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Reference Intervals for Clinical Laboratories

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Abstract

Reference intervals (RIs) are a fundamental tool in many medical disciplines for interpreting patient laboratory test results in clinical laboratories. Ideally, they enable the differentiation of healthy and unhealthy individuals. Clinical laboratories must establish accurate RIs, which is a very important process. Traditionally, RIs have been estimated using the 'direct' approach, which involves collecting laboratory test results from apparently healthy volunteers. An alternative approach is the 'indirect' approach, in which results from specimens collected for routine, screening, diagnostic or monitoring purposes are used to determine the RIs. When a laboratory receives an RI from the literature, manufacturers or another laboratory, the process of confirming its suitability for use is usually referred to as 'verification of RIs'. This raises questions about the transferability of RIs that need to be addressed. Common RIs can be obtained from multicentre studies, providing an opportunity to harmonise RIs within a given population. Clinical decision limits (CDLs) lead to the decision that individuals with values above or below the decision limit should be treated differently. There is still some confusion surrounding the difference between RIs and CDLs. The challenging groups, such as pediatric, geriatric and gestational age groups, as well as for uncommon sample types is a gap in the RIs studies. When individuality is a key factor, personalised RIs are far more effective than population-based RIs for monitoring individuals.

Keywords: Reference intervals, in clinical laboratories, clinical decision limits

INTRODUCTION

Laboratory medicine has long played a key role in diagnosing, treating and monitoring hospitalised patients. Every day, millions of laboratory tests are performed worldwide that need to be interpreted for clinical decision-making purposes. Reliable and accurate reference intervals (RIs) for laboratory analyses are therefore an integral part of correctly interpreting clinical laboratory test results (1).

Studies in this area began around six decades ago. In the mid-20th century, Gräsbeck et al. (2) published the initial paper entitled *Normal Values and Statistics*. In subsequent years, it was realised that the term “normal

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values" was inadequate and even partially incorrect. In 1969, Gräsbeck and Saris (3,4) launched the concept of the reference value(s) in a session devoted to normal values at a Congress of Clinical Laboratory Medicine, and the term "reference values" has since become widely accepted as an alternative to "normal values". From 1987 to 1991, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) published a series of six papers recommending that each laboratory follow defined procedures to produce its own RIs (5-10). Interest in this topic has been renewed as a result of the following regulatory initiatives of the last two decades. According to European Directive 98/79 on *in vitro* diagnostic medical devices, diagnostic kit manufacturers must supply their clients with the appropriate RIs for use with their assay platforms and reagents (11) and the International Organization for Standardization (ISO) 15189 standard for clinical laboratory accreditation states that each laboratory should periodically re-evaluate its own RIs (12).

Despite these requirements, RIs in most clinical laboratories are often out of date and incomplete due to the complex process of establishing them (13). Therefore, rather than developing RIs directly from an apparently healthy population, most laboratories obtain RIs for clinical use from various sources, such as manufacturers' package inserts, publications, textbooks, multicentre studies, published national or international expert panel recommendations and guidelines, local expert groups, or data mining of existing data. The laboratory is required to validate RIs from manufacturers or estimate appropriate RIs from the local population (13). The guideline entitled *Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory* (EP28-A3c) provides the necessary steps for selecting reference individuals and considers pre-analytical and analytical factors, as well as analysing reference values for RI establishment studies and transference and verification of RIs (14). However, in the present era of evidence-based medicine, there is still a significant discrepancy between theory and practice with regard to the application of RIs as decision-making tools, despite mandatory requirements.

Reference intervals are derived from the reference population value distribution, usually the central 95% interval, and describe a specific population using a minimum sample size of 120, as recommended by the Clinical Laboratory Standards Institute (CLSI) guideline EP28-A3c (14). The traditional method for establishing RIs, known as the direct approach, is based on collecting samples from members of a preselected reference population, making the measurements and then determining the intervals. An alternative approach is to perform analysis of results generated as part of routine pathology testing and using appropriate statistical techniques to determine RIs. This is known as the indirect approach (15). The methods and processes for determination of reference RIs using indirect methods have been in development for over 50 years. This approach is not only a useful adjunct to traditional direct methods but also has a number of significant benefits and advantages (15).

