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Dried Blood Spot Reference Intervals for Steroids and Amino Acids in a Neonatal Cohort of the National Children's Study

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BACKGROUND: Reference intervals from children are limited by access to healthy children and their limited blood volumes. In this study we set out to fill gaps in pediatric reference intervals for amino acids and steroid hormones using dried blood spots (DBS) from a cohort of the National Children's Study.

METHODS: Deidentified DBS annotated with age, birthweight, sex, and geographic location were obtained from 310 newborns aged 0–4 days and analyzed for 25 amino acids and 4 steroid hormones using LC-MS/MS. Nonparametric statistical approaches were used to generate the 2.5th–97.5th percentile distributions for newborns. Paired plasma/DBS specimens were used to mathematically transform DBS reference intervals to corresponding plasma intervals.

RESULTS: 10 of 25 DBS amino acid distributions were dependent on sex. There was little correlation with age, birthweight, or geographic location over the first 4 days of life. In most cases, transformation of DBS distributions to plasma distributions faithfully reflected independent studies of newborn plasma amino acid distributions. In general newborn steroid distributions were negatively correlated with age and birthweight over the first 4 days of life. Data distributions for the 4 steroids were not found related to geographic location, but testosterone concentrations displayed sex dependence. Transformation of DBS distributions to plasma intervals did not faithfully replicate other neonate steroid reference intervals determined directly with plasma.

CONCLUSIONS: These data demonstrate the feasibility and utility of deriving newborn reference intervals from large numbers of archived DBS samples such as those obtained from the National Children's Study biobank.

As many as 70% of clinical decisions to admit, discharge, and treat patients are based on clinical laboratory tests *(1)*. These test results are overwhelmingly interpreted in the context of a distribution of results from a healthy "reference" population. In adults, the use of a single reference population may be appropriate over many decades of life. Neonates, infants, toddlers, and adolescents, however, require the use of reference populations that reflect rapid physiologic changes associated with growth and development.

Generation of pediatric reference intervals is difficult because of the large numbers of participants required to define statistical boundaries, the requirement for as healthy a population as possible and the limited blood volumes available from children for inclusion. Despite these difficulties a number of initiatives are actively developing reference intervals for children. The University of Utah sponsored CHILDx (Children's Health Improvement through Laboratory Diagnostics) study (www.aruplab.com/pediatrics) is collecting hundreds of samples from children aged 6 months to 17 years with the goal of developing reference intervals for 144 analytes *(2, 3)*. The Canadian CALIPER (Canadian Laboratory Initiative on Paediatric Reference Intervals Database) project (www.sickkids.ca/caliperproject/) is actively enrolling participants from birth to 18 years of age and has so far developed intervals for more than 60 common analytes *(4–6)*. Both of these efforts are a valuable source of information but both efforts lack large numbers of very young participants including neonates and do not provide data for geographically diverse populations.

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The National Children's Study (NCS)[6] was conceived and implemented as a longitudinal study designed to prospectively address the impact of genetics and environment on the growth and development of 100 000 children from birth through the age of 21. Steroid hormones and amino acids are key mediators and indicators of child development. Steroid hormones, for example, play key roles in the synchronized development of secondary sex characteristics, bone and muscle growth, as well as neurite outgrowth, synapse formation, and myelination in the central nervous system. Amino acids are indicators of nutrition, metabolic state, liver disease *(7)*, and potentially cancer *(8)*, and diabetes *(9)*. The application of carefully crafted reference intervals for these compounds appropriate for age, sex, and geography may facilitate the diagnosis of large numbers of disturbances to physical, sexual, metabolic, and neurologic development.

Although this study was recently defunded, annotated blood, urine, and tissue samples from early participants in the NCS have already been collected. We obtained 310 dried blood spots (DBS) from the NCS biobank from neonates in the first week of life with the goal of building prospective reference intervals from the earliest days of life and continuing with the cohort through adolescence to adulthood. Application of multiplexed LC-MS/MS technology to these samples enabled large numbers of concurrent analytic profiles with minimal sample volume. The reference interval data for 4 steroid hormones and 25 amino acids derived from a geographically diverse population of neonates presented herein is the first step to providing the best biochemical parameters of healthy growth and development and enabling better recognition and detection of abnormal adrenal, reproductive, and metabolic pathology.

