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RESEARCH REPORT

Low Protein Formula: Consequences of Quantitative Effects of Pre-analytical Factors on Amino Acid Concentrations in Plasma of Healthy Infants

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Abstract *Objective*: Quantifying pre-analytical effects of postprandial sampling delay and daily protein intake on plasma amino acid concentrations in healthy infants fed formula with low protein content (1.8–1.9 g/100 kcal). Intake of formula with higher protein content bears a risk for later obesity (Kirchberg, J Clin Endocrinol Metab 100 (1):149–158, 2015). Formulas containing less than 1.8 g protein might be adequate but not safe (Fomon, J Pediatr Gastroenterol Nutr 28:495–501, 1999). With on-demand feeding reproducible controls of indispensible amino acid concentration cannot be made at trough level.

Methods: Data of 102 healthy infants aged 1 month and 79 aged 4 months fed formula with low protein content were obtained from a previous study (Haschke-Becher, J Inherit Metab Dis 39(1):25–37, 2016). They were analysed by multiple regression. Independent variables were the postprandial sampling delay from 2.25 to 4.5 h and the daily protein intake. Dependant variables were the amino acid concentrations. The combined effect was calculated with the natural logarithm of the amino acid concentration.

Results: Most amino acids fitted a significant exponential decrease due to the sampling delay, except of aspartate, citrulline, glutamine, glutamate, histidine, tryptophan and tyrosine at 1 month; and at 4 months except of citrulline, glutamine, glutamate, glycine and ornithine. Significant effects of protein intake were found for lysine and serine at 1 month and for glutamate at 4 months of age. Lowest limits of significant amino acid concentrations were calculated by extrapolation of sampling delay to 5 h and using the 10th percentile after back-transformation to μmol/L. A procedure to avoid the pitfall of overestimating amino acid concentration is presented.

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Introduction

When infants cannot be breast-fed formulas with low protein content (1.8–1.9 g/100 kcal) have been recommended in order to avoid an excessive protein supply or amino acid imbalance during organ development in infants (Koletzko et al. 2005). Furthermore, infant formulas with higher protein content are risk factors for later obesity (Kirchberg et al. 2015; Ong et al. 2009; Toschke et al. 2004). Formulas containing less than 1.8 g protein might be adequate but not safe as shown by Fomon et al. (1999). Frequent controls of amino acid concentrations in plasma of infants are thus needed when low protein formulas are exclusively fed in order to exclude that very low amino acid concentrations limit protein synthesis of infants during this phase of change of growth rate. For the interpretation of amino acid results the effect of pre-analytical factors must be taken into account; however, most often pre-analytical factors are not documented and/or communicated.

Relevant pre-analytical factors which modify the plasma levels are (1) the time elapsed between the end of the last

feed and the sampling of blood and (2) the actual daily intake of protein and its composition. For following the course of a patient or for excluding protein malnutrition blood sampling should be done at trough levels, i.e. between 4.5 and 5.5 h postprandial (p.p.) except of citrulline (Haschke-Becher et al. 2016; Windmueller and Spaeth 1981). However this is not always done or feasible. If blood is sampled during food absorption (i.e. until 2 h p.p.) the results are useless since no reference data of healthy infants exist. In practice samples are most often taken during the exponential decrease of essential and of some non-essential amino acids before trough level is reached, e.g. if the infants are fed on demand. Such results might, however, lead to pitfalls of interpretation: Most essential amino acids and some non-essential ones decrease exponentially after 2.25 postprandial (p.p.) hours until the trough level (>4.5 h) is reached; when blood is sampled during the exponential drop, the actual amino acid concentration determined will be higher than at trough level. If this is not taken into account in infants fed low protein formulas there is a risk of missing amino acid deficiencies and to interpret the amino acid concentration as safe level.

To our knowledge the effect of time of blood sampling and of the actual daily intake of formulas with low protein content on plasma amino acids has not been quantitatively estimated in healthy infants of 1 and 4 months of age. In this period of rapid growth the concentrations of indispensible amino acids in plasma should be safe, adapted to the needs and neither rate limiting for protein synthesis nor in excess.

We show quantitative data of plasma amino acid concentrations of healthy infants aged 1 and 4 months fed low protein formula on demand. We focus on the combined effect of the p.p. sampling delay from 2.25 to 4.5 h and of the individual daily protein intake on the amino acid concentrations in plasma. Furthermore we present low limits (the 10th percentile) of amino acid concentrations in plasma of healthy infants aged 1 and 4 months after extrapolating the time of sampling to trough levels (5 h p.p.). By comparison of patient results with these data the risk of amino acid deficiency might be evaluated in patients fed amino acid mixtures of low protein content.

