International Journal of Chemical and Biochemical Sciences
(ISSN 2226-9614)

Journal Home page: www.iscientific.org/Journal.html

Age Adjusted Reference Interval for Creatine Deficiency Syndrome Biomarkers by Gas chromatography- Mass Spectrometry

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Abstract

Creatine deficiency syndrome is a metabolic disorder affecting creatine biosynthesis or transport. There are three genetic defects associated with creatine deficiency syndrome include the two creatine biosynthesis disorders, guanidinoacetate methyltransferase deficiency and L-arginine:glycine amidinotransferase deficiency, and the creatine transporter deficiency. Guanidinoacetate (GAA) and creatine are reliable biochemical markers for creatine metabolic defects. The main aim of this study was to establish age adjusted reference interval for urinary GAA/ creatinine ratio and creatine/ creatinine ratio in a cohort of Egyptian children using gas chromatography-Mass spectrometry (GC-MS). The study was conducted on 150 subjects partitioned by age into two groups: less than 4 years old and from 4 to 15 years old. Metabolic profiling of urine samples was performed using GC-MS. The reference interval was calculated as values between 2.5th and 97.5th percentiles. The reference interval for GAA/ creatinine ratio was set as (20.3 - 223.3 µmol/mmol) and (15.3 - 209.8 µmol/mmol) for age groups (0 – 4 years) and (4 – 15 years) respectively; Regarding creatine/creatinine ratio, the reference interval was (0.047 – 1.45 mmol/mmol) for children aging less than 4 years old and (0.013 – 1.36 mmol/mmol) for age group (4 – 15 years). Both GAA and creatine decreased significantly with age (p= 0.039 and 0.001 respectively) denoting the importance of interpreting both biomarkers' results considering age group for proper diagnosis of creatine metabolic defects.

Keywords: Reference interval; creatine deficiency syndrome; reporting; gas chromatography-Mass spectrometry.

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1. Introduction

Creatine and phosphocreatine play an essential role in energy storage and transmission in several tissues [1]. Creatine is synthesized in the liver and pancreas by two reactions involving two enzymes: the first one is arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1) which transfers the amidino group from arginine to glycine, producing guanidinoacetate (GAA) and ornithine; the second enzyme is S-adenosyl-methionine:N-guanidinoacetate methyl- transferase (GAMT; EC 2.1.1.2), which transfers a methyl group to GAA producing creatine [2]. Creatine is then transported to various tissues by the creatine transporter and is non-enzymatically converted creatinine [3]. Three inherited defects in the biosynthesis and transport of creatine have been described: AGAT and GAMT deficiencies and defects in the creatine transporter SLC6A8. A common feature in all these disorders is the complete lack of creatine or phosphocreatine in the brain measured in vivo by magnetic resonance spectroscopy [4]. Clinical symptoms of the three defects are non-specific and vary in severity and include mental retardation with severe speech delay, autistic features, and seizures [5]. Some patients with GAMT deficiency exhibit a more complex clinical phenotype with extra pyramidal and abnormal movements [1]. AGAT and GAMT deficiencies, can be treated by creatine supplementation, while SLC6A8 patients do not respond to this treatment [6]. Biochemical detection of CDS relies on the determination of two main metabolites in biological fluids: creatine and GAA [7]. The accumulation of GAA in biological fluids in GAMT deficiency was demonstrated to be reliable diagnostic marker of this disease [8]. AGAT associated disorder is characterised by deficiency of both GAA and creatine. Finally, X-linked creatine transporter deficient patients are characterized by increased urinary creatine/creatinine ratio in urine and normal or slightly increased plasma creatine [9].

The main aim of the current study was to determine age adjusted reference interval for creatine/creatinine ratio

and GAA/creatinine ratio in a cohort of Egyptian children using gas chromatography/ mass spectrometry.

2. Materials and methods

2.1. Participants

One hundred and sixty Egyptian children were recruited to this study from Neurometabolic and Neurology paediatrics outpatient clinics at Cairo University Children Hospital during the period from February 2018 to February 2019. The study protocol was approved by the Research Ethics committee, Faculty of Medicine, Cairo University, Egypt as to be in accordance with Helsinki Declaration II, Finland. Written consents were taken from the guardians of all children after explaining the details of the study.

