Amino Acids and Acylcarnitines Reference Values for Neonatal Screening of Inborn Errors of Metabolism in Colombia by Tandem Mass Spectrometry

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Antonio Bermúdez[1], Dora Robayo[1], Gloria Porras[2] and María Amparo Acosta[3]

Abstract

Neonatal screening in Colombia has been carried out since 2000. The problem that most concerns is the absence of expanded screening. To stablish reference values for amino acids and acylcarnitines, in order to provide information to guide the implementation of expanded screening. Samples collected on Whatman 903 filter paper from 10284 newborns were processed by Tandem Mass Spectrometry System (Waters – Perkin Elmer), and the NeoBase™ non-derivatized MS/MS kit (PerkinElmer), which contains controls for 11 amino acids, and 31 acylcarnitine species. For each analyte the upper limit was set above the 99th percentile, while the lower limit was set below the 1st percentile. Comparison of full-term newborn amino acid concentrations with premature ones showed no significant differences in three of them: Glycine p-0.99574, Ornithine p=0.35274, Phenylalanine p=0.13499, neither in levels of 11 of the 31 acylcarnitines. Comparison of analyte concentrations in this study with previous reports showed significant differences for all amino acids and acylcarnitines (<0.05). Experience was gained in the pre-analytic stage of expanded screening and reference values were established, for the implementation of neonatal screening program in the country.

Keywords: Neonatal Screening, Inborn error of metabolism, tandem mass spectrometry, cut off values, amino acids, acylcarnitines.

Introduction

Newborn screening (NBS) in Colombia is a program that has been carried out since 2000, with national coverage and state funding for a single pathology, congenital hypothyroidism (CH). The epidemiological situation for the event is under constant monitoring by the National Epidemiological Surveillance System (SIVIGILA), which for epidemiological period XIII of 2019 [1] reported an incidence of 3.6 cases per 10000 births i.e. 1:2778 live newborns (NB). Information from NBS laboratories estimates that national coverage is 85%, mainly because there are regions that are very difficult to access for various sociodemographic reasons. In any case with this rate of CH it can be said that Colombia has an incidence at birth similar to those of other countries in the region. The problem that most concerns is the absence of expanded screening for Inborn errors of metabolism that can occur to 1:3600 NB.[2]

The Ministry of Health and Social Protection has been reviewing with experts the regulations to implement Law 1980 of 2019 [3], which provides the legal framework for the Neonatal Detection Policy of deafness, blindness, heart problems and inborn errors of metabolism (IEM), through the implementation of the NBS program.

The background is resolution 0412 of the year 2000 [4], by which NBS was initiated in the country, with the guideline of seeking CH, and then in 2013, with the evidence-based medicine strategy, the Ministry of Health and Social Protection developed guideline No. 03 for the management of NB with birth defects [5], which provides specific recommendations on which diseases should be fulfill to neonatal screening in Colombia, including CH. The focus is on diseases where preventive measures can

[1] National Institute of Health, Bogotá, Colombia.
[2] International Center Research Health, Comfamiliar Risaralda, Colombia.
[3] Cauca University, Health Sciences Faculty, Popayán, Colombia.

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Corresponding Author:
Antonio Bermúdez, Email: abermudez@ins.gov.co

be taken through genetic counseling, but especially where patients' quality of life can be improved through early diagnosis and timely treatment, as is the case with aminoacidopathies, organic acidemias, and fatty acid oxidation (FAO) disorders.[6]

It is complemented by the Decenal Public Health Plan 2012-2021 [7] and resolution 3202 of 2016 adopting the Comprehensive Health Care Routes, to achieve the cross-sectoral articulation between members of the health sector, and with the Comprehensive Health Care Program, created by Resolution 429 of 2016, with which it will be possible to implement what the Ministry of Health is obliged to do together with the National Institute of Health, for compliance with law 1980 of 2019.

This means having laboratory techniques for basic screening and expanded screening in the country. In general, most tests are immunoassays such as ELISA (Enzyme linked immunoassay) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) which allows simultaneous quantification of several metabolites such as amino acids and acylcarnitines for many IEMs.[8] The basis is the abnormal accumulation of certain amino acids in enzyme or protein defects in the metabolic pathways of amino acids or the alteration in acylcarnitines (ACs) concentrations that serve as biomarkers of organic acid metabolism errors, and fatty acid metabolism, including defects in mitochondrial metabolism.[9]

It is a national commitment as neonatal screening contributes to the fulfillment of the Millennium Development Goals [10], subsequently translated into the Sustainable Development Goals, one of whose goals is to reduce child mortality and reduce disability. The objective of this work was to analyze dried blood spots (DBS) from NB by tandem mass spectrometry, to know reference values of amino acids and acylcarnitines, in order to provide timely, valid and reliable information to guide the implementation of expanded screening, as required by law.[11]

Materials and Methods

Descriptive analytical study of NB in maternities chosen for convenience in several regions of the country, with the criterion of accessibility to samples with optimal quality and continuity, with the collaboration of the public health laboratories of the country.

Study Population: Between 2014 and 2018, heel DBS samples collected on Whatman N° 903 filter paper, to 10.284 NB in health care institutions, public and private, were included in this study. The inclusion criteria were NB of 24 or more hours of life. The exclusion criteria were NB with blood transfusion, treated with hormones and those DBS samples showing hemolysis, supersaturated, diluted, clotted, scratched, quantity insufficient or any other unacceptable characteristic.

Nurses were trained for sample management in accordance with the procedure in the Manual for Neonatal Screening of the National Institute of Health (INS).[12] The samples were allowed to dry at room temperature for 3 hours and then sent to the laboratory for processing. Upon receipt, samples were reviewed by expert staff to meet quality standards. They were registered in the INS sample information system for barcode handling, stored at 0-8°C, until processing and then at -20°C.

