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ORIGINAL ARTICLE

Reference values of amino acids and of common clinical chemistry in plasma of healthy infants aged 1 and 4 months

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Abstract
Objective To compare plasma levels of amino acids and clinical chemistry parameters in healthy infants at 1 and 4 months of age and to establish corresponding reference limits.
Methods Data of three multicenter studies assessing the safety of new infant formulas were used. During these studies infants of both age-groups were either breast-fed or received formulas of low or high protein content. All samples were analyzed centrally in the same accredited laboratory.
Results Plasma was collected from 521 infants in total, 157 boys and 135 girls aged 1 month and 121 boys and 108 girls aged 4 months. At the age of 1 month, 62 infants had received exclusively breast milk, 198 exclusively formula, and 27 both; in the 4-months age group corresponding numbers were 49, 158 and 18, respectively; for 9 infants, diet was unknown. Concentrations of most amino acids and clinical chemistry parameters differed significantly between both ages. Regardless of age, most plasma amino acid levels were comparable or lower in breast-fed than in formula-fed infants whereas at 1 month of age most clinical chemistry parameters were higher. While in breast-fed infants the plasma urea concentration decreased over 4 months of age, it increased in formula-fed infants. There were significant differences between infants fed a low and high protein formula. At both ages, high protein formulas resulted in significantly higher threonine, 2-aminobutyrate, and urea concentrations.
Conclusions For clinical use, age- and diet specific reference limits in infants are warranted.

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Introduction

Reference ranges are needed for medical decision making. They are used for suspicion, confirmation or exclusion of disease, its prognosis, follow-up, and treatment, as well as for the evaluation of safety of nutritional or pharmaceutical products. Reference limits should be transposable to other laboratories; for the interpretation of data additional information should be given e.g. on pre-analytical factors, on the analytical methods and the statistics used. Recommendations have been made by expert panels of the International Federation of Clinical Chemistry (IFCC) on the theory, production and use of reference intervals (IFCC 1987a, b). Recent guidelines have been approved by the Clinical and Laboratory Standards Institute (CLSI 2013) as well as recommendations for pediatric reference limits (Jung and Khosrow 2009).

In pediatrics, reference limits should be established in healthy populations of newborns, infants, and children. Partition in age groups takes into account that major physiological changes occur already within the first few months of life (Dupont 2003), due to growth (velocity), organ development, changing nutrient composition, and protein requirements. In particular, plasma composition in young infants might be affected by nutritional factors. However, formula

feeding has been modified in the last decades as reflected by the recommendations of the EFGHAN (Koletzko et al. 2005) in order to avoid an excessive supply or imbalance of protein composition and amino acids during early organ development. Reference limits need to adopt this and should generally allow distinction of breast and formula fed infants.

However the number of samples obtained from non-hospitalized healthy infants has often been too small to fulfill current international requirements for reference limits. Consequently, results of infants of 1 month up to 2 years of age have often been pooled (Lockitch et al. 1988; Applegarth et al. 1979; Lepage et al. 1997; Armstrong and Stave 1973a, b; Lepage et al. 1997; Ghoshal and Soldin 2003; Duran 2008) without further distinction of the post-neonatal period. Information on the reference population and pre-analytical factors is often missing and at least many older publications presented reference limits as mean values and standard deviation (SD), which is not adequate for parameters of which data are not normally distributed. To our knowledge age-related reference limits for plasma amino acids or common clinical chemistry parameters with sufficient numbers of infants below 1 year of age have not been published yet at all.

During three multicenter studies aiming the evaluation of safety of new infant formulas, we had the opportunity to measure plasma concentrations of amino acids and of common clinical chemistry parameters in healthy infants under highly-controlled feeding conditions. Collected data were sufficient to establish corresponding reference limits at one and four months of age and to test whether the results differ between these age groups.

Study populations and methods

Study populations

The target population in all three multicenter studies of Nestec Inc (Vevey, Switzerland) had to meet the same eligibility criteria including informed consent of the parents for the local or institutional ethical committees. The inclusion criteria were: healthy term infants, i.e. gestational age had to be between 37 and 42 weeks and birth weight between 2500 and 4200 g (>10th and <90th percentile for boys and girls). Infants were excluded if they had malformations, congenital coronary or vascular diseases, serious diseases of the gastrointestinal system, kidneys, liver, CNS and/or metabolic diseases; intensive care within the first 21 days of life or hospital admissions; evidently pathological biochemical markers; haemolytic plasma; gross outliers i.e. values below the 0.25 centile - 3 × interquartile range (IQR) or values above the 0.75 centile+(3 × IQR). Data from all three studies were pooled after verification that populations did not significantly differ in baseline criteria.

In all studies a control group was exclusively breast-fed at libitum since birth while the experimental groups received different formulas ad libitum. In the first study, formulas differed in protein quantity and quality (Räihä et al. 2002). The protein/energy content was either 2.2–2.3 g (control group) or 1.8 g protein/100 kcal (experimental groups). The second study used a whey-based formula of reduced protein content (1.8–1.9 g protein/100 kcal) along with pro- and/or prebiotics. In the third study, infants were fed a moderately hydrolyzed hypoallergenic formula (Beba Start HA®, Nestlé) with or without additional polyunsaturated fatty acids; the protein content was 2.3 g (control group) and 1.85 g/100 kcal (experimental group), respectively. The parents of formula-fed infants documented in diaries the consumption of formula during the three days preceding the control visits. The daily protein intake was averaged over these three days. In breast-fed infants the intake of milk was not assessed because the procedure of weighing before and after each feed would have been disruptive and stressful for mother and child.

Sample collection and handling

Blood samples of at least 2 ml were taken from a peripheral vein by butterfly needles and collected in heparinized tubes (Lithium heparinate monovettes, Sarstedt, Switzerland). Samples were centrifuged at 1500 g and 4 °C for 10 minutes within 30 minutes from draw. Plasma was removed and stored at -20 or -70 °C until dry ice shipment to the central laboratory every 2 or 6 weeks, respectively.

Analytical methods

All plasma analyses were done in the clinical chemistry laboratory at the CHUV (Lausanne Switzerland) which is accredited by the Swiss Federal Office of Metrology and Accreditation in concordance with standard ISO/IEC 17025.

Amino acids

Sample preparation: Lithium heparinate plasma which had been sent on dry ice was unfrozen and immediately deproteinized with a solution containing 5-sulfosalicylic acid (Merck; final concentration 32 g/L) and the internal standards. The plasma was vortexed for 60 seconds and left standing for 10 minutes before it was centrifuged twice (15 minutes 15600×*g* at 4 °C; Beckman centrifuge J2-21).