Study Population and Methods

In order to quantify and estimate the influence of postprandial sampling delay after the end of last feed and of daily protein intake on the plasma amino acids we used a population of healthy infants of 1 and 4 months of age fed exclusively formulas with low protein content (1.8–1.9 g protein/100 kcal). The population and the analytical methods used for the amino acid analysis including total plasma tryptophan have been published elsewhere (Haschke-Becher et al. 2016). For this study we included only those infants who had information on actual protein intake per day, on postprandial sampling delay (independent variables) and plasma amino acid concentrations (dependent variables). 102 samples were of infants aged 1 month and 79 of infants aged 4 months. We do not report data of the following amino acids: taurine (added to the protein mixture of the formulas), hydroxyproline (posttranslational product of proline), cystine (pre-analytical bias) and 1- and 3-methylhistidine (low precision of low values); as shown in Haschke-Becher et al. (2016) on Fig 1 and in its electronic supplemental material. We focussed on the kinetics of plasma amino acids in blood samples obtained between 2.25 and 4.5 h postprandial. We used the natural logarithm of amino acid concentrations and determined intercept and parameter estimates of both independent variables. Furthermore we used standardized Beta coefficients of the multiple regressions, i.e. the coefficients of the dependent variables transformed to a standard deviation of 1 for weighting the impact of the two independent variables.

Statistics

Sample distribution of amino acids was analysed with Shapiro-Wilk, Kolmogorov Smirnov and Cramer von Mises tests and quantile regression with SAS 9.4 TS1M2 software for Windows (SAS Institute Inc., Cary, NC). The fit of combined effects of both independent factors was calculated by multiple regression of the natural logarithm (ln) of amino acid concentrations in dependence of p.p. sampling delay and daily protein intake. Analyse-it 3 for Excel software was used for these calculations (Analyse-it Software Ltd., Leeds, UK). In addition, the fit of each dependant variable by linear regression was controlled separately. R software (Koenker and Bassett 1978) was used for computing the fit at the 10th percentile.

Values of $p < 0.05$ were considered significant. Half-life was calculated by: $T_{1/2} = -\ln(2)/\text{estimate of the regression coefficient of the sampling delay.}$

Results

Post-absorptive Kinetics of Amino Acid Concentrations in Plasma of Healthy Infants: Effect of p.p. Sampling Delay and Amount of Daily Protein Intake

The kinetic parameters of samples taken between 2.25 and 4.5 h p.p. differed among amino acids. The results of multivariate regressions are shown in Tables 1 and 2 for

Table 1 Kinetic parameters of plasma amino acids sampled at 1 month of age between 2.25 and 4.5 h p.p. fed formula of low protein content