Analytical method: For the quantification of amino acids, succinylacetone, carnitine and acylcarnitines, DBS from the NB's heel were processed by the Perkin Elmer Newborn Tandem Mass Spectrometry System (MSMS). The NeoBase™ non-derivatized MS/MS kit (PerkinElmer, Turku, Finland) was used, it contains controls for 11 amino acids, and 31 acylcarnitines (including free-carnitine) species, and internal standards stable-isotope labeled. It was also used the NeoBase Succinylacetone Solution. For the mass spectra a TQD equipment (Waters, Milford, MA, USA) was used, with the applications MassLynx™ and NeoLynx™ Software. The concentration is expressed in micromoles per liter (µmol/L).

Statistical Management

The results expressed in µmol/L for each analyte were organized into excel files (*.xls), exported from the MS/MS spectrometer. For central trend measures the Excel statistics module was applied, and the reference intervals were set with the values within the 1st and 99th percentile. Each of the boundaries was considered a cut-off point. For the normality test of the distribution of the concentrations for each analyte, the Bowman Sheldon test (Statistic B) was used. The null hypothesis in this test is data follow normal distribution, in accordance with the Jarque Bera test with skewness and kurtosis measurements. For the critical value of the F-distribution (ANOVA analysis) for analysis of variance was used Open Epi. [13] A p value < 0.05 was considered significant. For the handling of outliers, the algorithm "A" was applied with the Huber method according to ISO-13528 Annex C to obtain robust statistics.[14]

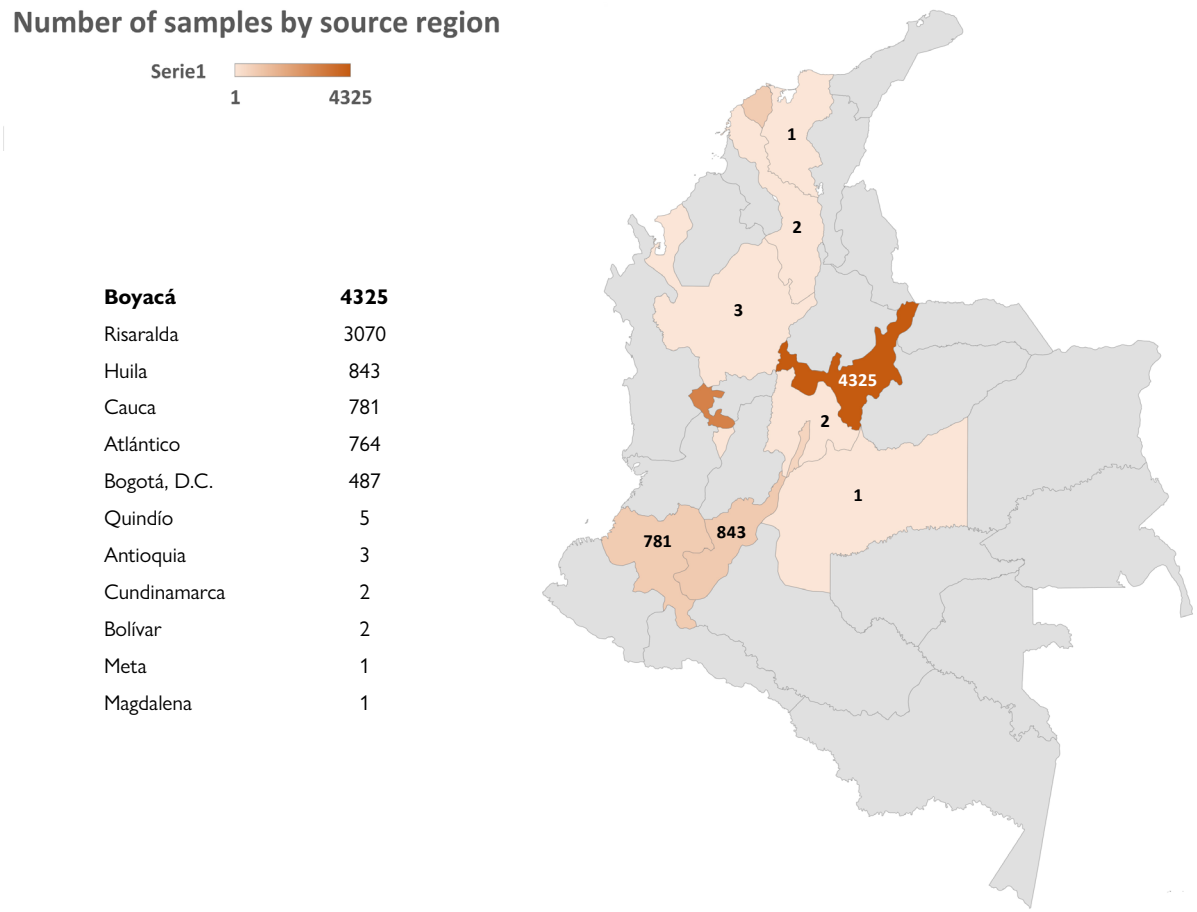
Ethical Considerations

For the completion of this work, the Helsinki declaration was taking into account and received the approval of the Ethics Committee of the National Health Institute (INS CEMIN 492019), Bogota Colombia.

