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In this issue

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Foreword of the editor

Editor in Chief: Gábor L. Kovács, M.D., Ph.D., DSc

Professor Edgard Delvin (Canada) was asked to be the guest-editor of the special issue on "Recent advances in pediatric laboratory medicine". After obtaining a PhD degree in biochemistry at the University of Montreal, Dr. Delvin pursued his post-doctoral training in Biochemical Genetics at the Montreal Children's Hospital (McGill University). In 1973 he joined the Shriners Hospital for Children, affiliated to McGill University, where he became senior investigator and Associate Director of the Genetics Unit as well as being Associate Professor in the Department of Experimental Medicine.

In 1992, the University of Montreal recruited him to chair the Department of Clinical Biochemistry at the University-affiliated Sainte-Justine Hospital. He also was appointed as full Professor of Biochemistry in the Department of Biochemistry of the Faculty of Medicine. After his retirement in 2012 he acted as consultant for the Montreal Children's Hospital, affiliated with McGill University until 2014.

He has served on the Paediatric Endocrinology Teaching Program of the University of Montreal until August 2012. After being Editor-in-Chief of the Journal Clinical Biochemistry from 2006 to 2012, he now is the Special Issues Editor.

In terms of international activities, he has been Editor-in-chief of the IFCC eNewsletter from 2006 till 2012 and now chairs the IFCC Committee for Public Relations (C-PR) as well as being Vice-Chair of the CPD-EC.

He has authored or co-authored 245 articles, published in internationally recognized journals, and 9 book chapters.

In 2003 he received the research Excellence Award from the Canadian Society of Clinical Chemists, and is a Member of the French Académie Nationale de Médecine.

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

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Recent advances in pediatric laboratory medicine

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EDITORIAL

Pediatric medicine that spans from the perinatal period through adolescence is a field in its own right, as it requires knowledge about early growth and development that are not present in adult medicine. Indeed, it is during these crucial years that adult health and wellbeing are being carved. This statement is supported by epidemiological studies and meta-analyses indicating that many adult diseases are grounded in early childhood from conception, and thus should be viewed as developmental disorders (1, 2). Clinical laboratories are instrumental in the detection of subtle metabolic impairment present often at the onset of ailments. Their efficacy in assisting attending physicians in their diagnostic and follow-up endeavours is intimately linked to availability of dependable analytical methods and reliable reference values. Paediatrics is particularly sensitive to this problem as rapid developmental changes associated with child growth, can impose rapid discontinuous changes on the physiology of individuals (3-5).

This issue of the eJIFCC presents 5 articles focused on pediatric laboratory medicine. The first 2 review

articles relate to childhood obesity and metabolic syndrome. The first (6) gives an overview of the relationship of obesity with genetic and environmental factors, and raises awareness on the applicability and limitations of important potential biomarkers used in preventive and predictive medicine. The second (7) reviews the pathophysiology, consensus definitions and laboratory assessment of pediatric metabolic syndrome as well as potential novel biomarkers.

The third (8) review is more generic and highlights the achievements and milestones of the Canadian CALIPER project, including the establishment of comprehensive bio-bank and database that have addressed several critical gaps in age- and sex-specific paediatric reference intervals impeding improvement of paediatric diagnostics. The fourth (9) article provides an overview of the diagnosis of primary neurotransmitters diseases (PNDs) through investigations using cerebrospinal fluid (CSF). It also calls for caution in sample management and analytical methodology as they potentially could affect diagnostic interpretation. The last short research article provides novel data on Pediatric Reference Intervals for Transferrin Saturation on a large CALIPER Cohort of Healthy Children and Adolescents (10).

These articles illustrate the effervescence in the field of paediatric laboratory medicine and invite professionals to continue providing state of the art data on this ever evolving subject.

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Pediatric obesity and cardiometabolic disorders: risk factors and biomarkers

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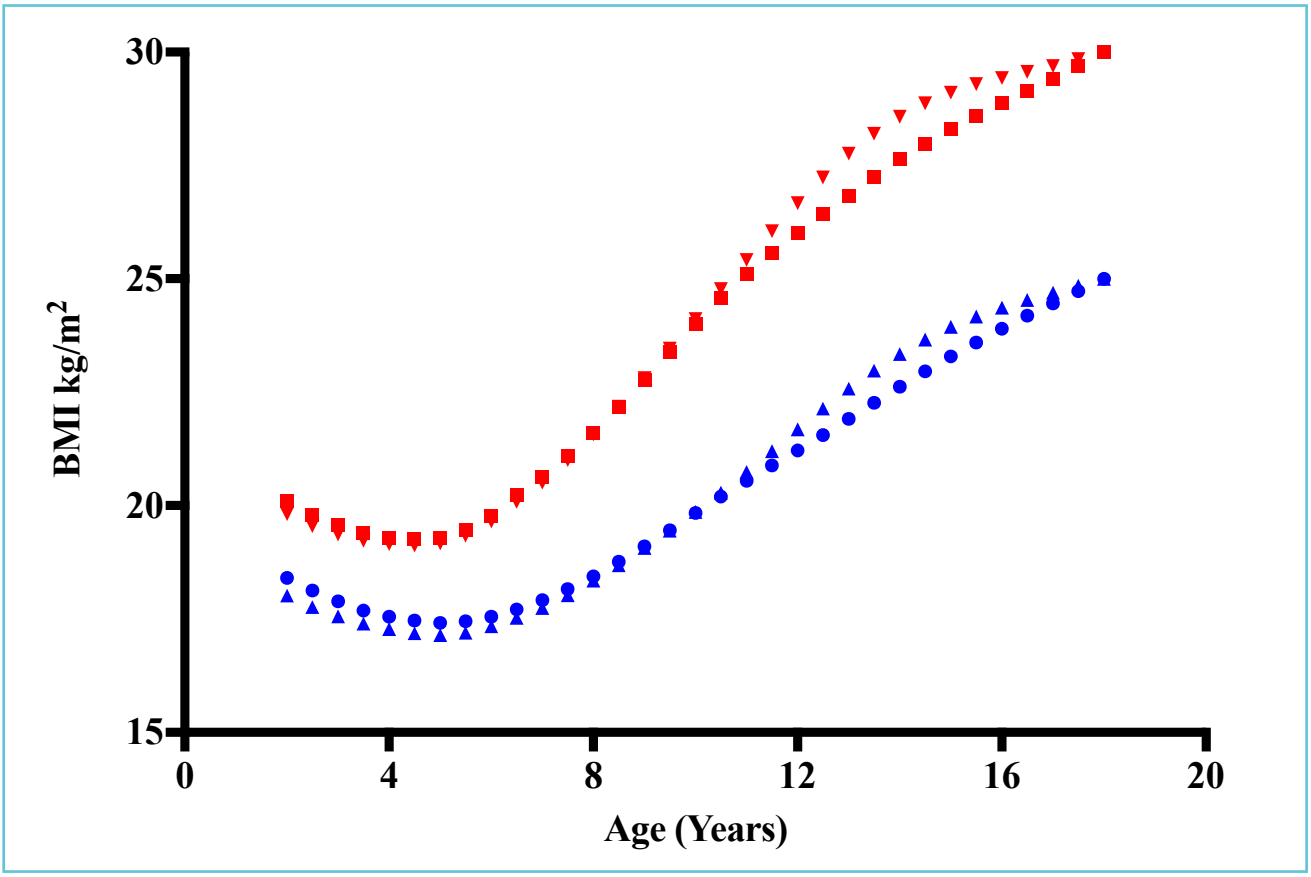
ABSTRACT

Obesity remains the most prevailing disorder in childhood males and females worldwide. Its high prevalence markedly predisposes children to insulin resistance, hypertension, hyperlipidemia and liver disorders while enhancing the risk of type 2 diabetes and cardiovascular diseases. In this review, the relationship of obesity with genetic and environmental factors will be described and the underlined causes will briefly be reported. As obesity in children constitutes an increasingly health concern, important potential biomarkers have been discussed for the diagnosis, treatment and follow-up of the wide range of overweight-related complications. Awareness about the applicability and limitations of these preventive and predictive biomarkers will intensify the research and medical efforts for new developments in order to efficiently struggle against childhood obesity.

INTRODUCTION

The prevalence of childhood obesity is rapidly increasing and presents a major public health concern in developed and developing countries (1-4), and assessment of obesity is of utmost importance to paediatricians. However, there are varying definitions of obesity in children and adolescents, along with ethnic-specific variations in body fat content and distribution, which complicate this undertaking (5). Moreover, these divergences may explain prevalence dissimilarities associated with cardiometabolic diseases (CMD) (e.g. insulin resistance, hypertension, dyslipidemia and diabetes) in adulthood (6-11). In the context of epidemiological studies, body mass index (BMI, weight/height^2) in adults is currently considered as a diagnostic test (separator variable) which is able to identify overweight (25 kg/m^2) and obese (30 kg/m^2) individuals and may predispose to increased CMD risk, morbidity and mortality (12, 13). However, no similar definite values can be used in childhood and adolescence because of the substantial changes in BMI, which occur naturally from birth to adulthood (14, 15), and because of the limited data in youth that relate BMI trajectory to cardiovascular events later in life. Age- and sex-specific BMI cut-offs were developed to define overweight and obese using different nationally representative age- and

Figure 1 International age- and sex-specific cut-off points for BMI for overweight and obesity



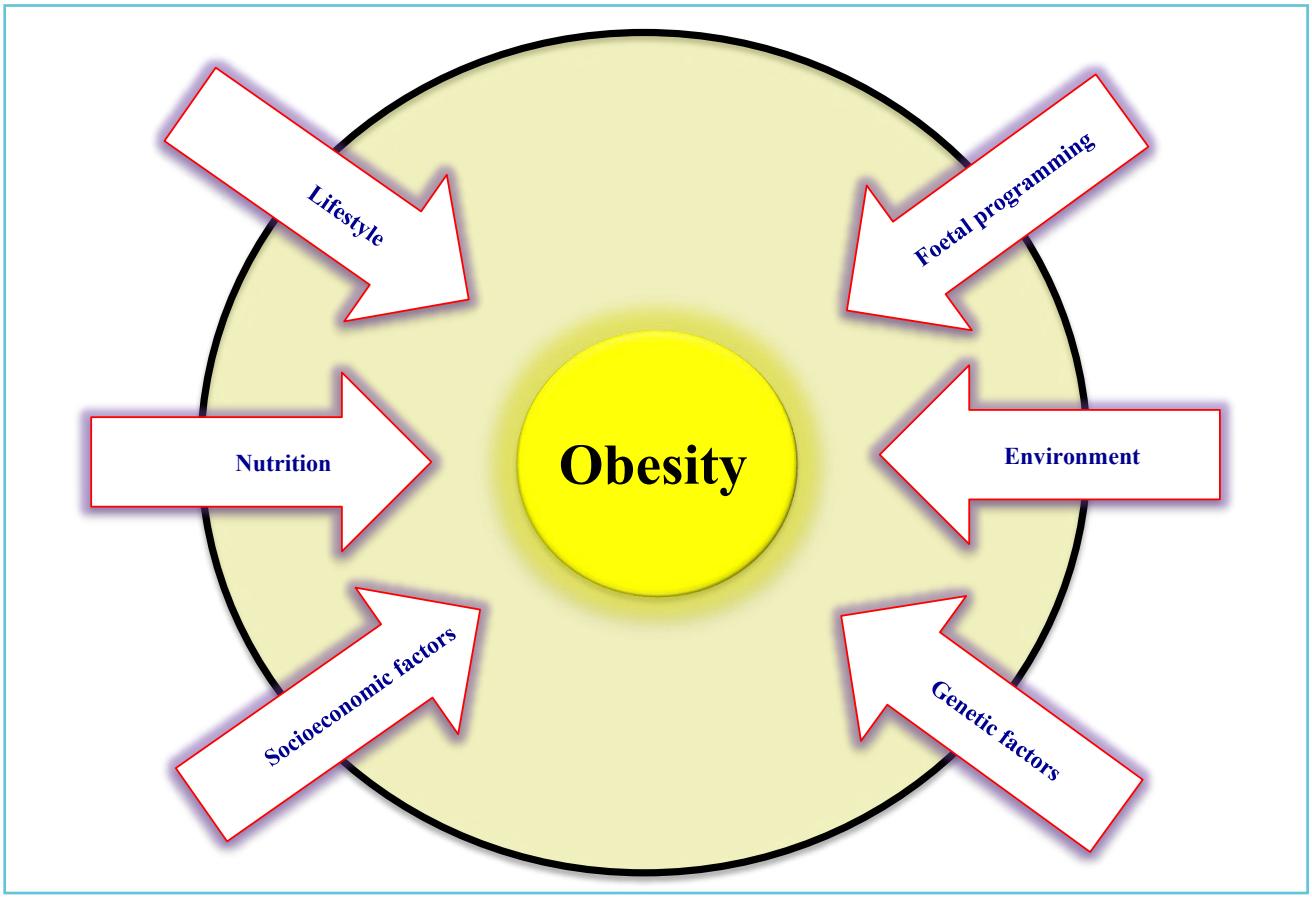
**Adapted with permission from data of Table 4 from Cole TJ et al. (16). Data obtained by averaging the national centiles.*

BMI: Body Mass Index. Filled circles: curve for overweight boys; filled square: curve for obese boys; filled upward triangles: curve for overweight girls; filled downward triangles: curve for obese girls.

sex-specific data sets, following recommendations from the International Obesity Task Force (16, 17). International age- and sex-specific BMI cut-offs for overweight and obese girls and boys are illustrated in Figure 1. Applying this concept to BMI trajectory, Attard et al. (18) demonstrated that the odds for diabetes were 2.35 higher for those with a BMI of 30 kg/m^2 relative to young male adults who had maintained a BMI of 23 kg/m^2 over an average of 12 years. These data suggest there is potential for improving the ability to assess the effect of paediatric obesity on development of diseases at a later time point. Secular trends demonstrate that the prevalence has plateaued in some countries (19) or even decreased (20), but has continued to rise in others, independent of how overweight and obesity are defined in childhood (1, 21-23). The apparent contradiction could partially depend on the span of the retrospective studies and on the years included. Nevertheless, the present high number of young adults with the stigmata of the metabolic syndrome (MetS), and the related non-alcoholic fatty liver disease (NAFLD) justifies that it be considered a major world public health issue (24). This review briefly describes the various potential causes of obesity in youth and underscores the available biomarkers for associated conditions.

Definite BMI thresholds to identify an increased risk for CMD cannot be used in childhood and adolescence. Age- and sex-specific BMI cut-offs to define overweight and obesity and predict trajectory into adulthood should be utilized using different nationally representative age- and sex-specific data.

Figure 2 Factors involved in the development of obesity



OBESITY AND LIFESTYLE

Lifestyle is broadly defined as the way or manner by which a person or a group of people lives. However, lifestyle can be influenced by a complex set of factors that are intertwined and can affect the quality of living and health (Figure 2). The socioeconomic position (SEP) stands out among these factors because it has a direct impact on the quality of nutrition and the living environment, including access to adequate physical activity facilities and education. Consequently, a comprehensive view must be adopted whenever addressing this topic but a majority of studies tend to focus in this area in a fragmented manner.

One such study, based on self-reports, demonstrated that poor children in the United States have worse health compared to wealthy children. This difference in health status diverged further as the children aged; thereby suggesting the adult health gradient had its origins in childhood. However, other than family income no other factors were considered which could explain these results (25). SEP may also impact the quality of nutrition. Darmon et al. (26) reported that higher-quality diets consisting of whole grains, lean meats, fish, low-fat dairy products, fresh vegetables and fruits were associated with greater affluence, whereas energy-dense and nutrient-poor diets (refined grains, added fats) are preferentially consumed by persons of lower SEP. Likewise, in a systematic review, Cameron et al. (27) reported that children of lower SEP had a steeper weight gain trajectory initiating at birth and led to a greater prevalence of obesity in children and adults. Pre-pregnancy maternal BMI, diabetes, pre-pregnancy diet, smoking during pregnancy, low birth weight, breastfeeding initiation and duration, early introduction of solids, maternal and infant diet quality, and some aspects of the home food environment were among the early-life predictors of later obesity and amid links with SEP. Furthermore, lack of physical activity is an additional risk factor for developing obesity. A longitudinal study involving repeated 7-day physical activity recall questionnaires over a 5-year period demonstrated that greater fluctuations in physical activity led to an increase in body fat in adolescent girls and boys (28). An interventional study supported these conclusions, demonstrating interruption of sedentary time with brief moderate-intensity walking resulted in an improvement of short-term metabolic function in non-overweight children without increasing subsequent energy intake (29). Despite the difficulty in directly comparing studies because of the variety of environmental factors and defined end-points, systematic reviews consistently highlight that better and safer access to physical activity resources are directly related to increased leisure time physical activity in children and adolescents, which subsequently decreases the risk of developing obesity (30-34).

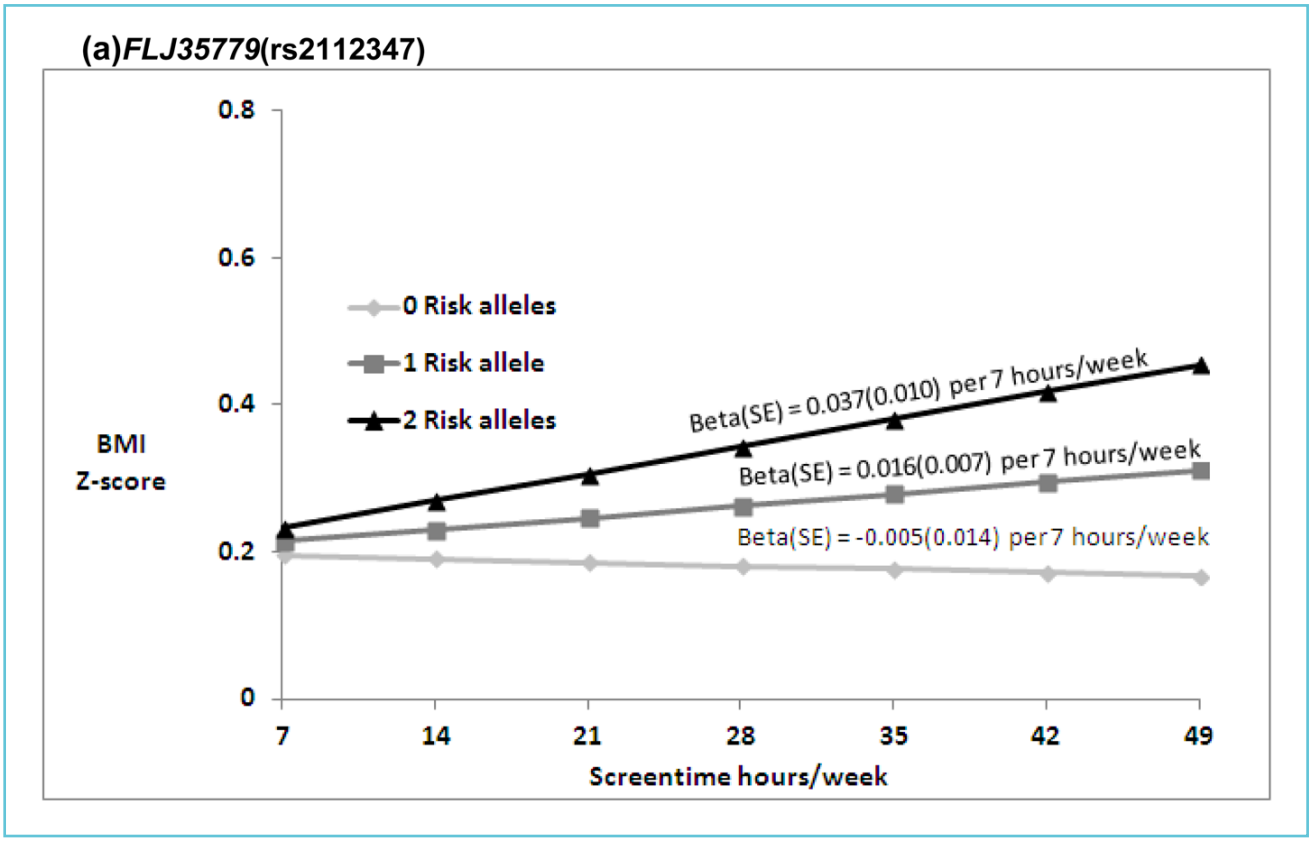
Access to physical activity resources is directly related to higher leisure time physical activity in children and adolescents and decreases the risk of developing obesity.

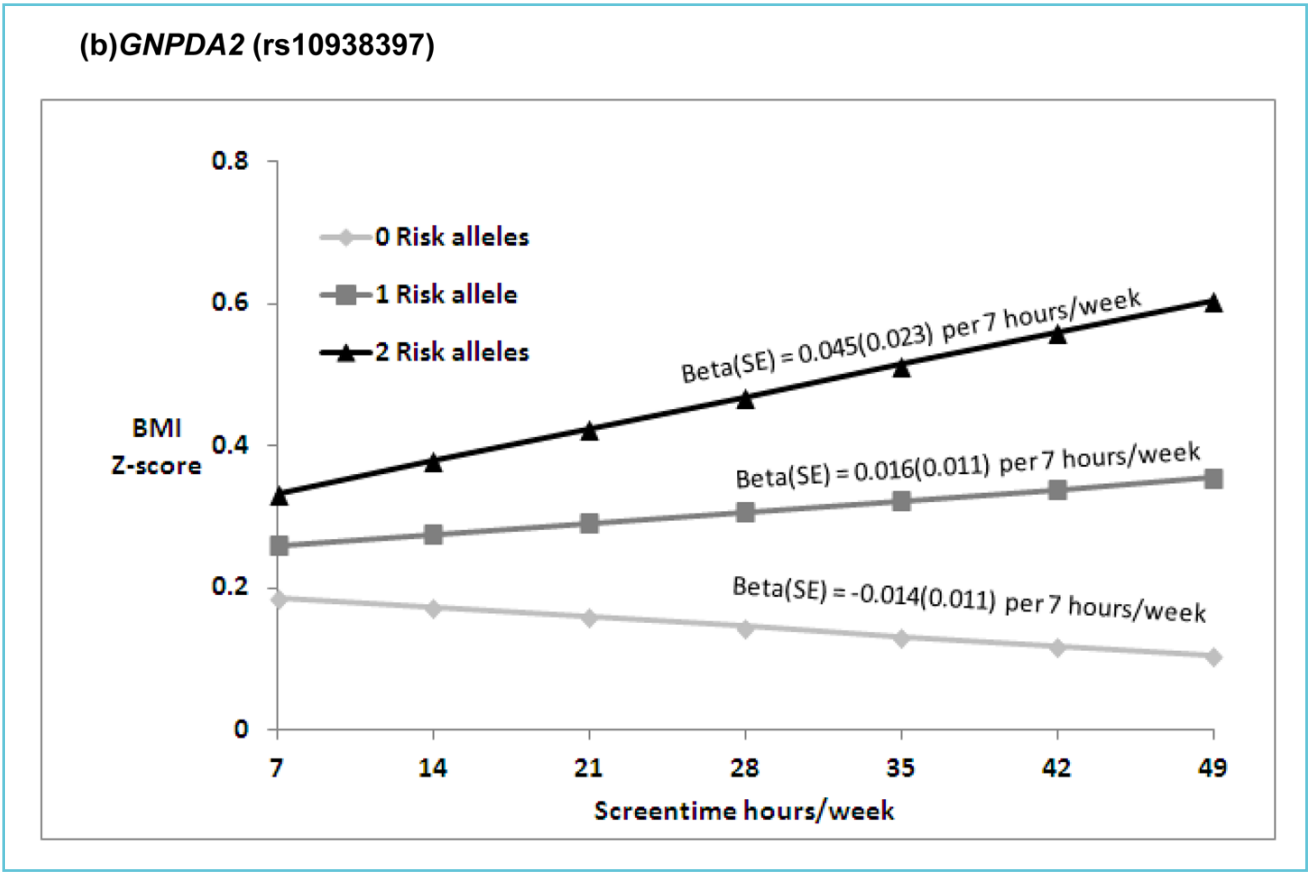
OBESITY AND GENETIC/EPIGENETIC FACTORS

In addition to the risk factors previously discussed, genetic background and foetal programming through epigenetic modifications are equally important in the development of obesity and related diseases. There is also increasing evidence suggesting synergetic effects between gene variant loci involved in metabolic traits and dietary or lifestyle factors. Maes et al. (35) compiled data from more than 25,000 twin pairs and 50,000 biological and adoptive family members and reported that genetic components contribute 40-70% to the

inter-individual variability in common obesity. Another study showed that parental obesity doubled the risk of adult obesity among both obese and non-obese children less than 10 years of age (36). Few studies have investigated the gene-environment interactions related to sedentary behaviour using large cohorts. The Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS cohort (IDEFICS) used a subsample of 4406 participants to demonstrate that the fat mass and obesity-related gene (*FTO*) polymorphism (rs9939609) could explain ~9% of the obesity variance, thereby suggesting the FTO gene was sensitive to the social environment (37). To date, genome wide association studies (GWAS) have provided evidence for a number of gene variants associated with the development of obesity in the youth. Willer et al. (38), based on a cohort of 11 year-old children, demonstrated significant and consistent association between BMI and variant loci (SNPs) located in or near the trans-membrane protein-18 (*TMEM18*), potassium channel tetramerisation domain containing-15 (*KCTD15*) and glucosamine-6-phosphate deaminase-2 (*GNPDA2*) genes. The high brain and hypothalamic expression of these factors, together with *FTO* and the melanocortin-4 receptor (*MC4R*), independently associated with adiposity and insulin resistance (39), supports the argument for a neuronal foundation in obesity. Whether these loci are modulated under neuronal influence by the environment or lifestyle remains to be elucidated. Graff et al. (40) provided a partial answer by establishing a dose-dependent

Figure 3 **Predicated BMI Z-score from model based coefficients per 7 hours/week of screen time in the presence of 0, 1 and 2 risk (T) FLJ35779 (rs2112347) alleles**





Legend: (p for interaction = 0.02) in EA (a), and 0, 1 and 2 risk (A) GNPDA2 (rs10938397) alleles, respectively (p for interaction = 0.03) in AA (b).

Abbreviations: BMI (body mass index), ST (hours per week of screen time), EA (European American), AA (African American).

Beta estimates are presented for the interaction model: Multivariable linear models of adolescent BMI Z scores regressed on SNP and ST (hr/wk), with SNP by ST interaction term, controlling for age, sex, current smoking (at least one cigarette every day for 30 days), geographic region, and self-reported heights and weights (n=39 EA, n= 12 AA), oversampling of highly educated African Americans (n=281; AA stratum only). Random intercepts allowed for individual, family and school with no sample weighting.

**Reproduced with permission from: Graff et al. Pediatr Obes 2013;8:doi:10.1111/j.2047-6310.2013.00195x.*

interaction leisure screen time (β = −0.014, 0.016, and 0.045/7h/week) with *GNPDA2* (rs10938357) SNP in Afro-American subjects for 0, 1 and 2 risk alleles. They observed a similar interaction for the *FLJ35779* (rs2112347) gene polymorphism (Figure 3). Although interactions are documented in each study, they are modest, and individually cannot explain the development of obesity or the onset of related diseases. Additional studies are required to probe the relationship between polymorphisms in multiple genes involved in energy management and the numerous environmental and lifestyle factors.