			Intercept at $t = 0$ (constant)			Regression coefficient of p.p. sampling delay (h)						Regression coefficient of daily protein intake				
ln amino acids	n	R^2	Estimate	Lower 95%CI	Upper 95%CI	Estimate	Lower 95%CI	Upper 95%CI	Beta	p	$T_{1/2}$ (h)	Estimate	Lower 95%CI	Upper 95%CI	Beta	p
2-Aminobutyrate	102	0.006	2.656	2.307	3.004	−0.0102	−0.099	0.079	−0.023	0.8204	68	−0.00749	−0.0275	0.0125	−0.075	0.4589
Alanine	102	0.225	6.377	6.092	6.662	−0.1904	−0.263	−0.118	−0.461	***<0.0001***	**3.6**	0.00760	−0.0088	0.0239	0.082	0.3588
Arginine	102	0.051	4.889	4.599	5.178	−0.0823	−0.156	−0.008	−0.217	***0.0294***	**8.4**	0.00425	−0.0124	0.0209	0.050	0.6131
Asparagine	102	0.144	4.128	3.912	4.343	−0.1068	−0.162	−0.052	−0.359	***0.0002***	**6.5**	0.00650	−0.0059	0.0189	0.097	0.2992
Aspartate	102	0.007	2.730	2.205	3.254	−0.0422	−0.176	0.092	−0.063	0.5328	16	0.00769	−0.0224	0.0378	0.051	0.6134
Citrulline	102	0.032	2.929	2.595	3.262	0.0762	−0.009	0.161	0.176	0.0786		0.00442	−0.0147	0.0236	0.045	0.6476
Glutamate	102	0.001	4.554	4.022	5.087	−0.0037	−0.140	0.132	−0.005	0.9571	188	0.00432	−0.0262	0.0349	0.028	0.7798
Glutamine	102	0.001	6.362	6.180	6.543	−0.0042	−0.050	0.042	−0.018	0.8577	165	−0.00084	−0.0112	0.0096	−0.016	0.8733
Glycine	102	0.011	5.586	5.392	5.781	−0.0269	−0.077	0.023	−0.107	0.2866	26	−0.00061	−0.0118	0.0106	−0.011	0.9140
Histidine	102	0.043	4.538	4.372	4.705	−0.0387	−0.081	0.004	−0.178	0.0733	18	0.00464	−0.0049	0.0142	0.095	0.3371
Isoleucine	102	0.208	4.594	4.300	4.889	−0.1865	−0.262	−0.111	−0.441	***<0.0001***	**3.7**	0.00824	−0.0087	0.0251	0.087	0.3363
Leucine	102	0.158	5.137	4.875	5.400	−0.1377	−0.205	−0.071	−0.377	***<0.0001***	**5.0**	0.00850	−0.0066	0.0236	0.103	0.2662
Lysine	102	0.178	5.594	5.378	5.810	−0.1086	−0.164	−0.053	−0.357	***0.0002***	**6.4**	0.01364	0.0012	0.0260	0.199	***0.0314***
Methionine	102	0.116	3.707	3.460	3.953	−0.1013	−0.164	−0.038	−0.302	***0.0019***	**6.8**	0.01035	−0.0038	0.0245	0.137	0.1501
Ornithine	102	0.135	4.885	4.575	5.196	−0.1458	−0.225	−0.066	−0.342	***0.0004***	**4.8**	0.01059	−0.0072	0.0284	0.111	0.2412
Phenylalanine	102	0.074	4.080	3.840	4.320	−0.0777	−0.139	−0.016	−0.244	***0.0135***	**8.9**	0.00757	−0.0062	0.0214	0.106	0.2781
Proline	102	0.117	5.477	5.241	5.713	−0.1097	−0.170	−0.049	−0.342	***0.0005***	**6.3**	0.00037	−0.0132	0.0139	0.005	0.9563
Serine	102	0.090	5.148	4.949	5.346	−0.0800	−0.131	−0.029	−0.301	***0.0023***	**8.7**	−0.00069	−0.0121	0.0107	−0.011	0.9050
Threonine	102	0.184	5.455	5.182	5.728	−0.1430	−0.213	−0.073	−0.371	***<0.0001***	**4.8**	0.01646	0.0008	0.0321	0.190	***0.0395***
Tryptophan	96	0.009	4.288	4.022	4.553	−0.0328	−0.102	0.036	−0.097	0.3486	21	−0.00048	−0.0155	0.0145	−0.007	0.9492
Tyrosine	102	0.027	4.564	4.270	4.857	−0.0625	−0.137	0.012	−0.164	0.1013	11	−0.00157	−0.0184	0.0153	−0.018	0.8541
Valine	102	0.102	5.303	5.058	5.549	−0.0997	−0.162	−0.225	−0.301	***0.0021***	**7.0**	0.00649	−0.0076	0.0206	0.087	0.3629
Aspartate + Asparagine	102	0.115	4.373	4.163	4.583	−0.0918	−0.146	−0.038	−0.322	***0.0010***		0.00566	−0.0064	0.0177	0.088	0.3547
Glutamate + Glutamine	102	0.0004	6.525	6.387	6.663	−0.0007	−0.036	0.035	−0.004	0.9698		−0.00080	−0.0087	0.0071	−0.020	0.8409

R^2 coefficient of determination, *Beta* standardized Beta, ***Italics*** significant result, *p.p.* postprandial (after end of last feed), $T_{1/2}$ half-life

Table 2 Kinetic parameters of plasma amino acids sampled at 4 months of age between 2.25 and 4.5 h p.p. fed formula of low protein content