Materials and Methods

SAMPLES

Deidentified DBS were obtained from the biobank of the NCS via a Supplemental Methodological Proposal approved by the University of Texas Southwestern Medical Center IRB. Recruitment criteria, techniques, and enrollment data are reported at www.nichd.nih.gov/research/NCS/Documents/NCS_Archive_Study_Description.pdf as well as by Montaquila et al. *(10)* and Baker et al. *(11)*. Blood cards were maintained desiccated at −80 °C before analysis. Under these conditions amino acids *(12, 13)* and select steroids *(14, 15)* exhibit minimal degradation over the course of a decade. The spots provided were from full-term male (53%) and female neonates ranging in age from birth to 4 days of age from 7 geographic regions. A summary of age, sex, and geographic distribution of the samples is provided in Table 1. A total of 308 of the original 310 spots were available for amino acid analysis in the Core Laboratory at St. Louis Children's Hospital. Data from 3 spots were excluded from analysis owing to erroneous age information. Data from 3 further spots were excluded owing to extreme amino acid concentrations suggesting sample contamination or metabolic disease. Data from 12 samples associated with birth weight <2500 g were also excluded, leaving 290 evaluable spots. Of the original 310 spots, 298 were subjected to steroid hormone analysis at Children's Medical Center of Dallas. Five spots failed to yield data and 12 additional samples were deleted owing to erroneous age information or low birthweight leaving 281 evaluable profiles. In light of scant information regarding the uniformity of "normal" newborn metabolism, no other data points were removed as outliers from the data set.

Table 1. NCS neonatal cohort demographics (n = 310).

Age in days, median (range)	2 (0–4)
Birthweight in grams, median (range)	3348 (596–5448)
Sex	
Male, n	163
Female, n	147
Geographic distribution	
Brookings County, SD	104
Duplin County, NC	30
Montgomery County, PA	39
Orange County, CA	31
Queens, NY	19
Salt Lake County, UT	83
Waukesha County, WI	4

MATERIALS

Standards, deuterated internal standards, mobile phase constituents, and other materials for amino acid analyses were as described in Dietzen et al. *(16)*. Unlabeled dehydroepiandrosterone-sulfate (DHEAS), and deuterated 4-androsten-3,17-dione (2,2,4,6,6,16,16-D_7) and DHEA (16,16-D_2) were obtained from Steraloids. Deuterated testosterone (16,16,17-D_3), 17α-hydroxyprogesterone (17-OHP, 2,2,4,6,6,21,21,21-D_8) as well as unlabeled androstenedione, testosterone, and 17-OHP were obtained from Cerilliant. Steroid-free serum and Optima MS grade water, methanol and acetonitrile were obtained from Thermo Fisher Scientific. 2-hydrazinopyridine, ethanol, trifluoroacetic acid, and formic acid were ob-

[6] Nonstandard abbreviations: NCS, National Children's Study; DBS, dried blood spot; DHEAS, dehydroepiandrosterone-sulfate; 17-OHP, 17α-hydroxyprogesterone; LLOQ, lower limit of quantification; CALIPER, Canadian Laboratory Initiative on Paediatric Reference Intervals Database; CAH, congenital adrenal hyperplasia.

tained from Sigma-Aldrich. Whatman 903™ sample collection paper was obtained from GE Healthcare. Hank's balanced salts without calcium, magnesium or phenol red, were obtained from Life Technologies, and an expired unit of packed red blood cells was obtained from the Children's Medical Center of Dallas blood bank.

DBS SAMPLE PREPARATION

Using the total volume of blood applied to the 13 mm spot and the fractional area represented by a 3 mm spot, the blood volume represented by a 3 mm punch was empirically determined to be 3.0 μL. This volume is consistent with the findings of Hall et al. *(17)*. For amino acid analysis, 3 spots (3 mm each) were punched from the larger 13 mm spot for analysis. Preliminary experiments demonstrated that the use of 3 punches reduced spot to spot variability whereas, the use of more than 3 punches yielded little additional improvement (see Fig. S1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue12). Amino acids were extracted by suspending the punched material in 250 μL of methanol containing internal standards followed by 10-min incubation at room temperature. Extraction efficiencies for amino acids under these conditions are displayed in online Supplemental Table S6. Extracted material was dried under N_2, butylated, and reconstituted in mobile phase (80/20 H_2O/acetonitrile/0.1% formic acid) for analysis. For steroid analysis, a single 7-mm punch was sonicated for 15 min at room temperature in 1.75 mL methanol and 0.25 mL of acetonitrile containing internal standards. Extraction was repeated with another 2.0 mL methanol. Pooled extracts were dried under nitrogen before analysis. 17-OHP, androstenedione, and testosterone were detected without derivatization following reconstitution in methanol:water (50:50 vol/vol). DHEAS was detected following derivatization with 15 mmol/L hydrazinopyridine/3 mol/L trifluoroacetic acid in ethanol for 1 h at 60 °C.

