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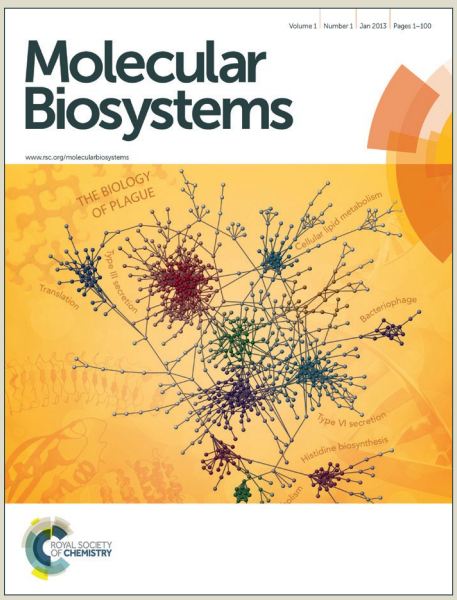

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Female and male human babies have distinct blood metabolomic patterns

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Abstract

A sex-gender approach in laboratory medicine is scarce; furthermore, the influence of sex on acylcarnitines and amino acids levels at birth has not been thoroughly investigated, even if sex impacts on newborn screening. We aimed to establish the influence of sex on amino acids and acylcarnitines levels in male and female newborns.

Amino acids and acylcarnitines were analysed in dried blood spots using tandem mass spectrometry in male and female newborns. Data were analysed before and after body weight correction also using principal components analysis.

This retrospective analytical study showed that females had small but significantly higher levels of amino acids and the correction for body weight amplified these differences. Acylcarnitines were overall higher in males before body weight correction with the exception of isovalerylcarnitine+methylbutyrylcarnitine (C5), which was significantly higher in females. Body weight correction decreased the sex differences in C5. Principal components analysis showed that both amino acids and acylcarnitines were necessary to describe the model for females, whereas only acylcarnitines were required for males.

These metabolomics data underline the importance of including sex as a variable in future investigations on circulating metabolites; the existence of sex differences highlights the need for setting distinct reference values for female and male neonates in metabolites concentration.

Keywords: neonates; metabolomics; amino acids; acylcarnitines; principal component analysis; sex differences

Introduction

Children and babies are not simply small adults, and female babies are not just leaner and smaller versions of male babies [1]. Moreover, it is well-established that sex differences start in utero [2-4], even if, many care providers are not aware of these differences and have not adopted a sex-gender approach in laboratory medicine that would provide appropriate diagnostic services for both sexes. In this context, it is not surprising that reliable reference intervals for males and females have not been extensively reported. However, a previous study showed that the baseline expression of toll-like receptor 4 in children from birth to 18 years of age is elevated in males compared with females [5]. Another study showed that S100B, a protein involved in cerebral development, is higher in the urine of female babies than in that of male babies [6].

A particular problem stemming from the lack of awareness of sex differences is reliability of newborn screening for inborn errors of metabolism, which is essential to reach a precocious diagnosis of aminoacidopathies, organic acidemias, and fatty acid oxidation disorders [7-9]. Reference ranges of blood amino acids and acylcarnitines are required for the diagnosis and management of inherited metabolic disorders and can significantly impact the clinical decision-making and quality of patient care. However, the influence of sex on acylcarnitines and amino acids levels at birth, to our knowledge, has not been thoroughly investigated [10-13]. The levels of amino acids and acylcarnitines are different in adult and healthy men and women, and oral contraceptive use in women can influence these parameters [14-16].

Therefore, our goal was to establish the influence of sex on amino acids and acylcarnitines levels from a dried blood spot (DBS) using LC-MS/MS. This diagnostic method permits the pre-symptomatic identification of several metabolic disorders before irreversible organ damage occurs [17]. Data were also subjected to principal components analysis (PCA), which

accounts for the loading coefficient of each variable and enables the correlation of the key components of a cluster [18, 19].

Results

As expected, male infants were heavier than female infants (median values: 3,340 g vs 3,150 g, respectively; $P < 0.001$),in line with data from the literature [20, 21]. Moreover, there was a significant positive correlation between weight and gestational age in both males and females (Fig.2 A, B).

Amino acid levels

Considering that males and females have significantly different body weights, the data are presented before and after body weight correction (Table 1). Overall, female newborns had a small but significantly higher level of amino acids than male babies when values were analysed before correction. In particular, female infants had significantly higher levels of alanine, methionine, glycine, valine, and tyrosine (4.4%, 2.9%, 1.9%, 1.8% and 2.2% increase, respectively) (Table 1). Leucine+isoleucine, phenylalanine, aspartic acid, arginine, glutamate, citrulline and ornithine were not significantly divergent between sexes (Table 1). The correction for body weight amplified the differences between males and females for all of the measured amino acids with the exception of arginine (Table 1).

Acylcarnitine profile

The serum levels of free carnitine (Fig.3A) and total esterified carnitine (Fig.3B) were significantly higher in males than females. The ratio of esterified/free carnitine did not significantly diverge (Fig.3C). When these previous values were corrected for neonate body weight, the sex differences were mildly reduced.

The saturated acylcarnitines, acetylcarnitine (C2), propionylcarnitine (C3), hexanoylcarnitine (C6), octanoylcarnitine (C8), decanoylcarnitine (C10), dodecanoylcarnitine (C12), tetradecanoylcarnitine (C14), hexadacanoylcarnitine (C16), octadecanoylcarnitine (C18), malonylcarnitine (C3DC), methylmalonilcarnitine (C4DC), glutarylcarnitine (C5DC), methylglutarylcarnitine (C6DC), octanedioylcarnitine (C8DC) and decanedioylcarnitine (C10DC), were significantly elevated in male infants compared with females, and the highest difference (-14%) was observed for C14 (Table 2). Isovalerylcarnitine/2-methylbutyrylcarnitine (C5) is the only saturated acylcarnitine that was significantly higher in female neonates (+22.2%), and butyrylcarnitine/isobutyrylcarnitine (C4) did not diverge between sexes (Table 2, bold). When these values were corrected for neonate body weight, the observed sex differences in C2, C3, C6, C18, C3DC, C4DC, C5DC, C6DC disappeared, and the sex differences in C2, C3, C5, C6, C16, C8DC, C10DC were reduced. Notably the correction for body weight evidenced a sex differences in C4 levels being higher in female neonates (+12.5%) (Table 2, bold).

