CALCULATION OF REFERENCE INTERVALS OF BLOOD PARAMETERS IN CHILDREN AND ADOLESCENTS: PROJECTS REVIEW

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The review of the currently existing projects focused on calculating the reference intervals of blood parameters in large samples of children of different gender and age discusses the urgent issues of calculating pediatric reference intervals of biochemical markers, the paper provides comparison of the reference intervals established within the framework of different projects. The limitations, future prospects and harmonization of pediatric reference intervals, including for juvenile athletes, are provided.

Keywords: reference intervals, blood parameters, pediatrics, children, juvenile athletes, sports medicine

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РАСЧЕТ РЕФЕРЕНТНЫХ ИНТЕРВАЛОВ ДЛЯ ПОКАЗАТЕЛЕЙ КРОВИ У ДЕТЕЙ И ПОДРОСТКОВ: ОБЗОР ПРОЕКТОВ

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В обзоре существующих на сегодняшний день проектов по расчету референтных интервалов показателей крови на больших выборках детей разного пола и возраста обсуждены актуальные вопросы расчета педиатрических референтных интервалов для биохимических маркеров, проведено сравнение значений референтных интервалов, полученных в разных проектах. Представлены ограничения, будущие перспективы и гармонизация педиатрических референтных интервалов, в том числе для несовершеннолетних спортсменов.

Ключевые слова: референтные интервалы, показатели крови, педиатрия, дети, несовершеннолетние спортсмены, спортивная медицина

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The child's organism is distinguished from the adult's organism not only by physical condition, but also by the organs maturity, features of metabolism, immune and endocrine responsiveness, etc. The dynamics of physiological processes that take place in the child's organism are accompanied by changes in the concentrations of many blood biomarkers, including hormones [1].

In fact, objective assessment and subsequent interpretation of the results of laboratory and instrumental tests considering certain age periods of the child are often complex and not always unambiguous. This negatively affects selection of the tactics for management of children and organization of treatment and preventive care [2, 3]. The reference intervals, being a fundamental medical instrument allowing one to correctly interpret blood test results and distinguish between normal physiological changes and the onset of the disease process in the child's organism, can help to solve this problem [2].

In terms of statistics, reference interval (RI) represents the limits of the range that includes the percentage (usually 95%) of values obtained from the healthy population. The reference limits are determined by calculating the 2.5th and 97.5th percentiles of the test results [2, 4, 5]. Hence, 5% of the results may be interpreted as abnormal.

According to the regulation, statistical methods for calculation of reference intervals are selected based on the distribution of reference values: parametric methods are used for the normally distributed data, while nonparametric methods are used in case there is no null hypothesis of the dataset distribution. Calculation of the 95% confidence interval is possible when the distribution of reference values is normal; conversely, nonparametric methods, specifically the rank test, are used when calculating reference intervals for the samples characterized by non-normal distribution of values obtained from the reference groups of healthy subjects [5].

When using the conventional "direct" approach, RI is usually defined as an interval denoted by two reference limits (2.5th and 97.5th percentiles) obtained from the sample represented by reference population [4, 5].

Currently, indirect methods of RI calculation that involve analysis of the laboratory parameter datasets have been widely implemented. The complex statistical algorithms that

Table. Projects focused on calculating the reference intervals of blood parameters in the populations of healthy children and adolescents

Project	Country	Age groups	Gender	Statistical methods, calculated parameters	Assessed biomarkers	References
AABC	Australia and New Zealand	All	M, F	Direct method, RI	Enzymes and ions	12, 13
Caliper	Canada	Under 18	M, F	Direct method, RI	Common blood analytes, endocrine markers, tumor markers, vitamins, biomarkers of metabolic disorders	8, 10, 14, 15–22
CHILDx	USA	0.5–17 years	M, F	Direct method, RI	Enzymes, hormones, vitamins, bone turnover markers, coagulation indicators	23–28
COPENHAGEN	Denmark	5–20 years	M, F	Direct method, RI	Common blood analytes	29
KiGGS	Germany	Under 18	M, F	Direct method, RI, median	Biochemical markers, immunological markers, thyroid hormones, noncommunicable disease markers	30–34
LOOK	Australia	8, 10, 12 years	M, F	Direct method, RI, median	Cardiac markers, common blood analytes	35, 36
NHANES	USA	all	M, F	Indirect method, 2,5; 25; 50; 75; 97.5 percentiles	Lipid profile, immunological and hematological markers, vitamins, inflammatory markers	37–44
NORIP	Scandinavian countries	Under 18	M, F	Direct method, RI	Tumor markers, common blood analytes	45–48
Referent-20	Russia	Professional athletes aged 14–17	M, F	Indirect method, RI; 5; 10; 25; 50; 75; 90; 95 percentiles	Common blood analytes, metabolic markers	11, 49, 50