AMINO ACID ANALYSIS

Following DBS extraction and butylation, amino acid analysis was performed essentially according Dietzen et al. *(16)*. Imprecision of amino acid analysis was monitored during each run with 2 stable liquid materials (RECIPE), and 2 DBS materials. One DBS control was prepared from pooled, EDTA-anticoagulated whole blood. A second EDTA-anticoagulated whole blood pool was centrifuged to pellet erythrocytes and the plasma was replaced by an equal volume of RECIPE control containing increased concentrations of amino acids. Both pools were spotted onto Whatman 903™ paper, dried overnight at room temperature, and stored for the duration of the study at −80 °C. Imprecision of DBS amino acid analyses was greater than imprecision observed with plasma despite the use of distinct punches from 3 areas of the spot. Imprecision in the normal control ranged from 10% to 34% over 20 analytic runs. Imprecision of the increased concentration control spots ranged from 9% to 33%. The greatest degree of imprecision was associated with those amino acids present at concentrations <10 μmol/L including homocitrulline, sarcosine, and γ-aminobutyrate.

STEROID ANALYSIS

For the DBS method, steroid calibrator and QC materials were prepared with steroid-poor whole blood prepared from equal volumes of expired blood bank erythrocytes and steroid-free serum. A 6 point calibrator material and 2 QC pools were applied to paper, dried, and stored at −80 °C before use. LC-MS/MS was carried out using an Acquity® UPLC system equipped with BEH C18 column (2.1 mm × 50 mm × 1.7 μm) coupled to a Quattro XE tandem quadrupole mass spectrometer (Waters). Androstenedione, testosterone, and 17-OHP were monitored in a single analytic run using a mobile phase variably consisting of 50%–100% methanol and employing primary transitions of *m/z* 287→97, 289→97, and 331→97, respectively. The hydrazinopyridine derivative of DHEAS was quantitated employing a mobile phase consisting of 0.1% formic acid and 0%–100% acetonitrile using a transition of *m/z* 460→362. The lower limit of quantification (LLOQ) for each of the steroids corresponded to the lowest concentration that was measurable with imprecision <20% CV. LLOQs for 17-OHP, androstenedione, and testosterone profiles were 0.3 nmol/L and 0.1 μmol/L for DHEAS. The upper limits of linearity for 17-OHP, androstenedione, testosterone, and DHEAS were 300 nmol/L, 40 nmol/L, 70 nmol/L, and 20 μmol/L, respectively. Total measurement imprecision for the 17-OHP, androstenedione, testosterone, and DHEAS assays ranged from 6.2% to 16.9%, 10.4% to 23.2%, 9.4% to 23.4%, and 10.4% to 21.1% across the dynamic range of the method over 20 runs. Full details of the DBS method and its analytic characteristics may be found in the online Data Supplement. Plasma steroid analyses were performed according to Guo et al. *(18)*.

DERIVATION OF PLASMA-EQUIVALENT REFERENCE INTERVALS

Pairs of DBS and plasma specimens from pediatric patients were prepared from EDTA-anticoagulated whole blood stored at 4 °C <24 h after collection. Each paired spot and plasma sample was subjected to amino acid analysis (n = 40) or analysis of androstenedione (n = 15), 17-OHP (n = 11), and testosterone (n = 12). Quantitative steroid comparisons were only performed when concentrations were greater than the LLOQ from both blood spot and plasma assays. The relationship of plasma to DBS concentration for each amino acid and steroid was derived from the ratio of the plasma to DBS concentration. DBS concentrations from NCS samples were

Table 2. Distribution of neonatal whole blood amino acid and steroid concentrations.