Results

Description of the Study Population

The samples correspond to NB from various maternities from 12 out of 33 (36%) departments or counties, the country's main geographic regions, most of the Cundiboyacense area (Figure 1), distributed in both sex (45% female, 48% male, 5% uninformed). The southeastern region of the country, which mainly belongs to the Amazon, was not included. The heel sample was taken in neonates of 7 days or less in 93%, an additional 4% between 7 and 30 days. 6% were premature and 0.4% transfused. 93% had a birth weight higher than 2500 g., 39.5% belongs to the subsidized health insurance scheme and 38.6% to the contribution scheme, the rest to special health insurance schemes like that of professors or private schemes for rich people. In the expectation of reducing



Boyacá	**4325**
Risaralda	3070
Huila	843
Cauca	781
Atlántico	764
Bogotá, D.C.	487
Quindío	5
Antioquia	3
Cundinamarca	2
Bolívar	2
Meta	1
Magdalena	1

Figure 1. Distribution of samples taken by department.

biases in reference values, transfused or treated with hormones NB were removed from the statistical analysis. After such exclusion the final database for the study was compliant by 9.601 term NB, with normal birth weight (> 2500 g) appropriate for the gestational age, feeding normally and 663 premature, and low birth weights NB, less than 2500 g.

Concentration Results

The quantification of amino acids and .acylcarnitines was performed in accordance with the methodology described and quality controls given by the CDC for tandem mass spectrometry, to obtain concentrations for 11 amino acids, 31 acylcarnitines, the ratio Phenylalanine/Tyrosine and Succinylacetone, thus allowing diagnosis of the different pathologies object of neonatal screening.

The results with the descriptive statistic are presented in Table 1, together with statistic B for Bowman Shelton Test to evaluate the normality of the distribution of concentrations for each analyte. The upper limit cut-offs were set at above the 99th percentiles, whereas the lower limit cut-offs were set at below the 1st percentiles as shown in Table 1.

The results of preterm and low birth weight children were analyzed separately, then compared to the population of full-term children and appropriate birth weight for gestational age. Concentrations of Glycine, Ornithine, and Phenylalanine showed no significant differences ($p>0.05$), nor is there a difference in Succinylacetone ($p=1$), or 11 ACs ($p>0.05$). This comparison is presented in Table 2.

We also compared our results to reference interval from previous reports. [15–17]. The comparison is presented in Table 3 for amino acids and Table 4 for acylcarnitines. The data of this study are shown along with the results of a study in southwestern Colombia, with population mostly of Afro-descendant origin [15], data from a global coverage study including Latin American information [16] and with data from Indonesia, very similar to this study in methodological design and a similar state of implementation of the screening in the country.[17] In general, the results of the different studies are comparable, however there is significant difference for all amino acids, while for succinilacetone the results are similar ($p=1$) although it is only compared to the global coverage study. For ACs, only data from three studies are compared and show significant differences in all molecules.

Table 1. Descriptive statistical central trends for amino acids and acylcarnitines, percentiles for reference intervals and normality test.

Analyte	Mean (µmol/L)	Median (µmol/L)	SD	Variance	1st percentile	percentile99th	Normality test	
							Statistic* B	*p*
Alanine	261,08	255,93	58,97	3476,92	160,78	356,90	19986,08	<0,05
Arginine	11,62	10,95	5,46	29,84	2,33	20,92	2269560,7	<0,05
Citruline	12,98	12,68	3,69	13,63	6,70	19,14	12430463	<0,05
Glycine	339,79	332,86	91,92	8450,17	183,42	488,60	5950,09	<0,05
Leucine+Isoleucine+Hydroxyproline	98,36	96,20	23,11	533,86	59,06	135,80	216910	<0,05
Methionine	12,27	12,20	3,88	15,07	5,68	18,72	11156	<0,05
Ornithine	81,63	78,81	22,70	515,13	43,03	117,77	11856,59	<0,05
Phenylalanine	47,92	47,07	10,05	100,97	30,83	64,28	32490	<0,05
Phenylalanine/Tyrosine	0,48	0,47	0,15	0,02	0,22	0,74	476	<0,05
Proline	172,06	167,83	38,14	1454,92	107,18	233,25	8222	<0,05
Succinylacetone	0,67	0,66	0,12	0,01	0,47	0,86	562,78	<0,05
Tyrosine	101,77	98,04	31,75	1008,36	47,76	152,68	559,23	<0,05
Valine	97,69	95,51	21,76	473,50	60,67	132,46	23184,84	<0,05
Free Carnitine	24,69	23,75	8,07	65,14	10,96	37,76	2,003,64	<0,05
Acetylcarnitine	23,21	22,17	9,27	85,90	7,44	37,87	8702,96	<0,05
Propionylcarnitine	2,22	2,11	0,85	0,72	0,77	3,58	16009,4	<0,05
Malonylcarnitine +Hydroxybutirylcarnitine	0,10	0,09	0,04	0,00	0,03	0,16	4598,93	<0,05
Butirylcarnitine	0,26	0,25	0,08	0,01	0,13	0,38	6569,48	<0,05
Methylmalonylcarnitine + hydroxyisovalerylcarnitine	0,18	0,17	0,05	0,00	0,09	0,26	764,47	<0,05
Isovalerylcarnitine	0,10	0,10	0,03	0,00	0,06	0,14	873828	<0,05
Tiglylcarnitine	0,01	0,01	0,00	0,00	0,01	0,01	13430,75	<0,05
Glutarylcarnitine/Hydroxyhexanoylcarnitine	0,12	0,12	0,04	0,00	0,06	0,19	2107,5	<0,05
Hexanoylcarnitine	0,04	0,04	0,01	0,00	0,02	0,06	14846,8	<0,05
Methylglutarylcarnitine	0,10	0,10	0,04	0,00	0,04	0,16	2872,9	<0,05
Octanoylcarnitine	0,05	0,05	0,02	0,00	0,03	0,08	3135,7	<0,05
Octenoylcarnitine	0,06	0,06	0,02	0,00	0,02	0,10	11191	<0,05
Decanoylcarnitine	0,07	0,06	0,02	0,00	0,02	0,10	1116,8	<0,05
Decenoylcarnitine	0,04	0,04	0,01	0,00	0,02	0,06	9072,5	<0,05

Table 1. Cont.