The epigenetic control of gene expression must also be considered in the understanding of the development of obesity. This concept stems from the early work of Barker et al. (41), who proposed that the tendency to store abdominal fat might be a persistent response to adverse conditions which initiated in the foetal life stage but persisted throughout infancy.

A myriad of peer-reviewed publications have confirmed this initial hypothesis (42-46). Lee et al. (47) suggest there is a gene-foetal environment interaction, one of which occurs through *in utero* exposure to maternal cigarette smoking and leads to a preference in adolescence for moderately enhanced fatty foods by silencing the opioid receptor mu-1 gene (OPRM1) involved in the brain reward system. Small gestational age (SGA) is also well recognized and linked to an increased risk for rapid postnatal weight gain and subsequent development of obesity and chronic metabolic diseases later in life. The Auckland Birth weight Collaborative Study demonstrated that smoking, low pregnancy weight, maternal short stature, maternal diet, ethnic origin of mother and hypertension are all "environmental" risk factors for SGA (48). A subgroup of the cohort later established that polymorphic *FTO* (rs9939609, intron), *KCNJ11* (rs5219, missense Lys23Glu), *BDNF* (rs925946, 9.2 kb upstream), *PFKP* (rs6602024, intron), *PTER* (rs10508503, 179 kb upstream) and *SEC16B* (rs10913469, intron) genes, were related to obesity, type 2 diabetes, and SGA which indicates the important interaction between genetic factors and fœtal environment (49). Finally, a prospective singleton normal pregnancy cohort study demonstrated a direct relationship between the maternal adipokines, leptin (a satiety factor) and adiponectin (an insulin sensitizer). The study included 339 healthy women without pre-existing diabetes who were evaluated at 24-28 and 32-35 weeks of gestation and the cord blood (foetal compartment) assessed at birth (50). Foetal insulin sensitivity was negatively associated with cord blood leptin and positively with pro-insulin concentrations, suggesting the maternal impact on foetal adipokines may be an early life pathway in maternal-foetal transmission of the propensity to develop obesity and insulin resistance later in life. These examples provide compelling evidence on the role and impact of the foetal environment and development of chronic diseases later in life.

Parental obesity more than doubles the risk of adult obesity among obese and non-obese children.

Gene-environment interactions are modest, and individually are not able to explain the development of obesity and the onset of related diseases.

There are compelling evidence highlighting the role of foetal environment and development of chronic diseases later in life.

Table 1 Caveats in assessing insulin sensitivity

Variable	Issue
Measurement of insulin	Wide inter-inter method (laboratory) variations Variation in standardization among methods
Measurement of plasma glucose	Pre-analytical quality of blood specimens Glycolysis at room temperature NaF inhibits enolase, a late glycolytic enzyme
Patient preparation	Poor assessment of patient's nutritional status Elevation of post-prandial glucose in malnutrition and low carbohydrate diets

Table 2 Cut-off points for defining insulin resistance (IR)

Insulin measurement	Population Studied	Age (years)	Gender	HOMA-IR 95th percentile	Ref
Immunoassay (Access, Beckman Coulter)	French Canadian	9 13 16	M/F	1.88/2.07 3.28/3.86 3.31/3.10	(76)
Fluoroimmunoassay (AutoDelfia, Pharmacia)	Brazilian	10-19	M/F	>2.93	(77)
Chemiluminescence Immunoassay (Immulite, Siemens)	American	11-14	M/F	≥2.7	(78)
Chemiluminescence Immunoassay (Cobas, Roche Diagnostics)	Spanish	8-18	M/F	≥3.6	(79)

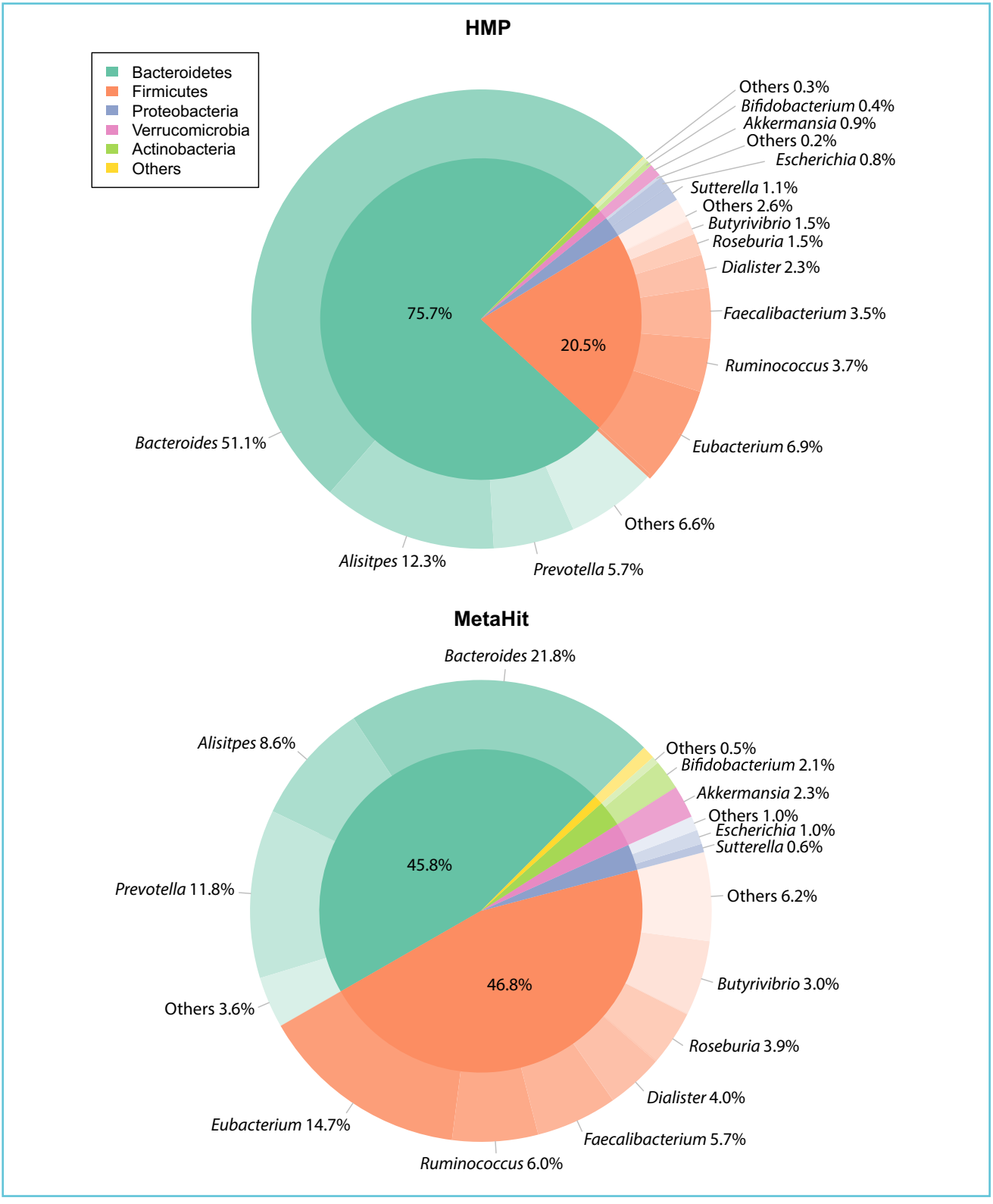
OBESITY AND MICROBIOTA

In addition to the above considerations, the gut microbiota may increasingly be shown to impact the course of metabolic diseases. This aspect is briefly reviewed. The synergistic relationship between the human body and the vast microbiotic environment present on all interfaces with the exterior, particularly the gut lumen, has become of major interest to the medical community. The microbiome cell number far outnumbers somatic or germ cells and represents a far more varied gene diversity than the human genome (51). The advent of high throughput genome sequencing technologies allowed the first meta-sequence of the human gut microbiome to be conducted, utilizing stool collected from 124 individuals, and characterized > $3X10^6$ genes from approximately 1000 different microscopic species (52-54). An excellent review by Arora et al. (55) discusses the composition of the gut microbiota and its association with metabolic diseases. Figure 4, taken from this review, shows that 2 phyla, namely Firmicutes and Bacteriodetes, constitute healthy adult gut microbiota and their relative proportions differ among populations.

Neonatal intestinal flora evolves according to its early environmental exposures, nutrition patterns (maternal or industrial milk), hygiene levels and therapeutic drug usage (56). Differences in intestinal flora patterns during the first six months of life may have potential impact and downstream consequences on the later development of chronic conditions such as type 2 diabetes and allergies (57, 58).

The gut microbiota has emerged as a new important player in the pathogenesis of obesity, potentially explained by the fact that each microbiotic species transforms the undigested and partially digested food into metabolites that may influence the physiological systems of the host. Therefore, a loss in diversity may lead to unwanted effects (55). This hypothesis is supported by the observation that composition of the gut microflora is globally less diverse in obese subjects, with a relative enrichment in Firmicutes and a impoverishment in Bacteroïdes (59). Moreover, detailed analysis

Figure 4 Quantitative comparison of faecal microbiota in two healthy populations



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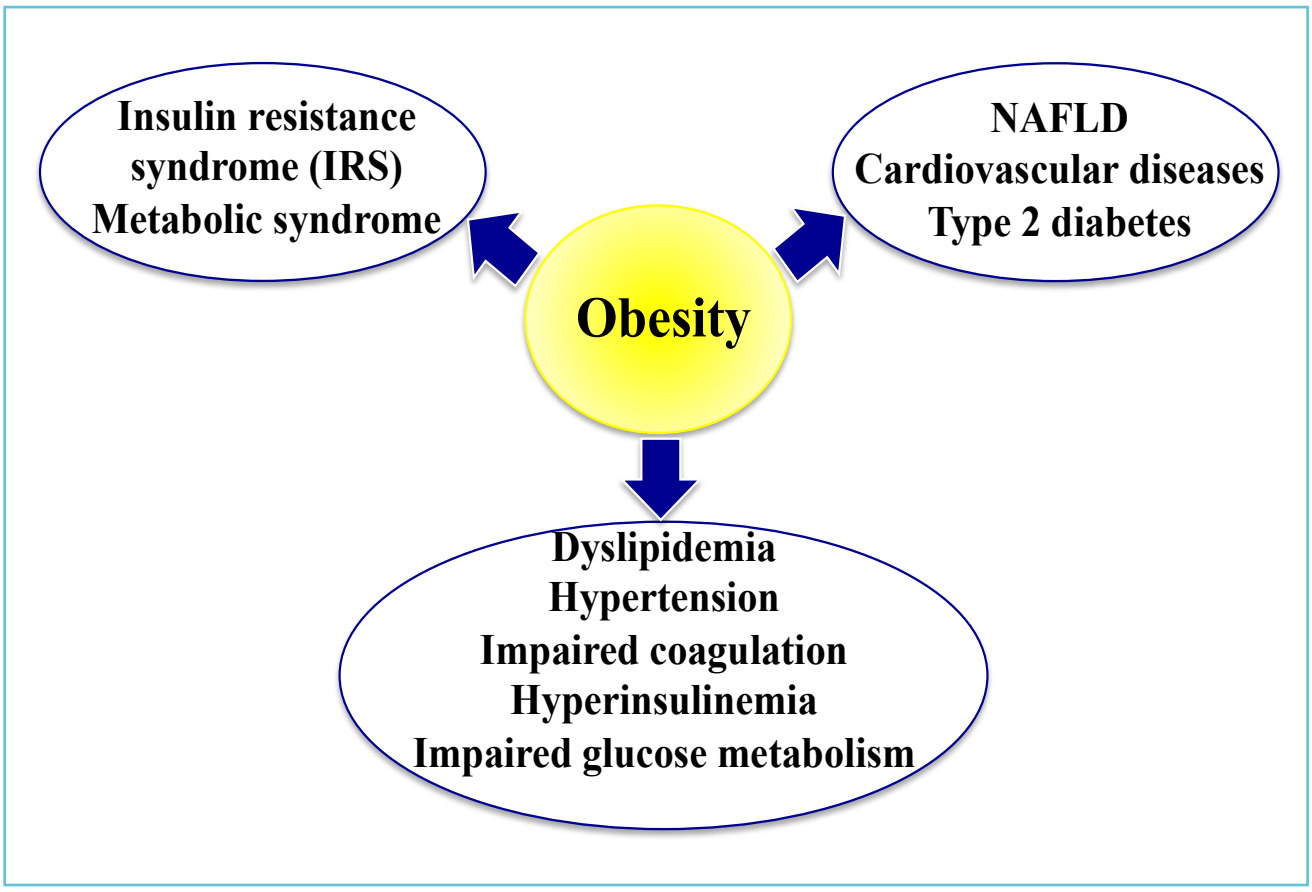
of the flora in obese subjects reveals a bimodal distribution: those with a low gene count (LGC) characterised by the predominance of 5 pro-inflammatory bacteria and a less diversified metagenome, and those with a high gene count (HGC) with a high percentage of 4 anti-inflammatory bacteria genii (60). The LGC group presents with insulin-resistance, dyslipidemia and low-level infiltration of adipose tissue with pro-inflammatory cytokine secreting immunity cells. It has recently been established that levels of butyrate-producing bacteria are reduced in patients with type-2 diabetes, whereas levels of Lactobacillus sp. are increased, thus the reduction of butyrate-producing bacteria may be causally linked to type 2 diabetes. The causal relationship for these differences in humans remains to be elucidated but opens the way to possible treatment of obesity via dietary manipulation. For example, a low calorie regiment composed of plant fibres, proteins and low carbohydrates potentially increases the microbiota diversity (61). Interestingly, bariatric surgery also increases the gut microbiota diversity (62, 63). As each microbiotic species transforms the undigested and partially digested food into metabolites that may influence the physiological systems of the host, a loss in diversity may lead to unwanted effects.

The gut microbiota, a new player in the world of obesity and cardiometabolic diseases, is increasingly called upon to elucidate findings related to these diseases and may eventually impact their course and treatment.

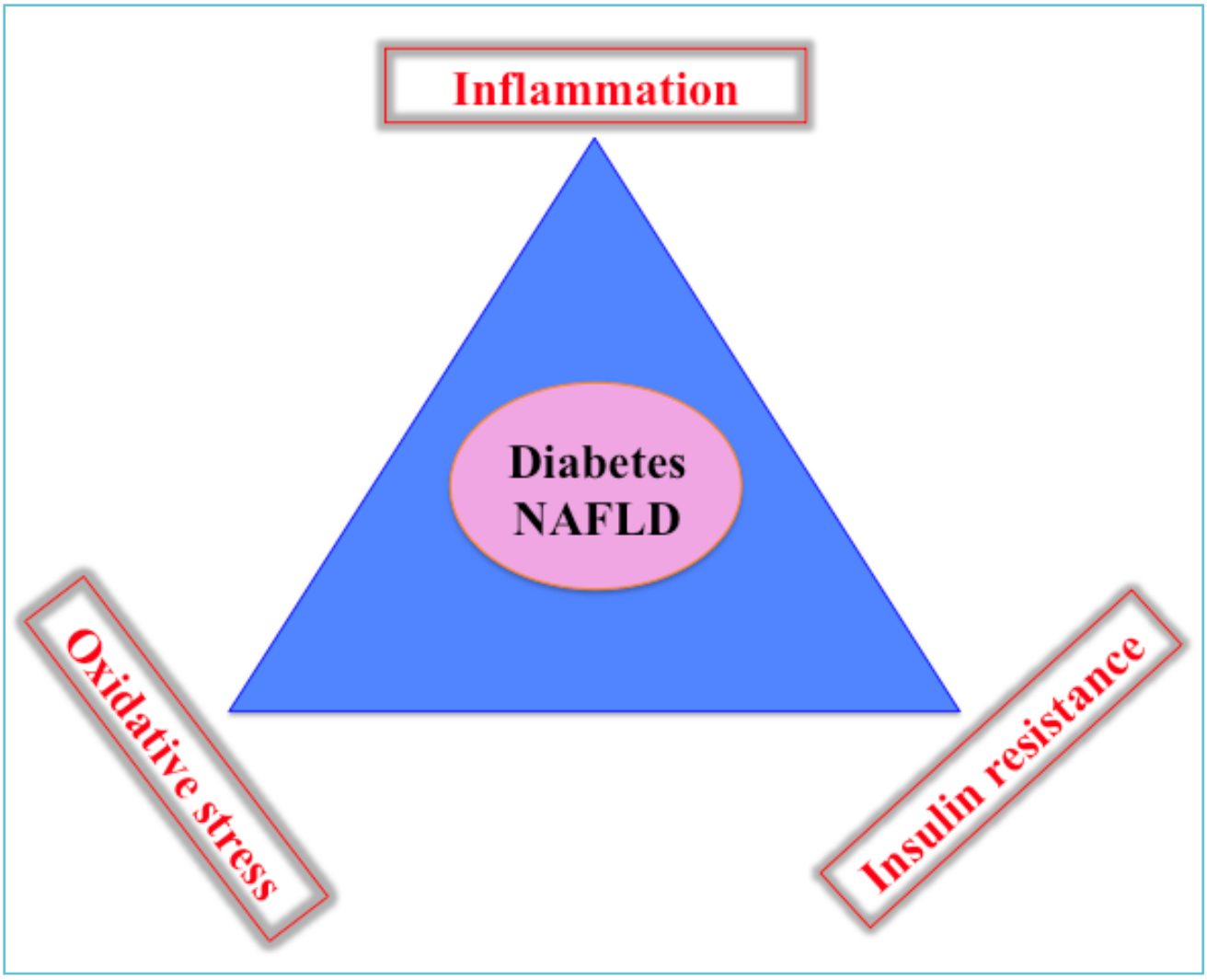
Figure 5 Impact of obesity on health status



BIOMARKERS

The status of metabolically healthy obese (MHO) individuals has been reported (64, 65) but obesity, particularly abdominal, remains a major risk factor for developing a series of complications (Figure 5) such as the metabolic syndrome, type 2 diabetes, early atherosclerosis and non-alcoholic fatty liver disease (NAFLD), the latter considered the hepatic manifestation of insulin resistance (66-68). Cellular redox potential imbalance, inflammatory processes and insulin resistance are central in the development of the complex chronic metabolic disturbances (Figure 6); hence measurement of related biomarkers to detect minor disturbances could help distinguish MHO from metabolically non-MHO individuals, and may result in establishing early primordial prevention programs. However, at the present time there is no international consensus as to the specific pathways that should preferentially be targeted in order to define the prevalence and severity of the conditions during childhood and adolescence.

Figure 6 Cellular redox potential imbalance, inflammatory processes and insulin resistance in the development of diabetes and non-alcoholic liver disease (NAFLD)



IMAGING TECHNIQUES

In the last decade, utilization of ultrasonography, transient elastography and magnetic resonance imaging (MRI) has increased significantly. In the context of the present review these techniques, except for MRI, are not suitable for the detection of metabolic disturbances and are primarily used to evaluate the extent of liver damage. Although widely available, ultrasonography is unable to accurately detect or quantify early liver fatty acid infiltrations. Furthermore, this technique is prone to observer- and operator-dependent variability and its use in obese patients is subject of debate (69, 70). Transient elastography, based on the assessment of liver stiffness, has also been shown to be useful in presence of significant fibrosis and cirrhosis (71). Liver magnetic resonance imaging–estimated proton density fat fraction (PDFF) is more sensitive and favourably comparable to histopathology scores (72). This technology is currently restricted to tertiary care institutions, is expensive, and demands experienced staff. In summary, these imaging techniques are useful in detecting steatosis, but they are relatively inefficient in determining early stage liver damage. Biomarkers easily measured in central laboratories are therefore of utmost importance and should center on insulin resistance, inflammation and oxidative stress, as this triad is the signature of NAFLD.

INSULIN RESISTANCE

The term insulin resistance (IR) frequently refers to a physiological state characterized by a diminished biological response to insulin. More precisely, IR refers to a holistic reduction of glucose uptake in response to physiological insulin concentrations, primarily in muscle tissue. The optimal assessment of IR in children and adolescents remains controversial. Following the Consensus Conference on Childhood IR in 2010, experts highlighted: 1) the paucity of data regarding cut-offs to define insulin resistance; 2) poor performance of surrogate measures such as fasting plasma insulin; and 3) lack of justification for screening children, even obese children, because there are no accepted treatments for euglycemic IR (73). However, the development of robust methods for assessing insulin sensitivity (IS) in paediatric populations remains of great interest, particularly for epidemiological studies to monitor metabolic trajectory into adulthood.

The hyperinsulinemic-euglycemic clamp is the gold standard for determining total-body IS (73). However, it is not applicable in the context of population screening or routine clinical work-up. In 2014 Brown and Yanovski (74) published an excellent review on this technique as well as surrogate measures and their pitfalls. The hyperinsulinemic-euglycemic clamp, as its name indicates, depends on repeated measures of both insulin and blood glucose, each having their own potential analytical pitfalls that may hinder inter-laboratory comparison (Table 1).

Reliable interpretation of hyperinsulinemic-euglycemic clamp studies is also dependent upon normal inter-individual biological differences such as insulin clearance rates and time required to reach a steady state. Alternative methods include the insulin tolerance test (ITT), the hyperglycemic clamp, the insulin-modified or frequently sampled intravenous glucose tolerance test (FSIGT) and the more frequently used oral glucose tolerance test (OGTT) (74).

FASTING INSULIN AND THE HOMA-IR

Assessment of IR or IS is frequently conducted using single measurements due to ease of availability and simplicity. Measurement of fasting insulin concentrations are considered representative of insulin hepatic sensitivity (low concentrations) or resistance (high

concentrations). In theory, this information is valuable and may alert clinicians to eventual liver function impairment but there are issues around defining an abnormal elevated fasting insulin concentration because the data on reference values in fasting insulinemia are scarce. In addition, the lack of standardization or harmonization between different insulin assays hampers direct comparison between laboratories, peer-reviewed publications, and impedes coherent measures for treatment guidelines. This was highlighted in 2007 by the IFCC Working Group on Standardization of Insulin Assays, in an evaluation of 12 commercial insulin methods (75). The within-assay CVs ranged from 3.7% to 39.0% and between assay CVs from 12% to 66% (75). In 2009 the working group reported that 4 out of 10 insulin assays, when re-calibrated with a purified recombinant insulin preparation, had ≥ 95% of the 39 individual donor sera results within 32% of the target value assigned by an isotope dilution-mass spectrometry assay. In addition, 7 of 10 assays had a bias >15% in 36 to 100% of individual samples. The consensus group concluded that agreement between assays would improve using an international reference material and a higher order mass spectrometry method (76). Subsequent high-throughput mass spectrometry immunoassays have been developed to quantitate human intact insulin as well as insulin analogs, which may allow an accurate definition of insulinemia to be determined (77, 78). Accurate measurement of plasma insulin is of paramount importance for establishing comparable Homeostasis Model Assessment of IR (HOMA-IR) reference values across laboratories, although variation between ethnic populations may be a confounding factor that should be taken into consideration. At the present time HOMA-IR cut-offs are still highly method dependent. Table 2 illustrates the distribution of published cut-off points for defining IR, and confirm the warning of Wallace et al. (79): "The HOMA model has become a widely used clinical and epidemiological tool and, when used appropriately, it can yield valuable data. However, as with all models, the primary input data need to be robust, and the data need to be interpreted carefully." To address this issue, the IFCC (http://www.ifcc.org/ifcc-scientific-division/sd-working-groups/wg-sia/), in collaboration with the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD), has created the Working group on Standardisation of Insulin Assays (WG-SIA) with the mandate of improving the standardization of assays for insulin by the development of a candidate reference method based on liquid chromatography-tandem mass spectrometry, and of a lyophilized recombinant human insulin preparation as primary reference material.

Although insulin resistance is a well-recognized clinical entity, there are currently no internationally accepted definition of its expression in children and adolescents. One well-characterized definition requires the presence of three or more factors which can be age-adjusted to define hyperinsulinaemia: Overweight, high systolic blood pressure, hypertriglyceridemia, low HDL-cholesterol and impaired fasting plasma glucose (84).

Data on normal reference intervals for fasting insulinemia are scarce.

Lack of standardized or harmonized insulin assays hampers comparison between laboratories and impedes coherent measures for treatment guidelines.

Distinguishing MHO young patients from those unhealthy bears a major clinical importance as they are, for reasons that are yet to be defined, resistant to develop CMD; hence follow-up and treatment differ (64). Low-grade inflammation

and cellular redox potential imbalance are, together with insulin resistance, key-role players in the development of the non-healthy state in obese subjects.

INFLAMMATION

Inflammation is the second cause in the development of CMD and NAFLD related to paediatric obesity. A number of biomarkers have been identified but primarily in the context of clinical trials, thus their specificity, sensitivity and predictive values have yet to be defined for screening and diagnostic purposes. C-Reactive Protein (CRP), a member of the pantraxin family involved in plaque instability, is the most commonly utilized inflammatory biomarker. Although the sensitivity of CRP is generally high, the specificity is low, particularly in the setting of potential low-grade inflammation. Nevertheless, discrete elevation in circulating CRP concentrations has been associated in the definition of the metabolic syndrome (84, 85). Its advantage resides in its wide accessibility by central laboratories. However, as for any other biomarkers, well-defined age-, sex- and ethnicity-adjusted reference values or thresholds have to be defined if they are to be used for clinical purposes. The analytical sensitivity, even for the high-sensitivity CRP (hsCRP) test, however, limits the definition of reference ranges. One European population-based study reported that 44% of the 9855 children tested exhibited serum CRP concentrations below the detection limit (0.2 mg/l) and confirmed our observation (85) to the effect that obesity influenced serum CRP concentrations (86).

C-Reactive Protein (CRP) is the most commonly utilized biomarker of inflammation. The specificity of CRP is questionable, particularly in the setting of low-level inflammation.

Well-defined age-, sex- and ethnicity-adjusted reference values or thresholds have to be defined if they are to be used for clinical purposes.

Visceral adipose tissue per se and its resident macrophages contribute importantly to systemic inflammation by secreting adipokines and pro- and anti-inflammatory cytokines. Indeed, clinical studies have consistently shown elevated blood concentrations of pro-inflammatory cytokines such as IL-6, IL-8, TNFα, PAI-1, resistin and amylin in overweight and obese insulin-resistant youth (87-90). However, this relationship does not imply unanimity. A recent report has noted that the relationship between pro-inflammatory and metabolic markers commonly observed in adults and pubertal adolescents is reversed in healthy black and white children before puberty, which warrants questions as to whether these inverse relationships modify the trajectory later in life (91). Population-based studies focused on evaluating pro-inflammatory and metabolic markers to determine which biomarkers constitute sensitive and specific tools in the context of a diagnosis of insulin resistance would be valuable.

OXIDATIVE STRESS

Oxidative stress is often a neglected cause of paediatric obesity-related morbidities, and no biomarkers have been successfully validated yet for routine clinical use. To our knowledge there are no clinical research studies demonstrating that circulating concentrations of malonyldialdehyde (MDA), Hydroxynonenal (HNE), advanced glycation end-products (AGEs) and 8-hydroxy-2-deoxyguanosine (8-OH-dG), which are surrogate markers for lipids, proteins and deoxyribonucleic acid damages respectively, are effective diagnostic tools for CMD in childhood and adolescence.