2.2. Inclusion and exclusion criteria

The inclusion criteria were as follow: 1) seizures of obscure aetiology; 2) global developmental delay; 3) autistic spectrum disorders as intellectual disability, hyperactivity associated with attention deficit, impulsive behaviour, and delayed speech. The exclusion criteria were: 1) identified metabolic disease by Tandem Mass Spectrometry; 3) seizures of known aetiology

All participants were subjected to full history taking, meticulous general and neurologic examination, and Brain MRI profile if dictated by the clinical picture of the patient. Moreover, basic laboratory investigations in the form of liver and kidney function tests, serum electrolytes, lactate, ammonia, extended metabolic screen and urine organic acid profile were performed before measuring urinary GAA and creatine by GC-MS.

2.3. Screening for CDS biomarkers

GAA/creatinine and creatine /creatinine ration were measured in the urine by GC-MS as described by Nasrallah et al, [6]. Briefly 100 µL of urine sample or standard were mixed with 100 µL of 2-phenylbutyrate, 50 µL of saturated aqueous sodium bicarbonate solution, 50 µL of hexafluoroacetylacetone and 600 µL of toluene, followed by heating the mixture at 80 °C for two hours, and then cooling. The toluene phase of the mixture was separated, 400 ml were taken and transferred to clean Eppendorf and allowed to dry by nitrogen flow at room temperature. Finally, 50 µL of BSTFA and 50 µL of chloroform were added to the dried residue then analysed by Thermo Trace GC Ultra gas chromatograph coupled with DSQ II mass spectrometer using non-polar capillary column. The instrument was operated using electron impact ionisation in full scan mode. The Xcalibur software was used for data acquisition. The specific ion selected for creatine was m/z 258, for GAA m/z 225, and m/z 221 for 2- pheylbutyric acid which was used as the internal standard.

Patients with positive screening biomarkers for creatine deficiency syndrome (CDS) namely creatine and GAA were excluded from the study as they were possible cases of CDS, while the remaining participants whose screening was negative for the two biomarkers were the cohort selected for reference interval determination.

2.4. Statistical analysis

Data was collected in Microsoft Excel 2019 MSO 64-bit then analysed using SPSS27 Statistics (IBM Corp., Armonk, N.Y., USA). Qualitative data was expressed as frequency and percentage. Quantitative data was expressed as median and percentiles as appropriate. For non-normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test. Spearman correlation method was used to test correlation between numerical variables. Differences were considered statistically significant when $p \leq 0.05$. Data were log transformed and the reference interval was calculated as the values between 2.5^{th} and 97.5^{th} percentiles [10].

3. Results and Discussions

3.1. Demographic data of the participants

One hundred and sixty participants were recruited in this study. Nonetheless, ten were excluded due to positive screening for CDS. The remaining 150 were partitioned by age, gender, and consanguinity as follow: 85 males and 65 females. Regarding consanguinity, they were 71 with positive consanguinity and 79 with negative consanguinity. The age was categorised into two groups as follow: the first group included 70 participants and aged less than four years old, and the second group included 80 participants aged from four to fifteen years old.

3.2. Age adjusted reference interval

Data for both GAA/creatinine and creatine/creatinine ratio were log transformed. The data were normally distributed, and the reference interval was set as the values between the 2.5^{th} and 97.5^{th} percentiles. GAA/creatinine was measured in µmol/mmol and creatine/creatinine was measured in mmol/mmol .There was statistically significant difference between the two age groups: <4 years old and 4-15 years old as illustrated in (**Table 1**) denoting the importance of interpreting CDS biomarkers regarding age. It was noted that gender has no statistical significance on urinary GAA/ creatinine ratio or creatine/creatinine ratio, $p = 0.71$ and 0.75.