In practice, this is very challenging because it is difficult to recruit a sufficient number of reference individuals, control pre-analytical variables and apply statistical methods appropriately (14). Therefore, the Committee for Reference Intervals and Decision Limits (C-RIDL) of the IFCC has emphasised the importance of common Rls and has conducted multicentre RI studies since 2009 (16). Where there are no apparent regional differences in reference values for any of the analytes and the assays are standardised nationwide, the reported RIs can be used throughout the country (17).

Clinical decision limits (CDLs) should be distinguished from RIs. While RIs describe the typical distribution of results seen in an apparently healthy reference population, CDLs are based on the diagnostic question and are obtained from specific clinical studies to define the probability of of a certain disease or another outcome (18).

This review describes the methodologies for establishing and verifying RIs, and provides a detailed evaluation of common RIs, CDLs, personalized RIs. The differences between these types of RI are explained (e.g., direct versus indirect RIs, RIs versus CDLs), to help readers avoid confusion. The review also discusses the importance of RIs for specific age groups, such as paediatric and geriatric patients.

REFERENCE INTERVALS

The concept of RIs is now well established and is based on including a fixed percentage of a reference population within the interval described withupper and lower reference limits (RLs). The reference population is generally made up of a statistically significant number of predefined condition-free subjects, but the concept can be applied to any defined population. Generally, it is the responsibility of laboratories to either validate a RI derived elsewhere or determine their own interval for use with their population and analytical methods. The pre-analytical, analytical, and post-analytical factors affect RIs (19).

Reference intervals are divided into two main subgroups: direct RIs and indirect RIs.

Direct Reference Intervals

Direct approach to RIs is the recommended process by

the EP28-A3c guideline, where subjects representing the reference population are selected and sampled and the specimen analyzed for this purpose (14). In this process, individuals from a population (the reference population) are selected for sampling based on defined criteria. Specimens are then collected from these individuals and analyzed for the selected measurands. This approach has been subdivided into a priori and a posteriori selection process. The a priori approach is to select individuals for specimen collection and analysis if they meet defined inclusion criteria and it is the more appropriate approach when the biology of an analyte is known. In the a posteriori approach, specimens collected from a population will be included in the analysis based on other factors such as clinical details or other measurement results, which were not used to define the collection. Thus, in the posteriori approach, not all specimens that were collected would be included in the reference population for further analysis. Ideally, a direct approach would use randomly selected members of the reference population; however, this is rarely achieved as the tested population is usually influenced by convenience and cost factors (20).

Pre-analytical and analytical aspects must be taken into consideration in the implementation of a RI study. Generally, the pre-analytical considerations involve biological (i.e. sampling time in relation to biological rhythms, fasting or non-fasting and physical activity) and methodological factors (i.e. sample collection techniques, type of additives, with or without tourniquet and sampling equipment, specimen handling, transportation, time and speed of centrifugation, and storage conditions) (14). For reproducibility and standardization, it is essential that the pre-analytical aspects are accurately defined and described as the preanalytical phase is known to have the highest error rate in the total test process (21).

Analytical aspects include the analytical variability of the method used for the measurement, equipment/instrumentation, reagents, calibration standards, and calculation methods. Different commercial methods may be used in a trueness-based approach to the reference measurement system, providing results traceable to the system and thus, comparable results can be produced in clinical laboratories. When performing a RI study, the reference measurement systems and standard reference materials are of great importance to ensure the traceability of the test results in comparisons (22).