Note: AACB — Australasian Association of Clinical Biochemists; CALIPER — Canadian Laboratory Initiative on Paediatric Reference Intervals; CHILDx —Children's Health Improvement through Laboratory Diagnostics; COPENHAGEN — The Copenhagen Puberty Study; KiGGS — German Health Interview and Examination Survey for Children and Adolescents; LOOK — Lifestyle of Our Kids; NHANES — National Health and Nutrition Examination Survey; NORIP —Nordic Reference Interval Project.

are essential for development and implementation of exclusion criteria for unhealthy subjects have become the main problem that restricts the use of indirect methods.

It should be noted that it is rather difficult to calculate and verify RIs. In 2008, specialists of the Clinical and Laboratory Standards Institute (CLSI) and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) prepared the guidelines (C28-A3) on the RI calculation and verification [6]. The accurately determined RIs are crucial for correct diagnosis and selection of the treatment method, while the general population-based RIs of blood parameters that have not been adapted for children may result in erroneous diagnosis, inadequate treatment, higher health expenditures, etc.

Analysis of the literature data on pediatric RIs has revealed the main directions of contemporary research focused on calculating RIs of blood parameters in children: bone markers [7], markers of cardiovascular disorders and the risk of metabolic syndrome [8], thyroid hormones and growth hormone [9], inborn features of metabolism [10].

In foreign countries, the results of several large-scale projects focused on calculating RIs of blood parameters in multiple samples of healthy children have been published over the recent years (Table). Unfortunately, there are still no such projects in our country. An exception is the project involving calculation of RIs of blood parameters in juvenile athletes that was implemented at the Federal Research and Clinical Center of Sports Medicine and Rehabilitation of FMBA in 2020. It is comparable with the abovementioned foreign projects in terms of the sample size [11].

KiGGS project

The KiGGS project launched at the Robert Koch Institute (RKI) in Germany has become one of the largest projects focused on calculating RIs of blood parameters in children and adolescents in Europe. RIs of multiple laboratory parameters of blood serum and urine were defined withing the framework of the KiGGS project using the sample of healthy children [30–34]. The sample consisted of 17,641 blood and urine samples obtained from children aged 0–17. A total of 43 blood parameters were assessed that were divided into three major categories based on the parameter association with nutrition, the risk of noncommunicable diseases, and immune status. The median values and RIs were calculated for such indicators, as total cholesterol, low density lipoproteins (LDL), high density lipoproteins (HDL), triglycerides, calcidiol. The median values, 25th and 75th percentiles were defined for thyroid hormones in 12,756 subjects over the age of three. KiGGS was also focused on studying the biomarker interplay. For example, a positive correlation between the thyroid-stimulating hormone and the concentrations of the lipid metabolism indicators, except HDL, was revealed.

The important objective of the KiGGS project was to enroll a large number of subjects (to obtain a representative sample) to be able to divide them into age groups for differential assessment with sufficient statistical significance. The fact that the sample included children and adolescents of different ethnic groups living in Germany was a challenge, since such a large population of these individuals could skew the results of the blood parameter RI calculation for the European population. After the sample was formed, the share of children and adolescents without German citizenship in the sample was 8.4%, which was insignificant. The authors plan to resume the project and calculate RIs of blood parameters in children stratified by gender, age, German federal state, migrant status, etc.

NORIP project

The Scandinavian NORIP project launched as early as in 1998 was the other project focused on calculating pediatric

reference standards [45–48]. The common inclusion criteria for the sample used to calculate RIs of blood parameters was healthy individuals under the age of 18. Blood serum, plasma and whole blood samples were obtained from 3036 healthy children in Denmark, Finland, Iceland, Norway, and Sweden. RIs (central 95% range) were calculated for 25 blood serum indicators, including enzymes. The sample was stratified by age and gender.