	Mean (all)	Median (all)	2.5%–97.5% (all)	2.5%–97.5% (male)	2.5%–97.5% (female)
Amino acid, μmol/L					
Phenylalanine[a]	54	51	30–97	29–108	32–74
Tyrosine	79	72	34–151	36–147	34–165
Isoleucine[a]	30	28	12–66	12–92	13–50
Leucine[a]	64	60	31–130	34–174	31–109
Valine[a]	100	95	46–224	41–246	50–184
Threonine	187	169	66–415	55–448	69–370
Serine[a]	249	234	130–472	127–462	138–550
Glycine[a]	350	329	182–637	163–493	229–691
Methionine	22	21	10–39	10–40	12–37
Glutamine[a]	495	489	238–808	203–773	257–850
Glutamate[a]	337	324	193–566	175–500	217–629
Citrulline[a]	13	12	5–23	6–21	5–25
Arginine	11	9	<1–36	<1–36	<1–39
Ornithine	48	45	19–105	21–111	18–103
Homocitrulline	1	1	<1–4	<1–4	<1–4
Alanine	201	188	104–394	98–400	115–394
Hydroxyproline	34	33	18–53	15–51	19–55
Proline[a]	159	155	94–245	92–251	94–244
Lysine	61	56	29–119	28–112	34–131
β-Aminoisobutyrate	4	3	<1–16	<1–15	<1–20
β-Alanine	8	8	4–15	5–15	3–15
Sarcosine	1	1	<1–4	<1–4	<1–5
γ-Aminobutyrate	2	2	<1–4	<1–4	<1–4
Histidine	48	46	24–98	25–101	22–98
α-Amino-n-butyrate	18	17	3–39	4–39	3–40
Steroid					
17-OH progesterone, nmol/L	2.4	1.3	<0.3–14.4	<0.3–16.7	<0.3–7.1
Androstenedione, nmol/L	2.4	1.9	0.4–7.3	<0.3–7.4	0.4–7.4
Testosterone,[a] nmol/L	1.8	0.6	<0.3–7.0	<0.3–7.3	<0.3–7.0
DHEAS, μmol/L	2.9	1.8	0.2–12.3	<0.1–12.6	0.2–10.8

[a] Distribution by sex is different by Kruskal–Wallis independent samples test, $P < 0.001$.

then transformed to yield plasma equivalent reference intervals.

STATISTICAL ANALYSIS

Descriptive and linear regression statistics were determined using Excel 2010 (Microsoft). Tests for normality of analyte distributions (Kolmogorov–Smirnov, Shapiro-Wilk) were carried out using SPSS 21 (IBM). Because analyte distributions were universally skewed, reference intervals were determined nonparametrically and correspond to the 2.5th–97.5th percentiles of the experimental distribution. Comparison of analyte distributions across categorical variables such as sex and geography were performed using the nonparametric Kruskal–Wallis independent samples test (SPSS 21). Correlation (Pearson) of analyte concentrations to continuous variables (age, birthweight) was likewise performed using SPSS 21.

Results

STEROIDS

Nonparametric reference intervals for the 4 steroid hormones are displayed in Table 2 (bottom). The influence of sex, age, geography, and birthweight was also exam-

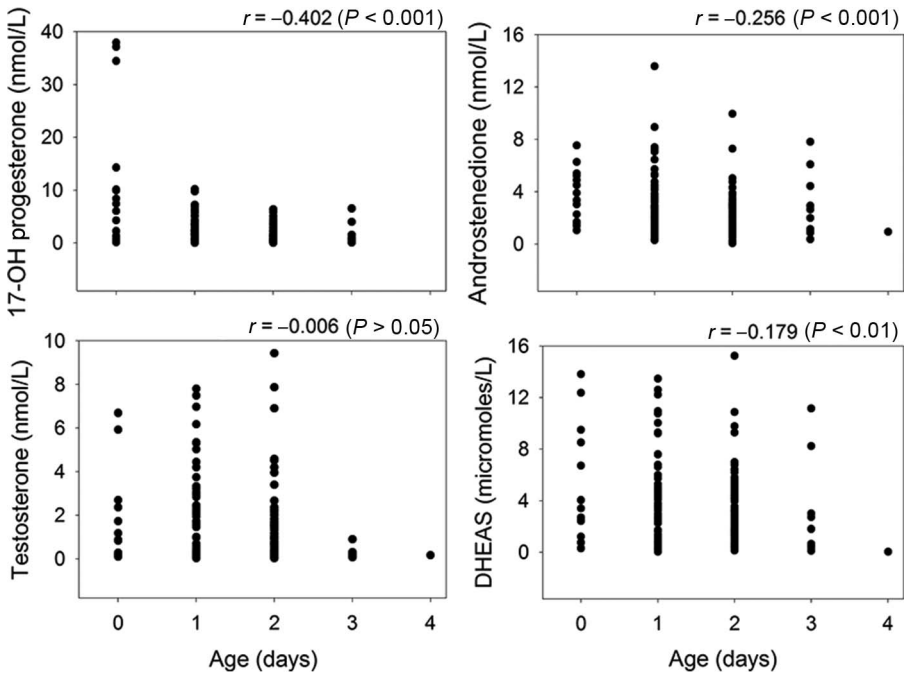

Fig. 1. Correlation of DBS steroid concentrations with age.