In an observational study performed on 35 children between the ages of 12 and 18 years,

Khelishadi et al. (92) reported that the age- and sex-adjusted changes in ox-LDL, waist circumference, CRP, MDA and body fat mass had the highest correlations with changes in coronary intima media thickness. More recently, in a population-based study, Galan-Chilet et al. (93) demonstrated a positive association of selenium at plasma concentrations above ~110 µg/L for 8-oxo-dG, but an inverse association with GSSG/GSH and MDA. They further identified potential risk genotypes associated with increased levels of oxidative stress markers with high selenium levels.

CONCLUSIONS

There is currently no single biomarker which can adequately define obesity-related CMD risk in paediatrics or adults. Prospective clinical trials should focus on devising a score based on well-characterized and appropriately validated biomarkers.

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In this issue: Recent Advances in Pediatric Laboratory Medicine

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Pediatric Metabolic Syndrome: pathophysiology and laboratory assessment

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ABSTRACT

Pediatric overweight and obesity is an emerging public health priority as rates have rapidly increased worldwide. Obesity is often clustered with other metabolic abnormalities including hypertension, dyslipidemia, and insulin resistance, leading to increased risk of cardiovascular disease. This cluster of risk factors, termed the metabolic syndrome, has traditionally been reported in adults. However, with the increased prevalence of pediatric obesity, the metabolic syndrome is now evident in children and adolescents. This complex cluster of risk factors is the result of the pathological interplay between several organs including adipose tissue, muscle, liver, and intestine with a common antecedent – insulin resistance. The association of the metabolic syndrome with several systemic alterations that involve numerous organs and tissues adds to the complexity and challenge of diagnosing the metabolic syndrome and identifying useful clinical indicators of the disease. The complex physiology of growing and developing children and adolescents further adds to the difficulties in standardizing laboratory assessment, diagnosis, and prognosis for the diverse pediatric population. However, establishing a consensus definition is critical to identifying and managing children and adolescents at high risk of

developing the metabolic syndrome. As a result, the examination of novel metabolic syndrome biomarkers which can detect these metabolic abnormalities early with high specificity and sensitivity in the pediatric population has been of interest. Understanding this complex cluster of risk factors in the pediatric population is critical to ensure that this is not the first generation where children have a shorter life expectancy than their parents. This review will discuss the pathophysiology, consensus definitions and laboratory assessment of pediatric metabolic syndrome as well as potential novel biomarkers.

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INTRODUCTION

The worldwide prevalence of pediatric overweight and obesity combined has risen by 47.1% between 1980 and 2013 (1). This alarming increase in pediatric obesity has become a global public health burden, evident by the World Health Organization (WHO) Health Assembly endorsement for the Comprehensive Implementation Plan on Maternal, Infant, and Young Child, Nutrition, which consisted of six global nutrition targets to be achieved by 2025, including "Target 4: no increase in childhood overweight" (2). Obesity is the most important risk factor for cardiovascular disease (CVD) and is often clustered with additional metabolic abnormalities including hypertension, dyslipidemia, and insulin resistance (3). These CVD risk factors tend to cluster, not only in adults, but more recently in children (4). This common cluster of major determinants of CVD led to the definition of what is known as the metabolic syndrome (MetS). The current paradigm of MetS was established by Reaven and colleagues (5) in 1988, originally termed Syndrome X. Reaven described MetS as the interrelation between insulin resistance, hypertension, type 2 diabetes (T2D), and CVD. Although this syndrome was not defined until the late 1980s, the relationship between obesity, hypertriglyceridemia, and hypertension was first recognized in the early 1980s (6). This was followed by the description of the central roles of insulin resistance and abdominal obesity in MetS in the late 1980s to early 1990s (7). Clinical definitions of MetS have been extremely variable, however almost all definitions require a partial combination of the following five elements: elevated triglycerides (TGs), reduced high-density lipoprotein cholesterol (HDL-C), increased blood pressure, elevated fasting plasma glucose, and increased waist circumference (3). Although MetS was once thought to be an adult-onset disease, this clustering of metabolic disorders is becoming increasingly prevalent in children and adolescents, making it a public health priority in the pediatric population as well. This review will discuss what is currently known about the underlying pathophysiology of pediatric MetS, particularly in regards to the major organs involved. Additionally, the difficulty in defining pediatric MetS, current definitions and laboratory assessment to define and monitor pediatric MetS, and potential novel biomarkers will be discussed.

In the Third National Health and Nutrition Examination Survey (NHANES III), conducted between 1988 and 1994 in the US, the prevalence of MetS in adolescents aged 12-19 years was 4%, increasing to 28.7% among strictly obese adolescents (8). A more recent analysis of NHANES data from 1999-2002, demonstrates that MetS prevalence in obese adolescents has since increased to 44% (9). If current trends continue, the World Health Organization (WHO) predicts that 70 million infants and young children will be overweight or obese by 2025. The prevalence of MetS directly increases with the degree of obesity and each component of the syndrome worsens with increasing obesity, independent of age, sex, and pubertal status (3).

Childhood obesity is also an early risk factor for adult morbidity and mortality (10,11) and 85% of obese children become obese adults (10,12). It is important to detect MetS early in childhood and adolescence to prevent further health complications in adulthood and minimize the global socio-economic burden of CVD and T2D. Unless action is taken, diabetes experts agree that this is the first generation where children may have a shorter life expectancy than their parents (13).

PATHOPHYSIOLOGY: UNDERSTANDING THE COMPLEX CLUSTER

The etiology of MetS is incompletely understood; however, insulin resistance is thought to be central to the development of MetS and play a role in the pathogenesis of its individual metabolic components. The World Health Organization (WHO) hypothesizes that the association and clustering of T2D, hypertension, dyslipidemia, and CVD arises from a common antecedent - insulin resistance (14). Insulin resistance is the decreased tissue response to insulin-mediated cellular actions.

Although hyperglycemia, the primary complication of insulin resistance, can result in substantial morbidity in T2D, CVD is the leading cause of death in T2D patients, mainly due to lipid abnormalities (15). This phenomenon is well-supported by results of the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study, in which attempts to tightly control glucose did not lead to an improvement in mortality (16,17). Insulin elicits peripheral effects on several organ systems, including adipose tissue, muscle, liver, and intestine. Therefore, in insulin resistant states, metabolic dysfunction across several organs occurs, together creating this observed interplay of several concurrent metabolic abnormalities.

Lipid partitioning and inflammation

It is widely accepted that obesity and the concomitant development of inflammation are the major components of insulin resistance (18). In obesity, adipose tissue storage capacity becomes saturated and insulin suppression of adipose tissue lipolysis is diminished (19). As a result, plasma free fatty acid (FFA) levels increase and this excess lipid can be stored in sites other than conventional subcutaneous adipose depots, including intraabdominal (visceral) adipose compartments and insulin-responsive tissues (i.e. muscle and liver). This altered lipid partitioning can shift the balance between adipocytokines, producing more inflammatory cytokines (i.e. TNF-α and IL-6) and fewer anti-inflammatory peptides (i.e. adiponectin). In addition to inflammatory effects of obesity, the increased FFA flux results in several metabolic dysfunctions. When the subcutaneous fat depot reaches its storage capacity and lipid is shunted to ectopic tissues (i.e. liver and muscle), peripheral insulin resistance occurs (20).

Derivatives of fatty acids (e.g. long chain fatty acyl-CoA and DAG) in hepatocytes and myocytes may alter the insulin signal transduction pathway, leading to this observed decrease in insulin sensitivity. Several studies support this theory, as lipid content in liver and muscle is increased in obese and T2D subjects and is a strong predictor of insulin resistance (21). Furthermore, obese adolescents with a high visceral to subcutaneous fat ratio demonstrate a markedly adverse metabolic phenotype of severe insulin resistance and alterations in glucose and lipid metabolism (22). Taken together, obesity results in increased inflammatory markers and FFA flux, subsequently reducing the insulin sensitivity of several organs (i.e. adipose tissue, muscle, liver, intestine). Insulin resistance across several organs results in the MetS phenotype which

includes dyslipidemia, subsequently increasing CVD risk by affecting endothelial function and the vascular system (23).

Adipose tissue insulin resistance and FFA flux

Adipose tissue enlargement (i.e. obesity) leads to a proinflammatory state in the cells, with reduced secretion of adiponectin and increased secretion of several inflammatory cytokines and chemokines (24). One of these chemokines, monocyte chemoattractant protein-1 (MCP-1), plays an important role in recruiting macrophages into adipose tissue (24). Macrophages infiltrate adipose tissue and contribute to adipocyte hypertrophy and further cytokine release (24,25). These cytokines can affect insulin action in other tissues, such as liver and muscle, but can also lead to local insulin resistance. Insulin inhibits lipolysis in adipose tissue, and therefore in insulin resistance, lipolysis is accelerated, leading to increased FFA release into the circulation (3). Therefore, insulin resistance further supports the proinflammatory state of obesity because its anti-lipolytic and anti-inflammatory effects are negated.

Muscle insulin resistance and glucose intolerance

Increased plasma FFAs, due to reduced insulin suppression of adipose tissue lipolysis, disrupt insulin-mediated glucose uptake by skeletal muscle, facilitating development of hyperglycemia (26). Insulin resistance in skeletal muscle may promote atherogenic dyslipidemia by diverting ingested carbohydrate towards hepatic *de novo* lipogenesis (DNL), rather than muscle glycogen storage (23). Young, lean, insulin-sensitive subjects store most of their ingested energy in liver and muscle glycogen, while young, lean insulin-resistant subjects have dysfunctional muscle glycogen synthesis and divert more of their ingested energy into hepatic DNL (27). This results in increased plasma TGs, lower HDL-C, and increased hepatic TG synthesis (27). Mouse studies further support these findings as muscle-specific inactivation of the insulin receptor gene results in increased plasma TGs and increased adiposity as a result of muscle-specific insulin resistance (28).

Hepatic insulin resistance and fasting dyslipidemia

The liver is a main target of insulin action and plays a major role in both carbohydrate and lipid metabolism. Two key hepatic insulin actions are reducing hepatic glucose output and inhibiting secretion of very low-density lipoproteins (VLDLs). To reduce hepatic glucose output, insulin phosphorylates FoxO1, preventing it from entering the nucleus, and consequently reducing the expression of genes required for gluconeogenesis (29). Postprandial insulin release enhances hepatic VLDL production by upregulating lipogenesis via activation of the transcription factor sterol regulatory element-binding protein (SREBP-1c) (30). SREBP-1c increases transcription of genes required for FA and TG biosynthesis, resulting in increased DNL. TGs synthesized by DNL and dietary lipids are packaged with apolipoprotein B100 (apoB100) into VLDLs. Although insulin increases substrate availability for VLDL production, it also acutely reduces VLDL secretion (31). This inhibitory action is thought to be due to an increase in apoB100 degradation, the main structural protein of VLDL (31).

Insulin has key metabolic regulatory roles in the liver, thus several metabolic abnormalities can clinically manifest with hepatic insulin resistance. Diabetic dyslipidemia is one such abnormality which is characterized by hypertriglyceridemia, increased small dense LDL (sdLDL) and decreased HDL-C (32). This phenomenon is the direct result of hepatic insulin resistance which results in impaired glucose homeostasis due to reduced FoxO1-mediated phosphorylation, and

enhanced hepatic DNL due to reduced SREBP-1 activation (33). Therefore, both hyperglycemia and hypertriglyceridemia are seen in hepatic insulin resistance. In addition to enhanced DNL, substrates for VLDL synthesis are increased due to elevated FFA flux from adipose tissue and increased hepatic uptake of chylomicron remnants (CM; lipoproteins secreted from the intestine) and VLDL remnants (34,35). Increased substrate availability for VLDL production and reduced apoB degradation can lead to VLDL overproduction and hypertriglyceridemia. As a result of hypertriglyceridemia, highly atherogenic sdLDL are also produced in insulin resistant states. sdLDL are produced from the action of cholesteryl ester transfer protein (CETP), which exchanges VLDL TG for LDL cholesteryl ester (CE), creating CE-depleted, TG-enriched, LDL particles (36). These particles become sdLDL after they are lipolyzed by lipoprotein lipase (LPL) or hepatic lipase (HL) (36). CETP action is thought to also contribute to reduced HDL-C levels in insulin-resistant subjects (36).

Hepatic steatosis, one of the main detriments to the liver in response to hepatic insulin resistance, is characterized by the accumulation of excess lipid in the liver, which can progress to inflammatory steatohepatitis, fibrosis, and even cirrhosis. This spectrum of diseases is collectively termed non-alcoholic fatty liver disease (NAFLD). Progression of NAFLD can cause liver failure, leading to the need for a liver transplant, even in adolescents (37). As the prevalence of pediatric obesity increases, NAFLD has also increased in prevalence, rapidly becoming the most common cause of pediatric liver disease (37). Furthermore, a pediatric study showed that every 1 cm increase in waist circumference is associated with a 1.97 and 2.08 fold increased risk of NAFLD in males and females, respectively (34). Although the pathological link between MetS and NAFLD is incompletely understood, the theory of the “two-hit model” is the most widely accepted (38). The first hit is insulin resistance which promotes the accumulation of hepatocyte lipid due to increased hepatic FFAs available for TG synthesis in an insulin resistant state (36). This results from insulin failing to block adipose tissue lipolysis, resulting in increased FFA release from adipose tissue. Increased circulating FFAs leads to increased FFA uptake by hepatocytes, increased TG synthesis and impaired FFA oxidation, producing excess lipid in hepatocytes (38,39). The second hit is injury from reactive oxygen species (ROS). Lipid accumulation in hepatocytes impairs the oxidative capacity of the mitochondria and can also lead directly to further ROS production (40). Increased susceptibility of hepatocytes to oxidative stress and subsequent lipid peroxidation by ROS promotes progression to nonalcoholic steatohepatitis (NASH). This is due to chemoattractants (i.e. by-products of oxidative stress and lipid peroxidation), which lead to fibrosis and the production of inflammatory cytokines (37).

Intestinal insulin resistance and postprandial dyslipidemia

In contrast to the numerous studies on insulin signaling in well-known insulin-sensitive tissues such as liver, muscle, and adipose, relatively little is known regarding intestinal insulin signaling and potential perturbations with insulin resistance (41). The intestine packages absorbed dietary fat into apoB-48-containing TG-rich lipoproteins, called chylomicrons (CMs), which transport TGs and fat-soluble vitamins to peripheral tissues (42). Similar to its actions in the liver, insulin has a key regulatory role in the production and clearance of TRLs produced from the intestine (43). Therefore, another defining feature of diabetic dyslipidemia is elevated postprandial levels of CM particles (44). The accumulation of CM particles in insulin resistance has been attributed to decreased clearance as

well as increased intestinal synthesis and secretion (45). Decreased clearance of CM and CM remnants in insulin resistance has largely been attributed to increased hepatic VLDL secretion (46), as intestinal and hepatic TRLs share common, saturable, removal mechanisms (47). Secondly, LPL activity is decreased due to diminished regulation by insulin (48), contributing to slow removal of CM and CM remnants in insulin resistance.

Although the intestine was conventionally regarded as a passive organ with respect to CM secretion, it is now evident that CM production can be actively increased in insulin resistant states (43). Insulin has been shown to directly decrease CM secretion from cultured human fetal jejunal explants (49) and to reduce CM production in healthy men following an insulin infusion (50). Mechanisms for CM overproduction in insulin resistance are unclear, yet may include increased apoB stability, increased mass and activity of microsomal triglyceride transfer protein (MTP; required for assembly of VLDLs and CMs), and enhanced DNL in the enterocyte (41,51). The inhibitory effect of insulin on CM secretion may also partly be due to its suppression of circulating FFAs (46,50,52), an effect that is blunted by insulin resistance (52) and T2D (53). Overall, human studies suggest that intestinal CM production is dysregulated in insulin resistance states, with diminished sensitivity to insulin's inhibitory effects, contributing to increased plasma CM levels. Intestinal lipoprotein production is particularly important as postprandial TG levels independently predict CVD (54). In addition, CM remnants are risk factors for atherosclerosis (55) and apoB-48 can be detected in atherosclerotic plaques (56).

The intestine is also involved in the pathogenesis of MetS through its important role as an endocrine organ. The intestine secretes several gut peptides with glucagon-like peptide 1 (GLP-1) playing a significant role in insulin secretion and signaling. GLP-1 is secreted by ileal enteroendocrine L-cells in response to a variety of nutrient, neural, and endocrine factors (57). This hormone has several biological actions on the pancreas, nervous system, gastrointestinal system, skeletal muscle, adipose tissue, and liver. As a result of the important roles GLP-1 plays in metabolism, agonists of GLP-1, as well as inhibitors of dipeptidyl peptidase-4 (DPP-4), the main protease in GLP-1 degradation, have been successful therapeutics for T2D (58). In the pancreas, GLP-1 stimulates glucose-dependent insulin secretion, improves the capacity of β-cells to sense and respond to glucose, increases β-cell mass, and inhibits glucagon and stimulates somatostatin secretion (57). The GLP-1 receptor (GLP-1R) and nerve fibers containing GLP-1 are located in the central nervous system and therefore several studies have examined central and peripheral actions of GLP-1. Central actions of GLP-1 include satiety promotion, reduced energy intake, and consequently decreased body weight (59). Additionally, the effects of GLP-1 on the pancreas may be mediated in part by a neural mechanism (60). In the intestine GLP-1 has inhibitory effects on lipoprotein secretion, gastric acid secretion and gastric emptying, which slows the transit of nutrients from the stomach to the small intestine, contributing to the normalization of blood glucose levels (61). The effect of GLP-1 on muscle, adipose tissue, and the liver, including stimulation of glucose uptake and inhibition of hepatic glucose production, remain controversial as to whether they are independent of changes in insulin or glucagon (57).

LABORATORY ASSESSMENT OF PEDIATRIC METABOLIC SYNDROME

An adult definition of MetS cannot simply be applied for use in the pediatric population because drastic changes in blood pressure, lipid levels, as well as body size and proportion occur

with age and development. Puberty also impacts fat distribution, insulin sensitivity, and insulin secretion (62). Children develop transient physiologic insulin resistance during puberty (63), with a 25-50% decline in insulin sensitivity which recovers upon completion of pubertal development (64). The dynamic physiological changes that occur in children and adolescents has led to the lack of standardized measures in pediatrics, including measurements of central obesity (3), which is a defining feature of adult MetS. Establishing a consensus definition of MetS in the pediatric population has therefore traditionally been a challenge. However, it is important to note that the MetS is not a disease, but a cluster of metabolic disorders. Therefore, applying any set of criteria to "define" the MetS truly reduces the complex reality of this cluster of components. Each component of the MetS is a continuous variable which gradually changes. This results in a continuum between a healthy and unhealthy metabolic profile, rather than a dichotomy of healthy and unhealthy states. However, an accepted definition of pediatric MetS is important as a diagnostic and monitoring tool to ensure standardization in clinical practice as well as in research to standardize clinical trials.

Rapid rises in obesity trends sparked the need to understand how to distinguish between children and adolescents at high risk of health complications and those with "simple" uncomplicated obesity. Traditionally, researchers have used several different definitions (65), resulting in the prevalence of metabolic syndrome varying between 0% and 60% in the same group of children, depending on the diagnostic criteria applied (66). This drove the International Diabetes Federation (IDF) to develop a universally accepted and easy to use definition for MetS in children and adolescents in 2007 (13). This definition was created with the intention to allow preventative measures to be taken before the child or adolescent develops T2D and/or CVD (13). The main component of the definition is waist circumference because it is an independent predictor of insulin resistance, lipid levels, and blood pressure (67,68). However, percentiles, rather than single cut-off points, must be used for this measure due to the dynamic metabolic changes that occur throughout the pediatric age range. A cut-off of the 90th percentile was chosen, as children and adolescents with a waist circumference ≥ 90th percentile are more likely to have multiple CVD risk factors (13).

The IDF consensus definition of MetS in children and adolescents is shown in Table 1. The definition excludes children who are younger than 6 years because of insufficient data for this age-group (13). For children aged 6-10 years, MetS should not be diagnosed, but those with abdominal obesity should be strongly advised to reduce their weight. For children age 10-<16 years, MetS should be diagnosed for those with abdominal obesity and two or more other clinical features including elevated triglycerides, decreased HDL-C, increased blood pressure, and increased fasting plasma glucose. For adolescents older than 16 years of age, it is recommended to use the IDF adult criteria. This IDF pediatric definition provides a standard that facilitated comparisons of study results, including prevalence estimates across studies.

However, the IDF definition of pediatric MetS is not without limitations. First, this definition does not provide criteria to diagnose children under the age of 10 years. Additionally, the blood pressure cut-off used in this definition is the same as that defined for adults and is thus too high for the pediatric population. This results in blood pressure contributing to a negligible proportion of children being classified as having the MetS using this definition (69). Lastly, rather than being based on evidence from the pediatric population, the IDF consensus definition is modified from a definition created for

Table 1 IDF consensus definition of the Metabolic Syndrome in children and adolescents

Age (years)	Obesity (WC)	Triglycerides	HDL-C	Blood pressure	Glucose
6-<10	≥ 90th percentile	Metabolic syndrome cannot be diagnosed, but further measurements should be made if there is a family history of metabolic syndrome, T2DM, dyslipidemia, cardiovascular disease, hypertension and/or obesity			
10-<16	≥ 90th percentile or adult cut-off if lower	≥1.7 mmol/L (≥150 mg/dL)	<1.03 mmol/L (<40 mg/dL)	Systolic ≥130/ diastolic ≥85 mm Hg	≥5.6 mmol/L (100 mg/dL) (If ≥5.6 mmol/L [or known T2DM] recommend an OGTT)
≥ 16 (adult criteria)	Central obesity (defined as waist circumference ≥ 94cm for Europid men and ≥ 80cm for Europid women)	≥1.7 mmol/L (≥150 mg/dL)	<1.03 mmol/L (<40 mg/dL) in males and <1.29 mmol/L (<50 mg/dL) in females, or specific treatment for these lipid measurements	Systolic ≥130/ diastolic ≥85 mm Hg, or treatment of previously diagnosed hypertension	Fasting plasma glucose ≥5.6 mmol/L (100 mg/dL), or previously diagnosed type 2 diabetes

**Table adapted from (13).*

WC: waist circumference; HDL-C: high-density lipoprotein cholesterol; T2DM: type 2 diabetes mellitus; OGTT: oral glucose tolerance test.

the adult population. A more recent MetS definition for European pre-pubertal children was proposed by the Identification and Prevention of Dietary- and Lifestyle-Induced Health Effects in Children and Infants (IDEFICS) Study which addresses these limitations.

The main factor contributing to the absence of a consensus MetS definition in children is the lack of reference values for MetS components in the pediatric population (70). Therefore, the IDEFICS study used reference values provided by their study of European children to classify children according to the different components of the MetS (69). They propose a definition with different cut-offs to classify children requiring either close monitoring (monitoring level) or an intervention (action level) (69). Using age-, sex-, and height- (in the case of blood pressure) specific

percentiles established from the IDEFICS cohort, percentile cut-offs are defined for the MetS components (shown in Table 2 for the monitoring level). Children are classified as requiring close monitoring of the MetS if three or more of these risk factors exceed the 90th percentile defined in the IDEFICS studies (69). If three or more of these risk factors exceed the 95th percentile, defined in the IDEFICS studies, an intervention is appropriate in affected children (69). They also created a simple web application (www.ideficsstudy.eu) to more easily classify an individual by entering individual measurement values and obtaining the appropriate percentiles. As a result of using percentile cut-offs established from a pediatric population rather than arbitrary cutoffs for MetS components, the IDEFICS definition provides a more equal weight to components of the definition, allowing a more equal contribution to the overall prevalence of the MetS. However, this definition is also not without limitations. In addition to only being applicable to children and not adolescents, the percentile cut-offs for each parameter is population-specific and therefore may differ for smaller, local populations. Also, clinically relevant, prospective outcomes related to the percentile cut-offs which would allow the assessment of disease risk in relation to defining the MetS are currently lacking.

In addition to proposing definitions to classify children as requiring monitoring or intervention for the MetS, the IDEFICS study also developed a quantitative CVD risk score. This was established using a z-score standardization to calculate a continuous score combining the MetS components, with a higher score indicating a less-favorable metabolic profile. A study by Pandit et al. supports the use a quantitative risk score, as this study suggested that a continuous MetS score was a better tool to assess atherosclerotic risk in children than cut-offs of individual MetS components (71). Rather than dichotomizing the population into children with a healthy and unhealthy metabolic profile based on cut-offs of each MetS component, the score provides a variable that accounts for gradual changes in these components. The continuous score better reflects the complex concepts of the MetS, where risk predictors lie on continuous scale and have complex interactions. The

Table 2 **IDEFICS definition of the Metabolic Syndrome in children –monitoring level**

Age (years)	Obesity (WC)	Triglycerides	HDL-C	Blood pressure	Glucose
2-<11 years	≥ 90th percentile	≥ 90th percentile	≤ 10th percentile	Systolic ≥90th percentile or diastolic ≥ 90th percentile	HOMA-insulin resistance ≥ 90th percentile or fasting glucose ≥ 90th percentile

**Table adapted from (69).*

WC: waist circumference; HDL-C: high-density lipoprotein cholesterol; HOMA: homeostatic model assessment.

continuous MetS score can be a useful tool in pediatric research and for evaluating interventions (69).

In addition to the parameters included in the consensus definitions of pediatric metabolic syndrome, the standard lipid profile aids in CVD risk assessment. A standard lipid profile includes fasting measurements of plasma or serum concentrations of total cholesterol, LDL-C, HDL-C, and triglycerides. Additional markers that have been added to the lipid profile in some clinical laboratories include non-HDL cholesterol, apolipoprotein B (apoB), apolipoprotein A1 (apoA1), and lipoprotein(a) (Lp(a)) (72). Non-HDL cholesterol, calculated as total cholesterol minus HDL-C, gives an indicator of the total cholesterol content of atherogenic lipoproteins. ApoB and apoA1 can also be used as alternatives to non-HDL and HDL cholesterol, respectively, where they indicate the particle number, rather than cholesterol content. Lastly, Lp(a) should only be determined in the same patient once as its concentration varies little over time.

POTENTIAL NOVEL BIOMARKERS IN LABORATORY ASSESSMENT OF PEDIATRIC METABOLIC SYNDROME

With the increasing public health burden of MetS, the identification and examination of novel biomarkers able to detect MetS and subsequently CVD risk early, with high specificity and sensitivity, is a clinical priority (73). Effective MetS biomarkers maximize the effectiveness of treatment in subjects who would benefit the most. The association of MetS with several systemic alterations that involve numerous organs and tissues adds to the complexity and challenge of identifying MetS biomarkers. A few categories of potential MetS biomarkers and nontraditional pre-analytical considerations that have recently been gaining interest will be discussed.

