ACYLCARNITINES' LEVEL IN THE DRIED BLOOD SPOT SAMPLES OF HEALTHY NEWBORNS IN SERBIA-THE PILOT STUDY

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ABSTRACT

Analysis of the acylcarnitines' (ACs) is the mainstay for screening for fatty acid oxidation disorders (FAOD). Data about the ACs profile in the dried blood spot samples of healthy newborns in Serbia are not at disposal. Therefore, we determined the ACs levels and established the cut-offs. Between August 2018 and August 2019 a total of 1771 samples had been analysed. Cut-offs, established using a non-parametric approach, were verified in comparison with the worldwide target ranges and the data for several Caucasian populations. The majority of ACs had comparable distribution in Serbian and the worldwide population. In case of discrepancy, the individual alterations had a frequency of less than 10%. Seventeen out of 25 established cut-offs were in the worldwide target range. Reliability of the cut-offs positioning out of the target ranges is not jeopardized, since alterations are negligible or similar findings were reported for other Caucasian populations. The established and verified set of cut-offs can be used in the future screening for carnitine uptake/transport defect, medium-chain acyl-CoA dehydrogenase deficiency, very long-chain acyl-CoA dehydrogenase deficiency, long-chain L-3 hydroxyacyl-CoA dehydrogenase deficiency, trifunctional protein deficiency, carnitine palmitoyltransferase deficiency Ia and II, as well as carnitine:acylcarnitine translocase deficiency.

Keywords: *Acylcarnitines, newborns, fatty acid oxidation, tandem mass spectrometry, Serbia.*

INTRODUCTION

Acylcarnitines (AC) have an essential role in the fatty acid oxidation (FAO), one of the main catabolic pathways in majority of human cells (1). Therefore, the fluctuations in the AC levels can mirror the presence of the FAO disorder (FAOD). These genetic conditions represent a heterogeneous group of more than 15 autosomal recessive traits, in which a deficiency of FAO enzymes and transporters profoundly depletes the potential for energy production (2). The FAOD are rare diseases, with the estimated joint frequency of 1: 5,000-10,000 newborns. Nevertheless, their manifestations can be extremely complex and often life-threatening. They are triggered by the prolonged fasting or/and increased energy demand; whereby clinical phenotype depends on the age of onset. The therapy includes lifelong nutritional support, avoidance of fasting and intensive therapy in situation of metabolic exacerbation (3).

Analysis of the ACs level is the mainstay of the FAOD diagnostics. Furthermore, the introduction of ACs analysis in the dried blood spot samples (DBS) from newborns, using tandem mass spectrometry, allowed a reliable, robust and cost-effective screening for FAOD. Additional positive outcomes followed-the diagnostic efficiency increased and created the path for timely diagnostic interventions to avoid metabolic crises and other highly deleterious sequels (3-5). Also, NBS results updated data on the FAOD epidemiology, what is the prerequisite for follow-up and genetic counselling (2). In line with that, the Recommended Uniform Screening Panel (RUSP), created by the American College of Medical Genetics (6), claims that every NBS should include the following five FAOD, marked as the "core": carnitine uptake defect/carnitine transport defect (CUD), medium-chain acyl-CoA dehydrogenase deficiency (MCADD), very long-chain acyl-CoA dehydrogenase deficiency (VLCADD), long-chain L-3 hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) and trifunctional protein deficiency (TFPD). The decreased levels of free carnitine (C0), acetylcarnitine (C2), palmitoylcarnitine (C16), stearylcarnitine (C18) and oleylcarnitine (C18:1) indicate the presence of CUD. Screening results showing the increased levels of hexanoylcarnitine (C6) and octanoylcarnitine (C8), accompanied with the increased C8/C2 ratio are presumptive of MCADD. A raise in concentration of tetradecenoylcarnitine (C14:1), tetradecanoylcarnitine (C14), together with increased C14:1/C2 and C14:1/C16 ratios, represent the positive screening results for VLCADD. Finally, LCHADD and TFPD share the screening features: increased hydroxy stearylcarnitine (C18-OH), hydroxy palmitoylcarnitine (C16-OH) and hydroxy oleylcarnitine (C18:1-OH), supported with a raise in C18-OH/C18 and C16-OH/C16. Besides "core", these ACs and ratios allow for screening three more FAOD: carnitine palmitoyltransferase deficiency Ia and II (CPTD-I, CPTD-II) as well as carnitine:acylcarnitine translocase deficiency (CACTD). Results indicative for CPTD-I are high C0, decreased C14, C16, C18, C18:1 and (C16+ C18:1)/C2, whereby C0/(C16+C18) raises. Apart of C0, which is not significantly changed, the screening pattern, mutual for CPTD-II and CACTD, is inverse to the pattern indicative for CPTD-I (7, 8).