Each point represents concentration from a single blood spot. Day 0 data are from participants less than 24 h of age. Pearson correlation statistics are indicated.

ined. The distribution of testosterone concentrations was skewed higher in male neonates (median = 1.42 nmol/L) than female neonates (median = <0.3 nmol/L) but the 97.5th percentile was similar in males (7.25 nmol/L) and females (7.00 nmol/L). There were no significant geographical differences in steroid distributions. The concentrations of 17-OHP, androstenedione, and DHEAS but not testosterone were negatively correlated with age over the first 4 days of life (Fig. 1). The collective *n* in each age bin was inadequate, however, to confidently calculate age specific intervals. Finally, the concentrations of some steroids were modestly and negatively correlated with birthweight from 596 to 5448 g (see online Supplemental Fig. 1). When samples from low and very low birthweight babies were excluded, correlation with birthweight was not significant for any of the steroids analyzed.

AMINO ACIDS

Nonparametric reference intervals for 25 amino acids are displayed in Table 2. Concentrations of 6 other amino acids including alloisoleucine, homocystine, cystathionine, cystine, argininosuccinate, α-aminoadipic acid, were not detectable (<1 μmol/L) in blood spot extracts. Concentrations of 10 amino acids were statistically different when examined by sex. Concentrations of 6 amino acids were significantly correlated with age over the first 4 days of life (Fig. 2). Phenylalanine, citrulline, alanine, and lysine concentrations decreased whereas hydroxyproline and β-aminoisobutyrate concentrations increased modestly. The concentrations of 5 amino acids, tyrosine, threonine, citrulline, arginine, and hydroxyproline were modestly and negatively correlated with birthweight and histidine concentrations were positively correlated with birthweight (see online Supplemental Fig. 2). Although statistically significant, the magnitude of the sex, birthweight, and age differences seemed insufficiently large to indicate the use of distinct reference intervals for clinical purposes.

Concentrations of 6 amino acids were statistically different based upon sampling location (Fig. 3). Samples from Utah were most commonly found to differ from other geographic regions. Glutamine and arginine concentrations from Utah were significantly greater than those from South Dakota, North Carolina, or California. Concentrations of α-aminobutyrate and β-aminoisobutyrate were also greater in blood spots from Utah than those obtained in North Carolina. Additionally, β-aminoisobutyrate concentrations in North Carolina were significantly lower than those obtained from South Dakota and Pennsylvania and α-aminobutyrate concentrations were also higher in Pennsylvania than in the North Carolina Cohort. Fi-