As shown in Table 3, the analysed hydroxylated acylcarnitines were significantly higher in males than in females; however, the sex differences were small and ranged from -2.7% to -10%. When these values were corrected for body weight, sex differences in 3-hydroxyisovalerylcarnitine/3-hydroxy-2-methylbutyrylcarnitine (C5OH), 3-hydroxyhexanoylcarnitine (C6OH), 3-hydroxydodecanoylcarnitine (C12OH), 3-hydroxyhexadecenoylcarnitine (C16:1OH) disappeared, sex differences in 3-hydroxybutyrylcarnitine (C4OH), 3-hydroxyhexadecanoylcarnitine (C16OH), 3-hydroxyoctadecenoylcarnitine (C18:1OH) levels were reduced, and sex differences in 3-hydroxytetradecanoylcarnitine (C14OH) increased from -6.9 to -11.1%.

The unsaturated acylcarnitines hexenoylcarnitine (C6:1) and octenoylcarnitine (C8:1) were significantly elevated in females compared with males (Table 4). In contrast decenoylcarnitine (C10:1), dodecenoylcarnitine (C12:1), tetradecenoylcarnitine (C14:1), tetradecadienoylcarnitine (C14:2), hexadecenoylcarnitine (C16:1), octadecenoylcarnitine (C18:1) and octadecadienoylcarnitine (C18:2) were significantly higher in male infants than female infants (Table 4). Tiglylcarnitine (C5:1) and decadienoylcarnitine (C10:2) did not diverge between sexes. The correction for body weight showed that the sex differences in C12:1 and C14:2 levels disappeared and the differences in C16:1, C18:1, C18:2, C14:1, C10:1 levels were reduced. The correction for body weight showed that C5:1 and C10:2 became significantly elevated in female babies.

Correlations

Free carnitine levels were positively related to gestational age both in males and females (Fig.4A, B). No correlation was found between free carnitine and neonate body weight in males or females ($r = -0.0396$, $P = 0.0879$ and $r = 0.0105$, $P = 0.654$, respectively).

Moreover, a multiple linear regression analysis revealed that C2, C3, C8, C10, C14, C18, C3DC, C5DC, C8DC, C6OH, C18OH, C5:1, C6:1, C8:1, C14:1, C14:2, C16:1, C18:1, and C18:2 predicted the values of free carnitine in the female population, and C2, C3, C5, C8, C5:1, C14, C18, C3DC, C14OH, C16OH, C6:1, C14:1, C14:2, C16:1, C18:1, C18:2, and C18OH predicted the values of free carnitine in the male population.

After body weight correction, free carnitine levels were positively correlated with gestational age only in males (Fig.4C, D). The multiple linear regression performed after weight correction revealed that multiple carnitines accounted for the ability to predict free carnitine in females, including C2, C3, C5:1, C8, C3DC, C10:1, C10, C5DC, C14:2, C14:1, C14, C8DC, C16:1, C10DC, C18:1, C18, C12OH, C6:1, C8:1, C18:2, C18OH, and

C16:1OH. C2, C3, C5:1, C5, C5OH, C8, C3DC, C14:2, C14:1, C14, C16:1, C10DC, C18:1, C18, C12-OH, C14-OH, C6:1, C18:2, C18OH, C6OH, and C16:1OH predicted the body weight-corrected values of free carnitine in the male population.

PCA

The simultaneous detection of amino acids and acylcarnitines enables us to perform a PCA analysis to identify the main components of the variance within each sex. The number of components, in both cases, was based on the scree plot. The components prior to the step of the scree plot were selected. In our sample set, we observed that the model was sufficiently described using 5 components for males (which explained 58.9% of variance) and 4 components (which explained 48% of variance) for females. In males, the PC1 (27% of explained variance) was described according to the value of carnitines (C2, C6, C8, C5:1, C3DC, C10, C10:1, C5DC, C12, C12:1, C6DC, C14, C14:1, C14:2, C8DC, C16:1, C16, C10DC, C16OH, C18, C18:1OH, C4OH, C12OH, C16:1OH, C10:2, C6OH, and total esterified carnitines) (Fig.5A). The weight of the variables on the other component was very limited. Confirming the findings of the univariate analysis, there was a clear difference between males and females in terms of acylcarnitine and amino acid concentrations.

In females, the first principal component (PC1) accounted for 24% of explained variance and was determined according to the value of carnitines (C0, C2, C4, C6, C8, C3DC, C10, C5DC, C12, C12:1, C14, C14:1, C8DC, C16:1, C16, C10DC, C16OH, C18, C18:1, C4OH, C12OH, C16:1OH, and total esterified carnitines) and the second principal component (PC2) was determined according to the value of amino acids (leucine+isoleucine, methionine, tyrosine, aspartic acid, glutamate, ornithine, citrulline and arginine) (Fig.5B).

The weight of the variables on the other two components was quite limited (data not shown).