Establishing of RIs involvels parametric and nonparametric calculation methods, detection of outliers, partitioning, and confidence intervals (CIs). In the parametric calculation method, the most suitable transformation method must be selected (e.g., logarithmic, Box-Cox power or some other function) and testing is then applied to establish whether the transformed reference values conform to Gaussian distribution (23). Box-Cox power transformation often has been used to transform data to a Gaussian distribution for parametric computation of RIs (23). In addition to the calculation of the RIs, detection, and exclusion of the outliers are very important to obtain reliable RIs. A simple but effective method for the detection of outliers is a visual inspection of the data. Although the method proposed by Dixon (24) is presented in the guideline, EP28-A3c (14), it is not very sensitive when there is more than one outlier. The Tukey method is a more sophisticated method, which includes Box-Cox transformation of the data to obtain Gaussian distribution followed by identification of the outliers in interquartile ranges (25). The latent abnormal value exclusion (LAVE) method proposed by Ichihara and Boyd (26) is a secondary exclusion method to exclude possibly abnormal results hidden within the reference values.

Stratification of RIs by age and gender is the minimum pre-requisite and other means include race, ethnicity, body mass index (BMI) or nutritional habits. The most widely-used partitioning method is that of Harris and Boyd (27), in which the means and standard deviations (SDs) of the subgroups are considered as a separate different SD that may produce different limits. A similar method was proposed by Lahti et al. (28) allowing the estimation specifically of the percentage of subjects in a subclass outside the RIs of the entire population in any situation. More recently, Klee et al. recommended a partitioning method on the basis of the magnitude of the SDs of test results named SD ratio (SDR). An SDR greater than 0.3 can be regarded as a guide for the consideration of partitioning reference values (29).

The CI is a range of values including the true percentile (e.g., the 2.5th percentile of the population) with a specified probability, usually of 90% or 95%, as the "confidence level" of the interval. It was recommended that RLs should always be presented together with their 90% CIs in the C28-A3 guideline. In the C28-A3 guideline, non-parametric CIs are given from the observed values corresponding to certain rank numbers from Reed et al. (30). Although one can theoretically determine 95% RIs with a lower number (as few as 39 samples), it is clearly recommended that at least 120 subjects are required to calculate the CIs of the lower and upper RIs in the guideline). Horn et al. (31) proposed a "robust method" method based on transformation of the original data according to Box and Cox followed by a "robust" algorithm giving different weights to the data, depending upon their distance from the mean (32).

Indirect Reference Intervals

An alternative approach is called the indirect approach where results from specimens are collected for routine purposes, which have been collected for screening, diag-

nostic or monitoring purposes and are used to determine the RIs. Data mining, or "big data", is the process of using previously generated data to identify new information. Routine pathology databases often contain many thousands or millions of results from many 100s or 1000s of patients, which can be used in this manner (15).

A key difficulty of standard statistical techniques is the high likelihood (or indeed expectation) of values from diseased individuals in the data set, which has been extracted from the pathology database, to influence RI results. As standard statistical techniques are strongly influenced by the extremes of the data set, and these extremes are those most likely to be from affected subjects, great attention needs to be given to outlier removal (15). There have been a number of examples of approaches to attempt to minimize the presence of results from diseased subjects in database extracts. For example, Ozarda et al. (33) applied data exclusion criteria to reduce contamination of the database by results from subjects with the disease. In this IFCC study, a two-step data cleaning process was applied as follows: 1) After excluding the test results of inpatients, only the results of outpatients were included, except for those ordered from outpatient clinics specialising in emergency care, oncology, anaesthesia and resuscitation, gastroenterology, and nephrology. 2) If a patient had multiple records in a given year, all records from that year were excluded except the first result, based on the assumption that the necessity for multiple testing implies a higher likelihood of an unhealthy status (33).

Standard parametric (mean and SD) or non-parametric statistics (percentiles), such as those used in direct RI studies, can also be used for indirect studies. This will involve outlier removal, either before or after transformation, followed by calculation of the mean and SD or median and relevant percentiles (33). The indirect RIs are usually determined by statistical methods based on identifying a distribution in the midst of the data such as Bhattacharya (34) and Hoffmann (35), rather than requiring assessment of all individual results in the database as belonging to the reference population or otherwise. Standard parametric or non-parametric processes have been used for indirect RI studies. This will involve outlier removal, either before or after transformation, followed by calculation of the mean and SD or median and relevant percentiles formation of the source data by use of Box-Cox formula. The truncated maximum likelihood (TML) method (36) and the truncated minimum chi-squared (TMC) method (37) are two indirect methods of estimating RIs. These methods use a software programme consisting of an Excel spreadsheet for the front end and an R script for the calculations. Both methods use an iterative algorithm to determine the optimal truncation segment of the reference value distribution and estimate the parameters of the corresponding distribution. The TML method is similar, except that it provides a more accurate estimation of λ and more reliable normality testing for the central truncated segment.