Adipocytokines

Recent literature has shifted the notion of adipose tissue as a nonfunctional energy storage site to an important secretory organ. Adipose tissue secretes low-molecular weight peptides, called adipocytokines, which have numerous functions including food intake regulation, glucose and lipid metabolism, and inflammation (74). More recently, studies have shown adipocytokines mediate obesity-associated metabolic disorders independently of other risk factors (75). One adipocytokine, adiponectin, is secreted primarily by the adipocyte and is actually decreased in plasma upon an increase in fat mass (76). Adiponectin has several functions including anti-inflammatory and anti-atherogenic effects, as well as insulin sensitization and lipid regulation (77). Pediatric studies have shown that plasma adiponectin concentration is inversely correlated with BMI, waist circumference (WC), fasting insulin concentration, and insulin resistance (78,79) and is 25% higher in healthy overweight youth compared to those with MetS (80). Additionally, a study of 5,088 adolescents showed that a decreased adiponectin concentration was associated with an increased risk of MetS, independent of age, BMI, WC, and total cholesterol (81).

Leptin, the first identified adipocytokine, is a product of the obesity gene and is known as the "satiety hormone" because it decreases food intake and increases energy expenditure. Leptin concentration has been shown to reflect body fat mass and, as a result, can be considered a reliable marker of fat mass and energy homeostasis in non-insulin resistant individuals (82). Not only do obese individuals tend to have elevated plasma leptin concentrations, but they are also leptin-resistant, negating the beneficial effects of leptin (83). Several studies have also shown this positive association between fat mass and leptin concentration in the pediatric population (84,85). Furthermore, leptin is

positively associated with insulin resistance in pre-pubertal children after adjusting for sex, age, and BMI, and for every 1 ng/dL increase in leptin levels, the odds of MetS increase by 3%, suggesting an important role for leptin as a marker of CVD risk (86).

As a result of several studies supporting the potential roles of both adiponectin and leptin as MetS biomarkers, studies to develop normative values for adiponectin were warranted. A study in 2012 established sex-specific reference intervals (2.5th and 97.5th percentiles of concentration distribution in healthy subjects) for total adiponectin in cord blood and for each one year interval from 0-14 years of age (87). Another study of 111 healthy children aged 0-10 years provided median, 25th and 75th percentile values for leptin (88). A more recent study established age- and sex-specific reference intervals for both serum adiponectin and leptin in pre-pubertal European children (ages 3-9 years) (89). Furthermore, studies have assessed the diagnostic potential of these biomarkers in the pediatric population. One study determined an adiponectin concentration of 6.65 µg/mL as a cutoff point to identify MetS with 64% and 67% sensitivity and specificity, respectively (75). Likewise, a recent study determined a leptin level of 13.4 ng/mL as a cutoff point to identify MetS with a sensitivity and specificity of 68% and 69%, respectively (86). Although further examination of these biomarkers is needed to determine their suitability in MetS detection, extensive progress has been made in the understanding of these adipocytokines in pediatric MetS.

Microalbumin

Microalbuminuria, an increased level of urine albumin, is thought to be the renal expression of vascular endothelial damage, particularly increased vascular permeability, as evidence suggests that glomerular leaking of albumin reflects general vascular damage (90–92). Therefore, microalbuminuria denotes preclinical atherosclerosis and can be used as an early atherosclerosis indicator (90–92). Obesity is strongly associated with the two most common causes of end-stage renal disease: diabetes and hypertension (93). Additionally, the MetS is suggested to be an independent risk factor for both chronic kidney disease and end-stage renal disease (94). Initially introduced into the criteria to define the MetS by the WHO in 1988 (14), microalbuminuria screening is now recommended to be added to the assessment of the CVD risk profile in adults (92). This is the result of well-established evidence of the relation between microalbuminuria and hypertension, central adiposity, the MetS, and CVD mortality (95). More recent studies have examined the association between microalbuminuria and obesity as well as other CVD risk factors in the pediatric population (93,96,97). A study of 150 obese children by Sanad M et al. found that obese children with microalbuminuria had a significantly higher blood pressure, triglyceride levels, LDL levels, as well as a higher prevalence of MetS, insulin resistance, and impaired fasting glucose levels, than those without microalbuminuria (93). Another study by Burgert T et al. found that 10.1% of an obese, non-diabetic pediatric cohort had a urine albumin to creatinine ratio in the microalbuminuric range (i.e. 2-20 mg/mmol), which is similar to the expected prevalence in an obese adult population (96). Even slight abnormalities in glucose metabolism may promote early vascular damage in pediatric obesity (96). Microalbuminuria has been suggested as a treatment target in adults (98,99), and now may also become an approachable treatment target in pediatric metabolic syndrome, potentially responsive to treatment (i.e. lifestyle intervention or pharmacotherapy) directed at improving insulin sensitivity and glucose tolerance (96).

Gut peptides

In contrast to the extensively studied adipocytokines, gut peptides, including GLP-1 and GLP-2, are more novel potential biomarkers that are gaining interest in parallel with the recently accepted metabolic role of the intestine. In addition to its well-known incretin action, GLP-1 also promotes satiety, inhibits gastric emptying, and regulates lipid metabolism (57). Studies have shown decreased GLP-1 secretion and blunted postprandial increase in GLP-1 in morbidly obese (83) and T2D individuals (100). This may be due to the decreased responsiveness of L-cells to nutrient intake in insulin resistant conditions (101). With the important incretin effect of GLP-1, it is evident that decreased GLP-1 secretion in an obese state would have implications on insulin action. Recent pediatric studies have shown that fasting total GLP-1 is reduced, but fasting active GLP-1 is elevated in obese compared to normal weight adolescent girls (102). Overall, GLP-1 secretion and plasma concentration in obesity remains controversial and pediatric studies of this phenomenon are extremely limited. GLP-2, encoded on the same gene and co-secreted in an equimolar amount with GLP-1, enhances intestinal lipoprotein production and nutrient absorption, as well as reduces inflammation (86). Recent studies in obese adults have shown an inverse relationship between GLP-2 secretion and insulin sensitivity, although the underlying mechanisms are still unknown (103). Studies on GLP-2 are even more scarce, particularly on obese pediatric subjects. Future studies examining the potential of GLP-1 and GLP-2 as MetS biomarkers in pediatric subjects are critical to understand their potential in laboratory assessment of pediatric MetS.

Lipoproteins and apolipoproteins

Although the standard lipid profile consists of lipids and lipoproteins, with some newly added apolipoproteins, there are additional lipoprotein subfractions recently receiving attention for CVD risk assessment. The first parameter, remnant lipoproteins (RLPs) are metabolic products of TG-rich lipoproteins (i.e. CMs and VLDLs). A study of 1,567 women from the Framingham Heart Study showed RLP-C was an independent risk factor for CVD in women, independent of TG (55). Postprandial RLP-C was shown to be an independent predictor of insulin resistance after adjusting for age, BMI, and other lipid profiles in a study of 78 adults (104). Pediatric studies have shown that RLP-C is significantly higher in obese subjects and strongly related to insulin resistance (91). Long-term prospective studies are needed to evaluate whether children and adolescents with high RLP-C are at greater risk of developing MetS. The second parameter is apoB-48 which is a specific marker of intestinal lipoproteins (i.e. CMs). As CMs are secreted in the postprandial state, apoB-48 can subsequently be used to examine postprandial lipoprotein metabolism (91). Adult studies have shown fasting apoB-48 is elevated in subjects with MetS (105) and T2D and is significantly associated with endothelial dysfunction (106). Recent studies in pediatrics determined that fasting plasma apoB-48 concentration is 2-fold higher in obese versus normal weight subjects (107). However, pediatric data on apoB-48, particularly in the postprandial state, is needed to understand the potential of apoB-48 as a MetS biomarker.

Assessment in the postprandial state

In addition to the recent exploration of novel MetS biomarkers, emerging pre-analytical conditions that may improve both the simplicity of laboratory testing and the relevance of the laboratory test results have been examined. In clinical practice, the lipid profile is traditionally measured in a fasting state even though the postprandial state predominates over a typical

24 hour day. Therefore, the lipid and lipoprotein content of a fasting sample does not accurately reflect the daily average concentration of these parameters. Additionally, evidence is lacking that a fasting sample is superior to a postprandial sample when evaluating for CVD risk assessment, and in fact, postprandial samples seem to be more advantageous (72). Some advantages include simplification of blood sampling for patients, particularly pediatrics, improving patient compliance with lipid testing, and decreasing the volume burden on laboratories in the morning. Several studies have found that postprandial lipid and lipoprotein measurements suffice for CVD risk screening, and in some cases are even better predictors (72). As MetS is a cluster of CVD risk factors, postprandial measurements may be more relevant for clinical guidelines. For example, a meta-analysis including over 300,000 individuals found that postprandial non-HDL cholesterol and calculated LDL-C were superior to fasting measurements for predicting CVD risk (108). Furthermore, the novel MetS biomarkers discussed here are more relevant following nutrient ingestion. For example, GLP-1 and GLP-2 concentrations are much more relevant in the postprandial state, as their concentrations in the fasting state are very low and their secretion is stimulated upon nutrient ingestion (95). Additionally, approximately 80% of the postprandial increase of TG is due to the increase in TG of RLPs (109) and apoB-48 is a marker of CMs (i.e. lipoproteins secreted from the intestine following a meal). Therefore, if MetS components lead to an alteration in these biomarkers, this change would be apparent in the postprandial, rather than fasting state.

CONCLUDING REMARKS

The clustering of CVD risk factors, termed the metabolic syndrome, is present in both adults and children. MetS is primarily driven by excess adipose tissue and subsequent insulin resistance. Insulin resistance manifests in several organs, including the muscle, liver, and intestine, and as a result is associated with several systemic complications including hypertension, dyslipidemia, and impaired glucose tolerance. The interplay of metabolic dysfunction in several organ systems leads to the development of atherosclerosis and consequent CVD complications. Defining MetS in the pediatric population has been controversial due to the difficulties of generalizing both a diverse syndrome and a diverse population. However, establishing a consensus definition is critical for identification and management of youth at a higher risk of developing CVD. As a result, the examination of novel MetS biomarkers in the pediatric population has been of interest to identify pediatric subjects with obesity-related metabolic complications early before CVD complications manifest.

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In this issue: Recent Advances in Pediatric Laboratory Medicine

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Pediatric reference intervals for biochemical markers: gaps and challenges, recent national initiatives and future perspectives

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ABSTRACT

Reference intervals provide valuable information to medical practitioners in their interpretation of quantitative laboratory test results, and are critical in the assessment of patient health and in clinical decision-making. The reference interval serves as a health-associated benchmark with which to compare an individual test result. While the concept of reference intervals and their utility appear straightforward, the process of establishing accurate and reliable reference intervals is considerably complex and involved. Currently, many pediatric laboratory tests are inappropriately interpreted using reference intervals derived from either adult populations, hospitalized pediatric populations, or from outdated and/or inaccurate technology. Thus, many pediatric reference intervals used in diagnostic laboratories are incomplete and may be inappropriate for clinical use. The use of inappropriate reference intervals impacts clinical decision-making and has potential detrimental effects on the quality of patient healthcare including misdiagnosis, delayed diagnosis, inappropriate treatments, and patient risk. These are critical gaps in pediatric healthcare and it is imperative to update and establish appropriate reference intervals for pediatric populations based on specific age- and

sex-stratifications. In the present review, specific issues, challenges and deficiencies in pediatric reference intervals for biochemical markers will be discussed. Early studies using hospitalized patients will be examined, followed by a review of recent national and global initiatives on establishing reference intervals from healthy pediatric population. We will highlight the achievements and milestones of the Canadian CALIPER project, including the establishment of a comprehensive biobank and database which has addressed several of these critical gaps. CALIPER's mandate is to establish and provide comprehensive, up-to-date pediatric reference intervals to all biochemical markers of pediatric disease. CALIPER has also begun knowledge translation initiatives to disseminate its data via peer-reviewed publication, an online database, and a smartphone application to allow greater access to CALIPER pediatric reference interval data. Finally, limitations, future perspectives and harmonization of pediatric reference intervals to improve pediatric diagnostics in Canada and worldwide will be discussed.

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INTRODUCTION

The measurement of disease biomarkers in clinical laboratories are used to screen, diagnose, and monitor a wide range of medical conditions. To interpret these laboratory test results, physicians compare patient test results with a reference interval, defined as the typical values derived from a healthy population [1]. Statistically, reference intervals are defined as the limiting values denoting a specified percentage (typically central 95%) of values from an apparently healthy reference population with 90% confidence. In the central 95% distribution model, the reference limits are determined by calculating the 2.5^{th} and 97.5^{th} percentiles of test results [2]. In this case, a total of 5% of values may be interpreted as abnormal or higher risk of disease and require further follow-up and investigation. In other cases, the reference interval definition may be modified, where a different percentile may be used, or either the upper or lower limit may be used if only a one-sided distribution is clinically significant [3]. Reference intervals are therefore a fundamental tool in test result interpretation and serve as a benchmark for health status [4].

Accurately established reference intervals are critical to clinical decision-making as a lack or inappropriate use of reference intervals may lead to adverse consequences including misdiagnosis, patient risk, inappropriate treatment, and/or higher healthcare costs, all of which impact the overall quality of patient healthcare. Many current reference intervals were determined decades ago with older and less accurate laboratory instruments and testing methodologies. Furthermore, as instrumentation and reagents are upgraded, reference intervals are not always appropriately updated [4]. With rapid advances in technology, there is also a lack of data on novel, emerging disease biomarkers. The lack of standardization and harmonization of assay methodology further contributes to variations of established reference intervals for both adults and pediatrics. These variations create confusion in the interpretation of test results for the same patient whose specimen may be tested in different laboratories.

Additionally, there are specific challenges related to the determination of pediatric reference intervals. The same reference intervals are sometimes used to interpret test results for both adults and children. However, *children are not small adults*; children have significant differences in physiology and metabolic state, physical size, organ maturity, bodily fluid compartments, and immune and hormone responsiveness when compared to adults [4]. Most notably, dynamic physiological changes, growth

and development profoundly influence biomarker concentrations. For example, it is well known that sex hormones, growth hormones, and bone alkaline phosphatase vary with a child's age and development [4]. Establishing appropriate pediatric reference intervals involves overcoming specific challenges mostly related to volume and quantity of healthy pediatric samples. Complex physiological factors may also necessitate the separation of reference interval (called partitions) to be age- and sex-specific, requiring a greater number of reference samples. Moreover, children also suffer from, or are more susceptible to, diseases that differ from adults, requiring unique or new biomarker reference interval determinations. To complicate this further, some of these diseases may be genetically inherited and occur at a lower frequency, posing challenges in acquiring adequate sample size for statistical calculations. Gap analyses of pediatric reference intervals have identified four major critical areas in pediatrics including bone markers [5], cardiovascular disease and metabolic syndrome risk markers [6,7], hormones of thyroid and growth hormone axes [8], and inborn errors of metabolism [9]. Data from published reference interval studies often suffer from limitations in design, small sample sizes, and the use of hospitalized patients [3]. Currently pediatric clinicians and laboratorians depend on scattered information and incomplete data from published (scientific journals and textbooks) and unpublished (hospital, private, reference laboratories) sources in laboratory test result interpretation. There is an urgent need to establish and update reference intervals for all populations and particularly pediatric populations.

While the concept and utility of reference intervals appear straightforward, the process to accurately establish or verify reference intervals is quite complex. The Clinical Laboratory Standards Institute (CLSI) and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) provide a guideline (C28-A3) on how to define, establish, and verify reference intervals [10]. When judging the validity of reference intervals, several integral factors should be considered including the use of a large healthy population, appropriate inclusion/exclusion criteria, awareness of physiological, pre-analytical and analytical factors that affect analyte concentrations, careful outlier exclusion, and appropriate statistical analysis.

The quality of reference intervals depends on the recruitment of a large number of healthy reference individuals within the age group(s) of interest and well-defined inclusion/exclusion criteria. Laboratories commonly verify and adopt a reference interval provided by a manufacturer or transfer a pre-existing reference interval with 20 healthy samples. One problem with verification and transference of pre-existing reference intervals is the quality of the original reference interval. Reference intervals supplied by manufacturers often lack information regarding the study's sample size, age, sex, and ethnic distribution, and often do not include pediatric populations. The success of transference also depends on comparability of the reference populations and analytical methods between the donor and receiving laboratories. To establish robust reference intervals, a *de novo* reference interval study with at least 120 healthy subjects per partition is needed. This is typically done for novel biomarkers. For a pediatric reference interval study that is divided into 5 age groups, it would require 600 healthy subjects or 1200 healthy subjects if also sex stratified [4]. This undoubtedly requires an enormous amount of effort, resources, time and cost. There are many inter-related variables that contribute to the validity of reference intervals including ethnic composition, geographic factors (climate), diet and food preferences, and lifestyle factors. Laboratories that serve ethnically

diverse population should evaluate and determine whether a single reference interval is valid or whether ethnic-specific reference intervals are necessary [4]. Inclusion and exclusion criteria require documentation of covariates (e.g. age, sex, and ethnicity) and assessment of general health using surveys. Pre-analytical factors include standardization of subject preparation (fasting, diurnal variation), specimen collection (posture, sample volume, tourniquet time), and specimen-handling, transport, and storage conditions [3]. Analytical factors include a detailed description of methodology including calibration traceability, imprecision, limit of detection, linearity, analytical measuring range, interferences, and variability factors. Outliers should be excluded through robust statistical methods including Dixon's [11] or Tukey's method [12] for outlier exclusion, parametric or non-parametric analysis, and covariate analysis. Overall, major challenges in the determination of pediatric reference intervals include the recruitment of a sufficiently large healthy pediatric population, the need for parental consent, and difficulties with pre-analytical variables (i.e. sample collection, sample volume, and a specialized phlebotomist with pediatric experience) [3].

In this review, the important milestones in addressing these gaps and challenges in pediatric reference interval determination will be highlighted. The early approaches to establishment of pediatric reference intervals derived from hospitalized patients as well as several recent global initiatives to close the gaps in pediatric reference intervals based on recruitment of healthy children will be discussed. The significant progress that the Canadian Laboratory Initiative on Paediatric Reference Intervals (CALIPER) project has achieved through pilot studies, the establishment of age- and sex-specific pediatric reference intervals for more than 100 biomarkers, and various sub-studies will be reviewed. Lastly, limitations, future perspectives, and goals for pediatric reference interval distribution and harmonization will also be discussed.

INDIRECT APPROACHES TO REFERENCE INTERVAL ESTABLISHMENT

Ideally, reference methods are determined based on a healthy population using a direct *a priori* approach where individuals are specifically selected for the study. However, the recruitment of many healthy individuals can be quite challenging, particularly in the pediatric population, as it is very costly and time consuming. Additionally, it is difficult to define an individual as "normal" or "healthy" as the potential for existing subclinical issues is unknown. Thus, indirect methods, also known as data mining, can be quite useful. The indirect approach uses existing data to establish reference intervals by identifying an acceptable reference population retroactively. One example of using the indirect approach is to identify a group of healthy individuals from hospital in- and/or out-patient populations to calculate reference intervals. In addition to having data readily available through the laboratory information system (LIS), indirect methods remove the need to recruit healthy individuals. This method represents a strong alternative to cases where laboratory markers are measured at high volume in community outpatient clinics and therefore should include a relatively healthy population.

The Hoffmann method proposed by Robert G. Hoffmann in 1963 [13], uses an indirect *a posteriori* method to determine reference intervals using available test results from hospital-based data from in- and out-patients. This approach makes two assumptions: 1) values obtained for a specific analyte follows a Gaussian distribution; and 2) majority of measurements made in the hospital represent normal individuals. Reference intervals are determined by plotting

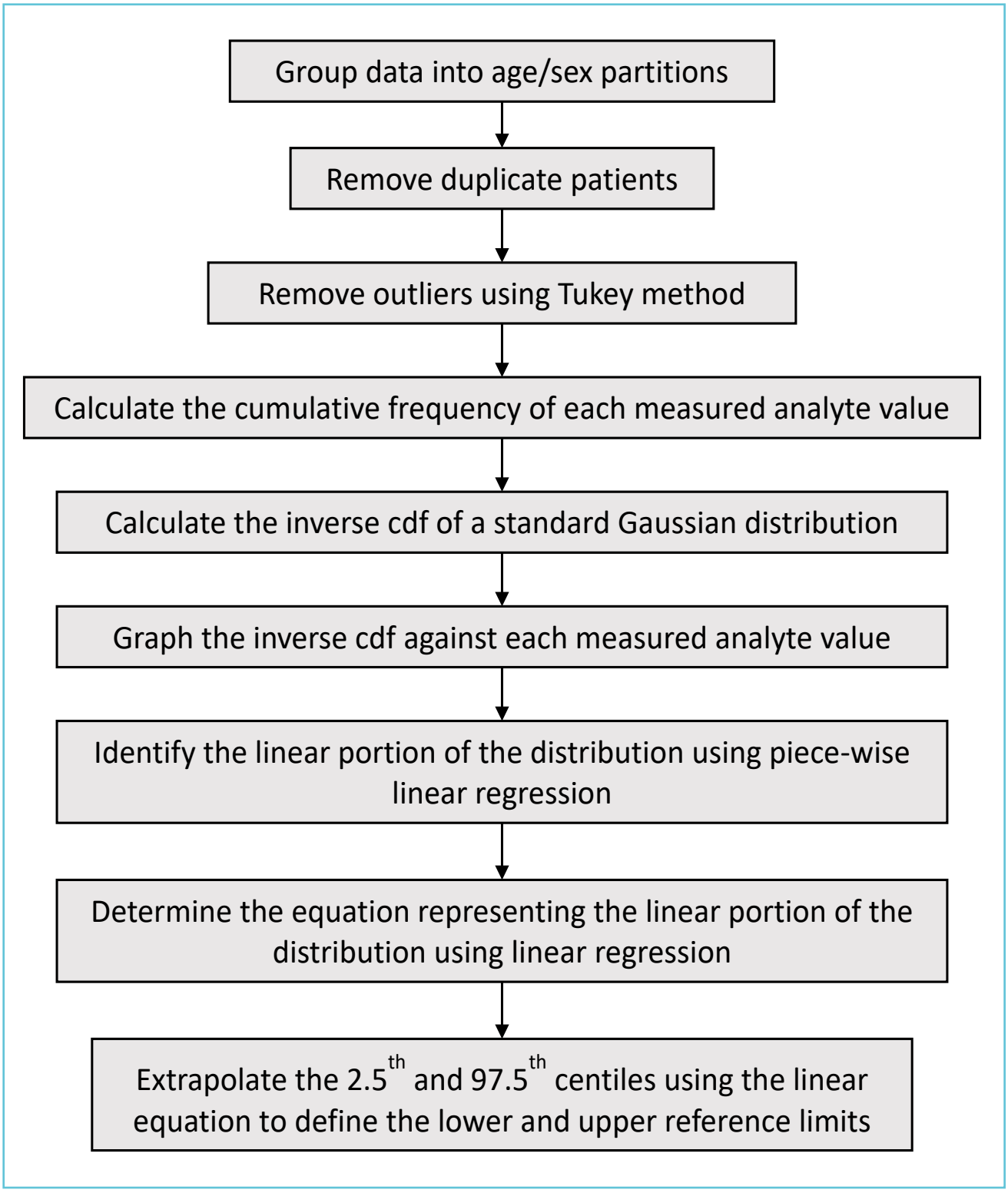
the cumulative frequency of a result against the analyte value. The linear portion of the resulting graph, centered on the 50th percentile, is chosen thereby giving these values the greatest weight. By extrapolating the linear portion of the graph, the 2.5th and 97.5th centiles are calculated, representing the normal range of values, if the assumptions are held true. Hoffmann used this approach with a relatively small number of patients (n=60) for glucose as a proof-of-concept. The application of this method using computer programs has eliminated much of the subjectivity of the Hoffmann approach and allows for the analysis of a very large number of samples.

Although the Hoffmann approach is highly cited, few authors have applied the Hoffmann method in their calculations. One notable exception is the study led by Steven Soldin at the Children's National Medical Center in Washington, DC, USA. Reference intervals were calculated using hospital-based data collected from patients from 1 day to 18 years old. This group reported reference intervals for several analytes including fertility hormones, thyroid hormones, adrenal hormones, and growth hormones [14]. As expected, age and sex-specific differences were identified for FSH, LH, estradiol, 17-hydroxyprogesterone, DHEA and testosterone. Sex-specific differences were also found for serum iron, homocysteine, IGF-1 and IgE levels. Lastly, age-related differences were found for free T4, TSH and 25-hydroxy vitamin D levels. Levels of 25-hydroxy vitamin D showed seasonal variation, with higher levels found in the summer months. This work, however, had some limitations. Much of the data was collected on a small number of Caucasian hospitalized patients and may not accurately reflect levels in a healthy multicultural population. Also, semimanual application of the Hoffman analysis of data, added subjectivity to the calculations.

To determine the accuracy of the Hoffmann method, Katayev *et al.* applied a computerized indirect Hoffmann approach to retrospectively determine reference intervals for hemoglobin, creatinine, calcium and thyroid stimulating hormone (TSH) and compared to previously published reference intervals [15]. Their method calculated cumulative frequencies of each test result and performed least squares analysis, applying a best-fit equation to the linear portion of the data. The statistical difference between the two methods was determined by calculating the reference change value (RCV), a factor representing within individual variation and analytical variation. They found that there was no statistically significant difference between the reference intervals calculated using the Hoffmann method and published reference intervals.

More recently, as part of the CALIPER initiative, Shaw *et al.* compared pediatric reference intervals calculated for 13 analytes (calcium, phosphate, iron, ALP, cholesterol, triglycerides, creatinine, direct bilirubin, total bilirubin, ALT, AST, albumin and magnesium) from hospital-based data using the Hoffmann approach (Figure 1) to reference intervals calculated by CALIPER [16]. They sought to determine; 1) whether the hospital-based reference intervals fell within the 90% confidence intervals calculated by CALIPER; 2) whether the hospital-based reference intervals fell within the RCV for each analyte; 3) by completing a reference interval validation study using reference samples from healthy children. None of the reference intervals calculated using the Hoffmann approach fell within the 90% confidence intervals calculated by CALIPER. When the RCV was used for comparison, only some of the Hoffmann calculated reference intervals fell within the RCV range calculated (creatinine 15 days–<1 year old and all phosphate partitions with the exception of 0–<14 days old). Given the wide biological variation in some analytes, it

Figure 1 Schematic of statistical approach to calculate reference intervals from hospital-based data based on the Hoffmann approach used in *Shaw et. al.* [16]



is not surprising that more of the Hoffmann-calculated reference intervals fell within the RCV than fell within the 90% confidence intervals calculated by CALIPER. Finally, all reference intervals were validated according to CLSI guidelines except for ALP (13–<15 year old male; 15–<17 year old male and female), creatinine (15–19 year old male), and iron (14–<19 year old female). Validation data for some age and/or sex partitions was not performed due to insufficient numbers. In this study, reference intervals calculated using the modified Hoffmann approach, were much wider than those calculated by CALIPER, suggesting that the use of hospital-based data may be limited in pediatrics, especially from a tertiary care center.

The main limitations of the Hoffmann approach are based on the assumptions made for the analysis. The method assumes that majority of measurements made in the hospital represent normal individuals, yet when using hospital-based data, the true proportion of unhealthy individuals included in the data set is unknown [17]. This is especially problematic when determining reference intervals for low volume tests that include a selective patient population, as the fewer available samples would likely skew the data due to the inclusion of unhealthy subjects [18]. Additionally, this approach assumes that the values obtained for a specific analyte follows a Gaussian distribution of healthy results which may not be true in all cases [13].