When the PCA was performed on the variables after body weight correction, it was observed that the model was sufficiently described using 5 components (which explained 70% of variance) for females and 6 components for males (which explained 66% of variance). In males, the PC1 explained 30.8% of the variance and was described according to the values of carnitines (C0, C2, C4, C5:1, C6, C5OH, C8, C3DC, C10, C10:1, C4DC, C5DC, C12, C12:1, C6DC, C14, C14:1, C14:2, C8DC, C16:1, C16, C16OH, C18, C18:1, C18:1OH, C4OH, C12OH, C14OH, C10:2, and total esterified carnitines). The weight of the variables on the other component was very limited (Fig.5C).

In females, the PC1 accounted for 40% of the variance and was mainly determined according to the value of carnitines (C5:1, C5, C6, C5OH, C8, C3DC, C10:1, C10, C4DC, C5DC, C12:1, C12, C6DC, C14:2, C14:1, C14, C8DC, C16:1, C16OH, C18:1OH, C4-OH, C12-OH, C6:1, C8:1, C10:2, C18OH, C6OH, and C16:1OH), methionine and arginine. The PC2 was determined according to values from alanine, leucine+isoleucine, phenylalanine, glutamate, glycine, C0, C2, C16, C18, C18:1 and total esterified carnitines (Fig.5D). For the other 3 components, the weight of the variables was quite limited (data not shown).

Discussion

The diagnosis and management of inherited metabolic disorders requires appropriate reference ranges of blood amino acids and acylcarnitines to improve clinical decision-making and the quality of patient care. The median values found for different acylcarnitines and amino acids are comparable to the standard reference values measured using tandem mass spectrometry and cited in the literature [10]. Here, we have extended

these previous findings because we show that many of studied parameters are sex-dependent and body weight-dependent. In particular, female neonates have higher levels of alanine, glycine, methionine, valine and tyrosine. Free carnitine and, overall, acylcarnitines, with the exception of C5, C6:1, C8:1, are higher in male neonates than in female neonates. The observed sex differences are statistically significant although relatively small. Some of sex differences have been previous reported in different populations [11-13].

Here we confirm that body weight is sex divergent for full-term newborns as body composition [22]. The observed sex differences in acetylcarnitines and amino acids are dependent on body weight. In fact, the correction for body weight increases the sex differences in amino acid levels with the exception of arginine. The fact that arginine is not influenced by sex confirms data obtained in umbilical vein plasma blood cords of premature neonates [23]. The correction for body weight exerted a more variable effect on acylcarnitines; however, it overall decreased the sex differences. In particular, before body weight correction, C2, C3, C8, C10, C14, C18, C3DC, C5DC, C8DC, C6OH, C18OH, C5:1, C6:1, C8:1, C14:1, C14:2, C16:1, C18:1, and C18:2 predicted the values of free carnitine in the female population. After body weight correction, the following acylcarnitines accounted for the ability to predict free carnitine in females: C2, C3, C5:1, C8, C3DC, C10:1, C10, C5DC, C14:2, C14:1, C14, C8DC, C16:1, C10DC, C18:1, C18, C12OH, C6:1, C8:1, C18:2, C18OH, and C16:1OH. In male neonates, C2, C3, C5, C8, C5:1, C14, C18, C3DC, C14OH, C16OH, C6:1, C14:1, C14:2, C16:1, C18:1, C18:2, and C18OH predicted the values of free carnitine before body weight correction. However, C2, C3, C5:1, C5, C5OH, C8, C3DC, C14:2, C14:1, C14, C16:1, C10DC, C18:1, C18, C12-OH, C14-OH, C6:1, C18:2, C18OH, C6OH, and C16:1OH predicted the body weight-corrected values of free carnitine in the male population. Previously, Gucciardi et al., 2014

[24] indicated that pre-term neonates showed a correlation between body weight and acylcarnitine. However, it is not known whether the differences due to birth weight are long lasting.

The influence of body weight on sex differences is confirmed by the observation that free carnitine levels are positively correlated with gestational age in the males, while there is a positive correlation in both males and females in the absence of a body weight correction.

For a comprehensive overview of the system as required by sex studies and to assess the quality of the data, we utilised multivariate data analyses. PCA is an analysis strategy that reduces the multidimensionality of a dataset. PCA correlations were modified by sex and weight in females, and both amino acids and acylcarnitines were necessary to describe the model. Only acylcarnitines were required to describe the model in males. These data also suggest that the free amino acids present in the blood, although they represent only a very small proportion of the body's total amino acid pool, play a special role in the development of sex differences, especially when body weight is considered. Indeed, males and females follow a divergent sex-specific trajectory, suggesting that metabolomic analysis combined with PCA are effective tools in differentiating sex differences in full-term neonates.

Conclusions

In conclusion, these results confirm that sex differences can start early in life [7-9, 25] and indicate that sex and sex differences should be considered in future investigations on circulating metabolites at any age. These emerging sex differences show that individual reference values for female and male neonates are very much needed for the improvement of our understanding of sex-related differences.

Experimental

Study population

A total of 12 amino acids and 37 acylcarnitines were measured using a dried blood spot from newborns to detect errors of metabolism. We constructed a database that included a total of 1856 male and 1824 female newborns by selecting neonates who were born between the 37th and the 42nd week of gestation and weighed between 2,430-4,050 g and 2,550-4,190 g for females and males, respectively. The above weights represent the 10th and 90th centile described in Ines Charts [26].

All experiments were performed in compliance with the relevant laws and institutional guidelines. Dried blood spots were analyzed in our laboratory, but they were collected from hospitals participating to the neonatal screening program of Campania Region and dealing with collecting the informed consent of the parents of newborns.

Reagents and internal standards

High performance liquid chromatography (HPLC) grade acetonitrile, formic acid and methanol were purchased from Mallinckrodt Baker B.V. (Deventer, Netherlands), and 3N HCl in n-butanol was purchased from REGIS Technologies Inc. (Morton Grove, USA). Labelled standards of amino acids and acylcarnitines were purchased from Cambridge Isotope Laboratories (Andover, USA).