Direct & Indirect Reference Intervals

Direct sampling techniques require a series of structured steps that together require significant resources (6,7). These steps include the following: definition of the reference population; locating/recruiting members of the reference population; obtaining informed consent; sample collection, processing and storage; sample analysis; statistical evaluation (including outlier exclusion); and development of RIs for routine use. The processes of identifying subjects, collecting specimens and performing analysis are, at the very least, expensive and time consuming. By contrast, the indirect approach is based on data that have already been generated as part of routine care, thus excluding the resource-intensive components, i.e. patient identification, recruiting, specimen collection and measurement, of the direct approach (7).

Important benefits of the indirect approach, relative to the direct approach, include that it is faster and cheaper. It is also based on the actual preanalytical and analytical conditions used in routine practice. Additionally, the reference population is the one from which a patient is actually being distinguished from, i.e., a person presenting to a health care service who does not have the condition under consideration is compared with the person attending for medical care of that condition (6).

There are however risks and difficulties associated with indirect approaches. The most important risk is the question as to whether the presence of diseased individuals influences the RIs. This will depend on the nature of the disease state, i.e. clearly separated or overlapping with the nondisease population, and the relative prevalence in the population. Data sets can be "biochemically filtered" to reduce the frequency of results from subjects where there is a higher likelihood of disease affecting the result. An additional recommended approach is to limit results to a single result per patient. As a diseased patient is more likely to be retested than a non-diseased patient, failure to do this is likely to lead to overrepresentation of results from unwell subjects. The removal of probable outliers from a data set can be a useful tool, even if more robust statistical processes are used. However, there is no consensus on the best statistical model to calculate the indirect RIs (33).

Table 1 involves the comparison of direct and indirect methods for RI determination showing mostly benefits of indirect methods. However, it should be born in mind that EP28-A3c still recommends the direct methods to establish RIs.

Table 1. Comparison of direct and indirect methods for reference interval determination.

Direct	Indirect
Ethical issues with sample collection and responses to new information identified on patient, obtaining informed consent may be difficult	No ethical issues with sample collection and no new information identified on patient
Costs of performing the study are high	Costs of performing the study are very low
Difficult and expensive to get statistically significant numbers	Significant numbers readily available
Difficult to define "healthy" status	Defining "health" is not required. Exclusion criteia would be heplful to exclude "unhealthy population"
Preanalytical and analytical conditions may not match routine conditions	Preanalytical conditions match routine conditions
Hard to perform direct RI studies	Easy to repeat indirect RIs
Recommended method by the guideline, EP28-A3c	Recommended especially for uncommon sample types and challenging groups (i.e., pediatric and geriatric patients)

VERIFICATION OF REFERENCE INTERVALS

Under optimal conditions, a laboratory should perform its own RI study to establish RIs specific for its method and local population. However, the process of developing RIs is often beyond the capabilities of an individual laboratory due to the complex, expensive and time-consuming nature of the process to develop them. Often, clinical laboratories lack the necessary resources to determine RIs adapted to their local patient population and therefore refer to manufacturers of laboratory devices and test kits.

Clinical laboratories may transfer adequate RIs from external sources. Assuming the original RI study was performed using robust methodology and statistical procedures, transferring an RI requires certain conditions to be fulfilled before it can be verified and accepted. There are two main scenarios in which RIs are transferred. First, reference values may originate from a different population or laboratory method than the receiving laboratory, and second, reference values may originate from a laboratory that shares the same laboratory method/population as the receiving laboratory (38).