Amino acids were analyzed using the automatic analyzer system 6300 (Beckman Instruments, Fullerton CA, USA) and corresponding standards for calibration. Peak surface integration was done with a TotalChrome, Nelson instrument v. 6.2.1 (Perkin Elmer AG, Schwerzenbach, Switzerland). Furthermore the retention time (CV% <0.4) and the ratio of the areas under the curve at 570 nm/440 nm were

calculated automatically for each peak and compared with the ratio of the standards to exclude co-elution of other ninhydrin positive substances.

For the measurement of total tryptophan concentration, plasma was deproteinized with 10 % trichloroacetic acid (TCA) in order to liberate the albumin–bound tryptophan; the TCA-solution also contained 2-methyltryptophan as internal standard. Upon addition of the TCA solution the sample was vortexed, cooled in ice water for 1 h and centrifuged as for the other amino acids. Total tryptophan was separated by HPLC (Hewlett Packard model 1090) using a RP-18 precolumn and a Superspher 10-RP-18 column (Merck, Darmstadt, Germany). For detection, a spectrofluorimeter LC 240 (Perkin Elmer) was used with excitation wavelength at 278 nm and emission at 363 nm. The quantification of peaks was done with Borwin Software v. 1.21.60 (JMBS Developments SA; Varian medical systems, Baden Switzerland).

General clinical chemistry

All parameters were analyzed on Hitachi 917 automatic analyzers (Roche Diagnostics, Basel, Switzerland) equipped with Roche reagents and calibrators (Electronic supplemental material; Table S7).

Quality assurance

Internal quality control pools of amino acids were prepared, aliquoted and stored at -80 °C. Pools, blanks, and calibrators were run every 10th analysis (at least once per week) and after each calibration or change of reagents. Inter-series precision (CV%) was <2.5 %, 3.5 %, and <4 % at amino acid concentrations of >100, 50–100, and 20–50 μmol/L, respectively; except for asparagine which was 5.0 % at 55 μmol/L. Below concentrations of 20 μmol/L, CV% was 8.8 % for hydroxyproline and 12.4 % for aspartate (at 10 and 4 μmol/L, respectively). The laboratory participated in external quality assessment programs: schemes of the European Research Network for evaluation and improvement of Screening, diagnosis and treatment of Inherited Metabolic Disorders (ERNDIM) were used for amino acids (8 samples/year) and that of the Centre Suisse de Contrôle de Qualité (CSCQ) was applied for the clinical chemistry (12 samples/year; for details see electronic supplemental material; Table S8–10).

Statistics

Data of breast-and formula-fed infants were analyzed separately and after pooling. Data distribution was evaluated with Anderson Darling and Kolmogorov Smirnov tests. Differences between groups were tested by the Wilcoxon-Mann-Whitney (WMW) test. The 0.025, 0.05, 0.1, 0.5, 0.9, 0.95, 0.975 centiles and interquartile ranges (IQR) were computed. For 90 % confidence intervals (CI) of centiles a distribution-free method was used (IFCC 1987a). We corrected the fact that 195 infants were tested both at 1 and 4 months by correcting the *P*-values by the method of Hochberg and Benjamini (1990). *P*-values<0.05 were considered significant.We used the Kolmogorov-Smirnov method for comparing the distribution of amino acids in infants aged 1 and 4 months fed formulas of low or high protein content. Statistical analyses were done using SAS 9.3 TS1M0 for Windows (SAS Institute Inc., Cary, NC) and Analyse-it 3 for Excel®, standard edition (Analyse-it Software Ltd., Leeds, UK).

Results

Plasma samples of 157 male and 135 female infants aged 1-month (23–43 days) were collected. Out of 292 of these infants, 62 were breast-fed, 197 formula-fed only, 28 were both breast- and formula-fed, and for 5 infants nutritional information was missing. At 4-months of age (113–133 days) plasma samples were collected from 121 male and 108 female infants. Of these 229 infants, 49 were breast-fed, 158 were formula-fed only, 18 were both breast- and formula-fed, and for 4 infants nutritional information was missing.

Tables 1 and 2 show the relevant upper and lower centiles of the analytes, median values and IQRs at both ages, for breast- and formula-fed infants combined (Tables 1 or 4) and separately for exclusively breast-fed (Tables 2, 5) and exclusively formula-fed infants (Tables 3, 6). The time elapsed from last feed until sampling was not significantly different for any two groups, neither age nor differences in diets or formulas. Reference limits and median values are given in bold. The distribution-free 90 % CI of the limits is given (in brackets) in Tables 1 and 4.

The data distribution of the amino acids and of most clinical chemistry parameters was not Gaussian (except for urea and total calcium); a log normal distribution fitted best the amino acid results with the exception of cystine (see below). Distribution of the reported protein intake (g/day) was Gaussian.

Differences between age groups

- All infants (Tables 1 and 4)

  At 4 month of age most plasma amino acid concentrations were significantly lower or not different than at 1 month of age except for 2-aminobutyrate (Table 4).

  Regarding the general biochemistry, plasma levels of ferritin, alkaline phosphatase (AP) and potassium were significantly lower at the age of 4 months than at 1 month whereas those of glucose, cholesterol, total protein and albumin were significantly higher. No differences between

Table 1 Reference limits (centiles) for one month of age. based on all infants breast- and formula-fed combined