Sample preparation for LC-MS/MS

The blood sample was collected between 48 and 72 h of life by a heel prick that was spotted on a Schleicher & Schuell 903 grade filter paper sampling card (Whatman, Dassel, Germany) and dried overnight at room temperature. DBS were delivered daily to the laboratory, and the blood samples were processed as previously described [17, 27-29]. Briefly,

a DBS was punched (3.2 mm) into a 1.5 mL tube and 200 µL of methanol containing labelled standards was added. The standard concentrations were in the 500-2500 µmol/L range for amino acids and in the 7.6-152 µmol/L range for acylcarnitines. To obtain working solutions, daily dilutions (1:200) were made using methanol. After 20 min at room temperature on an orbital shaking system, the samples were dried under nitrogen flow at 50 °C. The extracted acylcarnitines and amino acids were derivatised to butyl esters with 80 µL of 3N HCl in n-butanol at 65 °C for 25 min. After derivatisation, the samples were dried under nitrogen at 50 °C and then resuspended in 300 µL of acetonitrile/water (70:30) containing 0.05% formic acid. A 40 µl aliquot of the diluted samples were injected in the flow injection analysis mode for the MS/MS experiments.

LC-MS/MS analysis

An API 4000 triple quadrupole mass spectrometer (Applied Biosystems-Sciex, Toronto, Canada) coupled with an Agilent high performance liquid chromatograph from the 1200 series (Agilent Technologies, Waldbronn, Germany) was used. Three scanning modes were used for each examination. The Turbo Spray Ion source was operated in positive ion mode with a needle potential of +5900 V. Mass calibration and resolution adjustment on the resolving quadrupole were performed automatically using a 1-4 mol/L solution of polypropylene glycol (PPG) introduced via the built-in infusion pump. The peak width was set on both resolving quadrupoles at 0.7 Th (measured at half height) for all MS and MS/MS experiments. The declustering potential and collision energy were optimised for amino acids and acylcarnitines using Analyst 1.4 software. The mobile phase flow rate was 50 µL/min using acetonitrile/water (70:30) containing 0.05% formic acid. Data were quantitatively analysed with ChemoView v1.2 software by comparing the signal intensities of the analyte and its corresponding internal standard or the standard next to the spectrum.

Fig.1 shows a typical acquisition for each sample. The precursor ion scan (Fig.1A) was used for the quantification of acylcarnitines, the neutral loss scan was used for the quantification of amino acids (Fig.1B), and a reaction monitoring (MRM) experiment was used to quantitate glycine, ornithine, arginine, and citrulline (Fig.1C). Data from the analysis of the samples and disease ranges obtained from the Region 4 collaborative Project (www.clir-r4s.org) were used to determine cut-offs for each analyte and analyte ratio. The quality control samples were provided by the Centers for Disease Control and Prevention (Atlanta, USA).

Statistical analysis

Concentrations are reported as μM because this unit is commonly used by laboratories that conduct newborn screening. All data are displayed as the median value and the range. Statistical analysis was performed by comparing the 2 experimental groups using a Mann-Whitney rank sum test. The distribution of samples was evaluated using the Kolmogorov-Smirnov and Shapiro tests. A $P < 0.05$ was considered statistically significant. The strength of association between newborn weight and gestational age and between free carnitine and gestational age was analysed with the Spearman Product Moment correlation coefficient. A multiple linear regression analysis was performed to predict the association of free carnitine (dependent variable) with saturated, hydroxylated and unsaturated carnitines (independent variables) in the studied populations using SigmaStat software. Moreover, principal component analysis (PCA) was performed using R software. This multivariate statistical procedure enabled the analysis of the relationships between parameters and the behaviour of this multicomponent system as a network. In fact, when a large series of dosages are collected, the goal of PCA is to develop a model with a smaller number of

artificial analyses (principal component) that accounts for most of the variance in the set of observed variables.

Abbreviations

DBS Dried blood spot

HPLC High performance liquid chromatography

PPG Polypropylene glycol

MRM Multiple Reaction Monitoring

PCA Principal component analysis

C0 Free carnitine

C2 acetylcarnitine

C3 propionylcarnitine

C5 isovalerylcarnitine+methylbutyrylcarnitine

C6 hexanoylcarnitine

C8 octanoylcarnitine

C10 decanoylcarnitine

C12 dodecanoylcarnitine

C14 tetradecanoylcarnitine

C16 hexadacanoylcarnitine

C18 octadecanoylcarnitine

C3DC malonylcarnitine

C4DC methylmalonilcarnitine

C5DC glutarylcarnitine

C6DC methylglutarylcarnitine

C8DC octanedioylcarnitine

C10DC decanedioylcarnitine

C5 isovalerylcarnitine/2-methylbutyrylcarnitine

C4 butyrylcarnitine/isobutyrylcarnitine

C5OH 3-hydroxyisovalerylcarnitine/3-hydroxy-2-methylbutyrylcarnitine

C6OH 3-hydroxyhexanoylcarnitine

C12OH 3-hydroxydodecanoylcarnitine

C16:1OH 3-hydroxyhexadecenoylcarnitine

C4 OH 3-hydroxybutyrylcarnitine

C16OH 3-hydroxyhexadecanoylcarnitine

C18:1OH 3-hydroxyoctadecenoylcarnitine

C14OH 3-hydroxytetradecanoylcarnitine

C6:1 hexenoylcarnitine

C8:1 octenoylcarnitine

C10:1 decenoylcarnitine

C12:1 dodecenoylcarnitine

C14:1 tetradecenoylcarnitine

C14:2 tetradecadienoylcarnitine

C16:1 hexadecenoylcarnitine

C18:1 octadecenoylcarnitine

C18:2 octadecadienoylcarnitine

C5:1 tiglylcarnitine

C 10:2 decadienoylcarnitine

Author contributions

FF, IC and MR conceived and designed the experiments and wrote the manuscript. MR, ES and MC performed the metabolomic analysis. IC and VM performed statistical analysis All authors read and approved the final manuscript.