			Intercept at $t = 0$ (constant)			Regression coefficient of p.p. sampling delay						Regression coefficient of daily protein intake				
lnAA	*n*	R^2	Estimate	Lower 95%CI	Upper 95%CI	Estimate	Lower 95%CI	Upper 95%CI	Beta	*p*	$T_{1/2}$ (h)	Estimate	Lower 95%CI	Upper 95%CI	Beta	*p*
2-Aminobutyrate	79	0.046	2.922	2.636	3.208	−0.0374	−0.1162	0.0414	−0.106	0.3476	19	−0.0115	−0.0258	0.0027	−0.181	0.1113
Alanine	79	0.114	6.268	5.961	6.575	−0.1305	−0.2151	−0.0459	−0.333	***0.0029***	**5.3**	−0.0026	−0.0179	0.0127	−0.037	0.7341
Arginine	79	0.114	4.989	4.693	5.286	−0.1268	−0.2084	−0.0451	−0.334	***0.0028***	**5.5**	−0.0019	−0.0167	0.0128	−0.028	0.7947
Asparagine	79	0.169	4.101	3.851	4.351	−0.1341	−0.2030	−0.0651	−0.406	***0.0002***	**5.2**	0.0060	−0.0065	0.0185	0.100	0.3408
Aspartate	79	0.055	2.896	2.477	3.315	−0.0892	−0.2046	0.0263	−0.172	0.1281	7.8	−0.0140	−0.0348	0.0069	−0.149	0.1866
Citrulline	79	0.028	2.768	2.408	3.128	0.0716	−0.0275	0.1708	0.163	0.1544		0.0022	−0.0157	0.0202	0.028	0.8046
Glutamate	79	0.105	4.987	4.524	5.450	−0.0546	−0.1824	0.0731	−0.093	0.3972	13	−0.0325	−0.0556	−0.0094	−0.305	***0.0064***
Glutamine	79	0.028	6.250	6.051	6.449	−0.0258	−0.0807	0.0292	−0.106	0.3537	27	0.0060	−0.0039	0.0160	0.137	0.2303
Glycine	79	0.016	5.210	5.019	5.402	0.0004	−0.0525	0.0532	0.002	0.9895		0.0053	−0.0043	0.0148	0.126	0.2737
Histidine	79	0.089	4.574	4.384	4.764	−0.0590	−0.1114	−0.0065	−0.246	***0.0281***	**12**	−0.0067	−0.0161	0.0028	−0.154	0.1656
Isoleucine	79	0.202	4.564	4.298	4.830	−0.1599	−0.2333	−0.0865	−0.445	***<0.0001***	**4.3**	−0.0022	−0.0155	0.0111	−0.034	0.7428
Leucine	79	0.214	5.153	4.902	5.404	−0.1571	−0.2263	−0.0878	−0.460	***<0.0001***	**4.4**	−0.0013	−0.0139	0.0112	−0.022	0.8316
Lysine	79	0.197	5.542	5.297	5.787	−0.1453	−0.2127	−0.0778	−0.442	***<0.0001***	**4.8**	0.0049	−0.0073	0.0171	0.083	0.4248
Methionine	79	0.214	3.817	3.539	4.095	−0.1664	−0.2431	−0.0898	−0.441	***<0.0001***	**4.2**	−0.0078	−0.0217	0.0060	−0.115	0.2648
Ornithine	79	0.041	4.578	4.257	4.899	−0.0745	−0.1631	0.0140	−0.189	0.0976	9.3	−0.0042	−0.0202	0.0118	−0.059	0.6004
Phenylalanine	79	0.116	4.246	3.990	4.501	−0.0990	−0.1694	−0.0287	−0.303	***0.0064***	**7.0**	−0.0081	−0.0208	0.0047	−0.137	0.2103
Proline	79	0.112	5.476	5.206	5.746	−0.1011	−0.1755	−0.0267	−0.293	***0.0084***	**6.9**	−0.0088	−0.0223	0.0046	−0.142	0.1940
Serine	79	0.156	5.203	5.026	5.380	−0.0785	−0.1273	−0.0296	−0.338	***0.0020***	**8.8**	−0.0076	−0.0165	0.0012	−0.182	0.0884
Threonine	79	0.091	5.295	4.988	5.601	−0.1126	−0.1971	−0.0282	−0.291	***0.0096***	**6.2**	−0.0044	−0.0197	0.0108	−0.063	0.5651
Tryptophan	70	0.076	4.480	4.222	4.738	−0.0830	−0.1542	−0.0118	−0.274	***0.0231***	**8.4**	0.0026	−0.0100	0.0151	0.049	0.6817
Tyrosine	79	0.054	4.645	4.303	4.986	−0.0956	−0.1898	−0.0014	−0.226	***0.0467***	**7.3**	−0.0032	−0.0202	0.0138	−0.042	0.7089
Valine	79	0.152	5.377	5.165	5.588	−0.1041	−0.1623	−0.0459	−0.377	***0.0006***	**6.7**	−0.0039	−0.0144	0.0066	−0.078	0.4616
Aspartate + Asparagine	79	0.197	4.397	4.180	4.614	−0.1301	−0.1901	−0.0702	−0.445	***<0.0001***		0.0012	−0.0096	0.0120	0.023	0.8254
Glutamate + Glutamine	79	0.056	6.544	6.402	6.687	−0.0411	−0.0804	−0.0019	−0.233	***0.0404***		−0.0008	−0.0079	0.0063	−0.026	0.8159

R^2 coefficient of determination, *Beta* standardized Beta, ***Italics*** significant result, *p.p.* postprandial (after end of last feed), $T_{1/2}$ half-life

blood samples taken at 1 and 4 months of age, respectively. Biological half-life is also shown. The equations with the factors of multiple regressions are presented in Table 3. The sample distribution of amino acids was logarithmic; thus the natural logarithms (ln) of the amino acid concentrations were used for calculations. For infants of 1 month of age all the regression parameters of sampling delay of the ln of essential amino acids had a significant negative slope except for total tryptophan (Table 1). This was also found for the following non-essential amino acids: alanine, arginine, asparagine, ornithine, proline, serine and the sum of aspartate and asparagine. The regression coefficients of daily protein intake were significant and positive only for lysine and threonine at 1 month of age.

At 4 month of age the significance of regression coefficients of sampling delay differed from those at 1 month insofar as the regression coefficient of ornithine was not significant anymore ($p = 0.098$); the coefficients of histidine, tryptophan and tyrosine were significant as well as the sum of glutamine and glutamate (Table 2). At 4 months of age the sole significant coefficient of protein intake/d was that of glutamate ($p = 0.006$). Citrulline concentration increased during the post-absorptive phase; the lowest level was reached during the first hour after the end of last feed (Haschke-Becher et al. 2016).