	Units	n	*0.025*	*0.05*	*0.10*	*0.50*	*0.90*	*0.95*	*0.975*	*IQR*[a]
Birthweight	kg	290	2.600	2.655	2.799	3.380	4.111	4.348	4.514	0.680
Bodyweight (BW)	kg	286	3.390	3.483	3.600	4.120	4.815	4.998	5.159	0.670
Protein intake	g/day	194	5.4	6.3	6.8	10.0	13.2	14.1	15.0	3.8
Protein intake	g/(day*kgBW)	194	1.3	1.4	1.7	2.3	3.2	3.5	3.8	0.83
Sampling delay p.p.	h	283	1.25	1.50	1.80	3.00	4.0	4.5	5.25	1.25
Age at sampling	day	292	26	26	27	30	33	34	37	4.0
Amino acids										
2-Aminobutyrate	µmol/L	283	8 (7–8)[b]	8	9	13	20	22	24 (22–28)	5
Alanine	µmol/L	284	206 (200–222)	225	240	364	507	542	599 (554–644)	129
Arginine	µmol/L	284	65 (56–70)	71	77	105	138	156	167 (158–195)	31
Asparagine	µmol/L	284	30 (24–26)	34	38	49	63	67	69 (67–75)	12
Aspartate	µmol/L	284	7 (7–8)	8	8	12	23	27	31 (27–46)	7
Citrulline	µmol/L	284	13 (11–14)	15	16	23	34	38	40 (38–45)	9
Cystine	µmol/L	284	2 (1–2)	2	3	13	40	48	53 (49–56)	21
Glutamine	µmol/L	284	410 (377–434)	439	470	578	686	734	762 (748–801)	115
Glutamate	µmol/L	284	50 (46–52)	53	59	89	165	193	232 (196–300)	58
Glycine	µmol/L	284	164 (147–166)	168	187	233	279	289	329 (298–345)	52
Histidine	µmol/L	284	65 (61–67)	68	71	86	103	109	123 (110–130)	15
Hydroxyproline	µmol/L	284	37 (31–42)	43	46	62	76	80	86 (81–90)	15
Isoleucine	µmol/L	284	37 (33–37)	40	45	65	93	98	104 (102–118)	23
Leucine	µmol/L	284	82 (46–84)	85	94	126	167	185	194 (118–211)	45
Lysine	µmol/L	284	130 (119–144)	145	161	219	285	309	328 (311–365)	63
Methionine	µmol/L	284	21 (19–22)	22	24	32	43	47	49 (47–57)	10
1-Methylhistidine	µmol/L	284	3 (2–3)	3	4	7	14	16	17 (16–19)	5
3-Methylhistidine	µmol/L	282	1 (1–1)	1	2	2	3	4	7 (5–11)	1
Ornithine	µmol/L	284	55 (46–57)	58	66	90	126	142	155 (142–183)	32
Phenylalanine	µmol/L	284	33 (28–34)	35	39	49	63	71	79 (71–85)	12
Proline	µmol/L	283	124 (116–128)	129	139	179	242	256	276 (261–308)	53
Serine	µmol/L	284	100 (94–104)	105	112	139	169	182	192 (187–197)	30
Taurine	µmol/L	284	50 (49–53)	54	58	80	150	183	232 (281–329)	34
Threonine	µmol/L	284	100 (97–105)	107	118	176	242	267	291 (281–329)	62
Tryptophan, total	µmol/L	270	46 (39–48)	50	55	68	85	91	97 (91–106)	16
Tyrosine	µmol/L	284	45 (39–48)	49	57	80	107	115	125 (117–158)	28
Valine	µmol/L	284	100 (83–104)	107	119	158	213	235	244 (237–294)	46
Aspartate+Asparagine	µmol/L	284	42	46	49	64	79	85	89	16
Glutamate+Glutamine	µmol/L	284	527	557	581	681	796	830	859	104
Clinical chemistry										
Sodium	mmol/L	248	126	130	133	138	141	144	146	4.0
Potassium	mmol/L	250	4.4	4.5	4.7	5.2	5.9	6.1	6.2	0.60
Chloride	mmol/L	250	98	100	102	106	110	111	112	4.00
Calcium, total	mmol/L	247	2.30	2.37	2.42	2.58	2.70	2.75	2.84	0.16
Glucose	mmol/L	244	3.70	3.92	4.20	4.80	5.40	5.79	5.90	0.63
Creatinine	µmol/L	249	31	33	34	39	47	49	52	7.0
Urea	mmol/L	249	0.92	1.30	1.58	2.60	3.80	4.10	4.40	1.10
Urea/ Creatinine	mol/mol	249	23	32	38	63	96	110	117	30
Cholesterol	mmol/L	162	2.10	2.20	2.30	3.00	3.99	4.20	4.50	0.90
Protein, total	g/L	244	48	49	51	56.5	62	63	65	6.0
Albumin	g/L	162	34	35	37	43	46	47	48	4.8
Ferritin	µg/L	237	81	107	138	263	439	548	655	157
alkaline Phosphatase	U/L	79	192	212	241	310	454	481	500	118

[a] IQR: Interquartile range

[b] In brackets: distribution-free 90 % confidence intervals

Table 2 Reference limits (centiles) for one month of age. based on breast-fed infants

	n	*0.025*	*0.05*	*0.10*	*0.50*	*0.90*	*0.95*	*0.975*	IQR	p[a]
Birth weight	62	2.838	2.941	3.050	3.725	4.451	4.510	4.650	0.838	0.0002
Body weight	58	3.513	3.590	3.724	4.285	4.973	5.114	5.276	0.698	0.6094
Sampling delay p.p.	61	0.6	1.25	1.5	2.75	3.75	4.5	4.9	1.3	0.8634
Age at sampling	62	25	26	26	29	31	32	33	3.0	<0.0001
Amino acids										
2-Aminobutyrate	60	8	8	8	11	14	16	16	4.0	<0.0001
Alanine	60	216	226	233	311	445	460	534	102	0.0004
Arginine	60	61	65	73	100	122	129	132	29.5	0.0679
Asparagine	60	32	34	38	48	61	66	71	15.0	0.8634
Aspartate	60	7	7	8	10	16	18	20	3.0	<0.0001
Citrulline	60	10	12	13	19	26	29	31	6.3	<0.0001
Cystine	60	3	7	12	30	54	54	57	22.3	–
Glutamine	60	458	479	514	595	734	758	772	107	0.3990
Glutamate	60	53	59	62	80	136	142	151	40.5	0.3303
Glycine	60	149	158	165	207	268	270	274	53.0	<0.0001
Histidine	60	65	66	71	84	99	103	105	12.3	0.1859
Hydroxyproline	60	39	43	46	63	74	78	80	16.0	0.8634
Isoleucine	60	39	40	47	67	90	94	100	21.3	0.8634
Leucine	60	84	90	96	130	159	167	184	37.3	0.8634
Lysine	60	124	128	145	194	231	253	257	47.5	<0.0001
Methionine	60	19	21	22	29	36	37	40	7.3	<0.0001
1-Methylhistidine	60	3	3	4	8	16	19	20	8.0	0.6229
3-Methylhistidine	59	2	2	2	2	3	4	6	1.0	0.2233
Ornithine	60	55	58	62	89	117	124	127	28.3	0.8634
Phenylalanine	60	37	38	39	47	56	58	59	10.3	0.3999
Proline	59	164	170	174	212	274	280	296	44.5	<0.0001
Serine	60	107	110	116	152	188	196	204	31.3	0.0012
Taurine	60	50	54	56	71	111	125	157	24.5	0.0018
Threonine	60	95	97	101	133	171	191	208	39.3	<0.0001
Tryptophan, total	59	56	57	60	71	86	89	95	16.4	0.1575
Tyrosine	60	63	66	73	87	111	121	154	25.0	<0.0001
Valine	60	115	121	132	166	210	217	233	35.3	0.8634
Aspartate+Asparagine	60	42	46	47	60	73	78	85	15.5	0.0088
Glutamate+Glutamine	60	537	566	604	688	814	839	863	99.3	0.8634
Clinical Chemistry										
Sodium	55	130	131	133	139	143	145	148	3.0	0.8634
Potassium	55	4.50	4.60	4.64	5.10	5.70	5.90	5.90	0.6	0.4333
Chloride	55	98	99	101	106	111	111	115	4.0	0.8634
Calcium, total	55	2.30	2.37	2.38	2.62	2.79	2.86	2.90	0.2	0.0312
Glucose	54	3.70	4.10	4.40	4.90	5.47	5.74	5.94	0.58	0.7600
Creatinine	55	34	34	36	40	49.2	53	53.7	5.5	0.5007
Urea	55	1.4	1.57	1.9	2.80	3.8	4.13	4.3	1.0	0.2284
Urea/Creatinine	55	34	37	46	65	94	100	109	28	0.8634
Cholesterol	22	3.26	3.51	3.61	3.95	4.68	4.89	5.23	0.48	<0.0001
Protein. total	55	48	49	50	59	63	66	67	6.0	0.1220
Albumin	56	37	38	40	44	47	49	51	3	0.0203
Ferritin	52	57	112	150	296	583	721	828	165	0.0468
alkaline Phosphatase	21	199	231	233	310	449	479	489	98	0.8634