NATIONAL AND GLOBAL PEDIATRIC REFERENCE INTERVAL INITIATIVES BASED ON HEALTHY COHORTS

In contrast to a hospitalized patient population, the ideal reference population to establish population-based reference intervals is a group of well-defined individuals that are similar to the target patient in all respects other than the disease condition under investigation. Ultimately, the quality of a laboratory measurement depends on the quality of the reference interval that the value is compared with. In turn, the quality of reference intervals heavily depends on the selection and recruitment of a large number of appropriate reference individuals [2,19]. Although this is a challenging task, several national and international initiatives have recognized the critical gaps in pediatric reference intervals and the need to establish ranges that are robust and appropriate for the pediatric population (Table 1) [20,21].

The German Health Interview and Examination Survey for Children and Adolescents (KiGGS), performed by the Robert Koch Institute (RKI), is a national initiative aimed at providing information on several health aspects of German children and adolescents [22,23]. KiGGS has successfully determined reference intervals for numerous serum and urine laboratory biomarkers using healthy pediatric samples [20,23]. The baseline KiGGS study collected whole blood, serum and urine samples in addition to personal information (i.e. age, sex, socioeconomic status, geographical location, community population, and immigration status) from 17,641 pediatric subjects (aged 0-17 years) recruited from May 2003 to May 2006 [24]. Three main categories of analytes were examined depending on their relation to one of the following public health interests: nutrition, risk of non-communicable disease and immunization. 43 analytes were analyzed, for some of which median and 90% reference intervals were calculated, such as total, LDL and HDL cholesterol, triglycerides, and calcidiol, based on age, sex and other subjective information [24]. In an additional KiGGS study [25], the median, 25th and 75th percentile values for serum thyroid hormones and serum lipids were determined in 12,756 subjects ≥3 years of age. This study also examined biomarker relationships and identified a positive relationship between TSH and serum lipid biomarker

concentrations, except for HDL, even after adjusting for smoking status, age and sex [25].

The Nordic Reference Interval Project (NORIP) was established in 1998 to determine Nordic-specific reference intervals for common blood analytes [26,27]. Some of the general inclusion criteria included feeling healthy, aged ≥ 18 years, and individuals who are not pregnant, ill or hospitalized. Personal information obtained from subjects included age, sex, body mass index (BMI), cigarette and alcohol usage, ethnic origin and physical activity [28]. Serum, plasma and whole blood samples were obtained from 3,036 healthy adults (aged ≥ 18 years) in 102 Nordic laboratories in Denmark, Finland, Iceland, Norway and Sweden [21,26]. Reference intervals (central 95%) for 25 common serum biomarkers, including enzymes, were calculated based on IFCC recommendations, with 90% confidence intervals around upper and lower limits. Data were also partitioned based on age, sex and blood sample (plasma or serum). Interestingly, a 2013 study analyzed 21 blood biomarkers based on 1421 (596 males and 825 females) healthy Danish pediatric subjects between ages 5-20 who participated in The COPENHAGEN Puberty Study (from 2006-2008) [29], and the results were compared to the NORIP (adult) findings. This study used nonparametric statistics to calculate the central 95% RIs, and confidence intervals of upper and lower limits; the RIs were calculated for both sexes and 6 age groups. This paper compared the results from the oldest pediatric age group to the youngest NORIP adult results [29]. Although many of the results were similar, there were some differences between the two studies, some values being higher and others being lower in the pediatric study compared to the NORIP adult study. However, discrepancy in some of these analytes, such as alkaline phosphatase, lactate dehydrogenase and creatinine, was explained to be normal as these values are expected to increase or decrease by age (from adolescence to adulthood).

Children's Health Improvement through Laboratory Diagnostics (CHILDx) program has been establishing pediatric RIs based on a healthy cohort (6 months to 17 years) in Utah, United States since 2002 [30-32]. This group has determined RIs for a variety of analytes, such as vitamins [33], enzymes [32], hormones [34], coagulation tests [30] and bone markers [35]. A 2005 CHILDx paper describes reference intervals for seven common coagulation tests that were determined based on 902 healthy pediatric participants with ages ranging from 7 to 17 years [30]. Median, 95% reference intervals and 90% confidence intervals around upper and lower limits were determined for each parameter. The results were reported based on 3-year age groups. Another CHILDx study in 2011 focused on analyzing the serum levels of seven analytes, consisting of enzymes, prealbumin and uric acid, based on a healthy cohort of 1765 children and adolescents [32]. Participants were divided into 3-year age groups (except for the 6m-2y age group), and mean, median, and statistical difference between age and sex groups were determined. Subsequently, central 95% age-specific and sex-specific (sex differences for about one-third of analytes) RIs were determined using nonparametric statistics.

United States National Health and Nutrition Examination Survey (NHANES) evaluates the health status of pediatrics and adults in the U.S. population by receiving laboratory and interview details from their cohort, while recruiting thousands of additional participants each year [36-41]. The NHANES study examined the effects of age, sex, BMI, socioeconomic status and ethnicity on various health parameters, including biomarkers. For example, a study published in 2000, looked at the upper 95th percentile limit of C-reactive protein (CRP) concentrations in a sample size of more than 22,000 healthy

pediatric and adult individuals (from NHANES III) based on age, sex and ethnicity [42]. Females generally had a higher concentration compared to male counterparts. They also demonstrated that Caucasians and Hispanics have similar upper limit CRP values, compared to black adults. CRP levels were also higher in older adults compared to children. Furthermore, in 2004, NHANES III data were used to determine reference intervals for whole blood count based on approximately healthy 25,000 subjects ranging from pediatric to geriatric age groups (aged 10 to >75 years), partitioned based on age, sex and ethnicity (Mexican, white and black) [40]. Age-, sex- and/or ethnic-related differences were observed in some of the analytes. In 2012, NHANES data from 6062 healthy pediatric (ages 2-19 years) individuals were used to establish RIs for the same 3 ethnic groups (Mexican, white and black) [43]. In addition, 95% RIs, geometric means and statistical difference between the ethnic groups were determined for vitamins and lipids (total, LDL- and HDL-cholesterol). RIs were partitioned based on sex, age and other factors; this is interesting as it allowed for both genetic and environmental comparisons between the three ethnic groups. Overall, NHANES studies, along with other studies, highlight the importance of partitioning reference intervals based on sex, age and ethnicity since reference values are highly influenced by these factors [40,43,44].

The Lifestyle of Our Kids (LOOK) program is a longitudinal study that was initiated in Australia in order to study healthy children and adolescents, and the effects of physical activity on their health outcomes [45]. In one of the most significant studies done by this group, central 95% RIs and medians of 37 blood analytes were calculated from a sample of 852 healthy individuals [45]. Sex-specific as well as age-specific RIs were calculated for ages 8, 10 and 12 based on measurements in 2005, 2007 and 2009, respectively. Interestingly, they compared their results with those from other groups, including CALIPER RIs for ferritin, CRP, cholesterol, TSH and magnesium. In a 2012 paper, LOOK data from 854 pediatric individuals was used to calculate blood NT-proBNP RIs [46]. Median, 95% RIs and 90% confidence intervals were calculated based on sex (male, female and combined) and for the same 3 age groups (ages 8, 10 and 12). In addition, pairwise comparisons in concentrations between the sex groups and the 3 age groups were conducted with the use of Mann–Whitney U test. While no significant difference was observed between the sex groups of the same age, concentration differences were shown to be statistically significant between certain age groups.

Australasian Association of Clinical Biochemists (AACB) Committee for Common Reference Intervals and AACB Harmonisation Committee have been promoting the adaptation of common reference intervals, based on the values used in hospitals in Australia and New Zealand [47,48]. In a 2014 preliminary publication, reference intervals used by each laboratory, along with results of freshly obtained serum for various analytes, were reported by each of the 123 laboratories, partitioning analytes by sex when applicable [48]. This was used to identify differences in reference intervals used by each laboratory and analytical methods between the laboratories. Interestingly, it was demonstrated that for majority of analytes, reference interval variation was greater than analytical (i.e., measured sample) variation between the laboratories. Linear regression was also used to compare measured results against reported upper and lower reference limits reported by each laboratory [48]. Finally, the AACB group determined the location of the measured sample's value with regards to each laboratory's reference range, determining its relative location from upper and lower limits [48]; the

results were then compared between the laboratories. In another study published within the same year, harmonized 95% reference intervals were determined for 11 analytes in adults and 9 in pediatrics based on healthy subjects, while partitioning further based on age in pediatrics and sex in both pediatrics and adults (aged ≥ 18 years), where applicable [47]. Throughout the common reference interval selection process, many workshops and validation processes were held to identify analytical accuracy and biases, consider clinical importance, and compare the results to other studies (e.g. NORIP and Aussie Normals). Similar to other studies, this study highlights the importance of considering between-instrument/analytical method differences and the significance of local validation [44,49-51].

THE CANADIAN LABORATORY INITIATIVE ON PEDIATRIC REFERENCE INTERVALS (CALIPER) PROJECT

The Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER), is aimed at creating a comprehensive database of pediatric reference intervals to disseminate for use by other pediatric centres nationally and globally. This initiative was launched through the Pediatric Focus Group of the Canadian Society of Clinical Chemists (CSCC) and is a collaborative effort amongst pediatric institutions across Canada. CALIPER is an *a priori* prospective study that has been recruiting thousands of healthy community children and adolescents and establishing age- and sex-specific reference intervals for many routine and specialized biochemical markers.

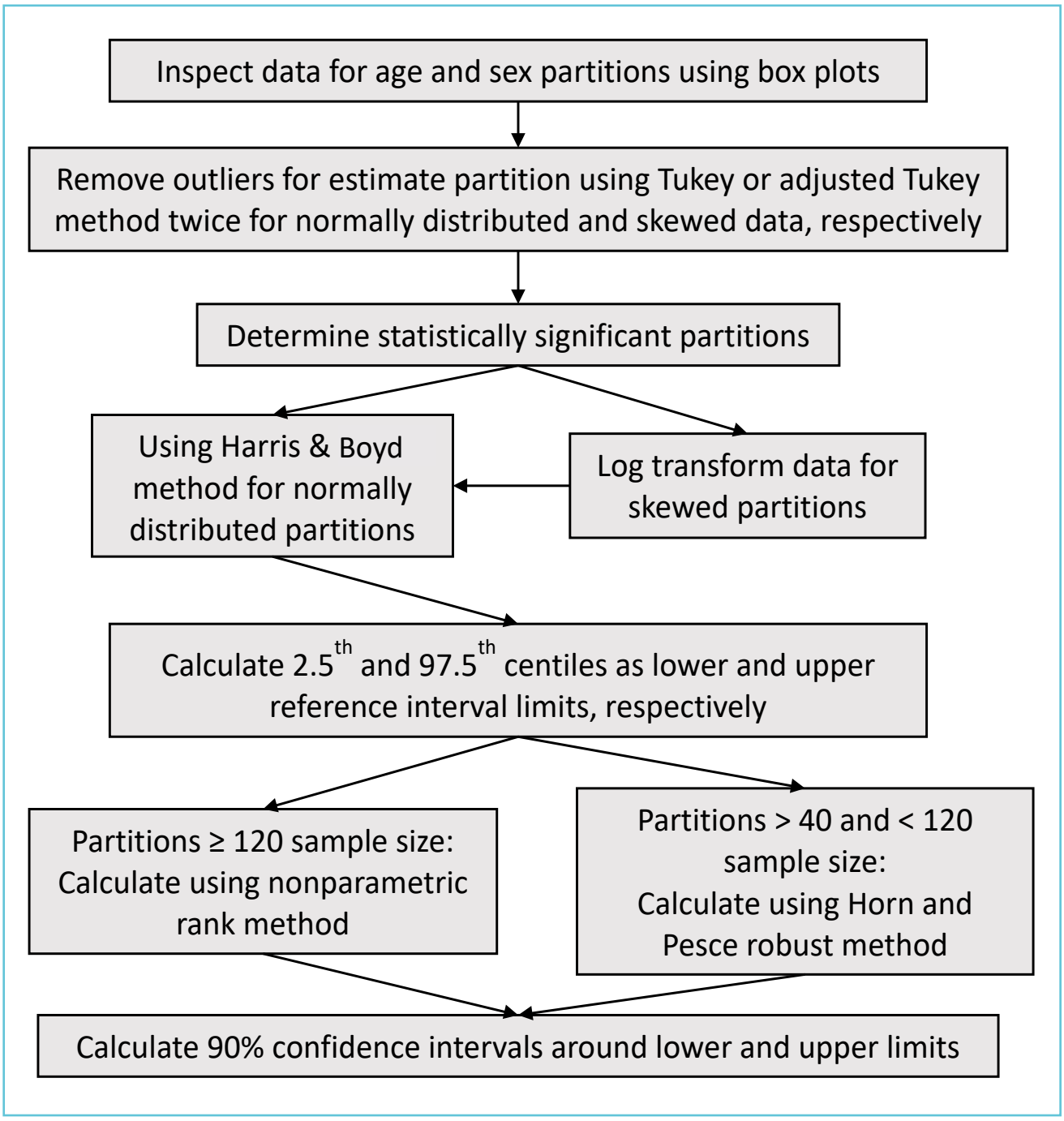
The early stages of CALIPER involved extensive planning to establish standardized procedures for pre-analytical, analytical and post-analytical aspects of the project. For example, blood collection, sample and statistical analysis were standardized to ensure consistency among different collection sites. A CALIPER team consisting of experienced research coordinators, project coordinators and volunteers was formed to help with promotion of the CALIPER campaign and recruitment of participants. Trained phlebotomists with expertise in pediatric sampling were also recruited to ensure ease and efficiency of collection.

Initially, preliminary pilot studies were conducted by CALIPER to refine project logistics and gain experience in sample collection, sample analysis, as well as statistical analysis and establishment of reference intervals. The initial CALIPER pilot studies included 2,809 serum and plasma specimens from apparently healthy and metabolically stable children from outpatient clinics. Overall, these initial studies analyzed over 50 chemistry and immunoassay biomarkers on the Abbott ARCHITECT ci8200 analyzer [52]. Using these data, CALIPER generated preliminary reference intervals according to CLSI and IFCC C28-A3 guidelines. Age and sex-specific reference intervals were established for five age groups. This was an important first step for CALIPER and formed the basis for more projects to come. However, as outlined by CLSI/IFCC C28-A3 guidelines, establishment of reference intervals should include recruitment of at least 120 healthy individuals per partition. Initial CALIPER pilot studies recruited apparently healthy children from outpatient clinics. According to CLSI guidelines, this was less than ideal since underlying disease in children from outpatient clinics could confound interpretation and establishment of reference intervals. Furthermore, a sufficient sample size of 120 patients per partition was not feasible in the initial CALIPER preliminary studies.

To address this limitation, subsequent CALIPER reference interval studies recruited healthy children and adolescents from Toronto and the Greater Toronto Area to establish a biobank

of healthy serum samples. With the help of the CALIPER team, over 9000 serum samples were collected from healthy children and adolescents allowing for appropriate sample size of 120 participants per partition, as per CLSI guidelines (Figure 2). Furthermore, to ensure adherence to CLSI guidelines and only inclusion of healthy participants, recruitment was limited to those without a history of chronic illness or acute illness within the previous month and without current

Figure 2 **Schematic of general statistical approach to calculate reference intervals in CALIPER study based on a healthy reference population [53,55]**

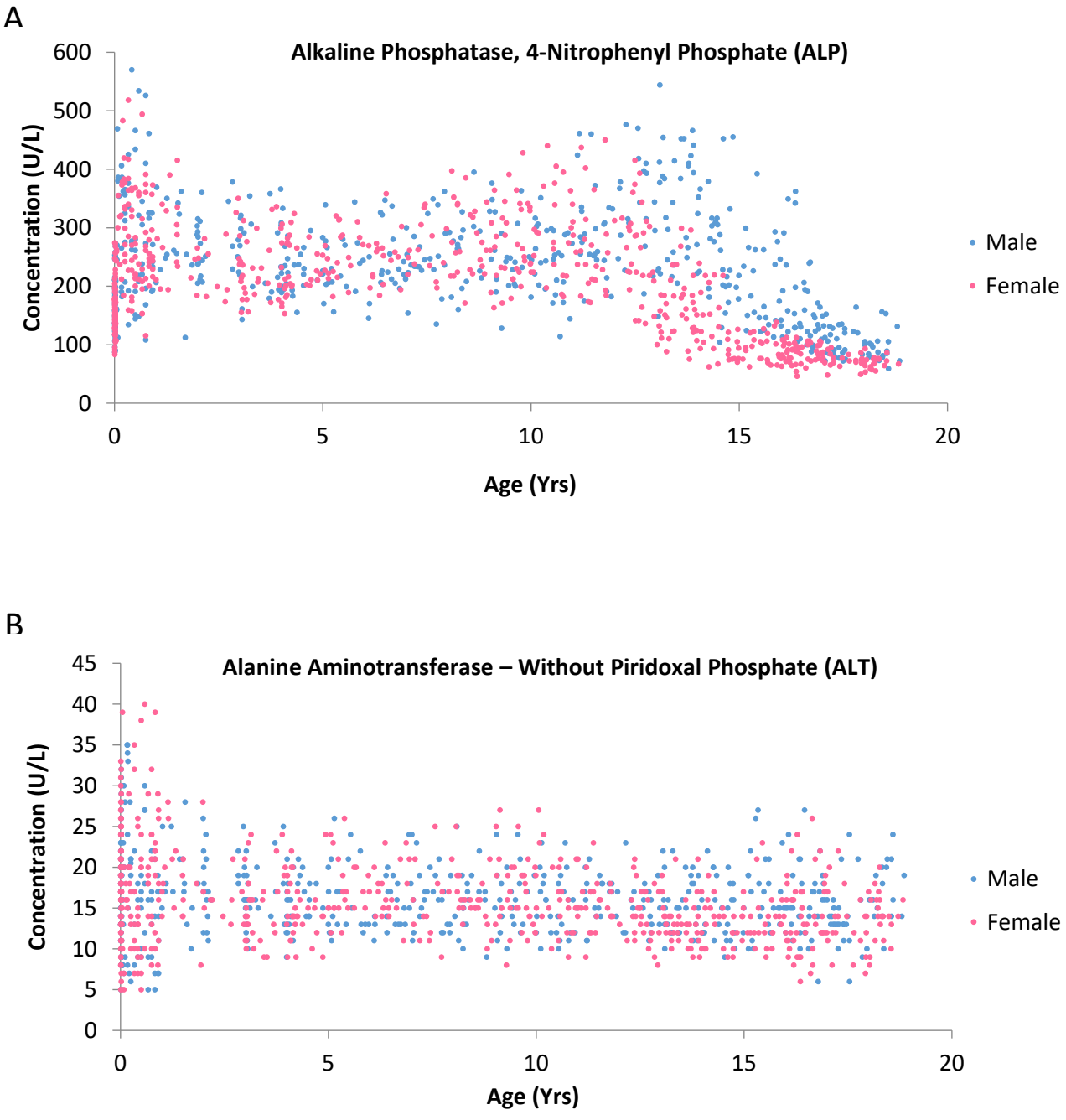


use of prescribed medication. Diverse collection sites, including community centres, day cares, churches and schools, further strengthened the CALIPER study and ensured that recruited participants accurately reflected the Canadian diversity. The first of these *a priori* studies, included the establishment of age-specific reference intervals for over 40 routine chemistry markers analyzed on the Abbott ARCHITECT c8000 system [53]. This hallmark study was the first of many CALIPER studies to begin to fill the gap in pediatric reference intervals for biomarkers routinely assessed in the pediatric population, including bone markers, markers of cardiovascular disease risk and metabolic syndrome. Findings from this study indicated that many analytes required multiple age partitions and the number of age partitions varied for each analyte. Oftentimes, developmental milestones are used to arbitrarily set age partitions. However, this study demonstrated that age partitions did not necessarily correlate with age developmental milestones, which was a paradigm shift from what was currently practiced. For example, alkaline phosphatase (ALP) required seven age partitions (0-14 days, 15 days - <1 yr, 1 - <10 yr, 10 - <13 yr, 13 - <15 yr, 15 - <17 yr, and 17 - <19 yr), as shown in Figure 3A, whereas alanine aminotransferase (ALT) required three age partitions (0 - < 1 yr, 1 - <13 yr, 13 - <19 yr), as shown in Figure 3B [53]. This study also performed preliminary analysis of differences between the major Canadian ethnic groups (i.e. Caucasian, South Asian, and East Asian). This analysis demonstrated biomarker concentration differences between ethnic groups for ALT, amylase, IgG, IgM, magnesium, total protein and transferrin [53]. While these three ethnic groups comprise a large proportion of Canada, future CALIPER studies will focus on expanding the analysis to other ethnicities.

As children grow and undergo pubertal development, there are significant changes in fertility hormone concentration. In 2013, CALIPER recruited healthy children and adolescents and measured 7 fertility hormones [54]. Age-specific reference intervals were required for all fertility hormones, and aside from prolactin, sex partitions were also required. This study also determined Tanner stage-specific reference intervals. Tanner staging is used to monitor progress of puberty in children. It is especially important to have Tanner stage-specific reference intervals for fertility hormones since every child enters puberty at various ages. Tanner staging is based on a 5-stage scale, with stage I correlating with pre-pubertal development and stage V correlating with adult development. Tanner staging was determined by providing participants with images of Tanner stages I to V and participants self-assessed their development relative to the diagram. Shortly following the examination of fertility hormones, pediatric reference intervals were established for additional endocrine and biochemical markers on the Abbott analyzer [55].

With the success of the initial CALIPER studies, the CALIPER initiative extended their studies to more specialized testing and moved beyond general automated analyzers. Age-specific steroid hormone reference intervals were completed on the AB SCIEX 4000 QTRAP mass spectrometer [56], HPLC analysis aided in establishment of vitamin A and vitamin E pediatric reference intervals [57], and 25-hydroxyvitamin D reference intervals were determined using LC-MS/MS analysis [58]. The 25-hydroxyvitamin D study also demonstrated elevated concentrations of 25-hydroxyvitamin D C3 epimer (C3-epi-25-OH-D_2 and C3-epi-25-OH-D_3) in neonates less than 1 year of age, which could interfere with 25-OH vitamin D measurements in this pediatric population [58,59]. Thus, caution should be exercised in measurement of 25-hydroxyvitamin D in neonates.

Figure 3 Scatter plot demonstrating the serum concentrations of (A) alkaline phosphatase and (B) alanine aminotransferase over the pediatric age range



*Adapted from: Colantonio et. al. [53].

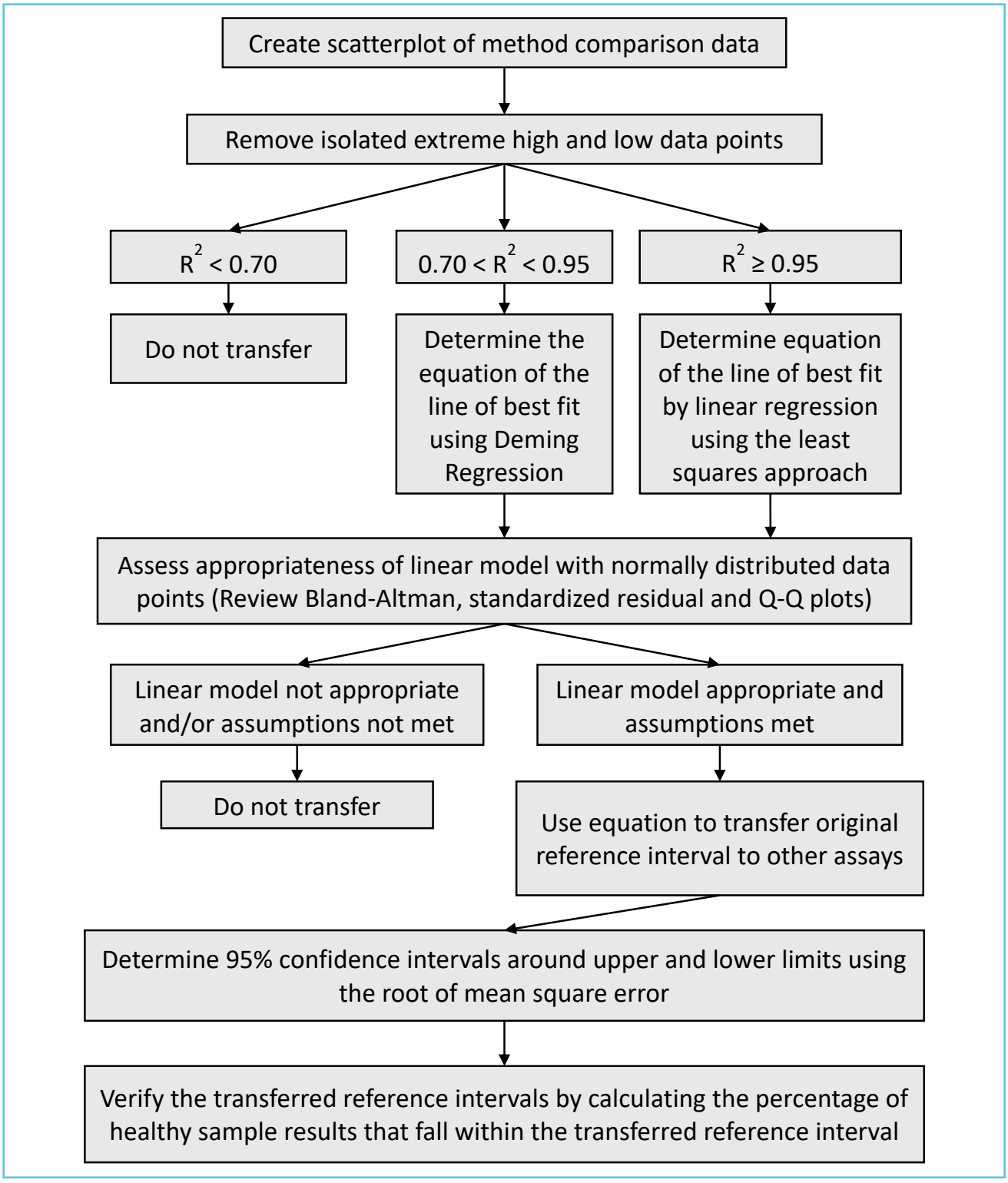
In addition to these large milestones, CALIPER allotted resources to smaller substudies. They have analyzed the effect of freezing conditions on samples and analyte stability [60], biological variation [61], and fasting on biomarker concentrations [62]. The stability of serum chemistry, protein and hormones were analyzed on three analyzers (Ortho Vitros Chemistry System, Roche Cobas Integra 400 Plus and Siemens Immulite 2500) using specimens that were frozen at -80°C [60]. The results of this study demonstrated that -80°C is a suitable storage method for serum samples since no significant deviations in analyte concentrations were observed. An understanding of biological variation is important for accurate laboratory test interpretation and for determining whether a change in biomarker concentration is clinically significant. This data is often lacking for many biomarkers, especially for the pediatric population, since it is often difficult to obtain sequential samples from one child. CALIPER analyzed the within and between-individual biological variation for the pediatric population. Four samples were obtained from 29 healthy participants, and over 30 analytes were analyzed to determine their biological variation [61].