There were not enough data for calculating the kinetics during the initial absorption of the gut (0–2.0 h) and after 5.5 postprandial hours when concentrations of many amino acids tended to increase presumably by incipient proteolysis.

Kinetic parameters of both independent variables calculated separately by univariate linear regression are presented in the electronic supplemental material (Tables 5–8).

Effect of the Independent Variables on the Mean of Amino Acid Concentrations

The standardized squared Beta values were higher for the p.p. sampling delay than for the protein intake/day. At 1 month of age the percentage of the total variance which exceeded 10% and can be attributed to the sampling delay was found for: alanine (21%), isoleucine (20%), leucine (14%), threonine, asparagine, lysine (13% each), ornithine and proline (each 12%). At 4 months the effect of sampling delay was 21% for leucine, 20% for isoleucine and lysine, 19% for methionine, 17% for asparagine, 14% for valine and 11% for arginine and alanine. The effect of protein intake per day on total variance was lower than 5% for all the amino acids, both at 1 and 4 months of age.

Univariate regression was done for each independent variable on the same set of data as used for the multivariate regression. As expected, most estimates differed between the two methods. The differences were, however, minor.

Procedure to Avoid the Pitfall of Overestimated Amino Acid Concentrations

The goal was to avoid the pitfall of overestimating amino acid concentrations sampled during the post-absorptive phase (2.25–4.5 h). We estimated by extrapolation to trough level (5 h) the ln of those amino acids which showed a significant p.p. drop in healthy infants fed formula with low protein content. We defined lower limits at the 10th percentile for such amino acids and back-transformed the logarithmic results into μmol/L in dependence of sampling delay during the exponential drop and at trough level (Tables 4 and 5). These data of healthy infants aged 1 or 4 months may be used for evaluating amino acid results (μmol/L) of controls made on patients aged 1 or 4 months fed low protein formula to make sure that there is no risk of protein malnutrition, e.g. if albumin is below the reference limits.

Discussion

The quantitative effects of p.p. sampling delay indicate that during the post-absorptive phase of 2.25–4.5 h the effect of sampling delay on the ln of essential amino acids (except total tryptophan) and of some non-essential amino acids leads to a significant negative slope, while several non-essential amino acids in plasma are not significantly affected by the p.p. sampling delay. Ornithine concentration is affected by the sampling delay at 1 month of age ($p < 0.001$), but not at 4 months of age ($p = 0.098$). This supports quantitatively the hypothesis that the flux direction at 1 month of ornithine to proline synthesis prevails (Haschke-Becher et al. 2016) due to the high demand of proline for collagen I synthesis. Since the proline content in the formula is lower than in breast-milk additional proline should be provided. Collagen I synthesis further requires glycine which is not affected by sampling delay in contrast to its precursor serine. Hydroxyproline, as a posttranslational product of proline is needed as well; the ln of proline and of hydroxyproline correlate positively (Spearman $\rho = 0.422$ and 0.454 at 1 and 4 months, respectively; $p < 0.001$ for both).

The effect of the actual daily intake of low protein formula is significant for lysine and threonine in infants of 1 month of age and at 4 months solely for glutamate (precursor of Δ1-pyrroline 5-carboxylate and proline or ornithine). It is not clear if the significant effects of intake of lysine and threonine at 1 month of age are due to a

Table 3 Equations of multiple regression of low protein formula-fed healthy infants

