENHANCING THE LABORATORY-CLINICIAN-PATIENT INTERFACE

It is a recognized reality that, while laboratory physicians are aware of the limitations of RIs, analytical variations, and potential errors in sample handling processes, clinicians often lack the same depth of understanding in these areas. This gap between clinicians and laboratory

personnel can exacerbate the already existing challenges raised by imperfect RIs. Enhancing communication between these departments, offering tailored courses on relevant laboratory information for clinicians, promptly notifying clinicians of changes in laboratory equipment or techniques, and providing regular updates are essential strategies for bridging this gap.

Clinicians should understand that laboratory results are merely puzzle pieces of the broader patient picture, rather than a direct reflection of their condition. Treating the patient and not the laboratory results should be a guiding principle. When analyzing a subsequent sample, its correlation with previous results often holds more significance than its comparison to the RI. Collaborating with the laboratory physician is a valuable resource that clinicians should utilize more frequently in situations of uncertainty. This ensures that patients are given the opportunity to receive the best possible medical decisions, taking into account all available information comprehensively.

On the flip side, it is just as crucial to ensure that patients are well-informed and educated about the complexities of medical decision-making. This means recognizing that, while laboratory reports are valuable tools, they should not be blindly accepted as absolute truths. Instead, patients should actively seek guidance from healthcare specialists, relying on their expertise and clinical judgment. Additionally, it is advisable for patients to consistently conduct their analyses in the same laboratory. This practice helps minimize variability that may arise from different methods, equipment, or personnel, ensuring more reliable and consistent results.

## FUTURE PERSPECTIVES

Despite facing major obstacles, significant progress has been made in establishing accurate pediatric RIs. Nevertheless, in the face of constant technological and clinical advancements, it is essential for national and international research initiatives to consistently evolve and better align with the changing landscape.

Standardization and harmonization in laboratory medicine are imperative for ensuring quality across every stage of the total testing process. Despite widespread calls for harmonization, achieving this goal remains the holy grail of laboratory medicine—a complex, costly, and time-consuming process demanding meticulous and systematic approaches at local, national, and international levels [33-35, 118, 119]. Comprehensive efforts are required across all phases of laboratory testing, from the pre-preanalytical stage (ensuring the appropriate test for the right patient at the right time) to the post-postanalytical phase (test interpretation and use) [120]. Harmonization is needed for standardized reporting

units, terminology, methodologies, RIs, medical decision limits, critical values, and clinical guidelines for test requesting and result interpretation. Global scientific organizations dedicated to the field of clinical laboratory science such as IFCC, CLSI, and EFLM should join forces and collaborate effectively to focus efforts on a unified initiative rather than separate projects, thereby providing support, and resources, and guiding the harmonization process.

Improvements can also be made regarding the method of reporting RIs. By including innovative visual aids, such as dynamic charts that continuously adjust according to patient data, clinicians can observe certain data patterns and make better-informed decisions. Also, percentile charts and z-scores, typically employed in pediatrics for anthropometric measurements, can improve the evaluation of laboratory results by visually depicting age-related changes and the transition of test results from healthy to pathological values [88]. These visual tools provide clinicians with valuable insights and can be easily integrated into electronic health records or accessed through mobile applications.

Integrating innovation to support the establishment of RIs is also an important future perspective. Utilizing big data and existing laboratory data libraries, alongside AI-powered analytics platforms, to analyze extensive datasets can significantly enhance the accuracy of RIs [121, 122]. This approach involves identifying patterns that might be missed by traditional methods, correlations, and outliers, while also optimizing algorithms. Additionally, machine learning techniques enable AI to continuously refine models with new data inputs, ensuring ongoing performance enhancement over time [123, 124]. Smart RI models and patient data could be accessible to clinicians through mobile applications designed with intuitive interfaces for their mobile devices, thereby enhancing their daily decision-making. Developing feedback mechanisms for clinicians is another valuable perspective worth exploring, as it could improve RI models based on clinicians' experiences and patient outcomes.

Furthermore, in light of the ongoing shift towards personalized, precision, and preventive medicine (3P medicine), RIs could also be refined and personalized using various tools (patient-specific data, genetic testing, molecular diagnostics) to ensure accurate interpretation of laboratory results for each patient [125-127]. Initiatives are already underway in this direction, utilizing previously measured test results and considering within-subject biological and analytical variations to develop algorithms for establishing personalized RIs [128]. However, this approach is in its early stages and requires further comprehensive research to advance towards a more individualized approach in clinical laboratory testing.

## CONCLUSIONS

Pediatric laboratory medicine is a challenging and unique segment that needs to continuously adapt and improve in order to respond to the specific needs of the pediatric population. Reference intervals play a crucial role in this process, by providing context for measured laboratory values and allowing the distinction between health and disease. Continuous research efforts must be made so that laboratories worldwide can provide accurate reference intervals that precisely reflect the health status of the studied populations, thereby improving diagnosing, monitoring, and overall patient care.

## ABBREVIATIONS

AHRIP – Australasian Harmonised Reference Intervals for Paediatrics

BMI – Body Mass Index

BV – Biological Variation

CALIPER – The Canadian Laboratory Initiative on Pediatric Reference Intervals

CDLs – Clinical Decision Limits

CHILdX – The Children's Health Improvement through Laboratory Diagnostics

CLSI – Clinical & Laboratory Standards Institute

EFLM – European Federation of Clinical Chemistry and Laboratory Medicine

HAPPi Kids – Harmonising Age Pathology Parameters in Kids

IFCC – International Federation of Clinical Chemistry and Laboratory Medicine

KiGGS – German Health Interview and Examination Survey for Children and Adolescents

LOOK – The Lifestyle of Our Kids

NORCHILD – Scandinavian Initiative for the Establishment of Pediatric Reference Intervals

NORIP – Nordic Reference Interval Project

PEDREF – Pediatric Reference Intervals Initiative

PRINCE – Pediatric Reference Intervals in China

RCV – Reference Change Values

RIs – Reference Intervals

SD – Standard Deviation

## AUTHORS' CONTRIBUTION

MM – Conceptualization, Formal analysis, Investigation, Writing (Original Draft).

MD – Conceptualization, Validation, Supervision, Writing (Review & Editing).

## CONFLICT OF INTEREST

None to declare.

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