Conflicts of Interest: The authors have no potential conflicts of interest to declare.

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Table 1. Amino acid levels stratified according to sex before and after body weight correction

	Male babies	Female babies	% of variation	Males after body weight correction	Females after body weight correction	% of variation
Alanine	125. 78 (56.54-393.26)	131.29 (39.30-288.51)**	+4.4	38.10 (15.5-124.8)	40.97 (12.3-115.1) **	+7.5
Leucine+ Isoleucine	94.24 (40.88-241.04)	94.44 (40.45-280.96)	+0.2	28.58 (12.82-80.88)	29.99 (11.68-98.58)**	+4.9
Methionine	11.49 (3.98-29.21)	11.83 (4.66-72.00) *	+2.9	3.47 (1.31-8.99)	3.74 (1.54-24.00)**	+7.8
Phenylalanine	30.07 (15.66-63.88)	29.79 (14.12-138.08)	-0.9	9.08 (4.26-19.02)	9.34 (4.09-40.73)**	+2.9
Glycine	192.15 (97.08-459.82)	195.82 (99.98-431.77)*	+1.9	57.91 (25.15-154.06)	61.58 (28.91-170.82)**	+6.3
Valine	91.68 (39.04-253.20)	93.37 (48.91-281.66)*	+1.8	27.84 (10.71-71.94)	29.46 (13.99-83.08)**	+5.8
Tyrosine	57.15 (19.53-232.17)	58.44 (15.84-271.29)*	+2.2	17.24 (5.59-72.75)	18.49 (4.95-86.40)**	+7.2
Aspartic acid	18.96 (7.25-68.10)	18.89 (7.25-91.85)	-0.4	5.68 (1.91-20.33)	5.94 (2.35-33.64)**	+4.6
Arginine	2.91 (0.65-19.99)	2.84 (0.69-69.10)	-2.4	0.87 (0.16-6.41)	0.91 (0.19-23.03)	+4.6
Glutamate	216.69 (90.88-582.18)	220.39 (80.94-614.93)	+1.1	65.68 (25.58-202.26)	70.57 (26.98-190.91)**	+7.4
Citrulline	7.57 (2.43-44.68)	7.74 (2.45-114.73)	+2.2	2.27 (0.81-15.56)	2.45 (2.28-36.93)**	+7.9
Ornithine	23.46 (7.14-111.68)	23.74 (7.39-107.99)	+1.2	7.12 (2.02-34.47)	7.58 (0.82-33.25)**	+6.5

Values (μM) are expressed as the median (range) for 1856 male neonates and 1824 female neonates.

* represents statistical significance: * P<0.05 and ** P < 0.001. The 5th and the 6th columns show the values of the parameters after correction for the weight of the neonates.

Table 2. Saturated acylcarnitine levels stratified according to sex before and after body weight correction

	Male babies	Female babies	% of variation	Males after body weight correction	Females after body weight correction	% of variation
C2	26.99 (69.79-9.60)	25.19 (6.63-90.24)**	-6.7	8.18 (2.88-23.09)	7.94 (2.21-27.43)	-2.9
C3	2.04 (0.29-10.26)	1.98 (0.40-9.66)*	-2.9	0.61 (0.09-3.36)	0.63 (0.12-3.02)	+3.3
C4	**0.27 (0.08-1.16)**	**0.27 (0.08-1.78)**	**0**	**0.08 (0.02-3.37)**	**0.09 (0.02-0.59)****	**+12.5**
C5	0.09 (0.03-0.62)	0.11 (0.03-1.32)**	+22.2	0.029 (0.01-0.20)	0.033 (0.01-0.44)**	+13.8
C6	0.04 (0.007-0.45)	0.038 (0.01-1.36)**	-5.0	0.01 (0.002-0.14)	0.01 (0.002-0.45)	0.0
C8	0.083 (0.02-0.88)	0.076 (0.01-1.16)**	-8.4	0.025 (0.006-0.28)	0.024 (0.006-0.39)*	-4.0
C10	0.1 (0.02-0.66)	0.09 (0.02-1.38)**	-10.0	0.030 (0.026-0.21)	0.029 (0.07-0.46)*	-3.3
C12	0.113 (0.62-0.03)	0.105 (0.02-1.36)**	-7.1	0.034 (0.01-0.19)	0.033 (0.008-0.45)*	-2.9
C14	0.21 (0.05-0.86)	0.18 (0.06-1.32)**	-14.3	0.062 (0.01-0.27)	0.057 (0.02-0.44)**	-8.1
C16	2.97 (0.72-8.26)	2.69 (0.64-7.69)**	-9.4	0.90 (0.21-2.80)	0.84 (0.22-2.72)**	-6.7
C18	0.84 (0.16-2.78)	0.79 (0.19-2.54)**	-5.9	0.25 (0.06-0.86)	0.25 (0.05-0.85)	0.0
C3DC	0.048 (0.01-0.58)	0.046 (0.0-1.04)**	-4.2	0.014 (0.003-0.18)	0.014 (0.00-0.35)	0.0
C4DC	0.23 (0.05-0.91)	0.21 (0.05-1.50)**	-8.7	0.07 (0.02-0.33)	0.07 (0.02-0.50)*	0.0
C5DC	0.053 (01-0.79)	0.050 (0.0-1.59)**	-5.7	0.016 (0.004-0.25)	0.016 (0.00-0.53)	0.0
C6DC	0.033 (0.0-0.49)	0.032 (0.0-1.26)*	-3.0	0.01 (0.00-0.16)	0.01 (0.0-0.42)	0.0
C8DC	0.037 (0.005-0.69)	0.035 (0.0-1.18)**	-5.4	0.0112 (0.002-0.22)	0.0110 (0.0-0.39)*	-1.2
C10DC	0.66 (0.0-1.85)	0.62 (0.0-2.36)*	-6.1	0.20 (0.0-0.66)	0.19 (0.0-0.79)*	-5.0

Values (μM) are expressed as the median (range) for 1856 male neonates and 1824 female neonates .