Infants aged 1 month	Infants aged 4 months
lnABA = 2.656 − (0.0102 × Sampling delay) − (0.007493 × Prot.Intake/d)	lnABA = 2.922 − (0.0374 × Sampling delay) − (0.01152 × Prot.Intake/d)
lnALA = 6.377 − (0.190 × Sampling delay) + (0.00760 × Prot.Intake/d)	**lnALA = 6.268 − (0.131 × Sampling delay) − (0.002617 × Prot.Intake/d)**
lnARG = 4.889 − (0.0823 × Sampling delay) + (0.00425 × Prot.Intake/d)	**lnARG = 4.989 − (0.1270 × Sampling delay) − (0.001935 × Prot.Intake/d)**
lnASN = 4.128 − (0.107 × Sampling Delay) + (0.00650 × Prot.Intake/d)	**lnASN = 4.101 − (0.134 × Sampling delay) + (0.005996 × Prot.Intake/d)**
lnASP = 2.73 − (0.0422 × Sampling delay) + (0.00769 × Prot.Intake/d)	lnASP = 2.896 − (0.08920 × Sampling delay) − (0.01396 × Prot.Intake/d)
lnCIT = 2.929 + (0.0762 × Sampling delay) + (0.00442 × Prot.Intake/d)	lnCIT = 2.768 + (0.0716 × Sampling delay) + (0.002234 × Prot.Intake/d)
lnGLU = 4.554 − (0.00370 × Sampling delay) + (0.00432 × Prot.Intake/d)	lnGLU = 4.987 − (0.0546 × Sampling delay) − (0.03251 × Prot.Intake/d)
lnGLN = 6.362 − (0.00419 × Sampling delay) − (0.000838 × Prot.Intake/d)	lnGLN = 6.25 − (0.0258 × Sampling delay) + (0.006032 × Prot.Intake/d)
lnGLY = 5.586 − (0.0269 × Sampling delay) − (0.000610 × Prot.Intake/d)	lnGLY = 5.21 + (0.00035 × Sampling delay) + (0.005289 × Prot.Intake/d)
lnHIS = 4.538 − (0.0387 × Sampling delay) + (0.00464 × Prot.Intake/d)	**lnHIS = 4.574 − (0.0590 × Sampling delay) − (0.006662 × Prot.Intake/d)**
lnILE = 4.594 − (0.187 × Sampling delay) + (0.00824 × Prot.Intake/d)	**lnILE = 4.564 − (0.160 × Sampling delay) − (0.002195 × Prot.Intake/d)**
lnLEU = 5.137 − (0.138 × Sampling delay) + (0.00850 × Prot.Intake/d)	**lnLEU = 5.153 − (0.157 × Sampling delay) − (0.001341 × Prot.Intake/d)**
lnLYS = 5.594 − (0.109 × Sampling delay) + (0.0136 × Prot.Intake/d)	**lnLYS = 5.542 − (0.145 × Sampling delay) + (0.004911 × Prot.Intake/d)**
lnMET = 3.707 − (0.1019 × Sampling delay) + (0.0104 × Prot.Intake/d)	**lnMET = 3.817 − (0.166 × Sampling delay) − (0.007815 × Prot.Intake/d)**
lnORN = 4.885 − (0.146 × Sampling delay) + (0.01060 × Prot.Intake/d)	lnORN = 4.578 − (0.0745 × Sampling delay) − (0.004225 × Prot.Intake/d)
lnPHE = 4.08 − (0.0777 × Sampling delay) + (0.00757 × Prot.Intake/d)	**lnPHE = 4.246 − (0.0991 × Sampling delay) − (0.008068 × Prot.Intake/d)**
lnPROL = 5.477 − (0.110 × Sampling delay) + (0.000374 × Prot.Intake/d)	**lnPROL = 5.476 − (0.101 × Sampling delay) − (0.008848 × Prot.Intake/d)**
lnSER = 5.148 − (0.0800 × Sampling delay) − (0.000687 × Prot.Intake/d)	**lnSER = 5.203 − (0.0785 × Sampling delay) − (0.007643 × Prot.Intake/d)**
lnTHR = 5.455 − (0.143 × Sampling delay) + (0.0165 × Prot.Intake/d)	**lnTHR = 5.295 − (0.113 × Sampling delay) − (0.004426 × Prot.Intake/d)**
lnTRP = 4.288 − (0.0328 × Sampling delay) − (0.000483 × Prot.Intake/d)	**lnTRP = 4.48 − (0.0830 × Sampling delay) + (0.00259 × Prot.Intake/d)**
lnTYR = 4.564 − (0.0625 × Sampling delay) − (0.00156 × Prot.Intake/d)	**lnTYR = 4.645 − (0.0956 × Sampling delay) − (0.003201 × Prot.Intake/d)**
lnVAL = 5.303 − (0.0997 × Sampling delay) + (0.00649 × Prot.Intake/d)	**lnVAL = 5.377 − (0.104 × Sampling delay) − (0.00391 × Prot.Intake/d)**
lnASP + ASN = 4.373 − (0.0918 × Sampling delay) + (0.00566 × Prot.Intake/d)	**lnASP + ASN = 4.397 − (0.130 × Sampling delay) + (0.001204 × Prot.Intake/d)**
lnGLU + GLN = 6.525 − (0.000674 × Sampling delay) − (0.0008038 × Prot.Intake/d)	**lnGLU + GLN = 6.544 − (0.0411 × Sampling delay) − (0.0008319 × Prot.Intake/d)**

Bold characters p significant (<0.05) for sampling delay, *Prot. Intake* intake of g protein/d, *sampling delay* postprandial delay (h) after end of last feed