In the first instance, comparing the laboratory methods serves as an instructive early screening tool to assess the suitability of the reference values for the receiving laboratory (13). Laboratory methods can be compared by a method comparison study between the method used during the development of the RI and the method used by the receiving laboratory to determine the statistical validity of an RI transfer (39). For a method comparison study, samples must be collected with an appropriate distribution of values spanning the RI, as aninsufficient range may underestimate and a range too large may overestimate the strength of the correlation. The correlation between the two methods is subsequently analyzed and, if appropriate, linear regression analysis is performed to determine the slope and y-intercept values of the best-fit regression line (40). These values are subsequently used to transfer the RI. According to the CLSI EP28-A3c guideline, the best-fit regression line should have a slope bias close to 1, a y-intercept close to 0 and a correlation coefficient (r^2) close to 1 (14). Furthermore, according to CLSI EP09-A3 guidelines, the scatter and bias plots should be examined for constant scatter to ensure there are no dramatic differences between the variation at the upper and lower ends of the range of values (41). To sufficiently assess the acceptability of the method bias, it is also important that the magnitude of the y-intercept is small compared to the range of the data and the RI. If the y-intercept is large compared to the RI, it is recommended to reject transference and establish an RI directly from a healthy reference population. If the preanalytical processes (e.g., preparation of reference individuals, specimen collection, transportation, and handling), the laboratory methods and the populations (e.g., a relatively homogenous population within the same geographical region) are very similar to those of the laboratory where the RIs originated, the method comparison study is still recommended to confirm the comparability, although the bias between the laboratory methods is expected to be very small (42).

Following transference, the CLSI EP28-A3c guideline recommends subsequently verifying the transferred RI.

It is important that laboratories verify their RIs before applying them for routine clinical care. This requirement applies to RIs derived using the indirect approach. This can be achieved by the conventional approach where the laboratory analyses samples from 20 subjects without the predefined condition in the reference population. The RIs is considered verified if two or less results out of 20 fall outside of the RIs that would correspond to a 95% probability (14). However, this procedure is not practical for clinical laboratories and is not often used for routine verification (38).

Alternatively, laboratories can assess if the given RI is appropriate for their testing patient population and analytical method by monitoring the percentage of abnormal results (that would be typically flagged by the laboratory information system) and comparing it with the expected percentage that may be easily derived from the original indirect study calculations. When a change in the flagging rate in any direction (increased or decreased) does not exceed a predefined expected value, the RI under evaluation is acceptable for use. This method does not require additional patient testing and may be programmed in the laboratory information system as a continuous quality control monitoring measure (15).

COMMON REFERENCE INTERVALS

Establishment of well-controlled, reliable RIs is an important mission for all clinical laboratories (43). Although direct RIs are most established using a well-defined and representative reference population, with sample analysis performed by a single laboratory, RIs can also be determined with the intention of serving a much broader population demographic and/or geographic location with sample analysis performed by a single platform or multiple platforms; these are termed common RIs. There are two types of common RIs. The first is objective RIs, which require many prerequisites (44) and defined by well-conducted multicenter studies (45). The second is subjective RIs, which are defined by the survey(s) and guidance from a group of experts using the harmonization approach (46).

The derivation of RIs on a national level by conducting a multicenter study that follows a common protocol, comprehensive standard operating procedures (SOPs), and secondary integration of the results on a global scale is probably the most effective way to establish globally applicable, or common RIs (47). The C-RIDL has published papers including a protocol and SOPs for multicenter RI studies (51), with indication of the utility of a panel of sera for the alignment of test results among laboratories in the multicenter studies (48).