Assessment of the ACs level in the population of healthy newborns is the initial prerequisite for the establishment of a FAOD screening programme (8). Currently, such data for the Republic of Serbia are not available (9). Accordingly, our aim was to determine the level of abovementioned ACs and ratios, as well as to establish and validate their preliminary cut-offs for the healthy newborns.

PATIENTS AND METHODS

Newborns

We were including apparently healthy newborns from the Clinic for Gynecology and Obstetrics, Clinical Centre of Serbia, Belgrade, Serbia during the period August 2018-August 2019. The exclusion criteria were premature delivery, parenteral nutrition and transfusion (4). Ethics Committee of Clinical Center of Serbia approved the study (Permission No. 747/33/19.07.2018) and mother of each newborn signed the informed consent.

Samples

DBS samples were collected from heel, following the approved standard recommendations (10). Time of collection was between 48 and 72 hours after the delivery (4).

Laboratory methods

After punching a 3.2 mm DBS disk, ACs were extracted and derivatized with the commercial kit [Amino Acids and Acylcarnitines from Dried Blood (Chromsystems, Grüfelfing, Germany)], according to the manufacturer's instructions. ACs were analysed on the modular line consisting of Nexera XR LC-20 AD pump with the associated devices (Shimadzu, Kyoto, Japan) and 3200 Q TRAP LC-MS/MS System (AB Sciex, Framingham, MA, USA). For quality assurance purposes, we used commercial control material [MassCheck® Amino Acids, Acylcarnitines, Succinylacetone Dried Blood Spot Controls Level I and II (Chromsystems, Grüfelfing, Germany)].

Statistical analysis

The normality of ACs and ratios distribution was analysed with the Kolmogorov-Smirnov test. The Tukey procedure identified outliers i.e. results below lower quartile minus 3 times the interquartile range, or above the upper quartile plus 3 times the interquartile range. Distribution of the ACs values was graphically compared with the 1%, 50% and 99%ile for the worldwide population of healthy newborns obtained in the Region 4 Stork (R4S) Study and Centers for Disease Control project (R4S) (7). Recommendations from the CLSI guidelines (8) were the basis for establishing the cut-off values. Following the

statistical approach lower cut-offs were set at the 0.1%ile and higher at 99.9%ile value. The calculation-based approach was applied for the higher cut-offs only. They remained at the 99%ile of the tested population when it was higher than the worldwide 99%ile. Otherwise, they were calculated as 50%ile plus two times the range between the 99%ile and 50%ile, if no outliers were present, i.e. plus three times the mentioned range in case of the outliers' presence.

RESULTS

The study included a total of 1771 newborns. None of the ACs and ratios had the normal distribution (Table 1-3). Comparison with the distribution in worldwide normal population (7) showed that in 1.30% of the tested newborns C0 was below 11 μmol/L (range: 10.03-10.95 μmol/L). For C2, 0.51% results were below 10 μmol/L (range: 8.93-9.99 μmol/L) and one result (58.26 μmol/L) was above the world 99%-ile. Just two samples had C6 concentration reduced under 0.02 μmol/L (0.017 and 0.019 μmol/L). In case of C8, 0.68% of values were lower than 0.02 μmol/L (range: 0.013-0.019 μmol/L), while distribution of C14 and C14:1 results fitted between the 1% and 99%-ile of the worldwide normal population (7). The C16 was lower than 0.8 μmol/L in only one sample (0.65 μmol/L). Also, 6.32% of the C16-OH results were below 0.01 μmol/L (range: 0.001-0.009 μmol/L). There were no C18 lower than 0.31 μmol/L and four results exceeded 1.70 μmol/L (range: 1.71-1.78 μmol/L). The C18-OH concentrations also corresponded to the range between 1% and 99%-ile esteemed on the worldwide level (7). However, 2.65% of the tested newborns had C18:1 higher than 2.5 μmol/L (range: 2.51-2.81 μmol/L) and 8.19% of the C18:1-OH results remained under 0.01 μmol/L (range: 0.001-0.009 μmol/L).