Analyte	Mean (µmol/L)	Median (µmol/L)	SD	Variance	1st percentile	percentile99th	Normality test	
							Statistic* B	*p*
Decadienoylcarnitine	0,01	0,01	0,00	0,00	0,01	0,01	1186,6	<0,05
Lauroylcarnitine	0,09	0,09	0,04	0,00	0,03	0,16	1147	<0,05
Dodecenoylcarnitine	0,07	0,06	0,04	0,00	0,00	0,13	3559,5	<0,05
Miristoylcarnitine	0,21	0,20	0,06	0,00	0,10	0,31	1122,19	<0,05
Miristoleylcarnitine	0,11	0,10	0,05	0,00	0,02	0,19	1101,28	<0,05
Tetradecadienoylcarnitine	0,02	0,02	0,01	0,00	0,01	0,03	21471	<0,05
3 Hydroxy (OH) Miristoylcarnitine	0,01	0,01	0,00	0,00	0,01	0,01	208,25	<0,05
Palmitoylcarnitine	2,88	2,84	0,89	0,79	1,37	4,35	1918,2	<0,05
Hexadecenoylcarnitine	0,20	0,20	0,08	0,01	0,07	0,33	1044	<0,05
3 Hydroxy (OH) Palmitoleylcarnitine	0,04	0,04	0,01	0,00	0,02	0,06	1528,15	<0,05
3 Hydroxy (OH) Palmitoylcarnitine	0,02	0,02	0,01	0,00	0,01	0,03	235,6	<0,05
Octadecanoylcarnitine	0,85	0,83	0,24	0,06	0,44	1,23	2468,9	<0,05
Octadecenoylcarnitine	1,26	1,24	0,35	0,12	0,68	1,82	2255,7	<0,05
3 Hydroxy (OH) Octadecenoylcarnitine	0,02	0,02	0,01	0,00	0,01	0,04	1760,3	<0,05
Linoleylcarnitine	0,16	0,15	0,07	0,00	0,05	0,26	10629	<0,05
3 Hydroxy (OH) Octadecanoylcarnitine	0,01	0,01	0,00	0,00	0,01	0,01	53,8	<0,05

*Bowman Shelton. Significancia: $p<0.05$

Table 2. Statistic comparison of mean concentrations per analyte, between full-term newborns vs. low birth weight or premature newborns.

Analyte	Preterm and low birth weight n=663		Full-term NB n=9601		ANOVA	
	Mean (µmol/L)	SD	Mean (µmol/L)	SD	F	*p*
Alanine	252,72	65,68	261,08	58,97	12,27	<0,05
Arginine	12,82	6,48	11,62	5,46	29,19	<0,05
Citruline	12,61	4,02	12,98	3,69	61,61	<0,05
Glycine	339,81	111,17	339,79	91,92	0,00	0,99574
Leucine+Isoleucine+ Hydroxyproline	102,59	27,91	98,36	23,11	20,18	<0,05
Methionine	12,96	4,79	12,27	3,88	18,97	<0,05
Ornithine	82,49	27,57	81,63	22,70	0,86	0,35274
Phenylalanine	48,53	11,67	47,92	10,05	2,23	0,13499
Phenylalanine/Tyrosine	0,46	0,17	0,48	0,15	10,83	<0,05
Proline	163,80	45,25	172,06	38,14	28,34	<0,05
Succinylacetone	0,67	0,11	0,67	0,12	0,00	1,00000
Tyrosine	110,16	42,93	101,77	31,75	41,11	<0,05
Valine	94,72	24,92	97,69	21,76	11,33	<0,05
Free Carnitine	27,41	9,37	24,69	8,07	68,90	<0,05
Acetylcarnitine	21,46	8,75	23,21	9,27	22,26	<0,05
Propionylcarnitine	2,21	1,00	2,22	0,85	0,0837611	0,77227
Malonylcarnitine+Hydroxybutirylcarnitine	0,09	0,04	0,10	0,04	38,76	<0,05
Butirylcarnitine	0,29	0,09	0,26	0,08	85,74	<0,05
Methylmalonylcarnitine + hydroxyisovalerylcarnitine	0,16	0,05	0,18	0,05	99,23	<0,05
Isovalerylcarnitine	0,14	0,05	0,10	0,03	989,00	<0,05
Tiglylcarnitine	0,01	0,00	0,01	0,00	0,00	1,00000
Glutarylcarnitine/ Hydroxyhexanoylcarnitine	0,12	0,04	0,12	0,04	0,00	1,00000
Hexanoylcarnitine	0,04	0,01	0,04	0,01	0,00	1,00000
Methylglutarylcarnitine	0,10	0,04	0,10	0,04	0,00	1,00000
Octanoylcarnitine	0,05	0,01	0,05	0,02	0,00	1,00000
Octenoylcarnitine	0,07	0,03	0,06	0,02	143,47	<0,05
Decanoylcarnitine	0,06	0,03	0,07	0,02	143,47	<0,05
Decenoylcarnitine	0,04	0,01	0,04	0,01	0,00	1,00000
Decadienoylcarnitine	0,01	0,00	0,01	0,00	–	–

Table 2. Cont.