Eight years ago, the C-RIDL performed a global multicentre study to evaluate the importance of age, BMI and levels of alcohol consumption and smoking as major sources of variations of reference values in various countries (ethnic groups). Multiple regression analysis was used to confirm differences related to ethnicity in BMI-related changes in reference values. This was done to confirm ethnicity-related differences in BMI-related changes in reference values. The aim was also to make a BMI-adjusted comparison of reference values among the countries and to delineate gender- and age-related profiles of reference values from a large number of datasets compiled from the 12 countries (49,50). This was a direct multicenter RI study with total recruitment of 13,386 healthy adults to determine global RIs of 25 analytes were measured chemically and 25 immunologically and an example of a well-conducted multicenter RI study, in which each laboratory acts as a central laboratory and sample analysis is performed using multiple platforms. In this type of multicenter study, it is essential to perform rigorous quality control monitoring to detect analytical deviations and use internationally accepted reference materials for standardized analytes to ensure traceability in each center. In addition to internationally accepted reference materials, the global IFCC, C-RIDL study is based on a common protocol (47) and the use of a panel of sera (48) to harmonize measurement results. This approach resulted in a method comparison and successful transference of the data obtained from the global study. As part of the global study, a multicenter RIs study was also performed in Türkiye, including seven geographical regions, using traceable materials and panel of sera from 40 reference individuals from the global study in the central laboratory, using a single platform, as an example of studies where the measurements were performed in one center acting as the central laboratory (51). With the lack of regional differences and the well standardized status of test results, common RIs for Türkiye have been derived from this nationwide study. Additionally, “cross-check testing” using at least 20 samples has been performed to compare results among the participating laboratories in Türkiye as recommended in the protocol for multicenter studies (47). Thus, common RIs were transferred from the multicenter study to each participating laboratory in Türkiye using the linear regression slope and intercept (45).

REFERENCE INTERVALS & CLINICAL DECISION LIMITS

Every laboratory request has a purpose, with specific questions. The question “Is the patient healthy or not healthy?” relates to RIs that describe the typical distribution of results seen in an apparently healthy refer-

ence population. However, the questions ("Is the patient at risk of a developing a disease, or is the patient diseased, or worsening?") are related to CDLs, where values above or below the threshold are associated with a significantly higher risk of adverse clinical outcomes or are defined as diagnostic for the presence of a specific disease (52).

Clinical decision limits are thresholds above or below which a specific medical decision is recommended and are derived from receiver operating characteristic (ROC) curves and predictive values (53). Reference intervals are focused on optimizing specificity (typically to 95%) while CDLs are also focused on optimizing sensitivity for the disease. The approaches to identifying CDLs can be categorized (52): 1) The Bayesian approach is probably the most evidence-based approach to modifying the management of the patient. Following these criteria, a value resulting from a diagnostic test that serves to distinguish between two clinical subgroups is based on stated assumptions regarding: (i) the clinical sensitivity of the diagnostic test; (ii) the clinical specificity of the diagnostic test; (iii) the relative distribution of individuals between the two subgroups; and (iv) the clinical costs of misclassification (54). 2) The epidemiological approach for defining CDLs is based on clinical outcome derived from population-based studies and is typically applied to lipid parameters (low density lipoprotein cholesterol, high density lipoprotein cholesterol, etc.) (55). 3) The physiopathological approach involves the use of "critical values" that represent a pathophysiological state with such variance from normal as to be life-threatening unless prompt action is taken. While many clinical endpoints can be difficult to define, the endpoint of mortality is clear and, because it defines the risk of dying or of major patient harm, it defines a particular set of high risk CDLs often called "critical values" (56).

There are two limits (upper and lower) of the RIs, conraversly there is only one CDL, which is usually an upper limit. However, according to the likelihood of various clinical situations or different clinical questions, multiple low and high CDL may be used. RIs are defined by laboratory experts using different methods (direct, indirect). Clinical decision limits sare defined by clinicians and laboratory experts. Consensus standards of RIs are well-defined (14) while than those of CDL's still to be developed.

As there are key differences between RIs and CDLs (see Table 2), it is important to note that RIs and CDLs should not be viewed as the same in clinical laboratories. Rls are generally considered as a distribution of test values in the predefined population, whereas CDLs are mostly determined by assessing the patients' outcomes or response to management change. Clinical decision limits are based on the diagnostic question and are obtained from specific clinical studies to define the probability of the presence of a certain disease or a different outcome (57). Reference intervals relate to studies based on apparently healthy individuals while CDLs are based mainly on clinical outcome studies (e.g., prospective cohort studies, meta-analysis), guidelines and consensus values. These limits lead to the decision that individuals with values above or below the decision limit should be treated differently. To avoid confusion, the EP28-A3c recommended reporting decision limits or RIs but not both, with a clear indication of which has been used (14).