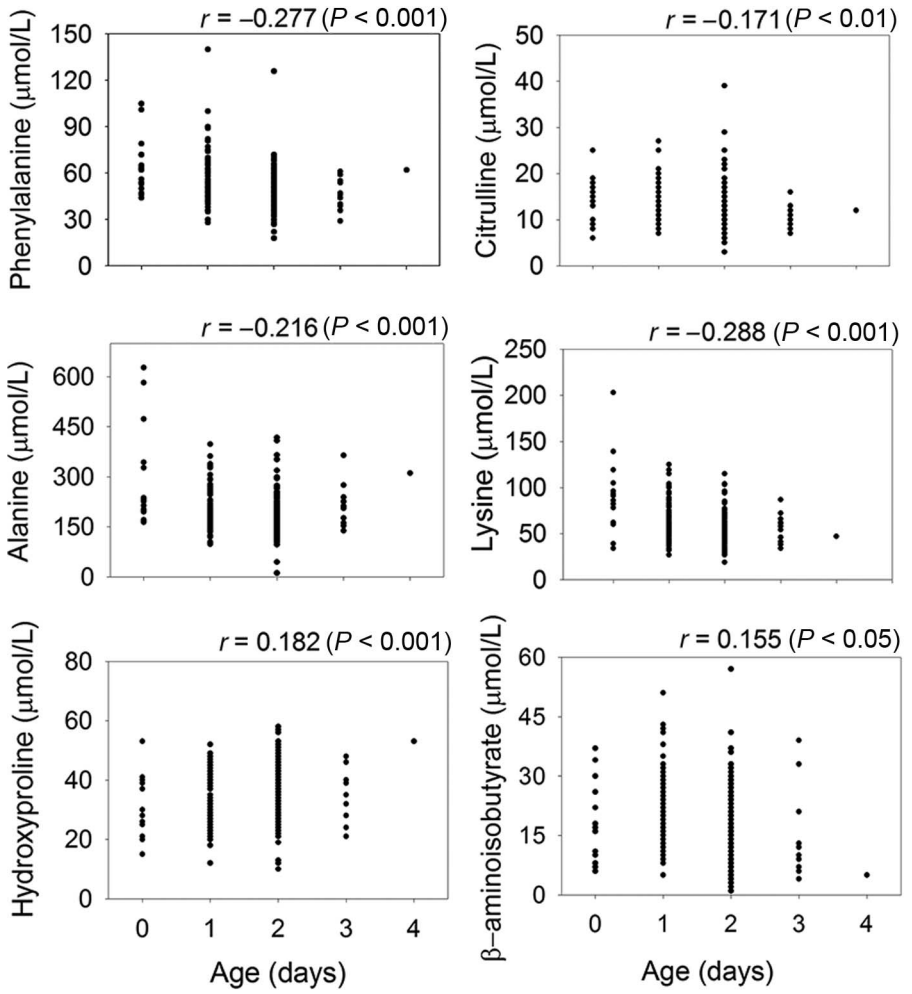

Fig. 2. Correlation of 6 amino acid concentrations with age.

Each point represents concentration from a single blood spot. Day 0 data are from participants less than 24 h of age. Pearson correlation statistics are indicated.

nally, methionine concentrations were significantly higher in South Dakota than Pennsylvania and threonine concentrations were higher in New York than in South Dakota. No conclusions can be drawn from the Wisconsin samples because only 4 blood spots were included in these analyses. Again, given the small magnitude of the geographic differences, the use of geography-specific reference intervals does not seem warranted without exploring the preanalytic mechanisms responsible for our observations.

TRANSFERENCE OF DBS INTERVALS TO PLASMA EQUIVALENT

Although blood spots are the currency of newborn screening, plasma is the primary specimen in pediatric acute care settings. As a result, studies to determine the feasibility of transferring the DBS reference intervals to plasma were carried out. Linear regression of plasma amino acids (n = 40) and steroids (n = 11–15) to their respective DBS concentrations revealed that differences were proportional and therefore, a plasma/DBS ratio was generated for each analyte that is presented in Table 3. This customized ratio was applied to the DBS data to generate a derived plasma interval.

Derived plasma amino acid concentrations were compared to those determined at St. Louis Children's Hospital using LC-MS/MS *(19)* and by the Canadian Laboratory Initiative on Paediatric Reference Intervals Database (CALIPER) group using precolumn derivatization, LC (liquid chromatography) separation, and UV (ultraviolet) detection *(6)*. With the exception of glu-

