Are All Breast-fed Infants Equal? Clustering Metabolomics Data to Identify Predictive Risk Clusters for Childhood Obesity

*Franca Fabiana Kirchberg, *Veit Grote, †Dariusz Gruszfeld, †Piotr Socha, ‡Ricardo Closa-Monasterolo, ‡Joaquin Escribano, §Elvira Verduci, §Benedetta Mariani, ||Jean-Paul Langhendries, ¶Pascale Poncelet, *Berthold Koletzko, and *Christian Hellmuth, for The European Childhood Obesity Trial Study Group

ABSTRACT

Objectives: Fetal and early life represent a period of developmental plasticity during which metabolic pathways are modified by environmental and nutritional cues. Little is known on the pathways underlying this multifactorial complex. We explored whether 6 months old breast-fed infants could be clustered into metabolically similar groups and that those metabotypes could be used to predict later obesity risk.

Methods: Plasma samples were obtained from 183 breast-fed infants aged 6 months participating in the European multicenter Childhood Obesity Project study. We measured amino acids along with polar lipid concentrations (acylcarnitines, lysophosphatidylcholines, phosphatidylcholines, sphingomyelins). We determined the metabotypes using a Bayesian agglomerative clustering method and investigated the properties of these clusters with respect to clinical, programming, and metabolic factors up to 6 years of age.

Results: We identified 20 metabolite clusters comprising 1 to 39 children. Phosphatidylcholines predominantly influenced the clustering process. In the largest clusters ($n \geq 14$), large differences existed for birth length (unadjusted $P < 0.0001$) and length and weight at 6 months (unadjusted $P < 0.0001$ and $P = 0.012$, respectively). Infants tended to cluster together by country (unadjusted $P < 0.001$). The body mass index (BMI) z score at 6 years of age tended to differ (unadjusted $P = 0.07$).

Conclusions: Our exploratory study provided evidence that breast-fed infants are not metabolically homogeneous and that variation in metabolic profiles among infants may provide insight into later development and health. This work highlights the potential of metabotypes for identifying inter-individual differences that may form the basis for developing personalized early preventive strategies.

Key Words: childhood obesity, developmental origins of health and disease, early programming, metabolomics, metabotypes

(*JPGN* 2019;68: 408–415)

What Is Known

- Stimuli or insults applied during early life can modulate later health outcomes.
- Effects of single factors such as infant nutrition have been found to alter metabolic pathways.

What Is New

- Clustering metabolic profiles of 6 months old breast-fed infants to capture complex multifactorial programming processes.
- Breast-fed infants are not metabolically homogeneous: country is a strong but not the sole predictor.
- Metabolic clusters at age 6 months show only limited prediction of obesity risk at early school age.

Pregnancy and early childhood represent a sensitive period of developmental plasticity. Early environment and lifestyle modify an infant's long-term health (1–5). So far, most studies investigate the effect of nutrition or environmental risk factors on alterations of metabolic pathways. There limitations of this approach: while not all risk factors may, however, be known; it is furthermore highly likely that the programming is a complex multifactorial process which may not be adequately reflected in risk factor analyses.

Received January 5, 2018; accepted October 2, 2018.

From the *Ludwig-Maximilians-Universität München, Division of Metabolic and Nutritional Medicine, Dr. von Hauner Children's Hospital, Munich, Germany, the †Neonatal Intensive Care Unit, Children's Memorial Health Institute, Warsaw, Poland, the ‡Pediatric Nutrition and Development Research Unit, Universitat Rovira I Virgili, IISPV, Reus, Spain, the §Department of Paediatrics, San Paolo Hospital, University of Milan, Milano, Italy, the ||Centre Hospitalier Chrétien St Vincent, Liège-Rocourt, Belgium, and the ¶Department of Pediatrics, University Children's Hospital Queen Fabiola, Université Libre de Bruxelles, Brussels, Belgium.

Address correspondence and reprint requests to Berthold Koletzko, Ludwig-Maximilians-Universität München, Dr. von Hauner Children's Hospital, Division of Metabolic and Nutritional Medicine Lindwurmstr. 4, D-80337 Munchen, Germany (e-mail: office.koletzko@med.lmu.de).

Supplemental digital content is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (*www.jpgn.org*).