The cut-offs established for ACs and ratios, together with the target ranges from the R4S project (7) are quoted in Table 1. The lower cut-offs for C0, C2, C0/(C16+C18) and (C16+C18:1)/C2 were in the agreement with the ranges, while in case of C16 (6.2%), C18 (6.4%) and C18:1(14.3%) the cut-offs were above the range. Among the higher cut-offs, when they were set using statistical approach, only the cut-off for C18 fitted into the target range. Much better concordance was present for the calculated cut-offs. Twelve of them was in the range: C0, C14:1, C16, C16-OH, C18-OH, C18:1, C0/(C16+C18), C8/C2, C14:1/C2, C14:1/C16, (C16+C18:1)/C2 and C18-OH/C18. Three cut-offs positioned above [C6 (16.7%), C18 (16.3%) and C18:1-OH (25.0%)] and the same number remained below the range [C8 (9.5%), C14 (8.0%) and C16-OH/C16 (3.0%)].

DISCUSSION

This paper reports the pioneer data for the Republic of Serbia, about the levels of ACs measured using tandem mass spectrometry in DBS of healthy newborns. In general, there is an acceptable concordance between the ACs distribution on the worldwide level (7) and in Serbian population. For the half of the ACs the levels fitted between the 1%ile and 99%ile of the worldwide population (7) or only individual deviations were present. Furthermore, in cases with more prevailing discrepancies between the distributions, the alterations' frequency of the individual ACs did not exceed 10%. Also, the intervals for the worldwide 1%ile i.e. 99%ile, calculated on the basis of the corresponding coefficient of variations (7), include the "extreme" values of all measured ACs except C16-OH, C18:1 and C18:1-OH. In the recent article about NBS experience in Turkey, the 1%-ile of normal population for C16-OH was 0 μmol/L [11], which is similar as in our study. For the purpose of screening for FAODs only increase in C18:1-OH levels represent a positive result (7), so the values that remained out of the worldwide distribution pattern, do not seem to merit a substantial attention. Nevertheless, the interpretation of C18:1 levels deserved additional caution, because it is the only AC with the distribution skewed to exceed the 99%-ile of the world population (7). In addition, the range of the "extreme" C18:1 results overlapped with the lower quartile of the values encountered in CACTD and CPTD-II (4, 7, 11). Since the results of other screening markers in the newborns with the "extreme" C18:1 results were not indicative for CACT or CPT-II (7), we attributed this finding to specificities of the tested population and set the higher cut-off for C18:1 at the 99%ile of the investigated population.

The applicability of the traditional statistical approach of setting the cut-offs at the 0.1% and 99.9%ile (8) was questionable. For the lower cut-offs the concordance with the worldwide target range (7) was rather acceptable. Nevertheless, that was not the case for almost all higher cut-offs, which were all below the range, thus raising the probability of false-positive results. Therefore, we referred to the recommendations for calculative adjustment of the cut-offs (7), similar to other authors (11), and achieved that more than a half of the calculated cut-offs were in the target range, established in the R4S project (7). This outcome was satisfactory, since McHugh et al. reported the overall agreement of 42% between the participants' individual cut-offs and corresponding range (7). Also, the differences in the structure and quantity of data between R4S project and our study, together with the consequential methodological specificities, could have an impact on the agreement rate. In R4S project the total amount of data, comprising almost 30 million of healthy and more than 10 000 newborns confirmed with certain metabolic disorders, allowed to calculate the target interval between the 99%ile of population with disorder and 1%ile of normal population in case of the low cut-offs or *vice versa* for the high cut-offs (7). However, we analyzed only samples from healthy newborns, so the additional calculative adjustments (7)were necessary to establish the cut-offs.

Even in cases when the calculated cut-offs remained out of the worldwide target, their applicability is not substantially jeopardized. Considering the low cut-offs for C16, C18 and C18:1, it is noteworthy that their decreased levels are not primary markers for any of the "core" FAOD (2, 4, 7,11-13). Also, almost all ratios including these ACs are within the

target ranges, thus additionally indicating the negligible significance of the discordance. Similar to our results, the cut-off for C6 in Italian population was 0.25 μmol/L (4), thus exceeding the R4S Project range (7). Also, the discrepancy with the target range was noted for C8 cut-off, likewise the population of the Republic of Slovenia, one of the countries neighboring the Republic of Serbia (14). The position of cut-off for C14 slightly below the target range may be attributed to the additional adjustments of the worldwide target range to improve specificity and minimize the probability of false positive results (7). In context of interpretation, the cut-off of C16-OH/C16, positioned below the target range, seems to be successfully "counterbalanced" with the cut-off of C16, placed within the corresponding range. C18 is the only of the investigated AC for which the preliminary high cut-off should be set at 99.9%ile. Finally, the cut-off for C18:1-OH slightly exceeding the rather narrow target range may not be surprising, specially taking into the account that the same cut-off is also used for NBS in Czech Republic (13) and Turkey (11).