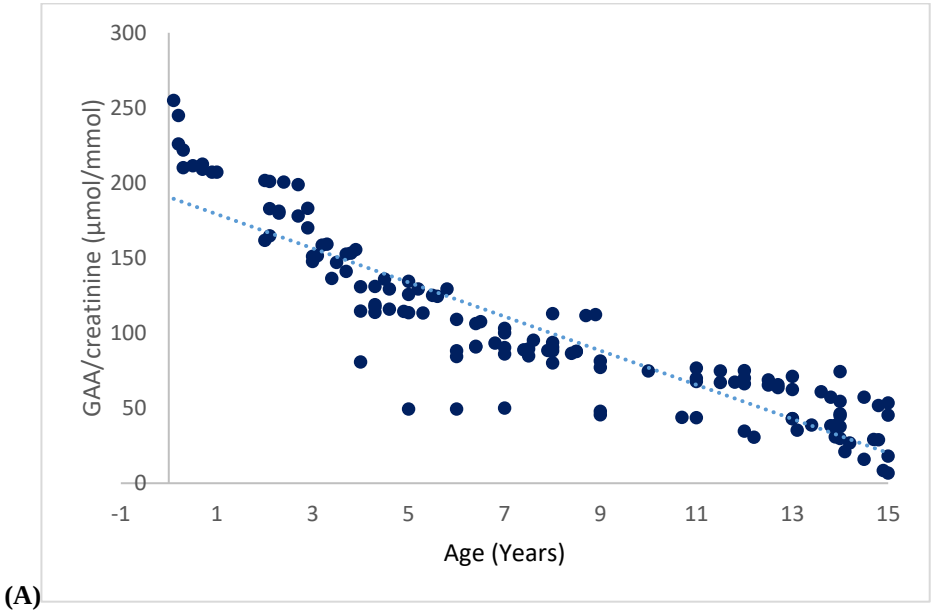
Reference interval determination is of paramount importance in patient care. Nearly 80% of physicians' medical decisions are based on information provided by laboratory reports. A test result by itself is of little value unless it is reported with the appropriate information for its interpretation. Typically, this information is provided in the form of a reference interval or medical decision limit [11]. No reference interval is completely "right" or "wrong." Most reference intervals in use today refer to the central 95% of the reference population of subjects meaning that 5% of all results will fall outside of the reported reference interval [12].

Table 1: Patients classification by age

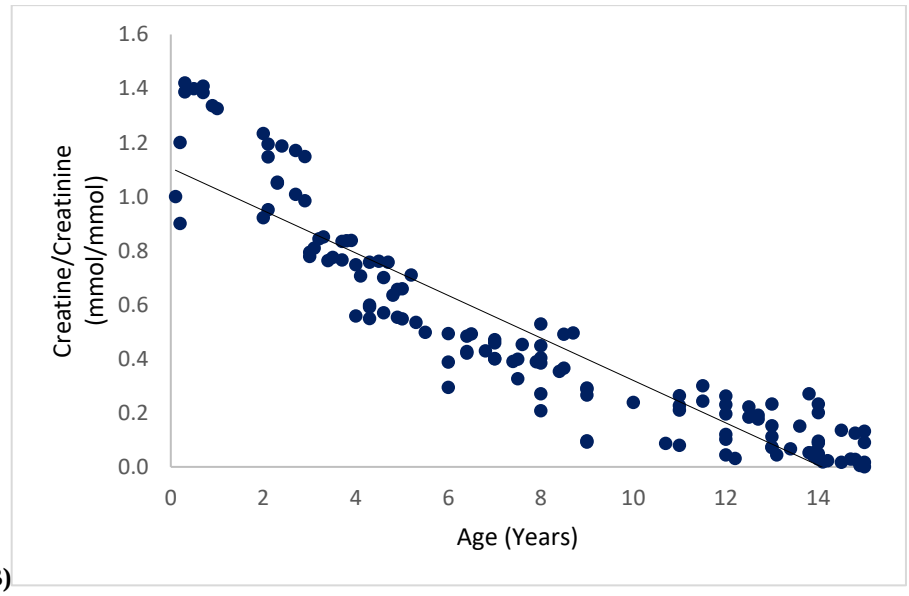
	Age	2.5th percentile	Median	97.5th percentile	*P* value
GAA/Crn by GC-MS (µmol/mmol)	**< 4**	20.3	114.8	223.299	**0.039**
	4 -15	15.3	89.6	209.815	
Cr/Crn by GC-MS (mmol/mmol)	**< 4**	0.047	0.570	1.416	**0.001**
	4 -15	0.013	0.365	1.353	