To further expand the CALIPER database, recent studies have established age- and sex-specific reference intervals on the Abbott ARCHITECT immunoassays for cancer biomarkers [63], metabolic disease biomarkers [64], testosterone indices [65], and specialized biochemical markers [66]. To expand the CALIPER database to additional analyzers, a series of transference and verification studies have been performed to transfer reference intervals for chemistry analytes established on Abbott assays to assays on other analyzers including Beckman, Ortho, Roche, and Siemens [44,49-51]. The statistical algorithm CALIPER uses to transfer and verify reference intervals is summarized in Figure 4. To date, CALIPER has created a comprehensive and robust age- and sex-specific pediatric reference interval database for over 100 biomarkers. Through knowledge translation initiatives, CALIPER has also disseminated reference intervals through peer-reviewed publications, development of a free online database (www.caliperdatabase.com), and a smartphone application easily accessible for physicians.

LIMITATIONS OF PEDIATRIC REFERENCE INTERVALS AND FUTURE DIRECTIONS

Despite several advances in addressing the critical gaps in pediatric reference intervals, limitations of the completed and ongoing studies leave additional gaps to be addressed. Current initiatives have extensively examined how analyte concentrations vary with age and sex. However, the influence of additional covariates, such as ethnicity and body mass index (BMI), on analyte concentrations remain to be comprehensively analyzed. Several studies suggest that analyte concentrations may vary by ethnicity. For example, Gupta *et al.* discussed significant differences in serum prostate-specific antigen (PSA) concentration between various ethnicities, with relatively higher concentrations in African American subjects and lower concentrations in Asian subjects [67]. Therefore, ethnic specific PSA reference intervals should be considered in accurate interpretation of test results in cancer diagnosis. Another study by Troy *et al.* measured hematologic and immunologic reference intervals in healthy Zimbabwean infants and compared them to those established using mainly Caucasian subjects [68]. Interestingly, majority of Zimbabwean subjects were considered to have adverse events and immunodeficiency based on hemoglobin and CD4% reference intervals established using Caucasian subjects, respectively. Thus, ethnic-specific and locally-validated reference intervals are required for accurate laboratory test interpretation. The influence of ethnicity on the

Figure 4 Schematic of general statistical approach to transfer original reference intervals to other clinical chemistry assays



**Adapted from: Estey et. al. and Araujo et. al. [44,49].*

concentration of several analytes may be due to several factors including genetic differences, environmental factors, and dietary patterns. Several national pediatric reference interval initiatives have established reference intervals based on a reference population comprised of a single ethnicity [69-72]. As Canada is comprised of a multi-ethnic population, the reference population used in the CALIPER studies proportionally represents the main ethnic groups of the nation (i.e. Caucasian, South Asian, East Asian). CALIPER performed analysis on these ethnic groups, however, the relatively small sample size of ethnicities, other than Caucasian, limited the scope of this study to strictly preliminary analysis [53-55]. Large, comprehensive studies are warranted for a thorough understanding of how analyte concentrations differ between ethnic groups and where warranted the establishment of ethnic-specific reference intervals.

With pediatric obesity becoming an important public health concern, it is also important to understand how analyte concentrations change with BMI [73,74]. A reference population should be comprised of subjects who are representative of the local population, and therefore this definition becomes challenging when covariates such as BMI is constantly changing in the general population. As the average BMI in the general population increases, reference intervals for analytes that change with BMI may also be shifted, or a substantial subset of the local population must be excluded from the reference population. Therefore, it is important to understand which analytes are significantly influenced by BMI and if these changes are physiological and not of clinical significance, or if this change is clinically significant and may be indicative of subclinical progression of a metabolic disease. Understanding how analyte concentrations change with BMI are critical for laboratory specialists and physicians to interpret blood tests from overweight and obese pediatric patients. Some studies have been performed to examine the effect of BMI on analyte levels in a healthy population, however these studies only included adults [75] or were performed for a very limited number of analytes [76]. Further studies are needed to comprehensively examine the influence of BMI on analyte concentrations in the pediatric population.

The ultimate end goal of pediatric laboratory medicine is to achieve harmonization. Laboratory test interpretation, based on reference intervals and decision limits, remain highly variable and poorly harmonized across laboratories. This leads to great potential for inappropriate patient care when laboratory test results on the same sample can be interpreted differently depending on the reference interval reported by the laboratory. Several groups have launched initiatives to harmonize reference intervals including the NORIP [26], the UK Pathology Harmony project [77], the Australasian Harmonised Reference Intervals for Adults (AHRIA) and Australasian Reference Interval for Paediatrics (AHRIP) [47]. In Canada, a Working Group on reference interval harmonization has also been initiated to identify the variation in reference intervals being used in clinical practice and establish/recommend practice guidelines on the use of harmonized reference intervals in clinical laboratories across Canada.

Major challenges have been overcome and significant advances have been made in the establishment and wide-spread dissemination of accurately established pediatric reference intervals. However, with the continuously evolving technological and clinical advances, national and international research initiatives need to ensure pediatric reference intervals continuously improve and adapt to the changing environment.

Table 1 Major pediatric reference interval studies based on healthy children and adolescent populations

Study	Country	Age range (years)	Sex	Statistical method	Examples of groups of biomarkers studied	References
AACB	Australia and New Zealand	All age groups	Both	Central 95%	Common blood analytes (mostly ions and enzymes)	[47,48]
CALIPER	Canada	0-18	Both	Central 95%	Common biochemical markers Endocrine markers Tumor markers Vitamins Metabolic disease biomarkers Testosterone indices	[4,6,8, 18,44, 49-52, 54-57, 60-62, 66]
CHILDx	United States	0.5-17	Both	Median, mean and central 95%	Enzymes Coagulation tests Hormones Vitamins Bone markers	[30-35]
COPENHAGEN	Denmark	5-20	Both	Central 95%	Common blood analytes	[29]
KiGGS	Germany	0-18	Both	Median and central 90%	Nutrient deficiency markers Non-communicable diseases and lipids Immunology markers Thyroid hormones	[20, 22-25]
LOOK	Australia	8, 10 and 12	Both	Median and central 95%	Cardiac Biomarker Common blood analytes	[45,46]

NHANES	United States	All age groups	Both	2.5th, 25th, median, 75th, or 97.5th	Lipid profile Immunology and hematologic markers Vitamins Inflammatory markers	[36-43]
NORIP	Nordic Countries (Denmark, Finland, Iceland, Norway and Sweden)	≥ 18	Both	97.5 percentile or central 95%	Tumor markers Common blood analytes	[21, 26-28]

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

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In this issue: Recent Advances in Pediatric Laboratory Medicine

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The utility of CSF for the diagnosis of primary and secondary monoamine neurotransmitter deficiencies

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ABSTRACT

Biogenic amine defects constitute a complex and expanding group of neurotransmitter disorders affecting cognitive, motor and autonomic system development, mostly in the pediatric age. In recent years different enzymatic defects have been identified impairing the tetrahydrobiopterin cofactor pathway and/or biogenic amine synthesis, catabolism and transport, with subsequent new disease entities described. The lumbar puncture, with subsequent withdrawal of cerebrospinal fluid (CSF), remains a key step in the diagnostic procedure. Due to the specific nature of CSF, timing of analysis, sample collection and storage, technical issues of the analytic process are still crucial for the diagnosis and follow-up of patients. A progressive approach to the diagnosis of biogenic amine defects is presented, pointing out criticalities and difficulties concerning sample collection and results interpretation, especially due to the increasing reports of secondary neurotransmitter alterations that, at present, constitute a challenge.

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Abbreviations

CSF: *cerebrospinal fluid*
PNDs: *primary neurotransmitters diseases*
Phe: *phenylalanine*
AD-GTPCH1: *autosomal dominant guanosin triphosphate cyclohydrolase 1*
AR-GTPCH1: *autosomal recessive guanosin triphosphate cyclohydrolase 1*
PTPS: *6-pyruvoyltetrahydropterin synthase*
SR: *sepiapterin reductase*
PCD: *pterin-4a-carbinolamin dehydratase*
DHPR: *dihydropteridin reductase*
TH: *tyrosine hydroxylase*
TPH: *Tryptophan hydroxylase*
AADC: *aromatic L-aminoacid decarboxylase*
MAO: *monoamine oxidase*
DβH: *dopamine beta hydroxylase*
VMAT2: *vesicular monoamine transporter*
DAT: *dopamine transporter*
HVA: *homovanillic acid*
5-HIAA: *5-hydroxyindolacetic acid*
3-OMD: *3 orthomethyldihydroxyphenylalanine*
5-HTP: *5-hydroxytryptophan*
MHPG: *3-methoxy-4-hydroxyphenylglycol*
BH4: *tetrahydrobiopterin*
BH2: *dihydrobiopterin*
Neo: *neopterin*
Sep: *sepiapterin*
Prim: *primapterin*

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INTRODUCTION

Monoamine neurotransmitter defects are included in the group of neurometabolic syndromes attributable to disturbances of neurotransmitter metabolism/transport and cofactors (i.e. tetrahydrobiopterin) synthesis/regeneration. Biogenic amines, serotonin and all catecholamines (dopamine, epinephrine and norepinephrine) are major neurotransmitters within the central nervous system (CNS). Their regulation is tuned and governed by the rate of neurotransmitter (NT) synthesis, packaging, release, re-uptake, degradation, and by receptor status. Clinically they are mainly characterized by a range of extrapyramidal manifestations including dystonia, hyperkinesia, chorea and oculogyric crisis (1,2). These defects are characterized by an elevation of phenylalanine, the presence of abnormal monoamines and deranged monoamine synthesis, degradation and transport (3) (Figure 1). Recently, abnormal neurotransmitter profiles have been reported in association with other non-metabolic and genetic diseases, and defined as secondary neurotransmitter abnormalities (4,5) (Table 1). Biochemical distinction between the two groups is difficult due to a considerable overlap in the concentrations of HVA and 5HIAA. Hence, a careful analysis of the pattern of all metabolites may be necessary to reach the correct diagnosis. Recent discoveries emphasizing the role of NTs in brain development have led to the possibility of treating these defects. Therefore an early and accurate diagnosis of biogenic amine disorder is paramount to an efficient therapeutic intervention (7).

In this review, we provide an overview of primary neurotransmitter diseases (PNDs) by cerebrospinal fluid (CSF) investigations. Sample management, analytical methodology, and diagnostic interpretation are described.

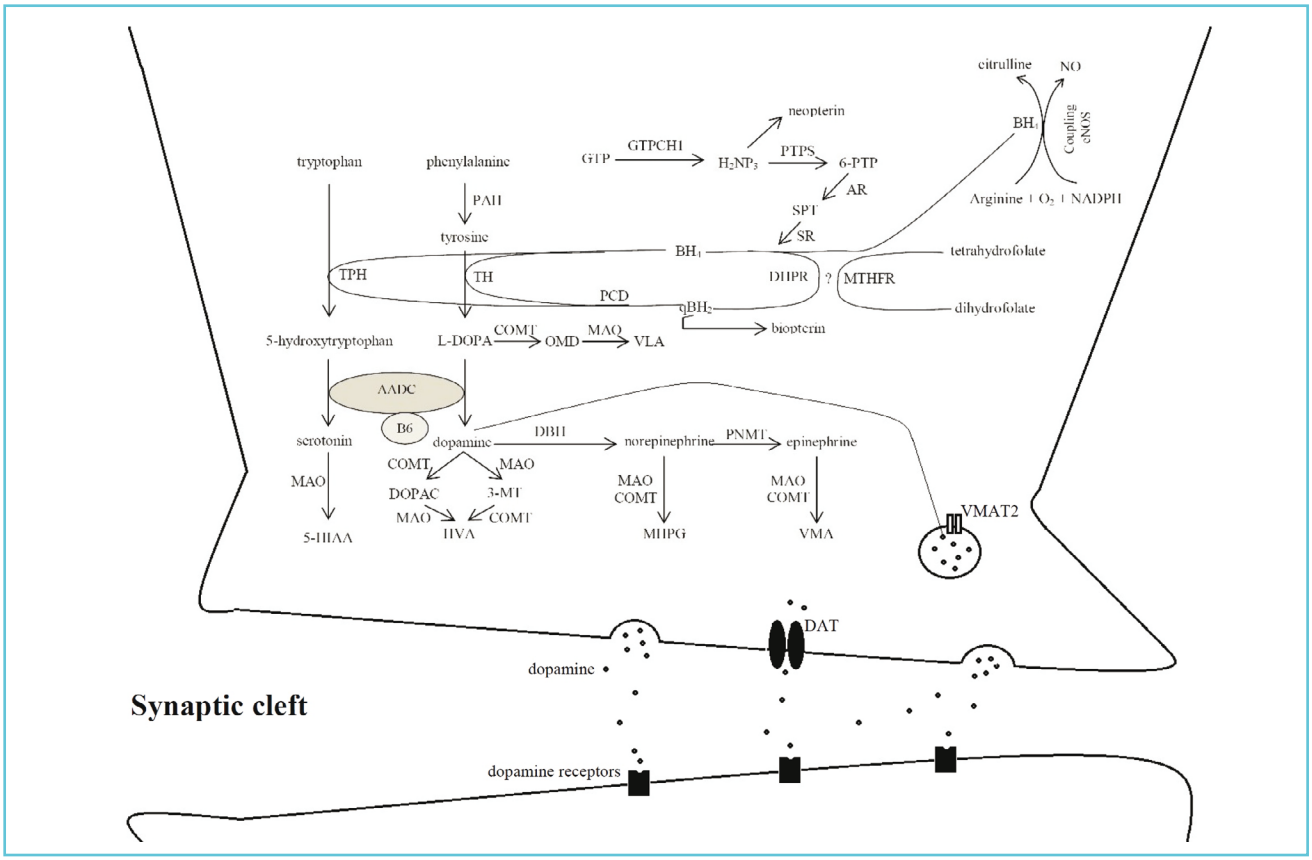
CLINICAL AND BIOCHEMICAL FEATURES OF MONOAMINE NEUROTRANSMITTER DISEASES

In all monoamine metabolic disorders, the clinical symptoms are strictly related to the effects of dopamine and serotonin deficiency. Signs of dopamine deficiency include Parkinsonism, dystonia, chorea, oculogyric crisis, ptosis, hypersalivation, and myoclonic epilepsy. The manifestations of serotonin deficiency are less well defined, and

include temperature instability, sweating, aggressive behaviour, irritability and sleeping disturbance. Non-specific symptoms include epileptic encephalopathy, mental retardation, microcephaly, swallowing difficulties, and pyramidal tract features mimicking cerebral palsy. A potential defect in biogenic amine should be considered in an infant presenting with any of the above symptoms, which may appear in isolation or together (8). Furthermore clinical monitoring of these patients could be challenging. Firstly, symptoms can fluctuate according to last medication with on-off effects. Secondly, high doses of L-Dopa inhibit postsynaptic receptors, resulting in symptoms (e.g., involuntary movements, dyskinesia, irritability, insomnia and opisthotonus) that are indistinguishable from the one resulting from under-treatment and/or dopamine deficiency.

Defect of tetrahydrobiopterin (BH_4) with consequent dopamine and serotonin deficiency are associated with an increased plasma phenylalanine (Phe) concentration (8). BH_4 acts as a cofactor or co-substrate in a range of biochemical reactions including the hydroxylation of aromatic amino acids (Phe, tyrosine and tryptophan) by the corresponding hydroxylases. *De novo* biosynthesis of BH_4 from GTP requires 3 enzymes (GTPCH, PTPS and SR), while dihydropteridine reductase (DHPR) regenerates BH_4 from q-dihydrobiopterin (qBH_2). Overall BH_4 synthesis, as such, is thus complex, and not completely understood as yet. In autosomal recessive and autosomal dominant GTP cyclohydrolase 1 (GTPCH1), 6-pyruvoyltetrahydropterin synthase (PTPS), and DHPR, the measurement of blood and CSF pterins facilitates the diagnosis. Noteworthy, all disorders

Figure 1 **Biochemical pathways involving dopamine, serotonin, epinephrine, norepinephrine, and the cofactor BH4**



Legend **Figure 1: Biochemical pathways (p. 66 above)**

The monoamines consist of catecholamines (for instance dopamine, norepinephrine and epinephrine) and serotonin. The amines are synthesized throughout a complex multienzymatic pathway which converts, tryptophan and tyrosine into serotonin and dopamine respectively, through reactions catalysed by tryptophan hydroxylase (TPH, EC 1.14.16.4), tyrosine hydroxylase (TH, EC 1.14.16.2) and aromatic L-aminoacid decarboxylase (AADC, EC 4.1.1.28). This latter enzyme acts as a common converging decarboxylating system for active neurotransmitter biosynthesis. In the case of AADC deficiency, the dopamine precursor (L-dihydroxyphenylalanine, DOPA) is metabolized into 3-Orthomethyldihydroxyphenylalanine (3-OMD) and vanillactic acid (VLA). Both TPH and TH require tetrahydrobiopterin (BH_4) as cofactor, while AADC needs vitamin B6 (pyridoxine). In noradrenergic neurons, dopamine is further converted by dopamine beta hydroxylase (DBH) into norepinephrine and epinephrine by phenylethanolamine N-methyltransferase (PNMT). Since BH_4 is crucial in serotonin and dopamine biosynthesis, a large subset of monoamine defects is to be referred to pterins build up and regeneration mostly presenting hyperphenylalaninemia as a characterizing hallmark. The biosynthesis and regeneration of BH4 is carried out by a complex system of enzymes starting from guanosine triphosphate cyclohydrolase 1 (GTPCH1), the rate limiting enzyme for BH4 biosynthesis, which is responsible for the hydrolysis of guanosine triphosphate into 7,8-dihydroneopterin triphosphate (H_2NP_3), thus releasing neopterin. H_2NP_3 is further metabolized into 6-pyruvoyltetrahydropterin (6-PTP) by 6-pyruvoyltetrahydropterin synthase (PTPS), the second critical enzyme for BH4 build up. 6-PTP is used to form BH_4 through sepiapterin (SPT) by a two step enzymatic pathway of aldose reductase (AR) and sepiapterin reductase (SR). BH4 is the basic cofactor of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) and once it links with this enzyme it is then released as tetrahydrobiopterin-4a-carbinolamine. Tetrahydrobiopterin-4a-carbinolamine is used to recycle BH_4 through the biosynthesis of quinonoid-dihydrobiopterin (qBH2) by pterin-4a-carbinolamin dehydratase (PCD) and dihydropteridin reductase (DHPR), which is also linked to folate metabolism via methylenetetrahydrofolate reductase (MTHFR) through an incompletely defined mechanism. This two step enzymatic regeneration (through PCD and DHPR) can be partly enzymatic partly not. BH4 is also related to nitric oxide (NO) metabolism through arginine, cytrulline and nicotinamide adenine dinucleotide phosphate (NADPH). This finding may explain the effect of BH4 in vasogenic control. The catabolism of the monoamine is then carried out by a two step pathway involving monoamine oxidase (MAO) and cathecol-O-methyltransferase (COMT) and major metabolites are represented by homovanillic acid (HVA), 5-hydroxyindolacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) and also vanillylmandelic acid (VMA). The three major end products are measured in the CSF reflecting overall dopaminergic, serotoninergic and noradrenergic activity. The process of monoamine neurotransmission requires, in summary, the biosynthesis of monoamines in the nerve terminal, their upload in the synaptic vesicles through the vesicular monoamine transporter (VMAT2) with the subsequent excytotic release, action at specific receptors in the postsynaptic interface and the termination of the effect either by degradation or by reuptake by dopamine transporter (DAT).

that present hyperphenylalaninemia are diagnosed by neonatal screening.

If all metabolic investigations are normal in plasma and urine and the patient is suggestive for a possible PNDs, a CSF puncture should be performed for determination of HVA and 5HIAA (9). These metabolites allow the diagnosis of the following defects: tyrosine hydroxylase (TH), aromatic L-aminoacid decarboxylase(AADC), sepiapterine reductase (SR), dopamine transport deficiency including β-hydroxylase deficiencies(DβH) and vesicular monoamine transporters defects (VMAT2).

MONOAMINE NEUROTRANSMITTER ANALYSIS: KEY FEATURES

Analysis of the CSF neurotransmitter metabolites includes the measurement of homovanillic acid (HVA), 5-hydroxyindolacetic acid (5-HIAA) and their ratio. The concentrations of 3-O-methyldopa (3-OMD), L-3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPA-C), 5-hydroxytryptophan (5-HTP), and 3-methoxy-4-hydroxyphenylglycol (MHPG) may be highly variable due to possible drug interactions.

Table 1 Acquired and genetic neurological diseases with abnormal biogenic amine values (data from literature and cases presentation)

Low HVA and low 5-HIAA
epileptic encephalopathies (SCN2A, SCN8A), Rett syndrome (FOXG1), organic acidurias (ACSF3), disorders of cholesterol synthesis (Smith-Lemli-Opitz Syndrome), brain tumours, leukemia (del6q21), perinatal hypoxia/ischaemia, preterm haemorrhagic injuries, thiamine metabolism disorders (SLC19A3), oligosaccharidoses, diseases of copper metabolism (Occipital Horn Syndrome), lysosomal disorders (Niemann-Pick type C), Lesch-Nyhan syndrome, pontocerebellar hypoplasia type 2, Steinert disease, stroke, dysautonomia, leukodystrophies, eye disorders (PITX3), acute necrotizing encephalopathy (RanBP2), intellectual disability (CASK), mitochondrial diseases (SDH), lysinuric protein intolerance (SLC7A2)
Low or normal HVA and normal 5-HIAA
Aicardi-Goutiéres syndrome (ADAR1, RNASEH2A, RNASEH2B), pontocerebellar hypoplasia (i.e. EXOSC3), seizure/epileptic encephalopathies (KCNQ2), chromosomal abnormalities (dup17p13.3), organic acidurias (ACSF3), alternating hemiplegia (ATP1A3), folate metabolism disorders (FOLR1, MTHFR, MTHFD1), mitochondrial diseases (NFU1, POLG, KSS, congenital myopathies (MTM1), , meningitis/encephalitis, malformative syndromes, astrocytoma
Normal HVA and low 5-HIAA
Hartnup disease (SLC6A19), chromosomal abnormalities (del8p23, tris12p23), post vaccine
High HVA high 5-HIAA
mitochondrial diseases (POLG)

SAMPLE COLLECTION AND STORAGE OF CSF

The measurement of biogenic amines can be misleading and hard to interpret if sample collection and handling do not follow strict procedures (10). There are several factors that can affect metabolite concentration, some playing a minor effect that are difficult to monitor, while others require careful and detailed instructions to minimize their impact. In should be mentioned that there is a rostrocaudal concentration gradient of monoamines in CSF. Therefore, CSF withdrawal should always be performed at the same spinal level (usually from a lumbar spinal tap). The first 0.5 mL aliquot is used for biogenic amine metabolite; the second can be stored at -70°C for further future analysis; the third 1 mL is either immediately frozen with dry ice at the bedside or filled with an antioxidant mixture ensuring BH_4 stability to measure pterins. The fourth 1 mL aliquot is used as a back-up sample. Finally, as blood contamination of CSF can cause a rapid metabolite oxidation (degradation of HVA and 5HIAA), the contaminated samples must be rapidly centrifuged and the supernatant transferred in new tubes before freezing. Therefore red blood cell count and proteins should always be measured, to exclude blood-brain barrier damage, and found normal for proper interpretation.

ANALYTICAL METHODS

The biogenic amine metabolites are tested using several different techniques including capillary electrophoresis (CE), GC-MS, HPLC with electrochemical (EC) detection or fluorescent detection (FD) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (11-17).

HPLC with Electrochemical detection (HPLC-EC)

HPLC-EC has been considered the gold standard for biogenic amine metabolite analysis because of its relatively low costs, and technical feasibility and simplicity, demonstrated in many procedures in which CSF was directly injected into the HPLC system without prior derivatization of the sample.

Chromatographic separation of analytes is in general performed by reverse-phase column in an isocratic condition. The elution order of biogenic amines is in order of increasing hydrophobicity. Acidic pH and ion pairing modifiers are commonly used in the mobile phase in order to suppress the positive charge of the catechol ring and optimize interaction with the stationary phase. Adapting the percentage of organic solvent (acetonitrile or methanol) retention times can be changed to obtain an accurate separation of analytes from other compound contents in CSF that can affect an accurate measurement. This is important especially for MPHG, DOPAC and DOPA that, as compare to HVA or 5-HIAA, are present in small amounts.

Since then, HPLC with electrochemical and/or fluorescence analysis has represented the gold standard in biogenic amine metabolite analysis and in defining biogenic amine metabolites age-related reference values. The fluorimetric method allows rapid sample preparation and the simultaneous determination of up to 7 biogenic amine metabolites in CSF. HPLC has also been largely tested in series of biological samples proving to be highly sensitive despite different concentration levels of biogenic amine metabolites in CSF. Nevertheless some limitations occur concerning the inability to pinpoint the interference of closely eluting metabolites regardless of the use of ion-pairing reagents to improve retention.

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

In the past ten years the application of LC-MS/MS has increased in routine clinical chemistry and inborn error of metabolism diagnostic laboratories. Detection by LC-MS/MS is based on structural characterization of small molecules. The application of this technique to biogenic amines overcomes the requirement of a highly resolved column separation necessary for other types of detectors (i.e. EC). Several LC-MS/MS methods have been recently introduced and standardized for the analysis of biogenic amine metabolites in bodily fluids (11-17). The high sensitivity acquired through positive ESI LC-MS/MS overcome the need of derivatization while improved separation and chromatographic resolution permit unequivocal identification of closely related compounds in a short time run, which is still a limit in HPLC analysis. The possibility to quantitate BH4, BH2, sepiapterin and neopterin simultaneously and the ability to detect conjugated neurotransmitters (such as glucuronides and sulfates) represents on one hand a clear advantage in differentiating pterin defects (most of all for DHPR and SR deficiency) and on the other hand the ability to speculate the role of conjugates in monoamine metabolism and brain neurotransmission.

QUALITY CONTROL

Since 2016, an established External Quality Control (EQA) scheme by ERNDIM is available that includes HVA, 5-HIAA, 3OMD and 5-HTP (http://www.erndim.org/home/start.asp). The scheme evaluates the quantitative performance and

assesses the ability of laboratories to diagnose inborn errors of neurotransmitter metabolism. The scheme consists of 8 spiked samples, paired two by two, of lyophilised pooled CSF (4 samples) and lyophilised artificial CSF (4 samples). Accuracy, recovery, precision, linearity and inter-laboratory CV are evaluated by ERNDIM. A high degree of harmonization between laboratories is important for the use of common reference values.

During the two-year pilot study period (2014-2015), a general agreement was observed in 5HIAA and HVA concentrations while a significant discrepancy was detected concerning 3OMD and 5HTP between different laboratories. Overall there was improvement of laboratory performance during these two years.

For internal quality control, commercial quality control samples are not available, but they can be easily prepared in-house. Aliquots of pooled "positive controls" CSF or spiked CSF with biogenic amine standards can be stored at -70°C and used to monitor the performance of the method (19).

AGE-RELATED REFERENCE RANGE

Despite precise internal collecting protocols, the recent increase in CSF biogenic amines measurement for the diagnosis of neurologically compromised children has resulted in challenging monoamine profiles difficult to interpret. As such, a critical role in the reliability of CSF biogenic amines interpretation is represented by the issue of reference values that can help to recognize misleading alterations or milder/moderate forms of deviations outside primary biogenic amine defects. Age-related reference ranges are important for differential diagnosis, management and monitoring (see Table 2) (20).