The utmost outcome of our study are the preliminary interpretative patterns, necessary for successful future implementation of NBS for FAOD in Serbia. In case of MCADD, the most frequently FAOD (15), the pattern consists of C6>0.28 μmol/L, C8>0.19 μmol/L and C8/C2>0.011. For CUD the primary screening marker will be C0<10.10 μmol/L, supported with C2<9.28 μmol/L, C16<0.85 μmol/L, C18<0.33 μmol/L and C18:1<0.56 μmol/L. Presence of VLCADD can be suspected when the results show C14:1>0.54 μmol/L, C14>0.46 μmol/L, C14:1/C2>0.024 and C14:1/C16>0.147. Confirmatory investigation of the remaining two "core" FAOD, LCHADD or TFPD, will be necessary in newborn with C18-OH>0.08 μmol/L, C16-OH>0.11 μmol/L, C18:1-OH>0.10 μmol/L, C18-OH/C18>0.108 and C16-OH/C16>0.032. The results showing C0>64.43 μmol/L, C0/(C16+C18)>27.55 and (C16+C18:1)/C2<0.1 enable differentiation of CPTD-I from CUD. CPTD-II and CACTD can be distinguished from VLCAD by the finding of C16>6.37 μmol/L, C18>1.75 μmol/L and C18:1>2.64 μmol/L. The C0/(C16+C18) <2.82 and (C16+C18:1)/C2>0.393 are the features providing the distinction of CPTD-II and CACTD from CPTD-I.

Significantly lower number of participating newborns in comparison with the R4S project (7) and several individual studies in Caucasian populations (11-13) may represent a limitation of our study. Nevertheless, number of analyzed samples fulfills the CLSI recommendations, quoting "hundreds or even thousands" as necessary (8). Also, the R4S project (7) and the abovementioned studies collate experiences of comprehensive NBS implementation lasting at least two years. On the contrary, only the population of healthy newborns was included in our pilot study, ACs and ratios were analyzed and their cut-offs established, thus paving the possibility for the future introduction of NBS for FAOD in Serbia. Their application in further, high volume testing will provide benefits for several healthcare areas (e.g. epidemiology of FAOD in the Republic of Serbia, treatment policies, genetic counseling, etc) (16).

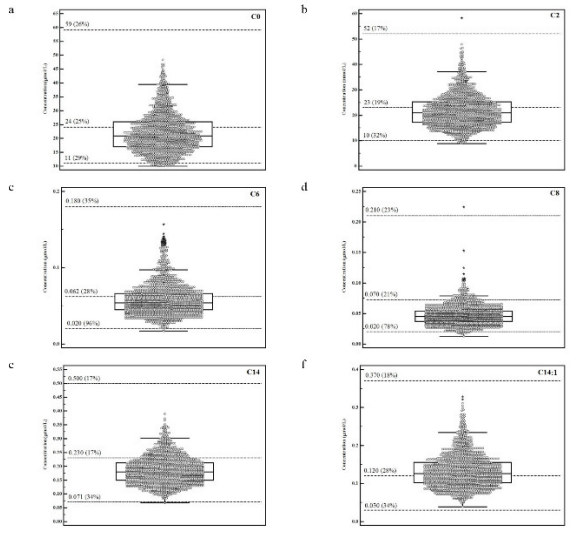

Figure 1. The concentration of (a) free carnitine (C0), (b) acetylcarnitine (C2), (c) hexanoylcarnitine (C6), (d) octanoylcarnitine (C8), (e) tetradecanoylcarnitine (C14) and (f) tetradecenoylcarnitine (C14:1). Box represents the values from the lower to upper quartile. The middle line in the box represents the median. A line extends from the minimum to the maximum value. Circles outside the minimum and maximum value are outside values i.e. below the lower quartile minus 1.5 times the interquartile range, or above than the upper quartile plus 1.5 times the interquartile range. Asterisks represent outliers. The dashed and dotted line gives 1%, 50%, and 99%ile, estimated for the worldwide population of healthy newborns, with the CV in parenthesis [7].