Analytical quality affects the reliability of both RIs and CDLs. The biological variability theory suggests that the

Table 2. Comparison of reference intervals and clinical decision limits.

	Reference intervals	Clinical decision limits
Population based on	General population	Clinical population
Method for derivation	95% central interval of the reference distribution	Clinical outcome studies, guidelines and consensus values, ROC curves, predictive values
Based on	Healthy population	Diagnostic question
Focused on	Optimizing specificity (typically to 95%)	Optimizing specificity and sensitivity for the disease
Data number	Two (lower and upper limits)	One or more, according to the likelihood of clinical situation or different clinical questions
Dependence	Type of population, age range, gender	Clinical problem, patient's category
Consensus standard	Well defined	Still to be developed
Defined by experts	Laboratory experts	Clinicians and laboratory experts

desirable bias for RI classification takes into account between-subject /intraindividual (CVI) and within-subject /interindividual variability (CVG) and that it will prevent an unacceptable increase in the proportion of healthy individuals flagged as outside RIs. Analytical quality will similarly affect the application of CDLs, although the impact is defined not by the statistics of the reference population distribution but by the clinical risk definitions as well as the prevalence of disease (58). Increasing measurement uncertainty generally causes greater clinical uncertainty; similarly, the impact of uncorrected measurement bias will lead to clinical bias. The traceability of method calibration is vitally important for both RIs and CDLs. Neither universal CDLs (e.g., for lipids and HbA1c) nor common CDLs (e.g., for routine analytes) can be clinically reliable without traceability and analytical quality standards (63).

CHALLENGING GROUPS FOR THE DETERMINATION OF REFERENCE INTERVALS

As the concentrations of many routinely measured analytes vary significantly with growth and development, the use of inappropriate pediatric RIs can result in misdiagnosis and misclassification of disease. It is well known that the determination of pediatric RIs is an extremely difficult task, primarily because of ethical limitations related to blood drawing in very young children and neonates. The most significant step in this area has been taken by Adeli et al. (45) in the Canadian Laboratory Initiative in Pediatric Reference Intervals (CALIPER) Project, which is a collaboration between multiple pediatric centers across Canada, that aims to address the current gaps in pediatric RIs and has established a database of age- and gender-specific pediatric RIs. The German Health Interview and Examination Survey for Children and Adolescents (Kinder- und Jugendgesundheitssurvey, KiGGS) is an another excellent example in this area (60). As these direct studies were well conducted and of large sample size, the current problems in pediatric RIs could be resolved through evaluation and application of the findings. However, as an alternative, indirect methods can be used for the pediatric group as recommended in the EP28-A3c (14).

The major difficulty in obtaining geriatric RIs is the selection of healthy individuals, as most elderly subjects do not meet the CLSI EP28-A3c guideline for inclusion in a healthy reference population (14). The width of the RI is altered by factors such as the regular use of medications or unrecognized subclinical diseases. Therefore, it becomes very difficult to differentiate the effects of age, aging, or a pathological condition. Although there has been increasing interest and studies in this subject (61,62), this issue remains inadequately addressed (63). It would be of great benefit to conduct a large, multicenter study with pediatric, adult, and geriatric reference individuals to develop common RIs, subsequently transfer them to local laboratories. They can be then verify them with respect to these specific age-groups using a limited number of healthy subjects and/or existing laboratory data (63).

Laboratory RIs during pregnancy, delivery, and the early postpartum period are another specific group as physiological changes during pregnancy may affect laboratory parameters and there is a need to establish reference values during pregnancy to recognize pathological conditions (64). Reporting the correct gestational age-specific reference values can also improve the sensitivity of the RIs.