[a] p of Wilcoxon Mann Whitney test after Hochberg-Benjamini correction (1990): breast-fed versus formula-fed infants of 1 month of age

both age groups were apparent for sodium, chloride, calcium, creatinine and urea (Table 4).

- Breast-fed infants (Tables 2 and 5)

  At 4 months of age no amino acid concentration was higher than at 1 month of age (p in Table 6). The difference between the two age groups was not significant for 2-aminobutyrate, alanine, asparagine, glutamine (and the sum of glutamine and glutamate), 1-methylhistidine, ornithine, taurine, tryptophan and tyrosine. All other amino acid concentrations were significantly lower at 4 months than at 1 month of age.

  Total protein, albumin and ferritin were significantly higher and potassium significantly lower at 4 months than at 1 month of age. No significantly differences were found for the other biochemical markers.

- Formula-fed infants (Tables 3 and 6)

  Most amino acids were significantly lower at 4 months than at 1 month of age except of 2-aminobutyrate which was higher at 4 months than at 1 month of age. Differences were not significant for alanine, isoleucine, 1-methylhistidine, phenylalanine, serine, total tryptophan, tyrosine, and valine.

  Regarding general biochemistry, beyond the significant differences revealed for the pooled data set, the formula-fed infants showed significantly lower potassium at 4 months than at 1 month of age. Urea, the urea/creatinine ratio, cholesterol, total protein and albumin were significantly higher at 4 months than at 1 month of age while ferritin and the alkaline phophatase were lower at 4 months as compared to 1 month of age (Table 6).

Differences between low and high protein formula

(Table S11 and 12 in electronic supplemental material)

Between infants fed formulas with high (2.2–2.3 g protein/100 kcal) and low protein content (1.8–1.9 g protein/100 kcal) the following significant differences were found for amino acids: infants of 1 month of age (Table S11) on high protein formulas showed higher threonine, 2-aminobutyrate and isoleucine but lower ornithine and arginine than infants fed low protein formulas. Infants aged 4 months (Table S12) on high protein formulas had higher threonine, 2-aminobutyrate, branched-chain amino acids and lysine, but lower aspartate, glycine and taurine than infants fed low protein formulas. As expected, infants fed with high-protein formulas had significantly higher urea concentrations than infants fed with low-protein formulas, regardless of age. The data distributions were tested with the Kolmogorov-Smirnov method by comparing the data at 1 and 4 months of age for infants fed formulas with a low or or a high protein content. There were no significant differences of data distribution except for 3-methylhistidine ($p<0.0001$) and ferritin ($p=0.0214$) for infants fed high protein and in those fed low protein formulas 1-methylhistidine ($p=0.0132$) and 3-methylhistidine ($p<0.0001$).

Differences between breast- and formula-fed infants

At 1 month of age most plasma amino acid levels were lower or about the same in breast-fed as compared to formula-fed infants (p in Table 2). The concentrations of proline, serine and tyrosine were significantly higher in breast fed than in formula-fed. At 4 months of age, only glutamine, proline and hydroxyproline were lower in formula-fed than in breast-fed infants; all other amino acids were as high or higher in formula fed infants (Table 6).

At 1 month of age, plasma levels of most of the clinical chemistry panel were higher in breast-fed than in formula-fed infants, most strikingly for cholesterol (median 3.95 versus 2.90 mmol/L). Only for potassium, chloride, and AP results were about the same in both groups.

At 4 months of age, higher levels in breast-fed infants persisted for glucose, cholesterol and ferritin whereas for urea and the urea/creatinine ratio they reversed to become significantly lower in breast-fed as compared to formula-fed infants. For the electrolytes, creatinine, total protein, and albumin, differences between both groups diminished or remained about the same.

Discussion

We propose pediatric reference limits of plasma amino acids and several clinical chemistry parameters for infants of one and four months of age. Centiles of Tables 1 and 4 can be used if the composition of the actual nutrition is unknown, e.g. in cases of emergency or if breast-milk is complemented with formula. In general, the adequate choice of centiles and tables depends on the clinical hypothesis, i.e. whether one aims at excluding, confirming or suspecting a disorder.

The reference population originated from three studies which tested the safety of new formulas on 3 cohorts of healthy infants. Amino acids were not assayed from whole blood or filter paper spots to avoid interference by changes in the aqueous volume of the sample due to the important variation of haematocrit (Bachmann 2000). We found that the concentrations of most amino acids were significantly lower at 4 months than at 1 month of age. Similarly, most of the usual clinical chemistry parameters showed significant differences between both age groups. This indicates that pooled reference of the two age groups should not be used for medical decisions. For both age groups, the proportion of infants receiving breast milk, a high or low protein formula only, and those fed on both were similar (at 1 month: 20 %,