Biogenic amines have been tested, to build up reference range values, both in CSF of healthy neonates and infants, and in mixed samples of healthy infants and infants with medical complications, and, most recently, in series of patients with several neurologic diseases. Metabolite concentration shows a decrement with age over the first few years, with peak values found during the first three months and with concentrations reaching a

Table 2 CSF HVA and 5HIAA age specific reference ranges. Reference ranges are established by our laboratory based on data from 100 individuals from our geographical area

Age	HVA (nmol/L)	5HIAA (nmol/L)
0 - 30 d	601 - 1397	382 - 949
1 m – 5 m	345 - 1111	206 - 922
6 m – 1 yr	302 - 797	120 - 345
2 yrs – 4 yrs	242 - 684	95 - 329
5 yrs – 10 yrs	130 - 573	80 - 183
11 yrs – 16 yrs	122 - 515	68 - 187
> 16 yrs	111 - 371	55 - 163

sort of plateau around five years of age. No correlation has been found between length of the child and age, i.e., for craniocaudal gradient (20). In adults a seasonal variation has been anecdotally described, but never confirmed in children. Reference intervals are critical. The available reference intervals in the literature are quite similar, but the differences may be related to the age of patients. This reinforces the concept that establishment of reference intervals specific to laboratories that perform neurotransmitter analysis is mandatory (18).

DIAGNOSTIC PATTERNS OF CSF MONOAMINE NEUROTRANSMITTER DISEASES

Table 3a summarizes the biochemical features of each monoamine neurotransmitter disorder with and without plasma hyperPhe. The biochemical classification of the disease is based on plasma phenylalanine as reported in the introduction section. In GTPCH1, DHPR and PTPS the determination of HVA and 5HIAA are reduced and pterin is abnormal. In SR, CSF analysis reveals decreased levels of HVA and 5-HIAA, normal to slightly

Table 3a Disorders of pterin metabolism with and without hyperphenylalaninemia

	HVA	5-HIAA	3-OMD	5-HTP	MHPG	MTHF	BH4	BH2	Neo	Sep	Prim (U)
with hyperphenylalaninemia											
AR-GTPCH1	↓↓	↓↓					↓		↓↓		
PTPS	↓↓	↓↓			↓		↓	n/↓	↑↑		
PTPS mild							↓		↑↑		
DHPR	↓	↓				↓↓	n↓	↑↑			
PCD							↓		↑		↑↑
without hyperphenylalaninemia											
AD-GTPCH1*	n/↓	n/↓					↓		↓↓		
compound heterozygotes ***AR-GTPCH1***	↓↓	↓↓					↓		↓↓		
SR	↓	↓					n/↓	↑		↑↑	

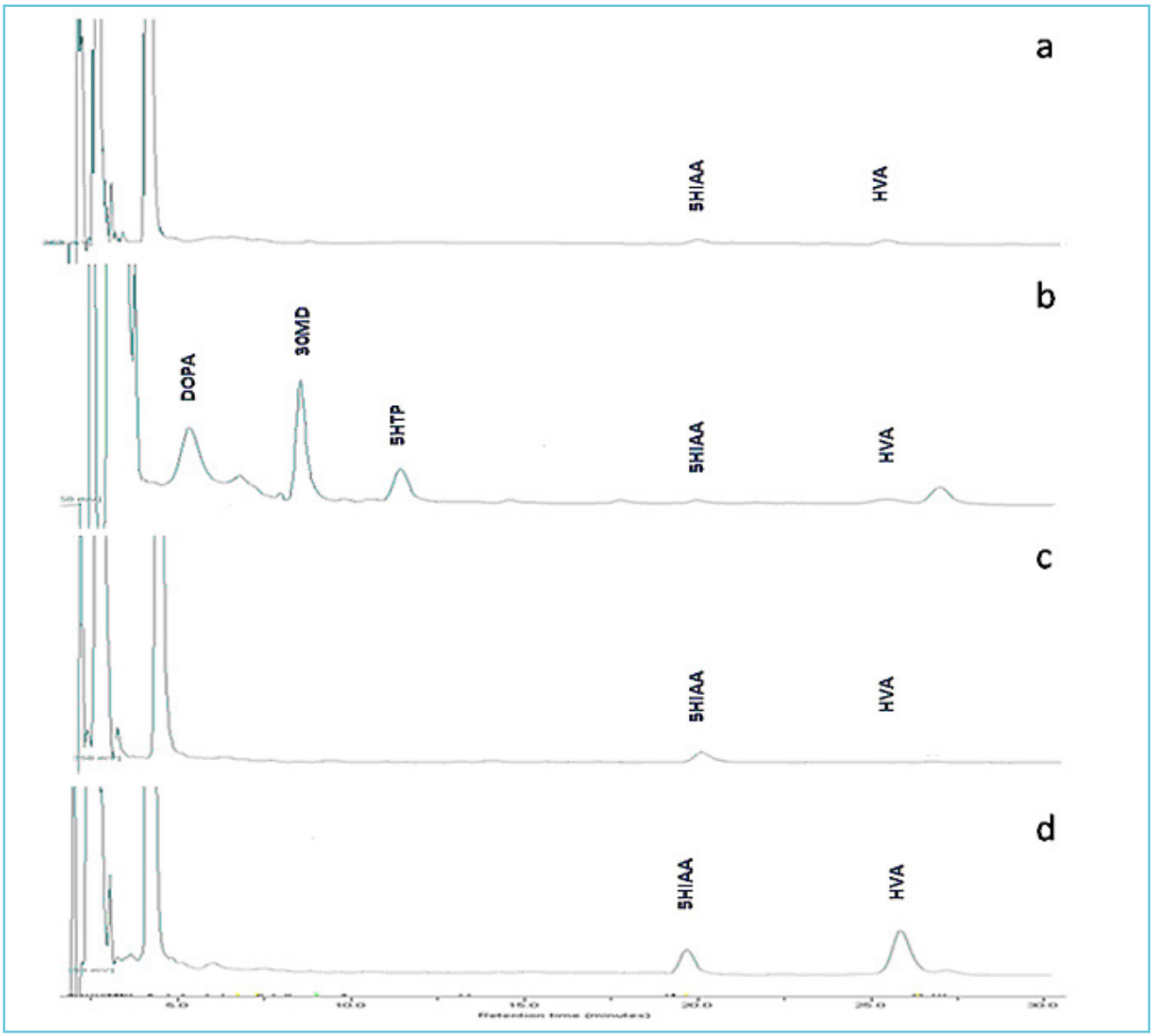
AD-GTPCH1: autosomal dominant guanosin triphosphate cyclohydrolase 1; AR-GTPCH1: autosomal recessive guanosin triphosphate cyclohydrolase 1; PTPS: 6-pyruvoyltetrahydropterin synthase; SR: sepiapterin reductase; PCD: pterin-4a-carbinolamin dehydratase; DHPR: dihydropteridin reductase; HVA: homovanillic acid; 5-HIAA: 5-hydroxyindolacetic acid; 3-OMD: 3 orthomethyldihydroxyphenylalanine; 5-http: 5-hydroxytryptophan; MHPG: 3-methoxy-4-hydroxyphenylglycol; BH4: tetrahydrobiopterin; BH2: dihydrobiopterin; Neo: neopterin; Sep: sepiapterin; Prim: primapterin; n: normal.

Note: Empty cells should be considered as normal values.

**AD-GTPCH1 present compromised Phe catabolism at Phe oral loading test.*

increased neopterin, and elevated total biopterin, dihydrobiopterin (BH2) and sepiapterin. Minimal abnormalities in random collections of plasma or urinary pterins are occasionally found but are not consistent. Oral phenylalanine-loading test (100 mg/kg) demonstrates abnormal and prolonged increase of phenylalanine levels due to impaired phenylalanine hydroxylation under loading conditions.

Figure 2 Example chromatograms



(a) *Patient with GTPCH1 deficiency; note the decrease in concentration of HVA and 5HIAA*

(b) *Patient with AADC deficiency; note the decrease in concentration of HVA and 5HIAA and the elevations of DOPA, 3OMD and 5HTP*

(c) *Patient with TH deficiency; note the decrease in concentration of HVA and the normal 5HIAA*

(d) *Healthy control with normal concentrations of 5HIAA and HVA. Chromatographic conditions: 50 µl CSF diluted with 50 µl of mobile phase was injected onto a 250 x 4.6 mm, 5 µm Spherisorb ODS1 column (Waters). Compounds were eluted under isocratic conditions at flow rate of 1.0 mL/min and mobile phase consisting of Acetate buffer (pH 3.5) containing sodium 1-eptasuphonate, EDTA and 10% methanol. Compounds were detected using a ESA Coularray 5600 electrochemical detector.*

Table 3b Disorders of biogenic amine biosynthesis and transport

	HVA	5-HIAA	HVA/5-HIAA	3-OMD	5-HTP	MHPG
TH	⇊		⇊			↓
TPH		⇊	↑		↓	
AADC	⇊	⇊		↥	↑	↓
VMAT2						
DAT	↥		↑			

TH: tyrosine hydroxylase; TPH: Tryptophan hydroxylase; AADC: aromatic L-aminoacid decarboxylase; VMAT2: vesicular monoamine transporter; DAT: dopamine transporter; HVA: homovanillic acid; 5-HIAA: 5-hydroxyindolacetic acid; 3-OMD: 3 orthomethyldihydroxyphenylalanine; 5-http: 5-hydroxytryptophan; MHPG: 3-methoxy-4-hydroxyphenylglycol; BH4: tetrahydrobiopterin; BH2: dihydrobiopterin; Neo: neopterin; Sep: sepiapterin; Prim: primapterin; n: normal.

Note: Empty cells should be considered as normal values

Figure 2 shows the CSF neurotransmitter chromatograms.

Table 3b includes monoamine neurotransmitter disorder due to enzyme deficiencies (TH, AADC) and defects of monoamine transport recently reported (Dopamine transporter deficiency syndrome, DTDS, and vesicular monoamine transporter 2 deficiency, VMAT2).

DTDS is associated with an increase of HVA while 5HIAA is normal leading to a raised HVA:5HIAA ratio. In VMAT2 both HVA and 5HIAA are normal.

TH affected patients have very low concentrations of HVA in CSF, and the HVA/5HIAA ratio is the most sensitive marker. In AADC deficiency, levels of HVA and 5HIAA are both low, but 3-OMD is highly increased.

CONCLUSIONS

The utility of CSF analyses for the investigation of monoamine metabolism abnormalities has been proposed in patients with progressive extrapyramidal movement disorders, especially parkinsonism-dystonia, chorea or mixed movement disorders combined with truncal hypotonia or in patients presenting epileptic encephalopathies of unknown origin. With the analysis of CSF, it is possible to appreciate the global functioning of monoaminergic neurotransmission. Homovanillic acid is a stable end product of dopamine and can be used as a marker of dopamine metabolism, whereas 5-hydroxyindoleacetic acid is a stable end product of serotonin and is a marker of serotonin turnover. Therefore, their quantification allows for identification of either defects of neurotransmitter biosynthesis (e.g. TH or AADC deficiencies) or defects of tetrahydrobiopterin biosythesis and regeneration (e.g. GTPCH1, PTPS, SR and DHPR deficiencies). However, abnormalities of biogenic amines are not only observed in PNDs but were recently reported in several acquired and genetic neurological diseases (see Table 1). Furthermore, HVA and 5-HIAA measurement can also be influenced by drugs that are

Table 4 Drugs that may interfere with monoamine measurement

Metabolites precursors or monoamines
3,4-dihydroxyphanylalanine (L-DOPA)/carbidopa, 5-hydroxytryptophan (5-HTP), dopamine
Monoamine oxydase (MAO) inhibitors
hydrazines, nialamide, isocarboxazid, bifemelane, pirlindole, toloxatone, rasagiline, selegiline, moclobemide, tranylcypromine, phenelzine, safinamide
Cathecol-O-methyltransferase (COMT) inhibitors
entacapone, tolcapone
Reuptake inhibitors
fluoxetine, citalopram, fluvoxamine, paroxetine, sertraline, venlafaxine, amantadine
Others
diazepam, chlorpromazine, pyridoxine, pyridoxal-5-phosphate, sapropterin, physostigmine, propranolol, phenotiazine, valproate, vigabatrin, 4-hydroxybutirrate, anticholinergic drugs

In some cases, drug interference is based on experimental models or mechanism of drug action.

No data are available on dopamine agonist drugs.

frequently used in neurological patients presenting hypokinetic movement disorders and impaired mood associated with dopaminergic/serotoninergic dysfunction. Therefore a complete documentation is mandatory. The clinical history, including neurological examination, should enclose all special features such as any deviation from the protocol, sample color, all medications and current clinical conditions such as fever. In fact, monoamine profile can be incorrectly interpreted due to the lack of information about pharmacological concomitant therapy that is not fulfilled in patient information schedules and the referring center is not completely aware about its effect on metabolite measurements. It is to be considered that, besides common drugs that acts upon dopamine and serotonin pathways, several others have been reported to possibly present biasing effects (see Table 4 for a detailed list of drugs that can impair monoamine CSF values). In the view of this, a drug-free wash out period is recommended for at least 1 week and up to 4 weeks before executing the lumbar puncture (21).

NT levels are age dependent with particular attention to the neonatal period. Preterm neurologically compromised neonates represent, in this sense, the most striking example of the difficulties of results interpretation. In this case, samples refer more frequently to severely affected patients. Patients sometimes present hypoxic/ischaemic encephalopathies or anoxia that can be a presenting symptom of several primary neurotransmitter disorders, but monoamine measurement in CSF can be impaired by brain atrophy itself. Furthermore, preterm neonates

can be hemodynamically unstable thus requiring inotrope and vasopressor support, which is frequently obtained using dopamine administration. The diagnostic process is further complicated by the fact that a marked and non-linear age dependency as well as a craniocaudal gradient of the CSF concentrations must be taken into account.

Nevertheless, laboratories have different age-related reference ranges requiring variations in the technique of CSF sampling and the precise aliquots used for analysis. As it is for other biochemical parameters, internal age-related reference ranges are thus recommended in laboratories that perform CSF monoamine metabolite measurements.

There may also be diurnal or catamenial changes, which are probably subtle (except in patients with Segawa syndrome) and not totally investigated.

Regardless of the growing interest in PNDs, the incidence of this cluster of disorders remains uncertain, with controversial results even among patients presenting with the most typical features of the disorders (i.e. movement disorders and/or epilepsy). This finding raises the question about the possibility of unrecognized patients presenting parkinsonian-like features even in adulthood. CSF neurotransmitter analysis, one of the few available direct measurements of brain neurotransmission, can help us to better understand brain pathophysiology and its potential connection with functional neuroimaging techniques.

In conclusion, a close collaboration between laboratory experts and clinicians is mandatory for the interpretation of CSF neurotransmitters levels.

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Pediatric reference intervals for transferrin saturation in the CALIPER cohort of healthy children and adolescents

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ABSTRACT

Background

Transferrin saturation reference intervals specific for age and sex have not been previously reported for the pediatric population. The reference values for transferrin saturation have been previously reported to be lower in children compared to adults, caused by a combination of low serum iron and high serum transferrin levels in children, warranting specific reference intervals.

Here we use the original iron and transferrin data from the CALIPER cohort to establish age- and sex-specific pediatric reference intervals for transferrin saturation.

Methods

Iron and transferrin concentrations were measured in serum samples from the CALIPER cohort of healthy children and adolescents on the Abbott Architect c8000. Transferrin saturation was subsequently calculated and statistically relevant age- and sex-partitions were determined.

After removing outliers, age- and sex-specific reference intervals with corresponding 90% confidence intervals were calculated using CLSI C28-A3 guidelines.

Results

Transferrin saturation required 3 separate age partitions, with an additional sex partition for 14-<19 year olds. Transferrin saturation was more variable during the first year of life, evident by a wider reference interval, which subsequently narrowed at one year until adolescence. Upon adolescence, a sex difference was apparent with females having lower percent transferrin saturation than males.

Conclusions

Age- and sex-specific pediatric reference intervals for transferrin saturation were established based on a large cohort of healthy pediatric subjects. Transference studies suggest that these intervals established using Abbott assays are comparable to those on Beckman, Ortho, Roche, and Siemens assays. Individual laboratories should however verify these reference intervals for their individual instrument and local population as per CLSI guidelines.

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INTRODUCTION

Transferrin is a plasma glycoprotein synthesized by the liver, that controls the level of free iron in the circulation. Transferrin has several functions related to iron activity and transport including rendering iron soluble, preventing iron-mediated free-radical toxicity, and facilitating iron transport into cells. One transferrin molecule can bind two Fe^{3+} ions and, together with ferritin, binds essentially all circulating plasma iron (1). The sum of these iron binding sites on transferrin, defined as the total iron binding capacity (TIBC), can be easily assessed by measuring serum transferrin concentration. The percentage of these iron binding sites occupied by iron is defined as the transferrin saturation. Determining levels of iron and TIBC, and calculating transferrin saturation are useful parameters in distinguishing between several conditions including iron deficiency, hemochromatosis, chronic illness, hemolytic anemia, and iron poisoning.

Reference intervals for these parameters are essential to correctly interpret patient laboratory test results. Traditionally, the normative range for transferrin saturation in adults has been reported as between 20-50%, with less than 20% indicating iron deficiency. However, this adult reference interval cannot be generalized to the pediatric population, as normal transferrin saturation levels have been shown to be lower in children than adults, caused by a combination of low serum iron and high serum transferrin levels in children (2). Unfortunately, in many cases pediatric laboratory test results are interpreted based on reference intervals established from an adult reference population. This is due to several challenges that are encountered in establishing pediatric reference intervals, including small sample volumes and collecting blood from a sufficient number of healthy children and adolescents to cover the extensive periods of growth and development (3).

To address these gaps, the Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER) program has established reference intervals for several analytes including common biochemical markers, protein markers, lipids and enzymes (4), specialty endocrine markers (5), fertility hormones (6), cancer biomarkers (7), vitamins (8), metabolic disease biomarkers (9), testosterone indices (10), and specialized biochemical markers (11). In our first publication of 40 routine biochemical markers we established age- and sex-specific reference intervals for serum iron and transferrin (4). Here we use the original iron and transferrin data from healthy children and adolescents to calculate transferrin saturation and establish age- and sex-specific pediatric reference intervals for this parameter.

METHODS

This study was approved by the Institutional Review Board at the Hospital for Sick Children, Toronto, Canada.

Participant recruitment and sample acquisition

Healthy children and adolescents (age 1 to < 19 years) were recruited from schools and community centres in the Greater Toronto Area. Samples from subjects <1 year were collected by heel stick from apparently healthy and metabolically stable subjects from outpatient clinics at the Hospital for Sick Children and Mount Sinai Hospital.

Participation in the study consisted of written informed consent, completion of a health questionnaire, anthropometric measurements (height, weight, and waist circumference) and donation of a blood sample. Prior to statistical analysis, participants were excluded if they were pregnant, had a history of chronic illness (including chronic low iron status) or metabolic disease, an acute illness within the previous month, or use of prescribed medication within the previous two weeks.

Blood samples were drawn into serum separator tubes (SST™; Becton, Dickinson and Company, Franklin Lakes, NJ, USA), centrifuged, separated and aliquoted within 4 hours of collection, and stored at –80 °C until testing.

Sample analysis

Serum samples were analyzed for iron, transferrin, and C-reactive protein (CRP) on the Abbott Architect c8000 analyzer.

Analytical methods were controlled according to the manufacturer's instructions using preventative maintenance, function checks, calibration, and quality control.

Statistical analysis and reference interval determination

Data were analyzed in accordance with Clinical and Laboratory Standards Institute (CLSI) EP28-A3c guidelines on defining, establishing, and verifying reference intervals in the clinical laboratory (12). Statistical analysis was performed using Microsoft Excel and R software. Transferrin saturation (%) was calculated by ((Iron (µmol/L)/Transferrin (g/L) x 25.1) x 100) (13). To determine if subjects with an elevated CRP value should be excluded from analysis, the Spearman's rank correlation coefficient between transferrin saturation and CRP concentration was calculated. Transferrin saturation and CRP were significantly negatively correlated, and therefore all subjects with an elevated CRP value (≥ 10 mg/L) were removed from the reference population. To determine if subjects taking oral contraceptives should be excluded from analysis, the Harris and Boyd method (14) was used to determine if transferrin saturation was significantly different between female adolescents using and not using oral contraceptives. If transferrin saturation was significantly different between these groups, those using oral contraceptives would be removed from the reference population. Subjects were chosen to ensure the ethnic composition of study participants was in accordance with the 2006 Canadian census data for the province of Ontario (15). The statistical approach for calculating reference intervals has been described previously (4). Briefly, scatter plots were generated to visually inspect the data and manually exclude extreme outliers. If data were not skewed, outliers were removed using the Tukey test (16). Conversely, if data were skewed, outliers were removed using the adjusted Tukey test by multiplying the interquartile range by a factor using the medcouple measure of skewness (17). The Harris and Boyd method was used to determine statistically relevant age and sex partitions (14).

Reference intervals for partitions with a sample size ≥ 120 participants were calculated using the nonparametric rank method. For partitions containing < 120 and > 40 participants, the robust statistical method of Horn and Pesce (18) was used to calculate the reference interval. For each reference limit, corresponding 90% confidence intervals were calculated.

RESULTS

Prior to calculating transferrin saturation pediatric reference intervals, the effects of CRP and oral contraceptive use on transferrin saturation values were determined. The Spearman's rank correlation coefficient between transferrin saturation and CRP concentration was -0.280 ($p < 0.0001$). As transferrin saturation and CRP were shown to be significantly (negatively) correlated, participants with an elevated CRP value (≥ 10 mg/L) were excluded. Using the Harris and Boyd method, transferrin saturation was not significantly different between female adolescents using oral contraceptives and those not using oral contraceptives. Therefore, females using oral contraceptives were not excluded from the reference population.

A total of 852 subjects had available data on both serum iron and transferrin concentrations, after excluding subjects based on the above stated criteria. Figure 1 shows a scatterplot of percent transferrin saturation plotted against age, with males shown in blue and females shown in pink. Age- and sex-specific pediatric reference intervals for percent transferrin saturation are shown in Table 1.

Transferrin saturation required 3 separate age partitions (0-<1 year, 1-<14 years, and 14-<19

Figure 1 Age- and sex-specific scatter plot of percent transferrin saturation in the CALIPER pediatric population

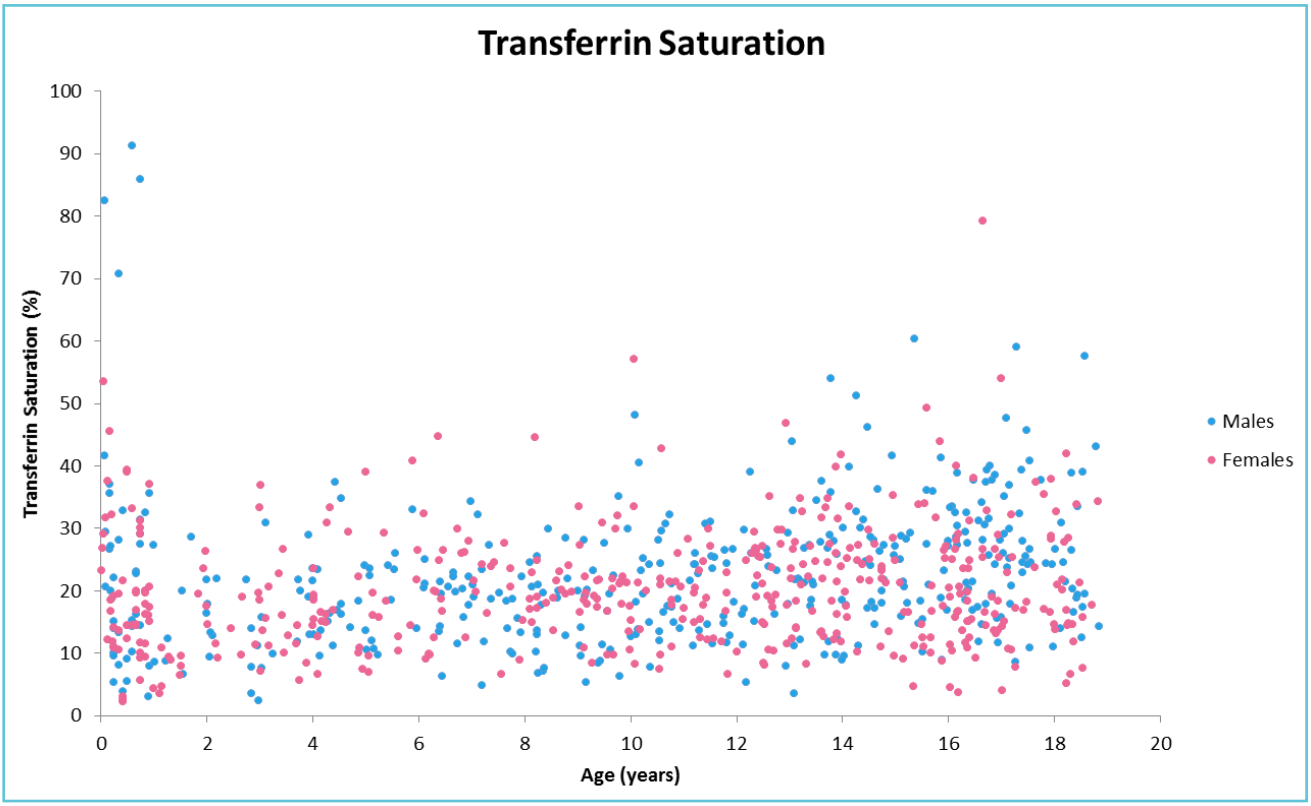

Table 1 Pediatric age- and sex-specific reference intervals for transferrin saturation

Transferrin saturation (%)	Males					
	Age range	n	Lower limit	Upper limit	Lower Confidence Interval	Upper confidence interval
	0-<1 year	92	4.1	59	(3.0, 5.3)	(51, 67)
	1-<14 years	473	6.5	39	(5.3, 7.1)	(35, 42)
	14-<19 years	136	9.6	58	(8.6, 11.7)	(43, 60)
Transferrin saturation (%)	**Females**					
	Age range	n	Lower limit	Upper limit	Lower confidence interval	Upper confidence interval
	0-<1 year	92	4.1	59	(3.0, 5.3)	(51, 67)
	1-<14 years	473	6.5	39	(5.3, 7.1)	(35, 42)
	14-<19 years	135	5.2	44	(4.6, 9.1)	(37, 54)

Underlined partition indicates sex-specific reference intervals.

years), with an additional sex partition for 14-<19 year olds. This parameter was shown to be more variable during the first year of life, evident by a wider reference interval, which subsequently narrowed at one year until adolescence. Upon adolescence, a sex difference was apparent with females having lower transferrin saturation than males.

DISCUSSION

Reference intervals for serum iron and transferrin were previously established by the CALIPER program (4). The present study is an extension to these previously determined reference intervals, by establishing age- and sex-specific pediatric reference intervals for transferrin saturation using Abbott assays.