* represents statistical significance: * P<0.05 and ** P < 0.001. The 5th and the 6th columns show the values of the parameters after correction for the weight of the neonates.

Table 3. Hydroxylated acylcarnitines levels stratified according to sex before and after body weight correction

	Male babies	Female babies	% of variation	Males after body weight correction	Females after body weight correction	% of variation
C4OH	0.104 (0.02-0.46)	0.097 (0.02-1.27)**	-6.7	0.031 (0.007-0.15)	0.030 (0.008-0.42)*	-3.2
C5OH	0.109 (0.04-0.54)	0.106 (0.03-1.42)*	-2.7	0.03 (0.01-0.17)	0.03 (0.009-0.47)	0.0
C6OH[a]	0.027 (0.0-0.32)	0.025 (0.007-1.19)**	-7.4	0.007 (0.0-0.10)	0.007 (0.0-0.40)	0.0
C12OH	0.027 (0.0-1.0)	0.025 (0.0-1.20)**	-7.4	0.008 (0.0-0.31)	0.008 (0.0-0.40)	0.0
C14OH	0.029 (0.0-1.16)	0.027 (0.0-0.54)**	-6.9	0.009 (0.0-0.36)	0.008 (0.0-0.19)**	-11.1
C16OH	0.029 (0.0-1.02)	0.027 (0.0-2.65)**	-6.9	0.0087 (0.0-0.32)	0.0085 (0.0-0.88)*	-2.3
C18OH	0.020 (0.0-0.96)	0.018 (0.0-2.29)*	-10	0.006 (0.0-0.30)	0.006 (0.0-0.76)**	0.0
C16:1OH[a]	0.044 80.0-0.39)	0.041 (0.0-2.47)**	-6.8	0.010 (0.0-0.11)	0.010 (0.0-0.82)	0.0
C18:1OH	0.025 (0.0-0.91)	0.023 (0.0-2.22)**	-8.0	0.008 (0.0-0.28)	0.007 (0.0-0.74)*	0.0

Values (μM) are expressed as the median (range) for 1856 male neonates and 1824 female neonates.

* represents statistical significance: * P<0.05 and ** P < 0.001. The 5th and the 6th columns show the values of the parameters after correction for the weight of the neonates. [a] n=1615 males and n=1569 females for C6OH and C16:1 OH.

Table 4. Unsaturated acylcarnitine levels stratified according to sex before and after body weight correction

	Male babies	Female babies	% of variation	Males after body weight correction	Females after body weight correction	% of variation
C5:1	0.03 (0.0-0.43)	0.03 (0.0-1.32)	0.0	0.007 (0.00-0.13)	0.008 (0.002-0.44)**	+14.3
C6:1	0.103 (0.0-0.48)	0.110 (0.0-1.26)**	+6.8	0.031 (0.0-0.15)	0.036 (0.0-0.42)**	+16.1
C8:1	0.100 (0.0-0.82)	0.105 (0.02-0.12)**	+5	0.030 (0.0-0.26)	0.033 (0.006-0.37)**	+10.0
C10:1	0.082 (0.01-0.74)	0.078 (0.02-1.42)**	-4.9	0.025 (0.003-0.23)	0.024 (0.008-0.47)	-4.0
C10:2	0.03 (0.0-0.72)	0.03 (0.0-1.47)	0.0	0.0096 (0.0-0.23)	0.01 (0.0-0.49)*	+4.2
C12:1	0.059 (0.005-0.60)	0.055 (0.004-1.37)**	-6.8	0.017 (0.001-0.19)	0.017 (0.002-0.46)	0.0
C14:1	0.115 (0.0-0.46	0.106 (0.0-1.22)**	-7.8	0.035 (0.0-0.15)	0.033 (0.0-0.41)*	-5.7
C14:2	0.049 (0.005-0.90)	0.047 (0.005-1.31)**	-4.1	0.015 (0.002-0.28)	0.015 (0.002-0.44)	0.0
C16:1	0.20 (0.03-1.55)	0.18 (0.03-2.96)**	-10	0.06 (0.01-0.48)	0.057 (0.008-0.99)**	-5
C18:1	1.42 (0.23-3.55)	1.32 (0.36-2.91)**	-7.0	0.43 (0.07-1.13)	0.42 (0.10-1.03)**	-2.3
C18:2	0.160 (0.009-0.86)	0.148 (0.0-0.68)**	-7.5	0.048 (0.003-0.27)	0.047 (0.0-0.24)*	-2.1

Values (μM) are expressed as the median (range) for 1856 male neonates and 1824 female neonates.

* represents statistical significance: * $P<0.05$ and ** $P < 0.001$. The 5th and the 6th columns show the values of the parameters after correction for the weight of the neonates.

Figure legends

Fig.1 Typical acquisition for each sample. LC-MS/MS was used to analyse amino acids and acylcarnitines. Three scanning modes were used for each examination: A precursor ion scan was used for the quantification of acylcarnitines (Panel A), a neutral loss scan was used for the quantification of amino acids (Panel B) and a MRM experiment was used to quantify glycine, ornithine, arginine and citrulline (Panel C). Data were quantitatively analysed by comparing the signal intensities of the analyte and its corresponding internal standard or, if not available, the standard next to the spectrum.