Table 3 Reference limits (centiles) for one month of age based on formula-fed infants

	n	*0.025*	*0.05*	*0.10*	*0.50*	*0.90*	*0.95*	*0.975*	IQR	p[a]
Birth Weight	198	2.600	2.650	2.797	3.355	4.009	4.120	4.456	0.635	0.6979
Body Weight (BW)	198	3.390	3.476	3.580	4.120	4.796	4.973	5.151	0.630	<0.0001
Protein intake g/day	194	5.5	6.3	6.8	10.0	13.2	14.1	15.0	3.70	<0.0001
Protein intake g/(d*kg BW)	194	1.35	1.42	1.69	2.34	3.17	3.48	3.84	0.88	<0.0001
Sampling delay p.p.	190	1.25	1.50	2.00	2.75	4.00	4.64	5.25	1.25	0.6979
Age at sampling	198	26	27	27	30	33	35	37	4	–
Amino acids										
2-Aminobutyrate	191	8	9	9	14	20	24	26	5	<0.0001
Alanine	192	205	223	245	376	511	553	602	120	0.3339
Arginine	192	68	74	78	107	146	160	174	34	0.0031
Asparagine	192	30	36	38	50	63	67	69	12	0.0007
Aspartate	192	7	8	9	14	24	26	31	7	<0.0001
Citrulline	192	15	16	17	25	36	39	42	9	<0.0001
Cystine	192	2	2	2	10	30	43	48	17	–
Glutamine	192	405	433	460	573	683	715	749	117	<0.0001
Glutamate	192	50	52	59	92	175	194	230	62	0.0178
Glycine	192	178	190	203	239	282	304	332	46	<0.0001
Histidine	192	66	70	71	87	103	110	123	16	<0.0001
Hydroxyproline	192	42	45	48	62	76	79	86	13	<0.0001
Isoleucine	192	35	40	46	65	93	100	103	24	0.6979
Leucine	192	81	85	94	126	171	187	193	47	0.0008
Lysine	192	142	160	174	231	293	312	344	61	<0.0001
Methionine	192	22	24	25	34	44	47	50	10	<0.0001
1-Methylhistidine	192	3	3	4	6	13	14	15	4	0.6979
3-Methylhistidine	191	1	1	2	2	3	5	8	1	<0.0001
Ornithine	192	57	61	67	92	133	145	157	32	<0.0001
Phenylalanine	192	33	35	39	50	64	71	79	14	0.0582
Proline	192	122	127	138	171	230	244	251	43	<0.0001
Serine	192	100	104	111	137	166	172	178	30	0.4311
Taurine	192	50	56	62	82	155	179	229	33	<0.0001
Threonine	192	112	120	139	190	250	282	299	58	<0.0001
Tryptophan, total	183	46	48	53	68	84	91	95	17	0.1061
Tyrosine	192	44	47	54	78	101	110	120	27	0.6907
Valine	192	94	105	116	156	215	236	247	48	0.6979
Aspartate+Asparagine	192	42	47	49	65	80	85	87	15	<0.0001
Glutamate+Glutamine	192	527	545	575	680	788	826	833	96	<0.0001
Clinical chemistry										
Sodium	166	126	130	134	138	142	144	146	4	1.0000
Potassium	170	4.40	4.50	4.70	5.30	6.00	6.10	6.20	0.68	0.0003
Chloride	170	98	100	102	107	110	111	112	4	0.9838
Calcium, total	167	2.26	2.35	2.42	2.55	2.68	2.72	2.75	0.17	1.0000
Glucose	166	3.81	4.00	4.20	4.80	5.40	5.80	5.90	0.75	1.0000
Creatinine	169	31	32	34	39	47	48	49	7	1.0000
Urea	169	0.90	1.24	1.48	2.50	3.82	4.10	4.30	1.1	0.0026
Urea/Creatinine	169	21	29	37	63	98	110	117	32	0.0283
Cholesterol	119	2.00	2.10	2.28	2.90	3.62	3.91	4.01	0.7	0.0029
Protein, total	163	48	49	51	56	61	62	64	5	<0.0001
Albumin	101	34	35	36	42	45	46	47	4	<0.0001
Ferritin	159	82	109	141	249	399	451	545	151	<0.0001
alkaline Phosphatase	58	199	212	248	312	455	478	513	120	<0.0001

[a] p of Wilcoxon Mann Whitney test after Hochberg-Benjamini correction (1990):formula-fed infants of 1 month of age versus infants of 4 months of age

Table 4 Reference limits (centiles) for age 4 months. based on all infants breast- and formula-fed combined