Analyte	Preterm and low birth weight n=663		Full-term NB n=9601		ANOVA	
	Mean (µmol/L)	SD	Mean (µmol/L)	SD	F	*p*
Lauroylcarnitine	0,08	0,04	0,09	0,04	38,76	<0,05
Dodecenoylcarnitine	0,05	0,03	0,07	0,04	159,55	<0,05
Miristoylcarnitine	0,21	0,08	0,21	0,06	0,00	-
Miristoleylcarnitine	0,09	0,04	0,11	0,05	101,59	<0,05
Tetradecadienoylcarnitine	0,02	0,01	0,02	0,01	0,00	1,00000
3 Hydroxy (OH) Miristoylcarnitine	0,01	0,00	0,01	0,00	–	–
Palmitoylcarnitine	2,55	0,97	2,88	0,89	84,24	<0,05
Hexadecenoylcarnitine	0,18	0,09	0,20	0,08	38,11	<0,05
3 Hydroxy (OH) Palmitoleylcarnitine	0,04	0,01	0,04	0,01	0,00	1,00000
3 Hydroxy (OH) Palmitoylcarnitine	0,02	0,01	0,02	0,01	0,00	1,00000
Octadecanoylcarnitine	0,83	0,27	0,85	0,24	4,23	<0,05
Octadecenoylcarnitine	1,29	0,40	1,26	0,35	4,47	<0,05
3 Hydroxy (OH) Octadecenoylcarnitine	0,02	0,01	0,02	0,01	0,00	1,00000
Linoleylcarnitine	0,22	0,10	0,16	0,07	426,96	<0,05
3 Hydroxy (OH) Octadecanoylcarnitine	0,01	0,00	0,01	0,00	–	–

Significance: $p<0.05$

Table 3. Comparison of amino acids concentrations (µmol/L) and reference intervals ((µmol/L) with previous reports.

Analyte	This report n=9601				southwest Colombia* n=891				Worldwide collaborative project** n=25000000				Indonesia*** n=993				F	p
	Median (µmol/L)	SD	Lower limit 1st percentile (µmol/L)	Upper limit 99th percentile (µmol/L)	Median (µmol/L)	SD	Lower limit 1st percentile (µmol/L)	Upper limit 99th percentile (µmol/L)	Median (µmol/L)	SD	Lower limit 1st percentile (µmol/L)	Upper limit 99th percentile (µmol/L)	Median (µmol/L)	SD	Lower limit 1st percentile (µmol/L)	Upper limit 99th percentile (µmol/L)		
Alanine	255,93	58,97	160,78	356,90	220,13	53,86	91,43	348,82	233,00	0,44	117,00	507,00	236,16	10,25	110,85	608,44	47987	<0,05
Arginine	10,95	5,46	2,33	20,92	28,24	11,48	4,40	52,07	8,70	3,31	2,30	32,00	9,04	0,52	0,71	53,00	11492	<0,05
Citruline	12,68	3,69	6,70	19,14	17,67	4,56	7,81	27,52	12,00	11,76	6,00	28,00	31,07	1,90	4,33	139,02	958	<0,05
Glycine	332,86	91,92	183,42	488,60	–	–	–	–	348,00	83,52	185,00	767,00	–	–	186,17	919,20	312	<0,05
Leucine+Isoleucine +Hydroxyproline	96,20	23,11	59,06	135,80	304,67	121,16	72,61	536,72	115,00	35,65	64,00	235,00	139,99	3,15	58,52	433,36	9432	<0,05
Methionine	12,20	3,88	5,68	18,72	56,46	19,71	11,00	101,91	21,00	6,93	11,00	44,00	7,01	0,44	1,66	34,22	14908	<0,05
Ornithine	78,81	22,70	43,03	117,77	–	–	–	–	–	–	–	–	161,95	2,30	44,35	703,98	13305	<0,05
Phenylalanine	47,06	10,05	30,83	64,28	51,43	11,78	23,92	78,94	54,00	11,88	33,00	97,00	51,69	1,17	29,90	99,68	1109	<0,05
Phenylalanine/ Tyrosine	0,47	0,15	0,22	0,74	–	–	–	–	54,00	8,10	–	–	–	–	–	–	419270	<0,05
Proline	167,83	38,14	107,18	233,25	–	–	–	–	–	–	–	–	172,83	4,96	93,68	363,58	17	<0,05
Succinylacetone	0,66	0,12	0,47	0,86	–	–	–	–	0,66	0,11	0,21	1,40	–	–	–	–	0	1
Tyrosine	98,04	31,75	47,76	152,68	119,14	34,72	37,03	201,25	80,00	14,40	34,00	207,00	105,45	3,89	53,80	203,03	7850	<0,05
Valine	95,51	21,76	60,67	132,46	131,59	32,10	59,18	204,00	103,00	22,66	57,00	212,00	95,93	2,54	41,39	249,47	852	<0,05

Significance:p<0.05

*Reference values of amino acids in newborn screening in southwest Colombia. [15].

**A worldwide collaborative project for clinical validation of cutoff target ranges in newborn screening [16].

***Profiles of amino acids in Indonesian Neonates [17].

Table 4. Comparison of acylcarnitines concentrations (µmol/L)in previous reports and reference intervals (µmol/L).