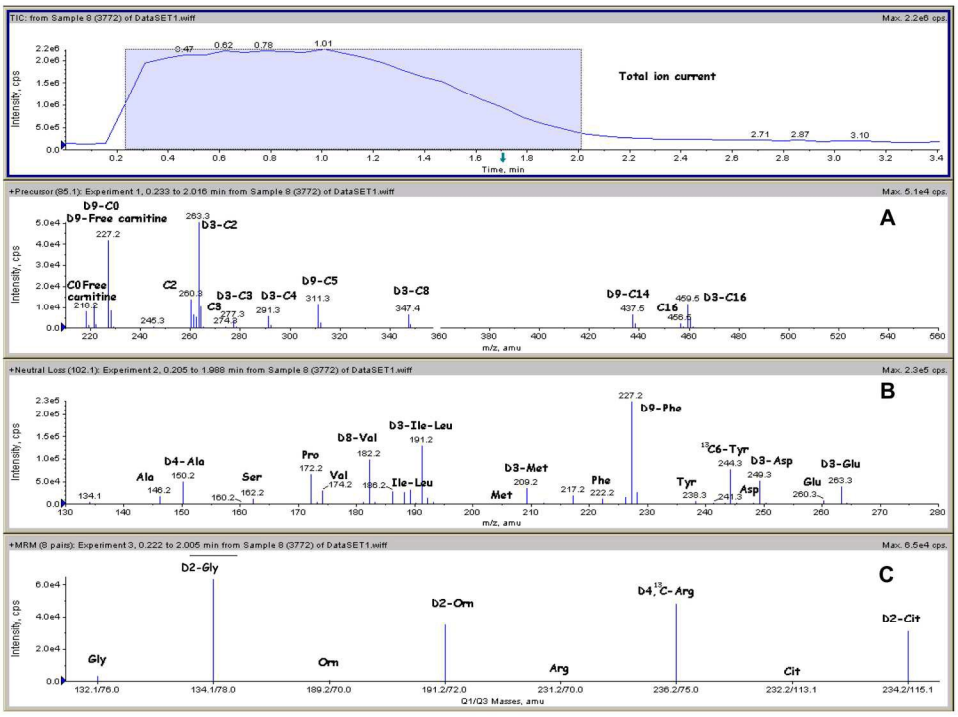
Fig.2 Correlation between neonates weight (kg) and gestational age (weeks) stratified by sex. Panel A represents correlation in males (▲, n=1856) and panel B in females (▄,n=1824). Each chart contains the equation of the line, the Spearman Product Moment Correlation coefficient and the P value.

Fig.3 Free carnitine, total esterified carnitines and esterified/free carnitine ratio levels stratified according to sex.

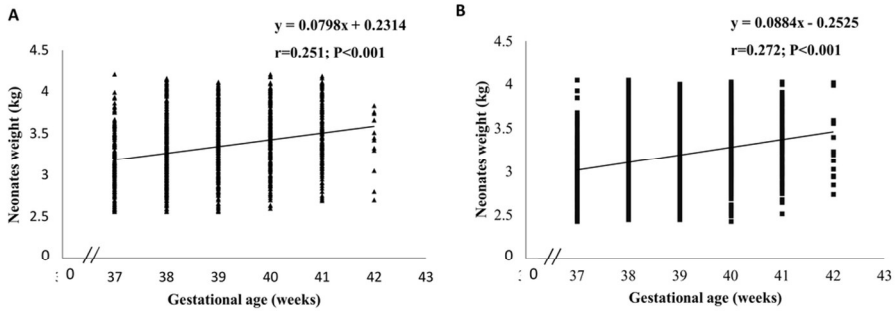
Box plot of free carnitine (A) and total esterified carnitines (B) measured in males (n = 1856, grey bars) and females (n = 1824, white bars) before and after body weight correction. (C) The esterified/free carnitine ratio was calculated in males (n = 1856, grey bars) and females (n = 1824, white bars) before and after body weight correction. The horizontal line across the box represents the median, and the box comprises the first and the third quartiles. The vertical lines represent the minimum and the maximum values. Connectors represent a significant difference between males and females.

Fig.4 Correlation between free carnitine (C0) and gestational age (weeks) stratified by sex. Panel A-C represent correlation in males before and after body weight correction (▲, n=1856) and panel B-D in females (▄,n=1824). Each chart contains the equation of the line, the Spearman Product Moment Correlation coefficient and the P value.

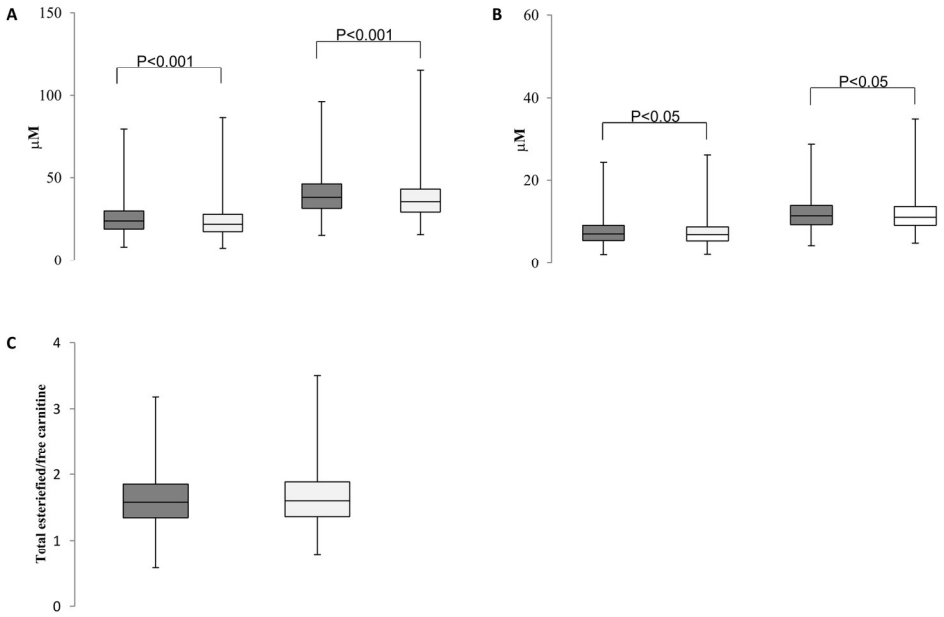
Fig.5 PCA analysis stratified according to sex. Panels A-C represent PCA loading plots in males before and after body weight correction (▲, n = 1856). Panels B-D represent PCA loading plots in females before and after body weight correction (▄, n = 1824).