	Units	n	*0.025*	*0.05*	*0.10*	*0.5*	*0.90*	*0.95*	*0.975*	IQR[a]	p[b]
Birthweight	kg	228	2.607	2.660	2.797	3.355	4.112	4.360	4.517	0.700	0.7347
Bodyweight (BW)	kg	228	5.357	5.550	5.684	6.540	7.443	7.640	7.790	0.990	<0.0001
Protein intake	g/day	154	6.9	7.5	8.5	11.9	16.6	18.6	20.6	3.89	<0.0001
Protein intake	g/(kgBW*day)	154	1.1	1.2	1.3	1.8	2.5	2.8	9.0	0.61	<0.0001
Sampling delay p.p.	h	222	1.50	1.50	2.00	2.79	4.25	4.99	5.50	1.25	0.7347
Age at sampling	day	229	115	116	117	120	124	125	126	4	<0.0001
Amino Acids											
2-Aminobutyrate	µmol/L	217	7 (5–8)[c]	8	9	15	21	24	27 (25–30)	6	0.0002
Alanine	µmol/L	217	213 (189–222)	227	253	336	463	533	565 (544–656)	121	0.3203
Arginine	µmol/L	217	56 (46–60)	61	67	95	126	134	146 (136–188)	30	0.0001
Asparagine	µmol/L	217	27 (24–28)	30	32	45	60	63	67 (64–69)	14	<0.0001
Aspartate	µmol/L	217	5 (5–6)	6	7	11	16	20	27 (23–34)	5	<0.0001
Citrulline	µmol/L	217	9 (7–10)	10	12	20	28	31	33 (32–37)	8	<0.0001
Cystine	µmol/L	217	1 (1–1)	1	1	7	34	41	46 (43–49)	19	–
Glutamine	µmol/L	217	409 (368–429)	431	451	541	669	705	728 (707–754)	108	0.0004
Glutamate	µmol/L	217	44 (26–47)	48	53	81	134	147	174 (159–280)	39	0.0012
Glycine	µmol/L	217	125 (108–130)	132	146	186	229	247	264 (249–289)	46	<0.0001
Histidine	µmol/L	217	56 (54–58)	59	63	76	91	96	100 (99–113)	14	<0.0001
Hydroxyproline	µmol/L	217	19 (15–21)	21	24	33	45	50	56 (51–63)	9	<0.0001
Isoleucine	µmol/L	217	37 (30–38)	39	41	60	82	93	99 (94–117)	24	0.0683
Leucine	µmol/L	217	67 (56–70)	70	75	108	148	167	181 (173–205)	41	<0.0001
Lysine	µmol/L	217	99 (94–109)	110	121	179	232	251	272 (264–308)	68	<0.0001
Methionine	µmol/L	217	15 (14–16)	17	18	25	33	36	39 (36–45)	8	<0.0001
1-Methylhistidine	µmol/L	217	3 (2–3)	3	4	7	16	17	20 (18–22)	6	0.7347
3-Methylhistidine	µmol/L	217	1 (1–1)	1	1	2	3	5	6 (6–8)	1	<0.0001
Ornithine	µmol/L	217	43 (36–46)	48	53	75	102	109	121 (115–148)	25	<0.0001
Phenylalanine	µmol/L	217	33 (30–33)	33	36	46	58	64	73 (67–79)	11	0.0011
Proline	µmol/L	216	107 (101–112)	116	127	161	226	251	264 (257–280)	47	<0.0001
Serine	µmol/L	217	98 (86–101)	101	110	134	165	176	183 (179–188)	26	0.3398
Taurine	µmol/L	217	42 (38–44)	44	47	73	141	179	262 (190–374)	40	0.0003
Threonine	µmol/L	217	72 (63–77)	77	91	133	194	215	234 (217–260)	56	<0.0001
Tryptophan, total	µmol/L	200	47 (41–51)	51	54	68	88	95	103 (97–111)	19	0.7347
Tyrosine	µmol/L	217	41 (38–45)	46	51	72	99	113	125 (114–153)	27	0.0027
Valine	µmol/L	217	104 (94–109)	110	116	152	194	214	240 (216–264)	44	0.1476
Aspartate+Asparagine	µmol/L	217	37	39	42	56	73	76	83	16	<0.0001
Glutamate+Glutamine	µmol/L	217	499	521	538	628	753	788	817	109	<0.0001
Clinical Chemistry											
Sodium	mmol/L	186	132	134	135	139	142	142	145	3.0	0.1111
Potassium	mmol/L	182	4.35	4.40	4.50	4.90	5.69	6.00	6.44	0.5	<0.0001
Chloride	mmol/L	181	99	100	102	106	109	110	112	4.0	0.2710
Calcium, total	mmol/L	180	2.36	2.39	2.44	2.59	2.74	2.78	2.80	0.16	0.7110
Glucose	mmol/L	178	3.84	4.00	4.27	5.00	5.50	5.62	5.80	0.70	0.0317
Creatinine	µmol/L	181	32.5	34.0	35.0	39.0	44.0	46.0	47.5	5.0	0.7347
Urea	mmol/L	181	1.25	1.50	1.80	2.70	3.60	3.80	3.85	0.90	0.4004
Urea/Creatinine	mol/mol	182	31	41	46	68	91	97	104	23	0.3432
Cholesterol	mmol/L	112	2.08	2.56	2.70	3.45	4.39	4.80	5.15	0.80	<0.0001
Protein, total	g/L	182	56	57	58	62	67	69	71	4.8	<0.0001
Albumin	g/L	135	38	41	44	48	54	55	57	4.0	<0.0001
Ferritin	µg/L	175	19	23	30	82	193	247	340	82	<0.0001
alkaline Phosphatase	U/L	57	167	183	195	284	344	403	440	79	0.0037

[a] IQR: Interquartile range

[b] In brackets: distribution-free 90 % confidence intervals

[c] p of Wilcoxon Mann Whitney test after Hochberg-Benjamini correction (1990): all infants of 4 months of age versus 1 month of age

Table 5 Reference limits (centiles) for age 4 months. based on breast-fed infants

	n	*0.025*	*0.05*	*0.10*	*0.50*	*0.90*	*0.95*	*0.975*	IQR	p [a]
Birth weight	49	2.900	2.948	3.019	3.660	4.460	4.506	4.742	0.900	0.9809
Body weight	49	5.560	5.604	5.668	6.410	7.318	7.684	7.764	0.675	<0.0001
Sampling delay p.p.	48	1.50	1.50	1.50	3.00	4.33	5.00	6.65	1.31	0.9809
Age at sampling	49	115	115	116	118	125	128	129	5	–
Amino Acids										
2-Aminobutyrate	49	5	7	7	11	16	19	19	6	0.9809
Alanine	49	200	210	228	306	401	415	444	89	0.9809
Arginine	49	51	60	66	87	112	120	124	26	0.0451
Asparagine	49	26	27	32	41	58	63	66	16	0.1284
Aspartate	49	5	5	6	8	13	15	17	3	0.0005
Citrulline	49	9	9	10	15	22	27	28	9	0.0039
Cystine	49	1	2	3	22	37	43	45	17	–
Glutamine	49	438	462	511	595	731	740	751	147	0.9809
Glutamate	49	31	43	48	68	111	127	136	29	0.0305
Glycine	49	110	120	126	157	211	213	214	42	<0.0001
Histidine	49	56	57	58	72	88	92	98	11	<0.0001
Hydroxyproline	49	22	25	26	35	48	52	56	12	<0.0001
Isoleucine	49	31	36	41	53	76	78	84	21	0.0039
Leucine	49	58	68	71	96	133	145	152	44	<0.0001
Lysine	49	94	95	97	142	196	209	216	60	<0.0001
Methionine	49	14	15	17	22	27	29	29	6	<0.0001
1-Methylhistidine	49	3	4	4	6	17	20	21	9	0.9809
3-Methylhistidine	49	1	1	1	2	5	7	8	0	0.0447
Ornithine	49	44	48	53	75	105	118	122	32	0.1287
Phenylalanine	49	31	33	33	41	52	54	54	9	0.0013
Proline	48	133	141	147	178	243	269	274	53	0.0012
Serine	49	99	101	107	141	180	183	185	42	0.3176
Taurine	49	40	44	46	66	108	123	138	32	0.8147
Threonine	49	67	70	80	107	139	142	153	27	<0.0001
Tryptophan, total	48	44	48	51	68	84	87	89	13	0.5218
Tyrosine	49	41	41	48	69	93	96	109	24	<0.0001
Valine	49	99	105	118	150	179	191	202	37	0.0155
Aspartate+Asparagine	49	34	36	39	51	66	72	76	14	0.0104
Glutamate+Glutamine	49	559	563	585	669	810	818	863	141	0.9809
Clinical chemistry										
Sodium	46	133	134	135	140	142	142	142	3.0	0.9809
Potassium	46	4.21	4.33	4.50	4.85	5.25	5.48	5.59	0.38	0.0008
Chloride	46	100	102	103	106	109	111	112	4.0	0.9809
Calcium, total	46	2.40	2.46	2.50	2.64	2.72	2.76	2.78	0.16	0.9809
Glucose	45	4.40	4.42	4.50	5.20	5.60	5.68	5.97	0.60	0.3624
Creatinine	46	35	35	36.5	40	44	44	45	4.0	0.9809
Urea	46	1.20	1.25	1.55	2.35	3.05	3.38	3.66	0.88	0.0504
Urea/Creatinine	46	30	35	41	57	77	79	83	19.3	0.1939
Cholesterol	17	3.26	3.42	3.56	4.20	5.18	5.38	5.54	0.9	0.9809
Protein. total	45	58.1	59	59	62	67	68	70	4.0	<0.0001
Albumin	46	45.1	46	47	49	54	55	57	5.0	<0.0001
Ferritin	46	18	20	40	115	282	403	513	88	<0.0001
alkaline Phosphatase	17	209	240	263	291	388	478	519	46	0.9809