Analyte	This report n=9601				southwest Colombia* n=891				worldwide collaborative project** n=25000000				F	p
	Median (µmol/L)	SD	Lower limit 1st percentile (µmol/L)	Upper limit 99th percentile (µmol/L)	Median (µmol/L)	SD	Lower limit 1st percentile (µmol/L)	Upper limit 99th percentile (µmol/L)	Median (µmol/L)	SD	Lower limit 1st percentile (µmol/L)	Upper limit 99th percentile (µmol/L)		
C0	23,75	8,07	10,96	37,76	46,19	13,71	13,63	78,74	24	6,00	11	59	6026	<0,05
C2	22,17	9,27	7,44	37,87	31,67	8,62	9,02	54,31	23	4,37	10	52	1858	<0,05
C3	2,11	0,85	0,77	3,58	2,14	0,73	0,23	4,05	1,75	0,35	0,57	4,74	5310	<0,05
C3DC+C4OH	0,09	0,04	0,03	0,16	–	–	–	–	0,12	0,07	–	–	1757	<0,05
C4	0,25	0,08	0,13	0,38	0,36	0,15	0,07	0,64	0,24	0,03	0,08	0,75	6540	<0,05
C4DC+C5OH	0,17	0,05	0,09	0,26	–	–	0	0	0,19	0,04	–	–	2364	<0,05
C5	0,1	0,03	0,06	0,14	0,34	0,09	0,09	0,59	0,12	0,02	0,05	0,39	57060	<0,05
C5:1	0,01	0,00	0,01	0,01	–	–	–	–	0,021	0,01	0,001	0,08	9116161	<0,05
C5DC+C6OH	0,12	0,04	0,06	0,19	–	–	–	–	0,095	0,05	–	0,02	2385	<0,05
C6	0,04	0,01	0,02	0,06	0,28	0,09	0,04	0,51	0,062	0,02	0,02	0,18	55788	<0,05
C6DC	0,1	0,04	0,04	0,16	–	–	–	–	0,07	0,04	0,022	0,17	5348	<0,05
C8	0,05	0,02	0,03	0,08	0,24	0,08	0,05	0,43	0,07	0,01	0,02	0,21	136479	<0,05
C8:1	0,06	0,02	0,02	0,1	–	–	–	–	–	–	–	–	–	–
C10	0,06	0,02	0,02	0,1	0,25	0,09	0,05	0,45	0,09	0,02	0,022	0,26	38630	<0,05
C10:1	0,04	0,01	0,02	0,06	–	–	–	–	0,06	0,01	0,02	0,18	38034	<0,05
C10:2	0,01	0,00	0,01	0,01	–	–	–	–	0,02	0,01	0,001	0,08	9599	<0,05
C12	0,09	0,04	0,03	0,16	0,45	0,16	0,08	0,81	0,14	0,05	0,04	0,41	21268	<0,05
C12:1	0,06	0,04	0	0,13	–	–	0	0	0,063	0,02	0,01	0,27	208	<0,05
C14	0,2	0,06	0,1	0,31	0,47	0,15	0,1	0,84	0,23	0,04	0,071	0,5	65306	<0,05
C14:1	0,1	0,05	0,02	0,19	–	–	–	–	0,12	0,03	0,03	0,37	4145	<0,05
C14:2	0,02	0,01	0,01	0,03	–	–	–	–	0,036	0,02	0,01	0,09	6129	<0,05
C14OH	0,01	0,00	0,01	0,01	–	–	–	–	–	–	–	–	–	–
C16	2,84	0,89	1,37	4,35	3,45	1,19	0,72	6,17	2,8	0,50	0,8	6	752	<0,05
C16:1	0,2	0,08	0,07	0,33	–	–	–	–	–	–	–	–	–	–
C16:1OH	0,04	0,01	0,02	0,06	–	–	0	0	0,05	0,01	0,011	0,13	9508	<0,05
C16OH	0,02	0,01	0,01	0,03	0,15	0,06	0,02	0,27	0,03	0,01	0,01	0,08	61811	<0,05
C18	0,83	0,24	0,44	1,23	1,15	0,36	0,3	2	0,81	0,13	0,31	1,7	3065	<0,05
C18:1	1,24	0,35	0,68	1,82	–	–	–	–	1,2	0,18	0,49	2,5	457	<0,05
C18:1OH	0,02	0,01	0,01	0,04	–	–	–	–	0,023	0,01	0,01	0,07	855	<0,05
C18:2	0,15	0,07	0,05	0,26	–	–	–	–	0,18	0,05	0,05	0,6	3392	<0,05
C18:OH	0,01	0,00	0,01	0,01	0,14	0,05	0,02	0,26	0,02	0,01	0,001	0,06	68158	<0,05

Significancia: $p<0.05$

* Reference values of amino acids in newborn screening in southwest Colombia. [15].

**A worldwide collaborative project for clinical validation of cutoff target ranges in newborn screening [16].

Discussion

Colombia has a diverse ethnic composition, with a majority of Hispanic ancestor population represented in this study by the Cundiboyacense, Bogota, Antioquia, and Quindio regions, on the other hand there are populations of Afro origin mainly on the Atlantic and Pacific coasts, represented in this study by samples of Atlantico, Magdalena, Bolivar and Cauca. The third component is the indigenous population of great importance because it is under government protection in the regions of amazonia, orinoquía and guajira mainly, but it is not represented in this study. It corresponds according to the national census carried out in 2018 [18], to 4.4% of the total population of the country, adding 1,905,617 indigenous peoples. Therefore, this population will be the subject of further study.

Concentrations of amino acids, show that Arginine has the lowest concentration with a mean of 11.62 (µmol/L) with a standard deviation of 5.46 and Glycine has the highest concentration with a mean of 339.79 and standard deviation of 92.92, showing a dispersion expected for the biological variability of amino acids in the population. In addition, it corresponds to what is reported in the global coverage study. The highest concentration ACs are Free Carnitine (C0) with mean 24.69 (µmol/L) and standard deviation 23.75 and Acetylcarnitine (C2) with mean 23.21 and standard deviation 22.17, as expected for the metabolic pathway for FAO disorders.[16] (Table 1).