COPENHAGEN project

In the COPENHAGEN project (2006–2008), the researchers assessed 21 blood parameters using 1421 blood samples obtained from healthy children (596 boys and 825 girls) aged 5–20 [29]. Nonparametric statistical methods were used to calculate the 95% RIs. The RIs were obtained for both genders and six age groups. Furthermore, RIs of the oldest group of children, who took part in the COPENHAGEN project, were compared with the results of the youngest group of the NORIP project. Many RI values calculated in these projects were similar. Some differences in such parameters, as alkaline phosphatase, lactate dehydrogenase, and creatinine levels were observed. These differences are quite natural, since the listed levels increase or decrease with age as the child grows. The feature of the COPENHAGEN project was that the sample of children included healthy school students with no complaints or deviations in health status, while in other projects blood was collected from children who were on outpatient treatment. Moreover, RIs calculated in this project were compared taking into account the measurement method.

CHILDx project

The CHILDx project had been implemented in the USA since 2002, in which pediatric RIs were calculated in the cohort of healthy children 6 months to 17 years of age [23–28]. RIs for a broad range of blood parameters were defined: vitamins, enzymes, hormones, coagulation parameters, and bone tissue markers. In 2005, blood samples of 902 healthy children and adolescents aged 7–17 were used to determine RIs of the coagulation parameters (prothrombin time; partial thromboplastin time; factors VIII, IX and XI; von Willebrand factor) within the framework of the CHILDx project. Eventually, several significant differences between the pediatric and adult RIs of coagulation blood parameters were found, which once again confirmed the need to calculate RIs adapted for children. For example, the median prothrombin time in children was 14.0 s, which was almost 1 s more compared to the median value obtained for adults (13.2 s).

The other CHILDx study conducted in 2011 was focused on calculating RIs of such blood parameters, as enzyme, prealbumin, and uric acid levels using blood serum samples of 1765 healthy children and adolescents [25]. The sample included different age groups. The mean values, median, and significant differences between age and gender groups were calculated (gender differences were defined for about 35% of parmeters). For example, gender differences at the age of 6–8, 12–14, and 15–17 were determined for aldolase levels. Only the levels of amylase enzyme showed no significant gender differences in any of the studied age group. At the same time, there were differences in the levels of ceruloplasmin and uric acid between groups of children aged 12–14 and 15–17. Significant gender differences in the creatine kinase levels were revealed in all age groups, except for the group aged 6–8.

NHANES project

The NHANES project that was also executed in the USA involved studying the impact of age, gender, body mass index, socioeconomic background, and ethnicity on various health parameters, including blood parameters. For that the data of laboratory studies and questionnaire surveys were acquired, and thousands of new participants were endolled every year [37–44]. For example, the study published in 2000 considered the upper 95th percentile of the C-reactive protein (CRP) concentration for the sample of more than 22,000 healthy children and adults taking into account their age, gender, and ethicity [43]. Women usually had higher CRP levels than men. CRP levels were also higher in older people than in children. Furthermore, in 2004 RIs of blood parameters were calculated using the sample of 25,000 healthy people aged 10–75, stratified by age, gender, and ethnicity, as part of this project [41]. Moreover, RIs of the levels of vitamins and lipid metabolism were determined for various age groups.

LOOK project

The large-scale Lifestyle of Our Kids (LOOK) study conducted in Australia was focused on assessing the impact of physical activity on the health of 3528 healthy children and adolescents. In of the most significant studies conducted as part of the project, the central 95% RIs and median values of 37 blood parameters were calculated using the sample of 852 healthy children stratified by gender and age [36]. Blood was collected from the same children at the age of 8, 10, and 12. The following important patterns were revealed:

– the alkaline phosphatase (AP) activity was higher in girls than in boys aged 8 and 10, however, in boys it became higher by the age of 12;

– the creatine kinase activity was higher in boys than in girls in all age groups;

– the cholesterol levels were higher in girls than in boys at the age of 10 and 12, while HDL levels were higher in boys in all age groups. The concentration of triglycerides was higher in girls in all age groups;

– the urate levels were significantly higher in boys at the age of 12;

– the ferritin levels were higher in boys than in girls at the age of 12 (the differences can be explained by the fact that 50 girls out of 256 menstruated at the time of blood collection);

– the glucose concentrations in boys and girls were almost the same, these progressively increased with age in children of both genders. The insulin levels were higher in girls than in boys and progressively increased with age.

In the LOOK study conducted in 2012, the data of 854 children were used to calculate the RIs of the blood NT-proBNP (N-terminal pro-brain natriuretic peptide B-type) levels [36]. The median, confidence intervals, and 95% RI were calculated based on gender for three age groups (8, 10, and 12 years). It was shown that the NT-proBNP concentration decreased between the age of 8–12 in healthy children.