[a] p of Wilcoxon Mann Whitney test after Hochberg-Benjamini correction (1990): breast-fed infants of 4 months versus breast-fed infants of 1 month of age

Table 6 Reference limits (centiles) for age 4 months. based on formula-fed infants

	n	*0.025*	*0.05*	*0.10*	*0.50*	*0.90*	*0.95*	*0.975*	*IQR*	p[a]
Birth weight	158	2.600	2.684	2.797	3.310	4.040	4.153	4.456	0.605	0.0026
Body weight (BW)	158	5.375	5.593	5.731	6.565	7.450	7.576	7.655	1.060	0.9296
Protein intake g/d	154	6.9	7.5	8.5	11.9	16.6	18.6	20.6	3.9	–
Protein intake g/(d*kgBW)	154	1.13	1.22	1.30	1.80	2.53	2.80	3.02	0.61	–
Sampling delay p.p.	152	1.50	1.75	2.00	2.75	4.25	4.61	5.11	1.25	0.9296
Age at sampling	158	116	116	117	120	123	124	124	3	–
Amino acids										
2-Aminobutyrate	146	10	11	12	16	22	25	27	5	<0.0001
Alanine	146	224	234	257	345	477	549	572	116	0.0028
Arginine	146	59	62	69	99	130	142	148	28	0.0054
Asparagine	146	28	30	34	46	61	64	67	14	0.6402
Aspartate	146	6	7	7	11	16	18	26	5	<0.0001
Citrulline	146	11	12	14	21	28	32	33	8	<0.0001
Cystine	146	1	1	1	5	27	40	43	9	–
Glutamine	146	391	428	445	516	621	646	662	99	<0.0001
Glutamate	146	46	50	57	84	131	145	174	35	0.0069
Glycine	146	133	147	156	191	233	249	267	37	<0.0001
Histidine	146	56	61	63	77	94	99	102	14	0.1742
Hydroxyproline	146	20	21	23	32	42	45	50	10	0.2319
Isoleucine	146	38	39	42	61	86	96	100	23	0.0394
Leucine	146	69	73	79	113	154	174	189	42	0.0174
Lysine	146	117	123	133	185	240	269	278	62	<0.0001
Methionine	146	16	17	19	26	34	36	39	9	0.0001
1-Methylhistidine	146	2	3	4	7	14	17	18	5	0.9296
3-Methylhistidine	146	1	1	1	2	2	3	6	1	0.0039
Ornithine	146	45	48	53	76	100	108	120	22	0.9296
Phenylalanine	146	33	35	37	48	61	67	73	11	0.0002
Proline	146	106	112	120	156	214	243	253	46	0.0003
Serine	146	97	102	110	132	162	167	175	24	0.9296
Taurine	146	43	44	48	74	137	174	209	34	0.6881
Threonine	146	76	83	101	146	208	224	242	59	<0.0001
Tryptophan, total	131	51	53	55	71	93	100	106	21	0.7082
Tyrosine	146	43	48	52	73	102	115	133	29	0.9296
Valine	146	109	112	116	157	198	235	246	40	0.4781
Aspartate+Asparagine	146	39	41	44	57	73	76	80	15	0.0180
Glutamate+Glutamine	146	491	515	527	612	718	728	743	97	<0.0001
Clinical chemistry										
Sodium	121	132	134	136	139	142	143	146	3	0.9296
Potassium	117	4.40	4.40	4.50	4.90	5.60	6.00	6.20	0.6	0.9296
Chloride	116	99	100	102	105	109	110	112	4	0.9296
Calcium, total	116	2.34	2.40	2.45	2.60	2.75	2.78	2.81	0.13	0.4985
Glucose	114	3.88	4.00	4.23	5.00	5.50	5.64	5.80	0.77	0.1572
Creatinine	116	31	33	35	39	45	46	48	5	0.5506
Urea	116	1.40	1.70	1.90	2.90	3.70	3.80	3.91	0.90	0.0002
Urea/Creatinine	117	41	45	47	73	95	101	106	23	<0.0001
Cholesterol	81	2.50	2.60	2.70	3.40	4.10	4.30	4.50	0.80	<0.0001
Protein, total	119	55	57	58	62	67	69	73	5	0.9296
Albumin	85	38	40	42	48	54	55	56	4	0.2684
Ferritin	111	21	26	31	70	161	200	235	69	0.0101
alkaline Phosphatase	40	158	183	188	266	342	354	401	83	0.3063

[a] p of Wilcoxon Mann Whitney test after Hochberg-Benjamini correction (1990): formula-fed versus breast-fed infants of four months of age

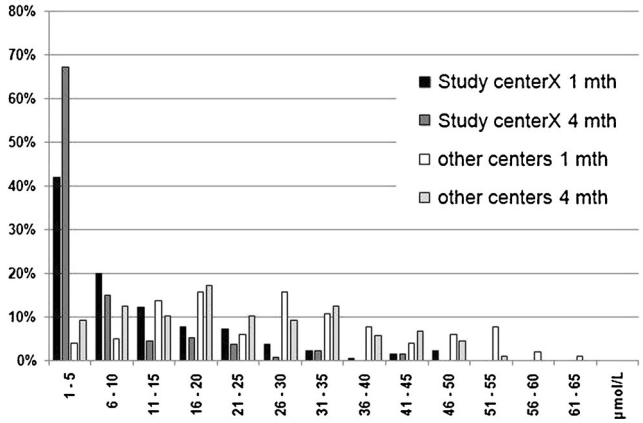

Fig. 1 Cystine: Predominance of very low values in one study center as compared to the other study centers. The distribution of cystine concentrations at one and four months is shown as percentage of the study center X and the other study centers

19 %, 51 %, 10 % and at 4 months 21 %, 21 %, 47 % and 11 %, respectively). Thus the lower amino acid concentrations at 4 months and the differences in reference limits of usual clinical chemistry parameters cannot be explained by a food effect, neither through different food quality nor through different times elapsing from the end of the last feed until sampling.