Table 4 Tenth percentile of significant amino acid concentrations (μmol/L) depending on p.p. sampling delays with 95 % confidence intervals (CI) in infants aged 1 month

p.p. sampling delay (h)	2.25	2.50	2.75	3.00	3.25	3.50	3.75	4.00	4.25	4.50	5.00[a]
Alanine	320[b] (312;328)	308 (302;328)	297 (291;302)	286 (281;290)	275 (270;280)	265 (259;270)	255 (249;262)	246 (238;253)	236 (228;245)	228 (218;238)	211 (200;223)
Arginine	85 (75;97)	84 (76;93)	83 (76;90)	82 (76;89)	81 (74;88)	80 (72;89)	79 (70;89)	78 (67;89)	77 (64;92)	76 (62;94)	74 (56:97)
Asparagine	43 (37;49)	41 (37;46)	40 (36;44)	38 (35;42)	37 (34;41)	36 (32;40)	34 (30;39)	33 (28;39)	32 (26;39)	31 (24;39)	28 (21;38)
Isoleucine	50 (48;53)	49 (49;51)	48 (46;49)	46 (45;48)	45 (43;47)	44 (42;46)	42 (40;45)	41 (38;44)	40 (37;43)	39 (35;43)	37 (32;43)
Leucine	102 (92;113)	99 (91;108)	97 (90;104)	94 (88;100)	92 (86;98)	89 (82;97)	87 (79;96)	85 (75;96)	83 (71;96)	81 (68;96)	77 (61;96)
Lysine	191 (169;215)	187 (169;206)	183 (169;198)	179 (166;192)	175 (161;189)	171 (155;188)	167 (149;188)	163 (142;188)	160 (135;189)	156 (128;190)	149 (116;193)
Methionine	27 (23;31)	26 (23;29)	26 (23;28)	25 (23;27)	24 (22;27)	24 (21;26)	23 (20;26)	22 (19;27)	22 (18;27)	21 (17;27)	20 (15;27)
Ornithine	77 (64;93)	75 (65;87)	73 (64;83)	71 (63;80)	69 (61;78)	67 (58;78)	65 (55;78)	64 (51;79)	62 (48;80)	60 (44;82)	57 (38;85)
Phenylalanine	40 (36;44)	40 (37;43)	40 (38;43)	40 (38;42)	40 (38;43)	40 (37;43)	40 (36;44)	40 (36;45)	40 (35;46)	40 (34;47)	40 (33;49)
Proline	138 (124;154)	137 (125;149)	135 (126;145)	134 (125;143)	132 (123;142)	131 (120;143)	130 (117;144)	128 (113;146)	127 (109;148)	126 (105;150)	123 (98;155)
Serine	113 (98;130)	111 (99;124)	109 (99;120)	107 (98;117)	105 (96;115)	103 (92;115)	101 (88;116)	99 (84;118)	98 (80;119)	96 (76;121)	92 (68;121)
Threonine	149 (126;177)	143 (124;164)	137 (122;154)	132 (119;147)	127 (113;142)	122 (106;139)	117 (99;138)	112 (92;138)	108 (85;137)	104 (78;137)	96 (66;138)
Valine	123 (107;141)	123 (110;138)	123 (112;135)	124 (113;135)	124 (113;136)	124 (111;139)	124 (108;143)	125 (105;147)	125 (102;152)	125 (99;158)	126 (93;169)

[a] Trough level
[b] Tenth percentile (lower; upper 95 % confidence limit)

Table 5 Tenth percentile of significant amino acid concentrations (μmol/L) depending on p.p. sampling delay in infants aged 4 months

p.p, sampling delay (h)	2.25	2.50	2.75	3.00	3.25	3.50	3.75	4.00	4.25	4.50	5.00
Alanine	270[a] (231;316)	266 (234:302)	261 (235;291)	257 (233;283)	253 (228;281)	249 (220;281)	245 (211;284)	241 (201;289)	237 (191;294)	233 (181;300)	226[b] (163;312)
Arginine	85 (77;94)	81 (74;88)	77 (71;82)	73 (68;77)	69 (64;74)	65 (60;71)	62 (56;68)	59 (52;66)	56 (48;64)	53 (45;82)	48 (39;59)
Asparagine	38 (34;42)	37 (34;40)	36 (33;38)	34 (32;37)	33 (31;36)	32 (30;35)	31 (28;35)	30 (27;34)	29 (25;34)	28 (24;34)	27 (21;33)
Histidine	66 (59;73)	65 (59;71)	64 (59;69)	63 (59;67)	62 (58;67)	61 (56;67)	61 (55;67)	60 (53;68)	59 (51;69)	58 (49;69)	57 (45;71)
Isoleucine	52 (48;57)	50 (47;54)	48 (45;51)	46 (44;48)	44 (42;47)	42 (40;45)	40 (37;44)	39 (35;43)	37 (33;42)	36 (31;41)	33 (27;39)
Leucine	99 (95;103)	95 (92;98)	90 (88;93)	86 (84;88)	82 (80;84)	79 (76;81)	75 (72;78)	72 (69;75)	68 (65;72)	65 (61;69)	60 (55;64)
Lysine	161 (140;186)	154 (137;172)	146 (133;161)	140 (128;153)	133 (121;146)	127 (114;142)	121 (106;139)	115 (98;136)	110 (90;134)	105 (83;132)	95 (71;128)
Methionine	23 (19;28)	22 (19;26)	21 (18;24)	20 (18;22)	19 (17;21)	18 (16;21)	17 (14;20)	16 (13;20)	15 (12;20)	15 (11;20)	13 (9;20)
Phenylalanine	39 (36;42)	39 (36;41)	38 (36;40)	38 (36;40)	37 (36;39)	37 (35;39)	37 (34;40)	36 (33;40)	36 (32;40)	36 (31;40)	35 (30;41)
Proline	137 (125;149)	133 (124;143)	130 (123;138)	127 (120;134)	124 (117;131)	121 (113;129)	118 (109;128)	115 (104;127)	113 (100;127)	110 (96;126)	105 (88;125)
Serine	122 (114;130)	119 (113;126)	116 (111;122)	114 (109;118)	111 (106;116)	108 (103;114)	106 (100;113)	103 (96;111)	101 (93;110)	99 (89;109)	94 (82;108)
Threonine	119 (114;124)	115 (111;119)	111 (108;114)	108 (105;110)	104 (101;107)	101 (97;104)	97 (93;101)	94 (90;99)	91 (86;96)	88 (82;94)	82 (75;90)
Tyrosine	54 (44;68)	54 (45;65)	54 (46;62)	53 (46;61)	53 (46;61)	53 (44;63)	52 (42;65)	52 (40;67)	52 (38;70)	51 (36;73)	51 (32;80)
Valine	131 (102;143)	128 (120;138)	126 (119;134)	124 (117;130)	121 (115;128)	119 (111;127)	116 (107;126)	114 (103;126)	112 (100;126)	110 (96;126)	106 (88;126)