AACB project

In the project on harmonizing the common RIs calculated using blood samples of healthy people at the hospitals of Australia and New Zealand (AACB), 123 laboratories presented RIs used by each laboratory and new test results stratified by gender in 2014 [12, 13]. These data were used to reveal the differences between RIs and analytical techniques applied in

the laboratories. Linear regression was used to compare the results of calculating the upper and lower RI limits. The AACB team defined the position of the values obtained for the new sample relative to the RI of each laboratory and its position relative to the upper and lower RI limits. Then the results of different laboratories were compared with each other. The project provided valuable information about the RIs of blood parameters used in Australia and New Zealand.

CALIPER project

The Canadian Laboratory Initiative on Paediatric Reference Intervals (CALIPER) is one of the large-scale projects focused on calculating pediatric RIs of blood parameters and aimed at building a database of pediatric RIs to be used in pediatric centers of the country and all over the world [8, 10, 14, 15–22]. In this prospective study involving thousands of healthy children and adolescents, RIs stratified by gender and age were calculated for many routinely assessed and specific biochemical markers. During the initial phase, the CALIPER project included 2809 blood plasma and serum samples obtained from apparently healthy children with stable metabolism who attended outpatient clinics. More than 50 blood parameters were assessed by biochemical methods and enzyme-linked immunoassay. Preliminary RIs were developed based on the data obtained in accordance with CLSI and the IFCC C28-A3 guidelines.

Initially, RIs were determined for five age groups stratified by gender. This phase provided the basis for further projects within the framework of CALIPER. However, the CLSI/IFCC C28-A3 guidelines stipulate that the sample for the RI determination must consist of at least 120 healthy people per parameter. The CALIPER initial pilot studies involved apparently healthy children who attended outpatient clinics and had some disease that could affect their blood parameters. Furthermore, the sample size was not enough for each parameter. Later the researchers improved the sites of blood collection: along with clinics, blood was collected in community centers, nursery schools, churches, and schools. The first of these studies was focused on determining RIs by age group for more than 40 most often assessed blood parameters. This phase of the project that represented the first of multiple CALIPER studies made it possible to start filling the gaps in pediatric RIs of blood parameters, including bone tissue markers, cardiovascular risk markers, and metabolic markers [8].

The study results showed that RIs of many blood parameters varied between the age ranges. However, the age ranges are not necessarily correlated to the generally accepted age-related development staged. For example, RI calculation has shown that it is necessary to divide the sample into seven age ranges for AP (0 to 14 days, 15 days to < 1 year, 1 year to < 10 years, 10 to < 13 years, 13 to < 15 years, 15 to < 17 years, and 17 to < 19 years), while three age ranges are enough for the alanine aminotransferase (ALT) (0 to < 1 year, 1 year to < 13 years, 13 to < 19 years) [8].

As for endocrine markers, a sample of healthy children was formed in 2013 within the framework of the CALIPER project in order to calculate RIs of seven reproductive hormones. In this study, RIs of reproductive hormones specific for certain Tanner stage (estradiol, testosterone, progesterone, sex hormone binding globulin, prolactin, follicle stimulating hormone, and luteinizing hormone) were determined. The Tanner stages are used to monitor the child's pubertal development. It is extremely important to have RIs of the hormones specific for Tanner stages, since all children enter puberty at different age. The Tanner stages are a five-item scale, where stage I corresponds to prepubertal stage, and stage V corresponds to postpuberty. The Tanner stage was defined by a subjective method: the study participants viewed the images of the Tanner stages I to V and assessed their own development relative to these images [14].

After determining the RIs of reproductive hormones, pediatric RIs of other biochemical markers, age-specific RIs of steroid hormones, and RIs of vitamins A, E [15], and D [16] were defined. Along with these large studies performed as part of the CALIPER project, some smaller but practically relevant studies were conducted. These were aimed to analyze the effects of freezing conditions on the samples and analyte stability, daily fluctuations of marker concentration in blood, and the effects of fasting on the concentrations of some biomarkers [17–19].

The age-specific and gender-specific RIs of tumor markers, biomarkers of metabolic disorders, testosterone indices, and specific biochemical markers were determined to further expand the CALIPER database. To date, the CALIPER has built a reliable comprehensive database of pediatric RI of more than 100 blood parameters considering age and gender [20–22].

The reviewed projects aimed at calculating RIs of blood parameters in children and adolescents explore a wide variety of blood parameter concentrations depending on age and gender. However, the impact of additional parameters, such as ethnicity, body mass index (BMI), amount of physical activity, etc., on the parameter concentrations is still poorly understood.