The reference values of cystine extend to very low levels. The data distribution is biased by the low cystine values of one study center (Fig. 1), which are most likely due to a pre-analytical error, i.e. a delay between sampling and centrifugation and freezing. Interestingly, such low values have been reported by others in children and adults (Armstrong and Stave 1973a; Mayo Clinic 2014). Since the bias through results of one study center leads to a multimodal distribution we did not make inferential comparisons for cystine. We

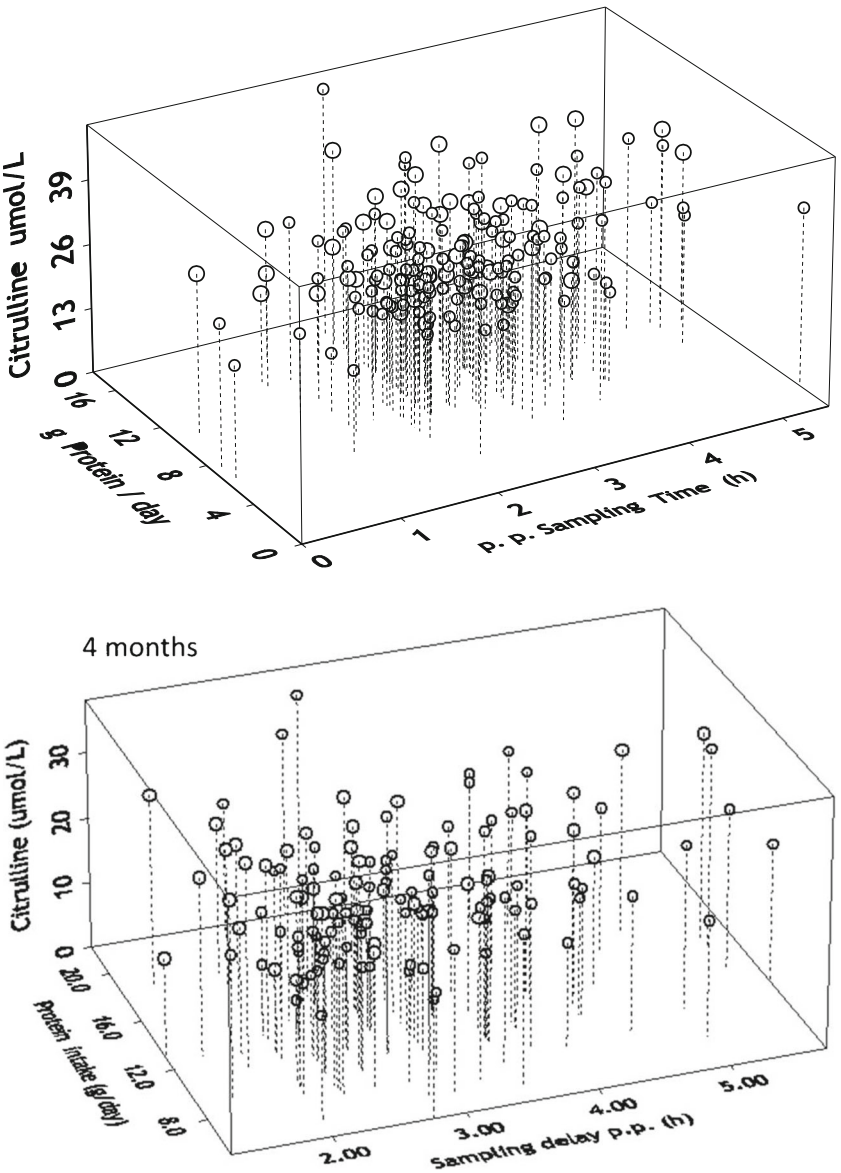

Fig. 2 Plasma citrulline concentration depends both on postprandial delay of sampling and on daily protein intake of age in formula fed infants aged one and four months. In contrast to other amino acids, citrulline concentrations in plasma not only increase with augmenting protein intake, but as well with increasing postprandial sampling delay

suggest that only the upper reference limit of cystine might be used, if at all.

Some high concentrations of potassium could be due to a mild hemolysis which could not be detected by inspection of the plasma.

The contribution of major organs and tissues which take up and release amino acids from and to the systemic circulation varies and has not been quantitated in infants. In addition to nutritional factors the postprandial time elapsed after the last feed affects some, though not all amino acid concentrations. Qualitative results concerning the postprandial absorption and elimination phase have been published (Bachmann and Haschke-Becher 2002; Bachmann 2003). When comparing nutritional effects between groups it is important to ensure that groups do not differ in times elapsing from last feed. One should note that the pattern of citrulline differs from other amino acids with trough plasma levels shortly after protein intake, followed by steady increase for up to 4–5 h (Fig. 2). This should be taken into account if low plasma citrulline is used as indicator of mitochondrial defects of urea cycle enzymes or transporters (Häberle et al. 2012) or control of enterocyte function after surgical resection. The pattern of the postprandial citrulline concentration in adults is not known.

Our findings of amino acid and urea concentrations in plasma at 1 and 4 months of age may shed some light on physiological mechanisms. Example (cp Tables S11 and 12 in the online supplemental material): formulas with low protein had a lower proline content (6.2–6.4 g aminoacyl/16 g N) than in breast milk (9 g aminoacyl/16 g N); Räihä et al. 2002). In infants aged 1 month fed low protein formula the median plasma urea concentration was the lowest compared to those of the other infant sub-groups; the median proline concentration in plasma did not differ between infants fed low or high protein formulas neither at 1 nor at 4 months of age. At 1 month but not at 4 months of age plasma ornithine was significantly lower in infants fed formula with low protein content compared to those fed high protein formula. This is compatible with a physiologically higher demand of proline and hydroxyproline (posttranslational modification) for procollagen synthesis at 1 month than at 4 months of age and with a flux direction of ornithine to proline. Findings in patients and animal experiments with some mitochondrial urea cycle disorders are in agreement with such an interpretation (Wang et al. 1995; Cleary et al. 2005; Ben-shalom et al. 2002; Baumgartner et al. 2005). In breast-fed infants however the median plasma urea and proline concentrations were lower at 4 months than at 1 month of age which is likely due to the higher proline content of breastmilk. This suggests that proline is a conditionally essential amino acid at 1–2 months of age. Increasing the proline content of low protein formulas to the level of breastmilk should perhaps be considered for that age group.

In conclusion, plasma concentrations of amino acids and clinical chemistry parameters in young infants are affected by age and diet. Both need to be taken into account through specific reference limits and standardization of pre-analytical procedures. Blood should be obtained from infants between 4.5 to 5.5 h after the last feed at trough levels to exclude deficiencies or imbalance of amino acids, except citrulline. After 6 h the plasma concentration of most amino acids is rising in infants probably due to endogenous protein catabolism.

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Compliance with Ethics Guidelines

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Informed consent All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the parents of all infants included in the study.

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