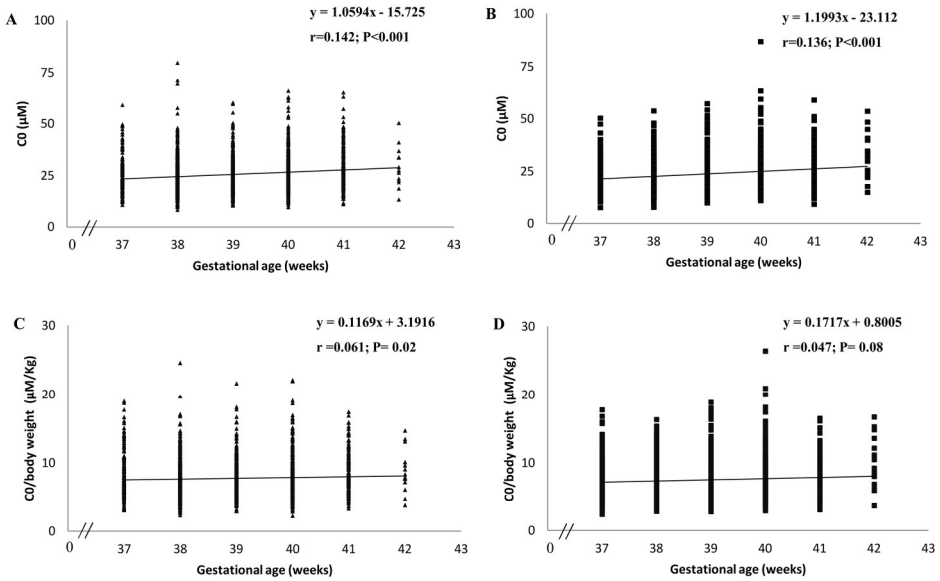
Typical acquisition for each sample
190x140mm (300 x 300 DPI)



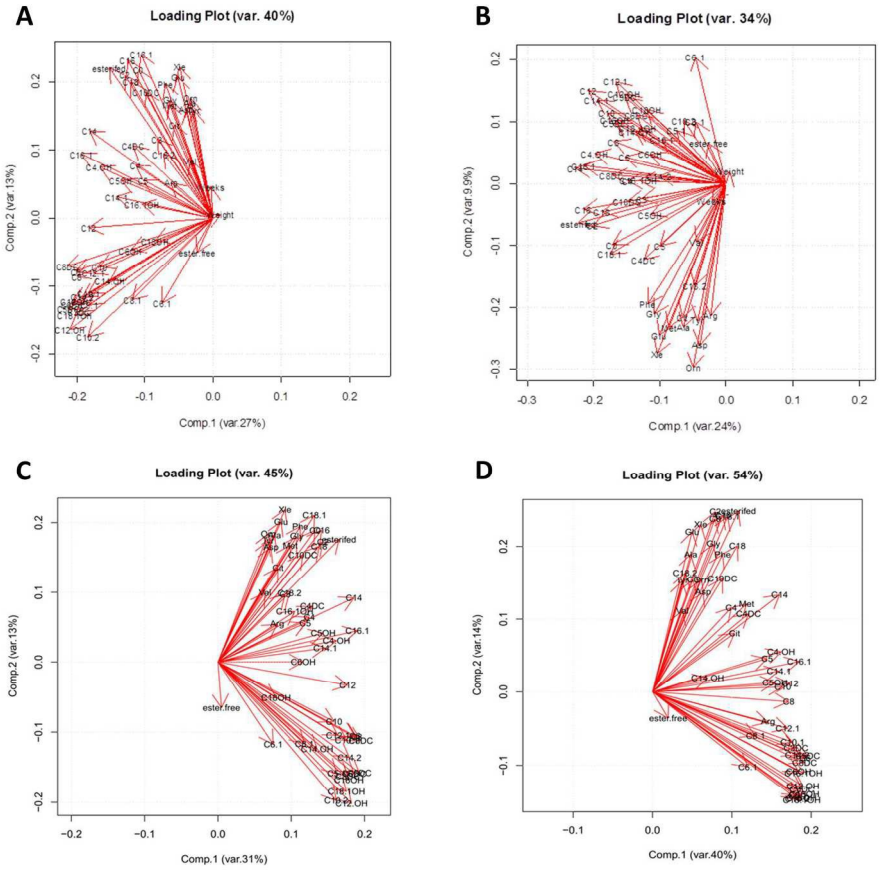
Correlation between neonates weight (kg) and gestational age (weeks) stratified by sex
102x41mm (300 x 300 DPI)



Free carnitine, total esterified carnitines and esterified/free carnitine ratio levels stratified according to sex
155x104mm (300 x 300 DPI)



Correlation between free carnitine (C0) and gestational age (weeks) stratified by sex
179x113mm (300 x 300 DPI)



PCA analysis stratified according to sex
202x189mm (300 x 300 DPI)