Childhood obesity in one more urgent issue related to calculating RIs. It is necessary to consider how the blood concentrations of substances change with BMI [51]. The reference population should consist of subjects that are representative of local population, however, some factors, such as BMI, change constantly with time. This makes it more difficult to obtain a representative sample. Blood parameters that depend on BMI can change with the increase in the average BMI of the sample. Therefore, it's important to know which blood parameters are affected by BMI and whether such changes are physiological or have some clinical significance (for example, as indicators of the metabolic syndrome subclinical progression) [51].

Referent-20 project

Among projects focused on calculating pediatric RIs of blood parameters, the Referent-20 project executed in 2020 at the Federal Research and Clinical Center of Sports Medicine and Rehabilitation of FMBA of Russia should be noted. The sample of thousands of professional juvenile athletes (2986 boys and 2181 girls), formed based on the results of in-depth medical examination of the Russian national team members that was performed in 2015–2019 in the clinic of the Federal Research and Clinical Center of Sports Medicine and Rehabilitation, was studied [11, 49, 50]. RIs of blood markers typical for certain components of metabolism and common blood analytes (a total of 26 parameters) were calculated within the framework of the project.

The athletes were divided into groups based on gender, age (14–15 and 16–17 years), and sports specialization: cyclic sports (the “endurance” group (distance runners) and the “speed + endurance” group (sprinters), speed-sthrength sports (technical types of track and field), complex coordination sports, team sports, and martial arts. When calculating RIs of the assessed blood parameters, the results of althletes not admitted to training based on the results of in-depth medical examination due to functional capabilities of the body and health status were excluded [11, 49].

After the sample was formed considering all exclusion criteria, the distribution of data was tested, outliers were excluded, and RIs of biochemical markers assessed during the in-depth medical examination were calculated. Considering the non-normal distribution of the data on multiple blood parameters, the nonparametric percentile-based statistics was used to determine appropriate RIs [11, 49].

Comparison of RIs of some blood parameters, for example, exercise tolerance markers, calculated for the sample of juvenile athletes, with RIs of the same parameters determined within the framework of foreign pediatric projects has made it possible to conclude that there are differences in both reference ranges and their minimum and maximum values [11]. For example, comparison of RIs of blood creatinine calculated for the sample of juvenile athletes (sports RI) with RIs calculated within the framework of the Caliper/Norip prject showed that in the sample of male athletes aged 14–15 the maximum values of RIs of this parameter were 30% higher than RIs in the Caliper/Norip boys, while in boys aged 16–17 these values were almost the same. In the sample of female athletes aged 14–15 the maximum values of RIs of creatinine were 13% higher, and in girls aged 16–17 these were 16% higher than RIs obtained in the Caliper/Norip projects. In sports, blood levels of creatinine are also used as an exercise tolerance marker. The increase in maximum values of RIs of creatinine in juvenile athletes compared to ordinary adolescents may be due to significant physical exertion and increased intake of protein foods [11].

This project has also shown that the minimum and maximum values of RI of cortisol calculated for the sample of juvenile athletes are 58–67% higher than in untrained children. Moreover, the calculated RIs of cortisol in boys and girls of the same age, who are involved in sports, are similar. When comparing RIs of cortisol calculated in the Referent-20 project and the Caliper/Norip projects, the following can be noted: boys aged 14–15, who are involved in sports, have maximum values of RIs of cortisol that are 64.7% higher compared to maximum values of RIs of cortisol in boys involved in the Caliper/Norip projects, while in boys aged 16–17 the maximum values of RIs of cortisol are 67.2% higher. Girls aged 14–15, who are involved in sports, have maximum values of RIs of cortisol that are 64.8% higher compared to maximum values of RIs of cortisol in girls involved in the Caliper/Norip projects, while in girls aged 16–17 the maximum values of RIs of cortisol are 66.7% higher. The finding of higher maximum values of RIs of cortisol in juvenile athletes may be due to stress they experience when engaged in professional sports [11].

CONCLUSION

Thus, the review of world's and domestic literature shows that successful attempts to determine RIs of blood parameters in the population of children and adolescents have been done in recent years. However, the question remains about calculating pediatric RIs of blood parameters in specific cohorts of children, for example, athletes, children of various ethnic groups or children with different BMI. Further research focused on determining RIs of blood markers in juvenile athletes based not only on gender and age, but also on the features of sports load, professional experience, and athletic performance, is required. The data obtained in these studies will help the sports medicine physicians to schedule and perform interventions aimed at optimizing the functional state in a timely manner.

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