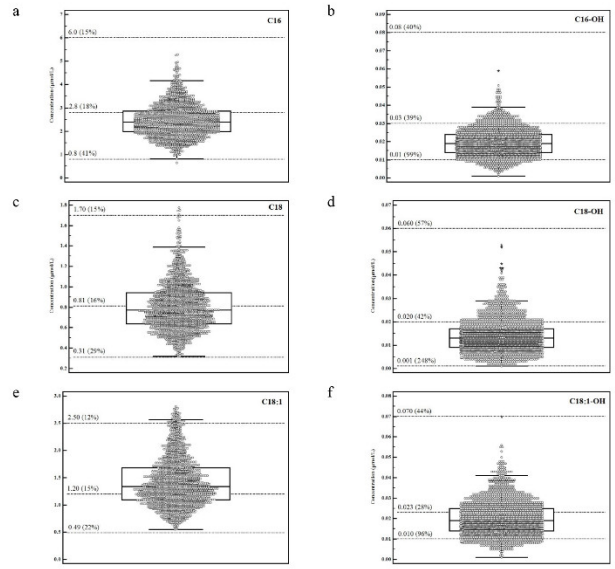

Figure 2. The concentration of (a) palmitoylcarnitine (C16), (b) hydroxy palmitoylcarnitine (C16-OH), (c) stearylcarnitine (C18), (d) hydroxy stearylcarnitine (C18-OH), (e) oleylcarnitine (C18:1) and (f) hydroxy oleylcarnitine (C18:1-OH). Box represents the values from the lower to upper quartile. The middle line in the box represents the median. A line extends from the minimum to the maximum value. Circles outside the minimum and maximum value are outside values i.e. below the lower quartile minus 1.5 times the interquartile range, or above than the upper quartile plus 1.5 times the interquartile range. Asterisks represent outliers. The dashed and dotted line gives 1%, 50%, and 99%ile, estimated for the worldwide population of healthy newborns, with the CV in parentheses [7].

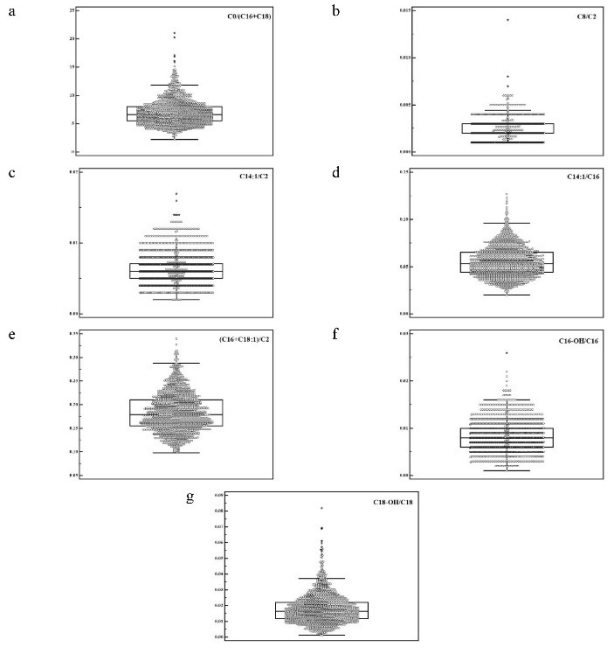

Figure 3. Acylcarnitines' ratios - (a) C0/(C16+C18), (b) C8/C2, (c) C14:1/C2, (d) C14:1/C16, (e) (C16+C18:1)/C2, (f) C16–OH/C16 and (g) C18–OH/C18. Box represents the values from the lower to upper quartile. The middle line in the box represents the median. A line extends from the minimum to the maximum value. Circles outside the minimum and maximum value are outside values i.e. below the lower quartile minus 1.5 times the interquartile range, or above than the upper quartile plus 1.5 times the interquartile range. Asterisks represent outliers.

CONCLUSION

The distribution of the ACs levels, measured using tandem mass spectrometry, in DBS from healthy newborns in the Republic of Serbia substantially matches the worldwide estimates. Also, the established cut-off values were successfully verified in comparison with the worldwide ranges and data from individual studies in several Caucasian populations.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Declaration of Helsinki and Ethics Committee of Clinical Center of Serbia approved the study (Permission No. 747/33/19.07.2018). Mother of each newborn signed the informed consent.

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None.

CONFLICT OF INTEREST

The all authors declare that there no conflict of interests.

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