The RIs for uncommon sample types (e.g., cerebrospinal fluids [CSFs], amniotic fluids) are usually interpreted on the basis of values reported in reference texts or handbooks; however, current reference texts either present normal CSF parameters without citation or cite studies with significant limitations. Recent developments to determine accurate, age-specific reference values for glucose tein concentrations and white blood cell counts in CSF, amniotic fluids and aspirations in a large population of neonates and young infants will bring literature up to date at a time when molecular tools are commonly used in clinical practice (65,66).

Integrating genetic and laboratory information would increase the accuracy of RIs by eliminating extreme results related to genetic variation. It has been reported that the use of genetic information to partition Rls could reduce the between-person variation and therefore with the reduced variance obtained from partitioning based on genetic differences, there could be potentially less misidentification of unusual test results caused by non-disease associated genetic variations. It has been reported that serum folate and homocysteine status are impaired by subgroup stratification of the rate of methylenetetrahydrofolate reductase (MTHFR) 677C > T i 1298A > C (67). However, there is often a lack of knowledge of the genetic status of the reference individuals. Integrating genetic information with RI values would improve the sensitivity of the RIs (20).

PERSONALIZED REFERENCE INTERVALS

Knowledge of major sources of variation inbiological quantities is a part of the concept of reference values.

There are many analytes that are affected by biological characteristics, such as age, gender, or pregnancy, or by factors, such as season or geographic location. Certain quantities have predictable cyclical biological variation (BV) (daily, monthly, seasonal) and the knowledge of the expected values throughout the cycle is vital for clinical interpretation of laboratory data (68).

When individuality is still a key factor, subject-based RIs are far more effective than population-based RIs for monitoring individuals (69). For clinicians, the main concern is whether the actual test result from a specific patient is indicative of disease or not. To answer this question, a personalized RI; i.e., an RI for that specific individual would be useful (70). The within-subject BV (CVI) describes the fluctuation of a measurand around its homeostatic set point in steady-state conditions in an individual, whereas the variation between the set point of different individuals is defined as the between-subject BV (CVG) (76). Many investigators have previously produced estimates for CVI and group CVG variation. However, there is now a better understanding of the need to produce and promulgate accurate estimates generated from significant sample sizes using the best statistical tools available (72). Important statistical considerations include determining the BV parameters, outlier removal, and their CIs (73).

Variations in the concentration of the analyte still within the RI can be significantly outside the subject's usual values, in which case it is useful to calculate if the reference change value has been surpassed or to calculate the statistical significance of a trend. The reference change value, which can be defined by absolute (±delta) or relative (±delta%) means, can help in the interpretation of the results of serial measurements (74). The progress of a disease or recovery from it is often reflected by the dynamics of test results (delta values/delta change). The example of absolute and relative kinetic changes of cTn in patients with acute coronary syndrome shows that serial measurements may assist in diagnosis and may be used to rule out non-ST elevation myocardial infarction (75).

The prerequisites to calculate delta changes from serial measurements are a well-accepted clinical algorithm with defined time points (e.g., baseline, 3 h, 6 h for cTn) and the knowledge of the intra-individual BV of the measurand CVI.

Although the source of BV data is typically from a healthy reference population, its application to disease assumes that BV is the same in chronic disease as in health (76), and this has been adopted as a surrogate for clinically significant changes.

CONCLUDING REMARKS

Interpreting the results of a clinical laboratory test requires comparison with a RI, a clinical decision point or previous results. Clinicians and laboratory experts should clearly distinguish between these concepts. Direct methods are still the gold standard for establishing RIs. However, this method is time-consuming and expensive for laboratories, and in many cases, laboratories prefer to use recommended RIs provided by manufacturers or modified RIs obtained from other sources. Indirect methods of deriving RIs are inexpensive, easy and fast. Although very important progress has been made over the last decade, there is still no consensus on the most effective model for establishing reliable RIs.

It should be borne in mind that RI is only an estimation. They involve uncertainties and assumptions that may or may not be true. Once a second sample has been collected, comparing it with the previous result may be more important than comparing it with the RI. Each patient should be assessed individually using all available clinical and laboratory data. Clinicians should realize that test result is not an absolute number but rather a range that is determined by a combination of analytical and BVs.

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