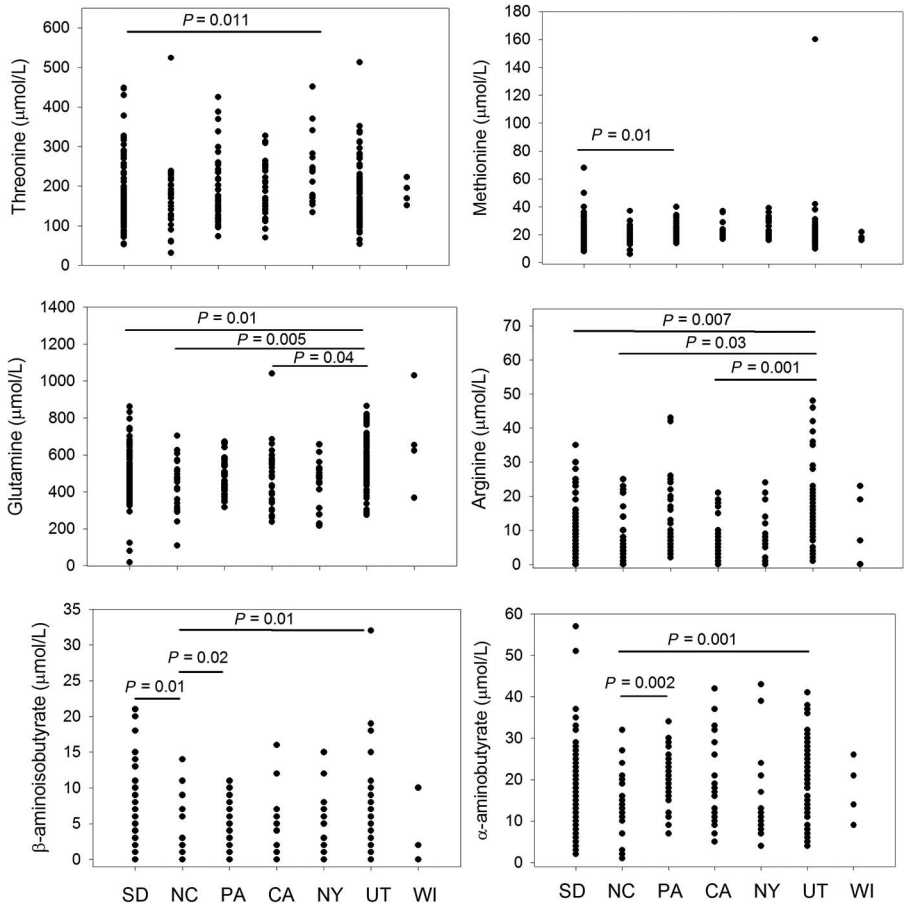

Fig. 3. Comparison of 6 amino acid distributions by geographic region.

Geographic regions are as indicated in Table 1. Each data point represents concentration from a single DBS. Statistical comparison performed by Kruskal–Wallis independent samples test.

tamine and glutamate, the derived plasma intervals are remarkably similar to those directly determined in plasma. Glutamine concentrations derived from DBS match the range of the CALIPER data quite well but are lower than those determined in our earlier plasma study. Likewise, glutamate concentrations derived from DBS agree with CALIPER ranges but are higher than our internal study of plasma. The most likely explanation for these observations is the age of the plasma specimens in reference 19 allowing for mild glutamine to glutamate conversion.

There was less agreement between newborn steroid hormone concentrations. The 17-OHP concentrations derived from DBS in the present study generally agree with those published by Mayo Medical Laboratories (mayomedicallaboratories.com) but are significantly higher than those from Esoterix (Table 3). Androstenedione concentrations are comparable across all 3 sources of reference intervals. The DBS-derived testosterone distributions in the present study do not faithfully reflect male and female distributions determined at the 2 reference laboratories. Finally, DHEAS intervals derived from DBS are slightly wider than those from 3-day-olds published by Esoterix. The reasons for these discrepancies are not immediately apparent but may be secondary to the small number of DBS to plasma comparison samples employed, differences in extraction efficiency, and differences in sample matrix (DBS vs plasma/serum), calibration, as well as sampling bias.

Discussion

Generation of pediatric developmental norms for circulating biomarkers suffers because of limited access to healthy control populations and the limited amount of blood available. The magnitude of the problem is greatest in neonates. For example, current techniques require 50–100 μL of plasma to perform a plasma amino acid profile

Table 3. Reference interval transfer from blood spots to plasma equivalent.

	Plasma/DBS[a]	Derived interval[b]	Direct interval[c]	Direct interval[d]
Amino acid, μmol/L				
Phenylalanine	1.30 (0.12)	39–126	35–130	49–107
Tyrosine	1.35 (0.16)	46–204	38–258	27–187
Isoleucine	1.43 (0.17)	18–94	17–102	25–129
Leucine	1.42 (0.15)	44–184	44–195	46–165
Valine	1.47 (0.21)	71–329	69–306	87–326
Threonine	1.42 (0.20)	94–589	129–550	81–313
Serine	1.09 (0.22)	141–514	117–545	199–843
Glycine	1.02 (0.22)	184–649	228–881	299–782
Methionine	1.48 (0.15)	15–57	19–71	13–44
Glutamine	1.55 (0.19)	369–1252	104–865	451–1113
Glutamate	0.94 (0.23)	182–532	105–954	91–401
Citrulline	1.38 (0.21)	7–32	6–40	9–44
Arginine	2.01 (0.75)	1–72	12–122	2–118
Ornithine	2.57 (0.92)	49–270	20–194	82–365
Homocitrulline	1.31 (0.17)	<1–5	<1–12	NR[e]
Alanine	1.31 (0.17)	136–516	110–585	175–427
Hydroxyproline	1.33 (0.18)	24–70	23–111	NR
Proline	1.15 (0.12)	108–282	105–319	127–292
Lysine	1.92 (0.28)	56–228	90–346	90–319
β-Alanine	0.62 (0.12)	2–9	2–11	3–27
Histidine	2.61 (1.04)	64–255	32–165	45–168
α-Amino-n-butyrate	1.49 (0.19)	4–58	4–45	7–42
Steroid			**Direct interval[f]**	**Direct interval[g]**
17-OH progesterone, nmol/L	1.01 (0.40)	<0.3–14.4	<2.4	<19.1
Androstenedione, nmol/L	0.88 (0.28)	0.3–6.4	0.4–9.7	0.7–10.1
Testosterone (male), nmol/L	0.71 (0.19)	<0.3–5.2	2.6–13.9	2.6–13.9
Testosterone (female), nmol/L	0.71 (0.19)	<0.3–5.0	0.7–2.2	0.7–2.8
DHEAS, μmol/L	1.19 (0.35)	0.2–14.6	2.4–9.6	NR*