Comparison of amino acid concentrations in full-term NB with premature ones showed that the biggest significant difference ($p<0.05$) was in Citruline, Tyrosine and Arginine, while no significant differences were observed in three of them: Glycine p-0.99574, Ornithine p=0.35274 and Phenylalanine p=0.13499. A non-significative difference was also observed in Succinylacetone, and non-significative differences were observed in levels of 11 of the 31 acylcarnitines. However, there was significant difference ($p<0.05$) in 20 of them and the main difference was presented in Free Carnitine and Acetylcarnitine. These differences could be expected because for Metabolic adaptation at birth, the NB make a transition from the transplacental supply of glucose to a fat-based fuel economy, but in prematurity or intrauterine growth restriction the patterns of metabolic adaptation are different to that of a full-term NB, even there is a recommendation of continuous evaluation at follow-up of the lipid profile, due to the increased cardiovascular risk.[19] Prematurity is multifactorial [20] and physiological response changes drastically according to gestational age. In this preterm 663 NB study, 84.5% were moderate to late preterm (32 to 37 weeks), 12.7% were very premature (28 to 32 weeks) and 2.9% were extreme preterm (less than 28 weeks), according to WHO criteria [21], therefore, under the consideration that from each 100 NB in Colombia, about 10 will have some degree of prematurity [22], neonatal screening needs to take into account specific reference values for preterm ones.

Comparison of amino acid concentrations in NB in this study with previous reports showed significant differences for all analytes, amino acids and acylcarnitines (<0.05) (Table 3). This comparison includes the study in the southwest region of the country, also made with MSMS spectrometry, but with an in-house development kit that can explain some differences. [15] The most notable are in the mean concentration of Leucine+Isoleucine+ Hydroxyproline of 304.67 (µmol/L) reported by them while in this study it is 96.20, similarly at the mean concentration of Methionine reporting 56.46 Vs. 12.20 and for Arginine they report 28.24 Vs. 10.95, but there are differences for most amino acids. Also included in the comparison was a population of Indonesian NB with which there were significant difference in Citruline 12.68 Vs. 31.07 in this study, but although more differences were expected because the ethnical origin, the results show similarity in amino acid concentrations. It draws attention because the cut-offs for amino acids and ACs in neonatal screening are highly influenced by various factors such as genetic background or geographical location of a population. [23] Another comparison group was an international multicentric study with more than 25 million NBs data [16] and there were no significant differences for the majority of the concentrations except that for Methionine, with mean of 12.20 (µmol/L) vs. 21.00 in this study. Nevertheless, the comparison between the four studies showed significative differences for all analytes, in the same way the ranges of values between the 1st and 99th percentile, shown differences in accordance with the observation of differences in the means. For example for Phenylalanine the range between the 1st and the 99th percentiles in this study is (30.83-64.28), in the southwest Colombia study (23.92-78.94), in the international study (33.00-97.00) and (29.90-99.68) for the Indonesian study. This overlap, but not coincidence at all, reflects the variability of the biochemical phenotype of each population, but also it could be explained because the international study cover millions samples while in this study we only have thousands of samples (25000000 vs. 10000). However, the results of the international study are applicable to the Colombian population, which is of great benefit because samples for the tandem mass spectrometry (MSMS) study in Colombia are often sent to laboratories abroad. The same can be said with respect to the comparison of acylcarnitines concentrations in NB from this study with previous reports, since significant difference was observed for all of them ($p<0.05$) (Table 4). However it is to take into account that the values of the two highest concentration carnitines, C0 and C2, are very similar in this study and the international study, the mean for C0 is 23.75 (µmol/L) Vs. 24 and for C2 is 22.17 Vs. 23 while values for the study in Colombia's southwest region are 31.67 for C0 and 54.31 for C2, and that same behavior is repeated for the other carnitines.

In addition to determining the reference intervals for amino acids and acylcarnitines, this study shown that separate reference values are required for premature NB or low birth weight NB,

as this study noted significant difference with full-term ones. The same analysis must be done for extreme premature, very premature, and moderate premature NB according to WHO criteria. On the other hand, the comparison with results of the study done in southwest Colombia showed significant differences, maybe because it was made in a mainly Afro descendant population. Colombia has ethnic diversity; therefore, it is expected to do the analysis of more samples in other regions and thus have more precise reference intervals for ethnic minorities. As for the comparison with international studies, the results indicate that it is possible to use the information of international reference values, established with a large data, especially useful for the diagnosis of IEM since we do not have our own information.

In conclusion, experience was gained in sample management and other aspects of the pre-analytic stage of extended screening, for instance the inclusion of indigenous people, as well as the ampliation of studies for afrodescendant people, in the perspective of implement NBS for all NB. Besides, reference values for amino acids and acylcarnitines were established, to contribute this experience to other researchers and government authorities for the implementation of the neonatal screening program in the country in accordance with the recently enacted law with the goal of helping to reduce child mortality.

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Authors' Contributions

MAA Made a substantial contribution to the concept and design, acquisition of data; revised the article critically for important intellectual content; Approved the final version to be published. GP Made a substantial contribution to the concept and design, acquisition of data; revised the article critically for important intellectual content; Approved the final version to be published. DR Made a substantial contribution to the concept and design, acquisition of data; revised the article critically for important intellectual content; Approved the final version to be published.

AB Made a substantial contribution to the concept and design, acquisition of data, analysis and interpretation of data;Drafted the article;Approved the final version to be published.

Declaration of Conflicting Interests

Authors report no conflicts of interest.