[a] Tenth percentile (lower; upper 95 % confidence limit)
[b] Trough level

relatively high concentration of these amino acids in formulas as compared to breast-milk and/or to reduced utilization for protein synthesis.

As shown in the equations of multiple regression (Table 3) the coefficients of sampling delay are more than 10 times higher than the coefficients of actual daily protein intake. Despite our demand to the supervisors of the original studies to obtain blood samples later than 3.5 h after the last feed, this recommendation was ignored. In fact 82% of the samples were taken before 3.75 h p.p. at 1 month and 80% at 4 months of age. This is due to feeding the infants on-demand. We wonder if after 3.5 h satiety was not reached any more with the low protein formula.

Conclusion

For the interpretation of plasma amino acid concentrations data on the postprandial delay of sampling must be obtained and taken into account; otherwise low amino acid concentrations could be missed in patients.

Take-Home Message

For the interpretation of plasma amino acid concentrations data of the postprandial delay of sampling must be obtained and taken into account.

Compliance with Ethics Guidelines

Conflict of Interest

Alexander Kainz was funded by the research fund of the central clinical chemistry laboratory (CHUV, Lausanne) for his statistical work.

All authors declare no conflict of interest.

The authors confirm independence from Nestec Inc. The content of the article has not been influenced by any company or institution.

Informed Consent

All procedures were in accordance with the ethical standards of the responsible committees on human experimentation (national and international) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the parents of all infants included in the original studies from which the data of this study were extracted.

Contribution of the Authors

C. Bachmann contributed by the conception, the planning, multiple regression statistics and analysis of the laboratory results, their interpretation as well as drafting and revising of the manuscript. He is the guarantor of the final manuscript.

A. Kainz contributed by the statistics including quantile regression, drafting and revising the manuscript.

E. Haschke-Becher contributed by the conception, the drafting and revision of the manuscript.

References

Fomon SJ, Ziegler EE, Nelson SE et al (1999) Infant formula with protein-energy ratio of 1.7g/100 kcal is adequate but may not be safe. J Pediatr Gastroenterol Nutr 28:495–501

Haschke-Becher E, Kainz A, Bachmann C (2016) Reference values of amino acids and of common clinical chemistry in plasma of healthy infants aged 1 and 4 *months*. J Inherit Metab Dis 39(1):25–37

Kirchberg FF, Harder U, Weber M et al (2015) Dietary protein intake affects amino acid and acylcarnitine metabolism in infants aged 6 months. J Clin Endocrinol Metab 100(1):149–158

Koenker RW, Bassett GW (1978) Regression quantiles. Econometrica 46:33–50

Koletzko B, Baker S, Clegholm G et al (2005) Global standard for the composition of infant formula: recommendations of an ESPGHAN coordinated international expert group. J Pediatr Gastroenterol Nutr 41:584–599

Ong KK, Langkamp M, Ranke MB et al (2009) Insulin-like growth factor I concentrations in infancy predict differential gains in body length and adiposity: the Cambridge baby growth study. Am J Clin Nutr 90:156–161

Toschke AM, Grote V, Koletzko B, von Kries R (2004) Identifying children at high risk for overweight at school entry by weight gain during the first 2 years. Arch Pediatr Adolesc Med 158:449–452

Windmueller HG, Spaeth AE (1981) Source and fate of circulating citrulline. Am J Physiol 241(6):E473–E480