P value is statistically significant when it is <0.05
GAA; Guandinoacetic acid, Cr; Creatine, Crn; creatinine, GC-MS; Gas chromatography- Mass Spectrometry

Data partitioning by age revealed that both GAA/creatinine ratio and creatine/creatinine ratio decrease with age as illustrated in **(figure 1).**



(A)



(B)

Figure 1: Scatter plot for : (A) GAA/creatinine ratio vs age, (B) Creatine/creatinine ratio vs age. Both GAA and creatine are inversely correlated with age.

There is an obvious requirement for health-associated reference values for quantities measured in the clinical laboratory. But the concept of health is problematic; much confusion may arise if the selection criteria for health are

not clearly stated for a specific project [13]. For example, when setting reference limits for cardiac-specific troponins, a "cardio-healthy" population is required that is in other ways similar to the patients who are likely to present with possible acute coronary syndrome for example: they should be of similar age and gender, and they may have hypertension or hyperlipidaemia [14,15]. Similarly, in the current study reference individuals were selected to match the clinical picture of CDS i.e.: they should have seizures or GDD and partitioned by age group for proper interpretation of the results. To the best of our knowledge, this is the first work to establish age adjusted reference interval for CDS in Egyptian children. CDS is underdiagnosed due to the wide spectrum of clinical presentation including seizures, GDD and autistic features which simulate the clinical picture of other neurological and neurometabolic disorders [3], denoting the importance of screening for CDS in suspected patients and interpreting the results in light of reference interval of matched population.

In the current study, age adjusted reference interval was established for both urinary GAA/creatinine and creatine/creatinine ratio in a cohort of Egyptian children partitioned into two groups: <4 years old and 4-15 years old. There was statistically significant difference between the 2 groups (table 1). Written reports should be provided and include the following information: units of measure; age-dependent, laboratory-specific reference ranges; and an interpretation. When abnormal results are detected, the interpretation should include an overview of the results and their significance, a correlation to any available clinical information, elements of a differential diagnosis, recommendations for additional biochemical testing and any available confirmatory studies (e.g., enzyme assay, molecular analysis), and a phone number to reach the reporting laboratory for additional questions. Recommendations for follow-up evaluation, including referral to a metabolic specialist, should also be included when appropriate [7].

4. Conclusions

CDS's biomarkers should be interpreted according to age for proper patient's screening and diagnosis. The established reference interval can be used in Egyptian children for CDS's biomarkers assay. Gender has no significant effect on both GAA and creatine in the urine in children under 15 years old. Therefore, setting different reference intervals for males and females aging less than 15 years old was not necessary. CDS diagnosis has been so far largely underdiagnosed, therefore, we highly recommended screening for it in individuals with global developmental delay and/or seizures of obscure aetiology along with the request for the diagnosis of organic acidurias and aminoacidopathies.

Abbreviations: Guanidinoacetate: GAA; gas chromatography-Mass spectrometry: GC-MS; arginine glycine amidinotransferase: AGAT; creatine deficiency syndrome: CDS.

Ethics approval and consent to participate:

The study protocol was approved by the local ethics committee of the department of Clinical and Chemical pathology, Faculty of Medicine, Cairo University (N-22-2018) as to be in accordance with Helsinki Declaration II, Finland. Written informed consent was obtained from all participants.

Competing interests: The authors declare that they have no competing interests.

Funding: This work was funded by Newton-Mosharrafa Research fund.

Authors' contributions: NM collected the data and samples, performed laboratory work-up, statistically analysed and interpreted the data and wrote the manuscript; NE and AA designed the work and revised the draft; DA collected patients' data; data and revised the script. All authors read and approved the final manuscript.

Acknowledgements: Newton-Mosharrafa Research fund.

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