[a] Data are mean (SD) (n = 40 for amino acids, n = 11–15 for steroids).
[b] DBS interval transformed by plasma/DBS ratio (2.5th–97.5th percentile).
[c] Intervals using Li-heparin plasma from full-term neonates (2.5th–97.5th percentile) per Oladipo et al. *(19)*.
[d] Intervals for serum (parametric) from youngest age bin per reference 6.
[e] NR, not reported.
[f] Serum steroid intervals for full-term neonates published by Esoterix (www.esoterix.com). DHEAS interval cited for full-term 3-day-old neonates.
[g] Serum steroid intervals for full-term neonates published by Mayo Medical Laboratories (www.mayomedicallaboratories.com). NR*, no neonatal DHEAS data cited.

requiring acquisition of 3–4 times that volume of whole blood at hematocrits common in newborns (50%–65%). In this study, we investigated the feasibility of using DBS for the generation of developmental reference intervals. A 13-mm blood spot requires only 60–80 μL of blood and is uniquely suited for extraction of a wide variety of molecules that can be analyzed by multiplex technologies, among them LC-MS/MS. From these spots, reference intervals for 26 amino acids and 4 steroids have been generated for a geographically diverse population of neonates from birth to 4 days of age.

Circulating amino acid concentrations are most commonly used to assess the possibility of inborn metabolic disease. The statistically significant but mild differences of amino acid concentrations based on sex, birthweight, and geography in this study have little consequence for the diagnosis of classic inborn metabolic diseases like phenylketonuria, maple syrup urine disease, or urea cycle disorders

like citrullinemia. Beyond this application, however, amino acids concentrations have the potential to convey information about energy utilization, nutrition, liver function, nitrogen balance, and connective tissue turnover. It is likely that such subtle differences may be more important as multivariate metabolomic profiles become more common in defining cancer and diabetes, for example. The mostly faithful transfer of amino acid distributions from blood spots to plasma-like intervals demonstrated in this study demonstrates that the current approach may be a feasible alternative to direct development of reference intervals using plasma.

Although this study did not target a broad complement of steroids the 4 that were the focus of this study provide key information regarding glucose metabolism, body composition, sexual differentiation, and fertility. 17-OHP is assessed as part of newborn screening programs to detect congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency. The false positive rate for this diagnosis is higher if samples are collected before 36 h of age, demonstrating the need for highly resolved age-specific reference intervals which will require additional, larger studies to define. Both 17-OHP and androstenedione are monitored during follow-up of patients with CAH. Accurate, prompt diagnosis and close follow-up can mitigate salt-wasting crises in all affected patients and assist with sex assignment in virilized female patients *(20)*. Other pathology associated with steroid metabolism typically presents at later stages of development. It is possible that such disorders may be detectable much earlier in life, maybe as early as the newborn period. Maternal or neonatal exposure so called endocrine-disrupting chemicals *(21, 22)* may be reflected in the measurement of these and other steroids, for example. The margin for error in defining normal distributions is likely to be small in such cases. Andersson et al. *(23)*, for example, detected a median testosterone difference of only 5 nmol/L between fertile males and those with idiopathic infertility. Normal distributions stratified by age, sex, geography, and birthweight, combined with clinical and maternal variables may increase the utility of circulating steroid measurements to predict and detect these developmental abnormalities.

To the best of our knowledge, these data are the first of their kind generated from the NCS biobank. The strengths of these data include the large sample of the youngest children and a level of geographic heterogeneity not previously achieved. The primary weakness is that these intervals were developed with a specimen type not commonly employed in clinical environments. Transference studies from blood spots to plasma may overcome this shortcoming for some analytes but not others. In aggregate, these data demonstrate the utility of exploiting the NCS resource to examine developmental biomarkers but the long term viability of this resource is not known. It was anticipated that the cohort would be retested on a regular basis for the duration of the NCS. Retesting the cohort through age 21 would enable a complete set of population reference intervals for amino acids, steroids, and many more analytes from infancy through puberty and into adulthood. In addition to population variability, maintaining as many members of the original cohort as possible would contribute toward resolving intraindividual variability throughout development as well. With or without the NCS resource, the continued collective pursuit of better pediatric reference intervals remains necessary to improve the medical care of children at all stages of development.

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