Our transferrin saturation reference intervals demonstrated a slight sex difference, with males having higher transferrin saturation than females after 14 years of age. Previous studies also showed a higher transferrin saturation levels in male adolescents (19,20), while others showed no difference (2,21). As iron has been shown to also be higher in males, but transferrin has no apparent sex difference, the transferrin saturation sex difference is most likely a result of higher iron levels in males (4). Although transferrin saturation is statistically different between sexes when comparing the mean and standard deviation of transferrin saturation between sexes, further examination is needed to determine if this difference is clinically significant. The upper limits reported for males and females differ by 27% and the lower limits differ

by 59%. To determine if these differences are clinically significant, they must result in different clinical decisions. Unlike statistical significance, wide-spread accepted criteria to judge clinical significance is lacking and is most often based on the judgement of the clinician (22). Additionally, similar to our study, others have demonstrated a small increase in transferrin saturation with age (2,19–21). CALIPER reference values were also similar to those published most recently by Aldrimer, et al, who defined reference intervals for 6 months – 11 years and 12 – 18 years as 6.2%-41% and 6.2%-48%, respectively (21). These values aligned closely with our reference intervals, which span from 4.1%-59% transferrin saturation.

To ensure that the established transferrin saturation reference intervals sufficiently represented the healthy pediatric population, those with a CRP concentration ≥ 10 mg/L were excluded. The acute phase response down-regulates the hepatic synthesis of transferrin, therefore decreasing the TIBC, meaning there are less available transferrin binding sites for iron (19). Additionally, inflammation is accompanied by lower concentrations of iron in serum (23). As a result, transferrin saturation may also be affected by inflammation. The calculation Spearman's rank correlation coefficient between transferrin saturation and CRP concentration was -0.280 ($p < 0.0001$). Although not very strong correlation, transferrin saturation and CRP were shown to be significantly (negatively) correlated. Therefore participants with a CRP concentration ≥ 10 mg/L were excluded to ensure falsely low transferrin saturation values were not included.

Estrogen greatly affects both iron and transferrin concentrations (24). Contraceptive medication can increase transferrin levels by as much as 15% (25). Additionally, contraceptives raise serum iron concentration and after cessation of contraceptive intake, serum iron concentrations decrease by as much as 30% concurrently with uterine bleeding (26). Another study showed serum iron, TIBC, and serum transferrin levels were significantly greater in users of oral contraceptives, while transferrin saturation was not different from the control group's level (27). The stimulatory effect of estrogen on the liver's biosynthesis of protein may explain the increase in serum transferrin and TIBC levels with oral contraceptive use (28). Additionally, increased serum iron concentration as a result of contraceptive use may be due to a decrease in menstrual blood loss. Oral contraceptive use did not have a significant impact on percent transferrin saturation in our pediatric reference population. Therefore, female adolescents using oral contraceptives were not excluded from the reference population prior to calculating reference intervals for transferrin saturation.

In this study, we establish pediatric reference intervals for transferrin saturation using an Abbott platform. However, transferrin saturation values obtained using Abbott assays should also be applicable to values obtained using Beckman, Ortho, Roche, and Siemens assays. Iron and transferrin, the two values used to compute transferrin saturation, were previously successfully transferred and verified from Abbott assays to Beckman, Ortho, Roche, and Siemens assay through a series of CALIPER transference studies (29–32). To perform transference, a method comparison was performed using 200 patient samples spanning the analytical measuring range. If there was a sufficient correlation between the methods and the residuals were normally distributed, the regression line equation was used to transfer the reference interval established using the Abbott assay to each of the other manufacturer assays. The transferred reference intervals were then validated if ≥ 90% of 100 healthy pediatric samples from the CALIPER cohort fell within the transferred reference interval. Therefore, these studies suggest that these intervals established

using Abbott assays are comparable to those on Beckman, Ortho, Roche, and Siemens assays.

This study provides age- and sex-specific reference intervals for percent transferrin saturation based on a multi-ethnic Canadian population aged 0-<19 years of age. These data will improve test result interpretation for pediatric patients. Prior to implementing these reference intervals into clinical practice, they should be validated by individual laboratories for their individual instrument and local population.

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Congenital hyperinsulinism caused by a *de novo* mutation in the ABCC8 gene – a case report

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LB- clinical diagnosis, collecting data, revising the manuscript;
LM, ÉB- molecular diagnosis;
JK- revising the manuscript;
IB- molecular diagnosis of hyperinsulinism, revising the manuscript

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ABSTRACT

Congenital hyperinsulinism (CHI) is a rare genetic disorder characterized by inappropriate insulin secretion and severe hypoglycaemia. There are two histological subtypes: diffuse and focal form. Diffuse form is most common in autosomal recessive mutations in ABCC8/KCNJ11 gene, while focal CHI is caused a paternally inherited mutation and a somatic maternal allele loss.

Here we report a case of a term male infant presented with severe hyperinsulinaemic hypoglycaemia. Gene panel testing was performed to give rapid genetic diagnosis. We detected the c.4415-13G>A heterozygous mutation in the ABCC8 gene. Targeted genetic testing of the parents proved the *de novo* origin of the mutation. The mutation has been previously described. The infant received octreotide treatment and is prepared for 18-fluoro-dopa PET-CT examination in order to localize the lesion.

Rapid genetic testing might be crucial in the clinical management strategy, with decision algorithms taking into account of the genetic status of the patient.

INTRODUCTION

Congenital hyperinsulinism (CHI) is the most common cause of nonketotic persistent severe hypoglycaemia in neonates, and is caused by an inappropriate insulin secretion from pancreatic ß cells. The estimated incidence of CHI is 1 in 50,000 live births (1). A number of different genetic causes has been associated with CHI. K_{ATP} channel subunits (ABCC8, KCNJ11) alterations are considered to be channelopathies, while metabolopathies are enzyme defects, transcription factor defects or metabolic defects (GLUD1, SLC16A1, HNF4A, GCK, HNF1A, HADH, UCP2) (2).

CHI shows considerable genetic heterogeneity, it can be inherited in a recessive or dominant manner (3). Of the several genes implicated, the most common ones are ABCC8 (ATP binding cassette subfamily C member 8) and KCNJ11 (potassium voltage-gated channel subfamily J member 11) being responsible for 40-50% of the cases. ATP sensitive K^+ (K_{ATP}) channels are composed of eight subunits from two different gene products, ABCC8 encoding SUR1 and KCNJ11 encoding Kir6.2 subunit (2). The K_{ATP} channel is a key component in the regulation of insulin secretion in response to elevating glucose level. The channels close as a consequence of glucose metabolism (increased ATP level) leading to membrane depolarization which ultimately results in the opening of a voltage-gated Ca^{2+} channel. The rise of the intracellular Ca^{2+} concentration stimulates exocytosis of insulin-containing granules from the pancreatic ß cell (Figure 1). The K_{ATP} channel is a Janus-faced protein as gain-of-function mutations will lead to neonatal or maturity-onset diabetes while loss-of-function mutations will cause CHI (4).

Histologically 2 different types of congenital hyperinsulinism can be distinguished. In diffuse forms all ß cells are affected, which is a result of an autosomal recessive CHI. In focal forms a localized hyperplasia is present in the otherwise normal pancreatic tissue. These focal lesions might be the results of two different events. One possible event is a paternally inherited mutation in the ABCC8 or KCNJ11 gene that would remain silent, but if there is an acquired somatic deletion of the maternal allele, focal CHI will develop because of the loss of heterozygosity. The ABCC8 and KCNJ11 containing chromosome region also contains several imprinted genes, which are responsible for cell replication. The paternally expressed genes are involved in cell replication, proliferation. Normally they are compensated by the maternally expressed gene products, but in case of maternal allele loss, ß cell hyperplasia will develop (8).

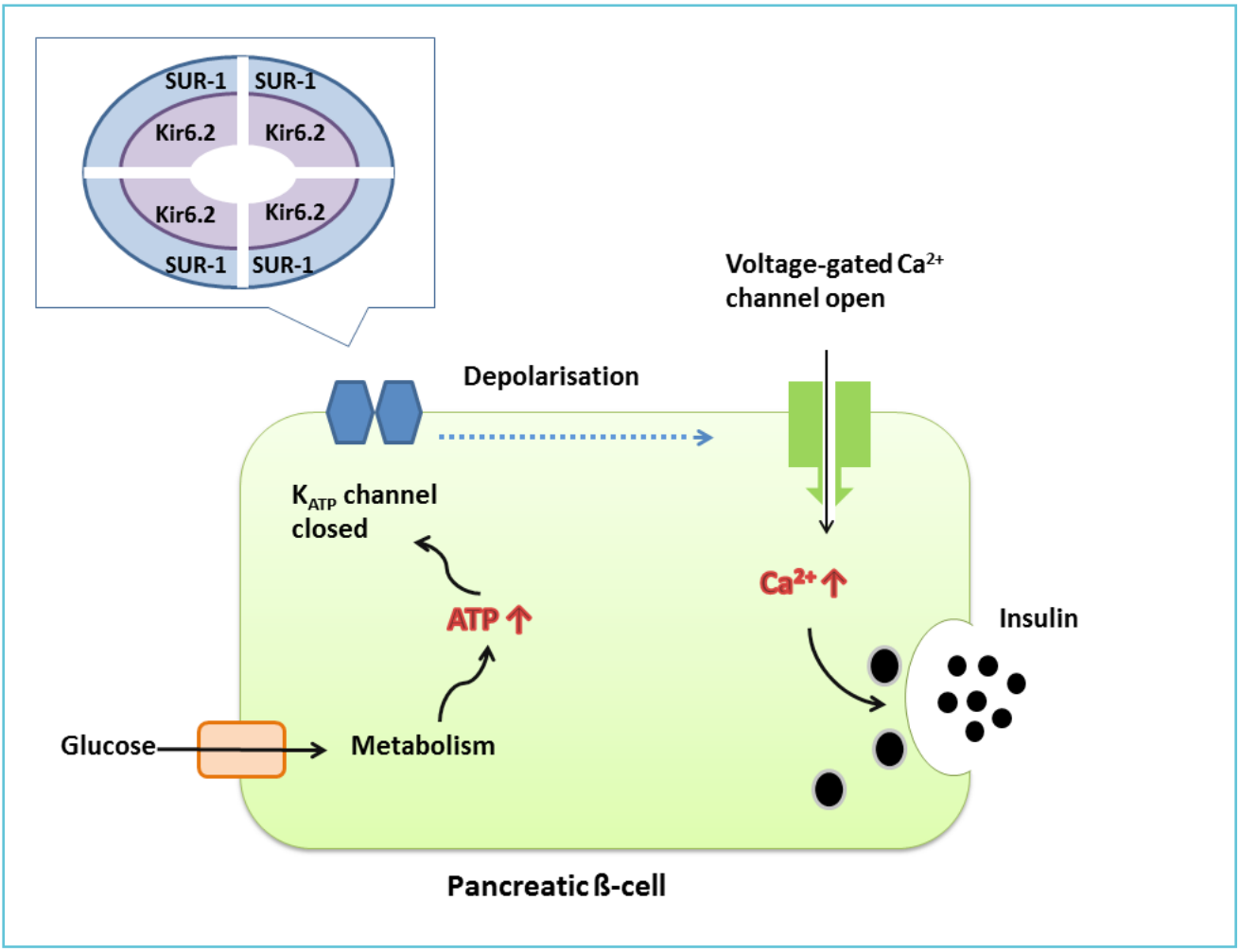
Diffuse CHI is suspected when the detected alterations are homozygous, compound heterozygous or in the case when only maternal heterozygous mutation is found.

Focal CHI is most commonly associated with paternal heterozygosity, but focal CHI has also been reported with mutations arising either *de novo* or of unknown parental origin. Although paternal heterozygosity has a higher probability for focal CHI, additional investigation such as 18-fluoro-dopa PET-CT scanning is necessary to localize the lesion in focal CHI (Figure 2) (9). According to some reports a proportion with paternal heterozygous mutations might have diffuse CHI which could be explained by dominant inheritance or inability to identify a maternal mutation in a recessively-inherited disease (2).

Rapid, high-throughput techniques for analysis of candidate genes have helped the care of patients with CHI. Genotyping guides further investigation of the patient and, because of the strong genotype-phenotype correlation, also the therapy (10,11).

The main goal of CHI treatment is to prevent brain damage. The nutritional approach includes glucose infusion and enteral feeding.

Figure 1 Mechanism of the K_{ATP} channel regulated insulin secretion in pancreatic β-cell. Model of the K_{ATP} channel protein subunits



Medical treatment includes administration of diazoxide, octreotide, nifedipine and glucagon. Diazoxide acts on the SUR1 subunit of the K_{ATP} channel. It keeps the channel open, halting insulin secretion. Octreotide is a somatostatin analogue that inhibits insulin secretion. Glucagon stimulates glycogenolysis and gluconeogenesis. Growing evidence of published cases suggest a novel treatment option, sirolimus therapy (12). When CHI patient fail to respond to medical treatment, surgery should be considered. For patients diagnosed with focal form, surgical resection of the lesion is the appropriate cure. In case of diffuse CHI subtotal pancreatectomy may be necessary in severe, medically unresponsive cases (1).

CASE REPORT

A term male infant was born after uneventful pregnancy. His birth weight was 4050 g (95-97 percentile), Apgar scores at birth were 9 at 1 min and 10 at 5 min. The infant did not have any dysmorphic features or congenital anomalies. The mother was primipara and had no diabetes during pregnancy. Due to being large for gestational age blood glucose monitoring begun, and hypoglycaemia was detected at one hour of life.

Table 1 Glucose, insulin and C-peptide levels during treatment

	Upon admission	Day 1	Day 2	Reference range
Blood glucose (mmol/L)	1.6	2.1	5.6	3.6-6.0
Insulin (mU/L)	16.65	4.46	N.D.	2.6-24 (in normoglycaemia)
C-peptide (ng/mL)	2.43	0.76	N.D.	0.48-5 (in normoglycaemia)
Therapy	glucose infusion	diazoxide	octreotide	-

N.D.: not determined

Figure 2 Clinical diagnostic algorithm for management of congenital hyperinsulinism integrating molecular genetic testing

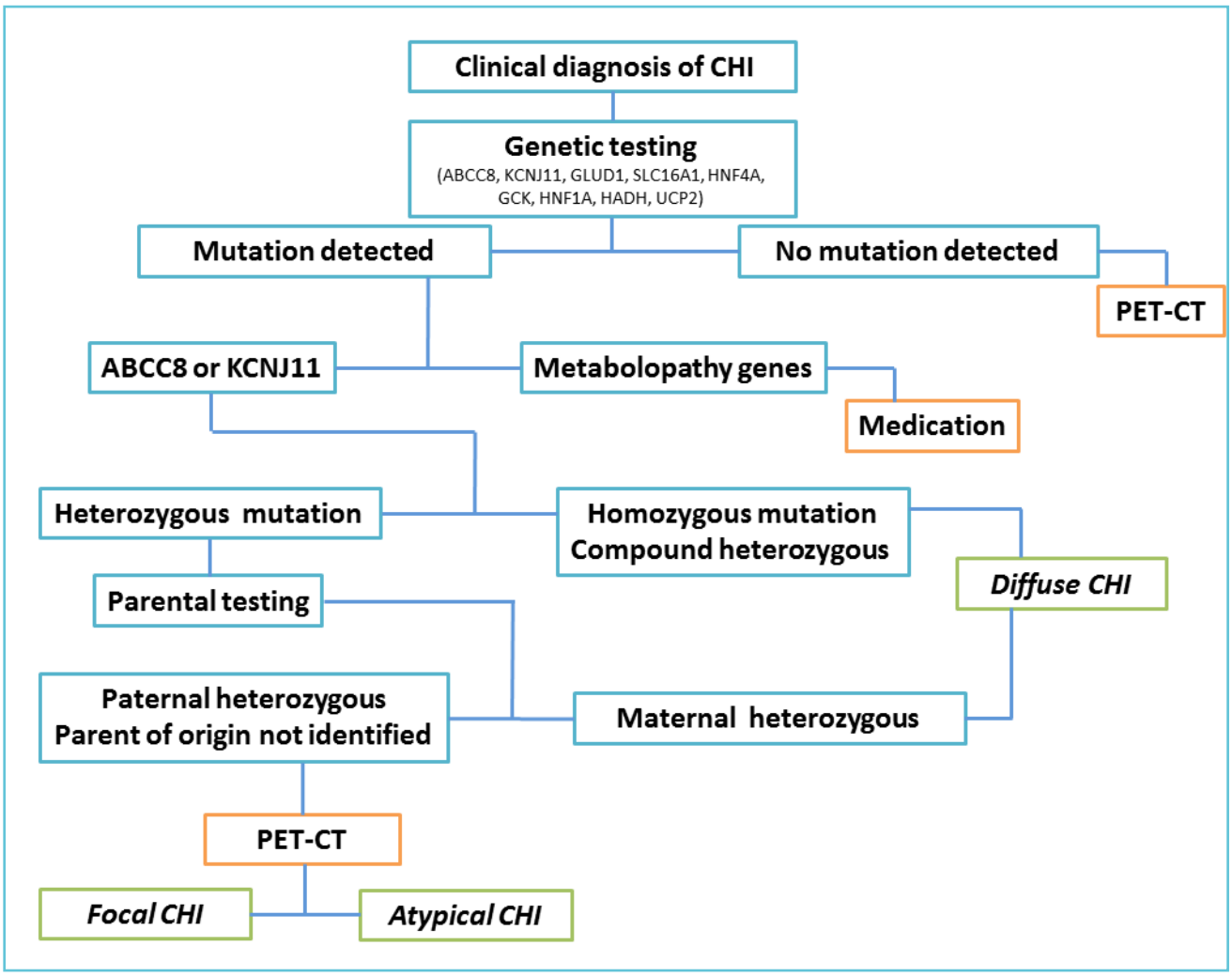
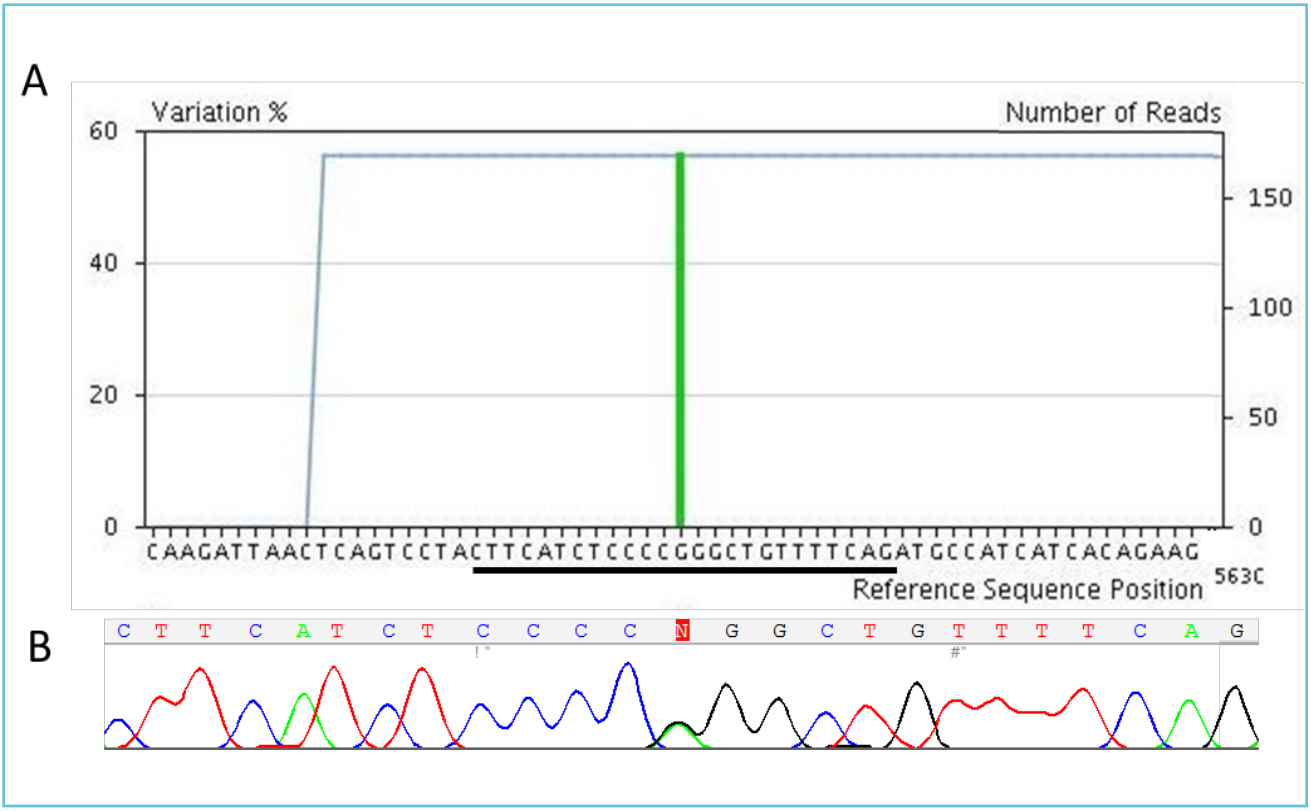


Figure 3 **Genetic analysis revealed a heterozygous c.4415-13G>A mutation of the ABCC8 gene**



Part A: *Next-generation sequencing result*
Left Y axis shows the percentage of the mutant allele
Right Y axis and the blue line show the coverage
Region of the ABCC8 gene containing the mutation site is shown

Part B: *Sanger sequencing electropherogram*

He was treated with i.v. glucose infusion and hydrocortisone. Adrenal insufficiency was excluded (cortisol level: 398 nmol/L, reference range: 140-690 nmol/L). After the sixth day of life the blood glucose normalized, the infant did not require any medication. A diagnosis of transient hypoglycaemia was made. At the age of two months, he was admitted to hospital because of frequent myoclonic movements of the lower and later the upper limbs, sweat and pallor was observed with episodes of hypoglycaemia (glucose: 1.1-1.9 mmol/L). In time of hypoglycaemia (glucose: 1.6 mmol/L) the insulin level was 16.65 mU/L, and C-peptide level was 2.43 ng/mL. Normoglycaemia could only be maintained by continuous i.v. glucose load up to 20mg/kg/min. Insulin and C-peptide levels were elevated during hypoglycaemia.

Oral diazoxide was administered because of the hyperinsulinism at 13 mg/kg/day in divided doses. He had poor response to diazoxide, the glucose infusion was further administered. With this therapy the blood glucose could be maintaned at 2.1-2.3 mmol/l. Insulin was 4.5 mU/L, C-peptide was 0.76 ng/mL (Table 1). Genetic testing was initiated. After starting octreotide therapy his blood glucose begun to improve. Subcucatenously introduced octreotide was successfully replaced by lancreotide every four weeks.

Genomic DNA was extracted from peripheral blood leukocytes. Next-generation panel gene sequencing was performed on a Roche GS Junior DNA sequencer using a Multiplicom MODY MASTR Kit which contains the following genes: ABCC8, GCK, HNF1A, HNF4A, HNF1B, INS and KCNJ11. The detected mutation was confirmed by Sanger sequencing.

We detected the c.4415-13G>A heterozygous mutation in the ABCC8 gene (Figure 3). Targeted genetic testing of the parents proved the *de novo* origin of the mutation. The c.4415-13G>A mutation has been previously described, and was associated with octreotide responsive, focal histological forms (4). Although there is no functional study analyzing the consequence of the mutation, the c.4415-13G>A has been shown to be present in many CHI patients (5,6,7).

The infant received octreotide treatment and could be prepared for 18-fluoro-dopa PET-CT examination.

DISCUSSION

Early diagnosis and treatment is crucial for the appropriate management of CHI (1). The primary goal of treatment is to maintain normoglycaemia in order to prevent neurological damage. Rapid gene mutation analysis with short turnaround time is a key part of the clinical management strategy, as it is shown in the decision algorithm (Figure 2). Importantly, the finding of a paternal heterozygous mutation or a *de novo* mutation is a key step before 18-fluoro-dopa PET-CT scanning and the subsequent possible finding of a solitary focal lesion in the pancreas that can be cured by lesionectomy (8,9,10). Genotype-phenotype correlations is also helpful in the management of the patient (3,11). The treatment algorithm recommends the K_{ATP} agonist, diazoxide as first line therapy. Our patient was also octreotide responsive, similarly to the patient in the first literature report (4).

TAKE-HOME MESSAGES/LEARNING POINTS

1. CHI is a cause of severe hypoglycaemia in neonates.
2. The main pathophysiological characteristic of CHI is that insulin secretion is independent from the glucose concentration, showing elevated insulin and C-peptide levels even in the presence of hypoglycaemia.
3. Loss of function mutations in the ABCC8 or in the KCNJ11 gene can lead to CHI.
4. Rapid genetic testing in patients with CHI is crucial and guides further investigations.

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Hemochromatosis, erythrocytosis and the *JAK2* p.V617F mutation

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LETTER TO THE EDITOR

Hereditary hemochromatosis (HH) is an inherited iron overload disorder, particularly prevalent in Irish and Scandinavian populations (1), characterised by abnormal iron metabolism, leading to excess iron deposition in the parenchymal cells of the liver, heart, and endocrine organs. HH is commonly associated with mutations in the *HFE* gene and in other genes such as *HJV*, that regulate the biology of hepcidin, a key regulator of iron homeostasis, and *TRF2* that is responsible for uptake of transferrin bound iron (2, 3). A rare but recurrent hematological manifestation of HH is a raised hematocrit due to an excess of red cells (erythrocytosis) possibly an important clue to an underlying hepatoma (4). Therapy is based on the removal of excess iron by phlebotomy or erythrocytapharesis, with ferritin levels used to monitor treatment effectiveness (5). The most common cause of acquired primary erythrocytosis is the myeloproliferative neoplasm polycythemia vera (PV). Approximately 95% of PV patients harbour the *JAK2* p.V617F mutation. In hematopoietic stem cells, this mutation leads to constitutively activated, intracellular JAK-STAT signalling resulting in increased production of red and white blood cells and platelets. For many years the mainstay of PV therapy has been

phlebotomy and cytoreductive agents to prevent thrombotic events and transformation to acute leukemia, however recently developed targeted therapies have shown considerable efficacy (6).

A sporadically observed trigger for requesting *JAK2* p.V617F analysis is for investigation of an erythrocytosis or raised hematocrit in patients with known HH. In order to address the clinical value and laboratory impact of such requests, a retrospective audit was performed on all *JAK2* p.V617F requests received at a molecular diagnostics centre for hematological malignancies. From January 2006 to December 2016 inclusive, 14617 diagnostic requests for *JAK2* p.V617F mutation analysis were received. Of these, 48 requests (0.3%) were received from known HH patients with accompanying clinical details of a raised hemoglobin and/or hematocrit or erythrocytosis. Using an allele-specific PCR screening assay capable of detecting 2% mutant alleles, the *JAK2* p.V617F mutation was not detected in any of these 48 patients.

The role of *HFE* genotypes as risk factors for development of a myeloproliferative disorder remains somewhat confounding (7, 8). However, considering the above data and, while the number of requests for *JAK2* p.V617F mutation in patients with HH does not considerably impact on overall laboratory workload, routine screening for the PV-associated *JAK2* p.V617F mutation in patients with HH with raised red cell counts appears inappropriate.

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