References

1. SIVIGILA. Informe epidemiológico de evento Defectos congénitos, con corte a PE XIII 2019. https://www.ins.gov.co/buscador-eventos/informesdeevento/defectos%20cong%c3%89nitos_2018.pdf
2. Yang N, Gong L, Zhao J, Yang H, Ma Z, Liu W, Wan Z, Kong Y. Inborn errors of metabolism detectable by tandem mass spectrometry in Beijing. *J Pediatr Endocrinol Metab*. 2020;33:639-645. doi:10.1515/jpem-2019-0420
3. República de Colombia. Ley 1980 de 26 de julio de 2019. http://www.suin-juriscol.gov.co/viewDocument.asp?ruta=Leyes/30036695
4. República de Colombia. Ministerio de Salud. Resolución 412. 2000. http://www.saludcolombia.com/actual/htmlnormas/Res412_00.htm
5. República de Colombia. Ministerio de Salud y Protección social. *Guía de práctica clínica. Detección de anomalías congénitas en el recién nacido* – Guía 03. 2013. https://www.minsalud.gov.co/sites/rid/lists/bibliotecadigital/ride/inec/iets/gpc_ptes_ac.pdf
6. Cambra A, Martínez L, Ortiz A, *et al*. Análisis de casos positivos de cribado neonatal de errores congénitos del metabolismo en la Comunidad de Madrid. *Rev Esp Salud Publica*. 2020;16:94
7. Ministerio de Salud y Protección Social. Viceministerio de Salud, Dirección de Epidemiología. Informe del Plan decenal de Salud Pública 2012 – 2021. 2012. https://www.minsalud.gov.co/sites/rid/lists/bibliotecadigital/ride/vs/ed/psp/imp_4feb+abcminsalud.pdf
8. Lampret BR, Remec ŽI, Torkar AD, *et al*. Expanded Newborn Screening Program in Slovenia using Tandem Mass Spectrometry and Confirmatory Next Generation Sequencing Genetic Testing. *Zdravstveno varstvo*. 2020;59:256–263. doi:10.2478/sjph-2020-0032
9. Vissing CR, Dunø M, Wibrand F, Christensen M, Vissing J. Hydroxylated Long-Chain Acylcarnitines are Biomarkers of Mitochondrial Myopathy. *J Clin Endocrinol Metab*. 2019;1:104. doi:10.1210/jc.2019-00721
10. Naciones Unidas. Objetivos de desarrollo del milenio. Nueva York: Naciones Unidas; 2015. https://www.undp.org/content/dam/undp/library/mdg/spanish/undp_mdg_report_2015.pdf

11. Valera Antequera D, Montealegre Páez AL, Bermúdez A, García Robles R. Importancia de una propuesta para la implementación de un programa de tamizaje neonatal expandido en Colombia. *Rev Med*. 2019;27(2):1. doi:10.18359/rmed.4195

12. República de Colombia. Instituto Nacional de Salud. *Tamizaje Neonatal Vigilancia por el laboratorio. Actualización de recomendaciones técnicas y operativas para el laboratorio*. Bogotá: Imprenta Nacional; 2014.

13. Dean AG, Sullivan KM, Soe MM. *OpenEpi: Open Source Epidemiologic Statistics for Public Health*. Version 3.01 – Released April 4 and revised April 6, 2013. https://www.openepi.com/menu/oe_menu.htm

14. NTC-ISO 13528:2015. *Statistical methods for use in proficiency testing by interlaboratory comparison*. 2015. https://www.iso.org/standard/56125.html

15. Céspedes N, Valencia A, Echeverry CA, *et al*. Reference values of amino acids, acylcarnitines and succinylacetone by tandem mass spectrometry for use in newborn screening in southwest Colombia. *Colomb Med (Cali)*.2017;48(3):112-118. doi:10.25100/cm.v48i3.2180

16. McHugh D, Cameron CA, Abdenur JE, *et al*. Clinical validation of cutoff target ranges in newborn screening of metabolic disorders by tandem mass spectrometry: A worldwide collaborative Project. *Genet Med*. 2011:13(3):230-254. doi:10.1097/GIM.0b013e31820d5e67

17. Timan IS, Sjarif DR, Pasaribu MM, Anandari L. Profiles of Amino Acid in Indonesian Neonates. *Research Square*. 2020;preprint. doi:10.21203/rs.3.rs-26435/v1

18. República de Colombia. Departamento Administrativo Nacional de Estadística (DANE). Población Indígena de Colombia – 2018. 2019. https://www.dane.gov.co/files/investigaciones/boletines/grupos-etnicos/presentacion-grupos-etnicos-2019.pdf

19. Viera CS, Barreto GMS, Silveira R, *et al*. Biochemical predictors for metabolic syndrome in preterm infants according to weight ratio. *Arch Endocrinol Metab*. 2020;64(5):567-574. doi: 10.20945/2359-3997000000237

20. Blencowe H, Cousens S, Oestergaard M, *et al*. National, regional, and worldwide estimates of pretermbirth rates in the year 2010 with time trends since 1990 forselected countries: a systematic analysis and implications. *Lancet* 2012;379:2162–2172. doi:10.1016/S0140-6736(12)60820-4

21. Organización Mundial de la Salud. *Nacimientos prematuros*. 19 de febrero de 2018. https://www.who.int/es/news-room/fact-sheets/detail/preterm-birth

22. Ospino Guzmán MP, Mercado Reyes MM. *Comportamiento de la prematuridad en Colombia durante los años 2007 a 2016*. Instituto Nacional de Salud, Dirección de Investgación en Salud Pública, Grupo de Salud Materna y Perinatal. 2018. http://fundacioncanguro.co/wp-content/uploads/2018/11/Comportamiento-de-la-prematuridad-en-Colombia-durante-los-a%C3%B1os-2007-a-2016.pdf

23. Suprovath KS, Tarikul I, Aparna B, Golam SB, Rosy S, Nusrat S. Age-Specific Cut-off Values of Amino Acids and Acylcarnitines for Diagnosis of Inborn Errors of Metabolism Using Liquid Chromatography Tandem Mass Spectrometry. *Bio Med*. 2019;2019:3460902. doi:10.1155/2019/3460902