Clinical Biochemistry 48 (2015) 828–836

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Highlight Article

# Dynamic biological changes in metabolic disease biomarkers in childhood and adolescence: A CALIPER study of healthy community children

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ARTICLE INFO

*Article history:*
Received 17 March 2015
Received in revised form 29 April 2015
Accepted 5 May 2015
Available online 12 May 2015

*Keywords:*
Pediatrics
Reference intervals
Acylcarnitines
Amino acids
Inborn errors of metabolism

ABSTRACT

**Background:** Understanding age- and sex-specific biological changes in metabolic disease biomarkers is essential for their appropriate utilization in management of children with inborn errors of metabolism (IEM). The CALIPER program aimed to establish pediatric reference values in healthy community children for common metabolic biomarkers and determine the effects of key covariates including age and sex across the pediatric age.

**Methods:** A cohort of 500 healthy children and adolescents from birth to 19 years were initially recruited to establish pediatric reference intervals according to the CLSI C28-A3 guidelines. Serum samples were used to measure 37 amino acids by ultra-performance liquid chromatography, 32 acylcarnitines, as well as free and total carnitine by tandem mass spectrometry, and β-hydroxybutyrate and free fatty acids using the Vitros 5.1 chemistry analyzer. P ediatric reference intervals were calculated using non-parametric statistics and partitioned based on age- and sex-distributions.

**Results:** Approximately 80% of all analytes required 2 to 4 age-dependent partitions, with over 50% of amino acids and over 70% of acylcarnitines exhibiting significant physiological changes during the neonatal period. Also, 21% of all analytes required partitioning during puberty and adolescence, half of which produced sex-specific distributions.

**Conclusions:** A comprehensive reference interval database for metabolic disease biomarkers established in this study will improve detection of IEMs by providing appropriate age- and sex-related information in the pediatric population. It will also aid newborn screening programs and guide the management of patients with known metabolic diseases, especially pubertal and adolescent boys and girls that display sex-specific concentrations.



## Introduction

Pediatricians and other healthcare professionals depend on objective laboratory data to assess medical concerns in children and adolescents. Normative data (also referred to as reference intervals, RIs) determined from a cohort of healthy children are critical to the accurate interpretation of biochemical markers used in the diagnosis and monitoring of pediatric disease. A reliable protocol to establish RIs has been documented in the Clinical Laboratory and Standards Institute (CLSI) C28-A3 guidelines [1] and outlines detailed protocols for determining RIs, and determining if age and/or sex partitions are necessary for the particular study population. According to the C28-A3 guidelines, a minimum of 120 healthy individuals per partition are required. Thus, establishing RIs is challenging due to the large number of individuals required, as well as the overall feasibility and logistics of the sample collection and data analysis.

*Abbreviations:* IEM, Inborn errors of metabolism; Eth, Ethanolamine; Asa, Argininosuccinic acid; Sarc, Sarcosine; C2, Acetyl carnitine; C3, Propionyl carnitine; C3-DC, Malonyl carnitine; C4, Butyryl/isobutyryl carnitine; C4-DC, Methylmalonyl/succinyl carnitine; C5:1, Tiglyl carnitine; C5, Isovaleryl carnitine; C5-DC, Glutaryl carnitine; C5-OH, 3-Hydroxy-isovaleryl carnitine; C6, Hexanoyl carnitine; C6-DC, Adipoyl carnitine; C8:1, Octenoyl carnitine; C8, Octanoyl carnitine; C10:1, Decenoyl carnitine; C10, Decanoyl carnitine; C12:1, Dodecenoyl carnitine; C12, Dodecanoyl carnitine; C12-OH, 3-Hydroxy-dodecanoyl carnitine; C14:2, Tetradecadienoyl carnitine; C14:1, Tetradecenoyl carnitine; C14, Tetradecanoyl carnitine; C14:1-OH, 3-Hydroxy-tetradecenoyl carnitine; C14-OH, 3-Hydroxy-tetradecanoyl carnitine; C16:1, Palmitoleoyl carnitine; C16, Palmitoyl carnitine; C16:1-OH, 3-Hydroxy-palmitoleoyl carnitine; C16-OH, 3-Hydroxy-palmitoyl carnitine; C18:2, Linoleyl carnitine; C18:1, Oleoyl carnitine; C18, Stearoyl carnitine; C18:2-OH, 3-Hydroxy-linoleyl carnitine; C18:1-OH, 3-Hydroxy-oleoyl carnitine.

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http://dx.doi.org/10.1016/j.clinbiochem.2015.05.005
0009-9120/

Additional challenges for establishing RIs for children is that they must be stratified to appropriately reflect changes in body size, which is constantly increasing from birth to adolescence, and changes in body organs which continue to mature after birth. This process is further complicated by the limited sample volume that can be collected, as well as obtaining access to an appropriately healthy pediatric cohort for sample collection. Currently, the majority of laboratories are using alternate methods to establish pediatric RIs, which include using their own laboratory acquired data, an approach known as the Hoffman method [2], or collection of a limited number of samples from healthy children. The former approach uses sufficiently large pools of data from laboratory databases, but there is a high probability that the individuals included in the population are not healthy [3]. The latter approach requires unique statistical analysis because the limited sample size may skew the data and not reflect the true healthy population from which the intervals were established [4]. RIs can also be adopted from the manufacturer, but are often limited to small sample sizes and adults only [4].

The Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER) program is a national initiative to establish comprehensive pediatric RIs based on an *a priori* approach. To date, CALIPER has recruited over 8500 healthy children and adolescents from across Southwestern Ontario in Canada, ranging in age from birth to 19 years [5]. After a series of gap analyses to identify analytes requiring new pediatric RIs, CALIPER has established RIs for well over 85 analytes including most routine biochemical markers, endocrine hormones, and many special chemistry assays. In 2006, a detailed gap analysis in the area of inborn errors of metabolism (IEM) revealed incomplete RIs for a number of metabolic disease biomarkers [6]. Gaps included either incomplete or unspecified age ranges within the full pediatric population and limited sample sizes. Although a recent report described establishment of a comprehensive global database for amino acid and acylcarnitine cutoff values for various IEMs, the study used dried blood spots only from healthy and diseased newborns [7].

IEMs are a collection of rare diseases that are genetic in nature and result in enzyme defects that affect several metabolic pathways including amino acid metabolism, fatty acid oxidation, and carbohydrate metabolism [8]. In Canada, the incidence of IEMs is approximately 1 in 2500 [8]. These diseases often present in the early weeks of life and can have devastating outcomes if unrecognized or untreated. In the present study, we have used a cohort of approximately 500 healthy individuals from the CALIPER study population to establish pediatric RIs for four important classes of IEM markers, including serum amino acids, acylcarnitines, total and free carnitine, and free fatty acids and β-hydroxybutyrate. These analytes are essential for both the detection and management of patients with IEM and will aid in improving the reliability of newborn screening results across Canada and globally.

To the best of our knowledge, comprehensive pediatric RIs for amino acid and acylcarnitine profiles in an exclusively healthy and non-hospitalized pediatric cohort have not yet been established across the entire pediatric age range (birth to 19 years). Given the importance of early detection of IEM in newborns, we have focused on this age range, collecting data from over 100 healthy neonates to establish robust RIs during this critical period. Detailed RIs were also established for children of all ages up to 19 years of age.

## Materials and methods

### *Participant recruitment and sample collection*

Healthy volunteers ranging in age from 1 day to 19 years from the Greater Toronto Area were recruited based on defined inclusion and exclusion criteria as described previously [5]. Whole blood specimens were collected in serum separator tubes, centrifuged, separated, and stored at −80 °C for on average less than 3 months for neonatal samples and one year for the remaining samples before being analyzed. A cohort of approximately 500 healthy children samples from both sexes and across the pediatric age range (neonatal to 19 years of age) were selected from the CALIPER biobank for analysis of amino acids, acylcarnitines, free and total carnitine, β-hydroxybutyrate and free fatty acid.

### *Sample analysis for metabolic markers*

Serum amino acid analysis was performed using the MassTrak AAA on Waters Ultra-performance liquid chromatography (UPLC). Briefly, samples were derivatized according to manufacturer's protocol. Physiological amino acids were separated by reverse-phase chromatography and detected by UV spectrophotometry. Underivatized free and total carnitines were analyzed by a liquid chromatography and tandem mass spectrometry (LC-MS/MS) assay developed in-house and based on the method previously reported by Stevens et al. [9]. Acylcarnitine analysis was performed using electrospray LC-MS/MS on the API4000 mass spectrometer. The method has been developed in-house and is based on the method described by Chance et al. [10,11]. β-Hydroxybutyrate and free fatty acids were analyzed on the Vitros Chemistry System 5.1 Fusion Series. Both of these assays were developed in-house using reagents from Randox and have been implemented on the User Defined Assay module of the Vitros 5.1 Fusion Series Chemistry System (Ortho-Clinical Diagnostics). Analytical performance including imprecision (coefficient of variance, CV) and detection limits are shown in Supplemental Table 1 for all methods.

### *Statistical analysis and reference interval determination*

Statistical analysis was performed in accordance with CLSI C28-A3 guidelines on defining, establishing, and verifying RIs in the clinical laboratory [1] and as previously described [5]. The data were analyzed using the statistical computing software, R (http://www.r-project.org/). Following visual examination of data distributions, possible partitions were determined based on age and/or sex and statistically evaluated using the Harris and Boyd test, which uses the standard deviation (SD) and a modified z statistic for 2 groups to statistically determine if each group is different and whether they can be partitioned separately [12]. If two groups did not warrant partitioning, they were combined and reevaluated. For each partition, normality was checked using probability plots and the Shapiro–Wilk test [13]. If normality was not achieved, the data was transformed using Box–Cox transformation, which ranks the values using power functions to achieve a normal distribution [14]. Depending on the result of the normality test for the transformed data, outliers were removed from each partition using the Tukey test or adjusted Turkey test, which is similar to a T-test such that it compares the mean of one group to the other groups and identifies differences between the two means that is greater than the expected standard error [15].

Following removal of outliers, RIs were calculated as outlined by CLSI C28-A3 guidelines [1]. For partitions with a sample size greater than 120, the non-parametric rank method was employed based on sample quantiles, with values ranked and the 2.5th and 97.5th percentiles calculated. For partitions with a sample size less than 120 but more than 40, the robust statistical method developed by Horn and Pesce was used to calculate the RIs, which is a method used on small sample sizes that prevents the data from being adversely influenced by outliers and involves transforming the data to achieve normality, then downweighing values further from the center of the distribution [16]. Lastly, 90% confidence intervals around the lower and upper reference limits were calculated to estimate the range of values, which act as good estimates of the limits.

To minimize the pre-analytical effect of ornithine metabolism to arginine after blood collection at room temperature, thereby falsely lowering ornithine and falsely elevating arginine [17], we excluded individual samples on the basis of the following criteria: ornithine >400 μmol/L and arginine <5 μmol/L. This resulted in only 4 outliers excluded.