Berthold Koletzko and Christian Hellmuth contributed equally to this work.

www.clinicaltrials.gov registration number: NCT00338689

The metabolomics analyses were financial supported by the Commission of the European Communities 7th Framework Programmes Early Nutrition (FP7-289346), DYNAHEALTH (H2020-633595) and LIFECYCLE (H2020-SC1-2016-RTD), and European Research Council Advanced Grant ERC-2012-AdG—No. 322605 META-GROWTH. Partial financial support from Polish Ministry of Science and Higher Education (2571/7.PR/2012/2). This paper does not necessarily reflect the views of the Commission and in no way anticipates the future policy in this area. Additional supported by, German Ministry of Education and Research, Berlin (Competence Network Obesity, Nr. 01 GI 0825). The authors report no conflicts of interest.

DOI: 10.1097/MPG.0000000000002184

In our work, we follow the idea that metabolomics data contains the information of the programming processes and that we can group children into metabolite clusters that are predictive for certain metabolic trajectories and later health outcomes. Metabolomics, the study of small molecules which are substrates, intermediates and end products of cellular regulatory processes, offers the possibility to explore potential molecular mechanisms. The metabolome is the product of genes and environment, including lifestyle, diet, and gut microbial activity (6). The term ''metabotype'' (7) describes a characteristic metabolic profile reflecting the physiological state of an organism, and therefore also considers metabolic consequences of genetic modifications or disease processes that may not be directly observable in the phenotype. One way to define such metabotypes is cluster analysis where individuals with similar metabolite patterns are grouped together in classes/clusters (8). This concept has been applied in many research areas of personalized medicine (9,10).

We investigate whether the metabolome of 6 months old healthy breast-fed infants contains information on multifactorial perinatal programming processes and the adaptations of the metabolism to pre- and early postnatal conditions. We aim at identifying characteristic metabotypes predicting a high risk for later childhood obesity. The longitudinal European Childhood Obesity Project trial (CHOP) offers great opportunity to investigate this research question. It has been shown previously that formula-fed infants are at higher risk for obesity later in life (11) and that the metabolite profile differs between formula-fed and breast-fed infants (12). Data on differences within groups of breast-fed infants are, however, missing; rather populations of breast-fed infants are often considered to be metabolically homogenous. This work investigates the opportunity of metabolomics for early assessment and targeted prevention of obesity by examining metabolic differences among breast-fed infants.

METHODS

Study Design

The data evaluated were collected as part of the European Childhood Obesity Project. This double-blind, randomized, multicenter intervention trial funded by the European Commission was conducted in 5 countries: Germany, Belgium, Italy, Poland, and Spain (13). Shortly after birth but no later than 8 weeks after birth, parents of infants who met the inclusion criteria were invited to participate in a study on the effects of dietary protein on obesity and growth. Eligible for study participation were apparently healthy, singleton, term infants who were born between October 1, 2002, and July 31, 2004. Infants of mothers with a hormonal or metabolic disease or illicit drug addiction during pregnancy were not included. Breast-feeding was encouraged during the recruitment process. If nevertheless parents decided to formula-feed, the infants were randomly assigned to either a higher or lower protein content infant formula. Children in the breast-fed group had to be fully breast-fed since birth and at least during the first 3 months. To exclude the influence of the randomized formula intervention, we only included breast-fed infants in this article (12). The study was approved by the ethics committees of all the study centers, and written informed parental consent was obtained for each infant (trial registration: ClinicalTrials.gov; identifier: NCT00338689).

Anthropometric Measurements and Early Programming Factors

Birth weight and length were obtained from hospital data. Height- and weight-for-gestational-age percentiles were calculated using a German reference dataset of births between data of 2007–2011 (14). Since this is a European study, caution should be applied when interpreting the percentiles in an absolute way with respect to the reference population rather than using them for comparison purposes within this study. All other anthropometric measures were obtained at visits to the study centers: at baseline as well as at 3, 6, 12, and 24 months of age. Thereafter, measurements were taken every 6 months until 6 years of age. The time of the visits was planned to be within 14 days of the targeted age until the 2 years visit and within 3 months for all later time points. Obesity at 6 years was defined according to the International Obesity Task Force criteria (15,16): girls and boys were classified as obese if they had a body mass index (BMI, kg/m^2) >19.7 or 19.8, respectively. Weight, height, and BMI were transformed to age- and sex-specific z scores according to the WHO growth standards (17,18). We furthermore calculated the quartiles of birth weight and allocated each child to 1 of the 4 quartile categories. Change in weight-for-age (WFA) was defined as the absolute difference in the WFA z scores. Data on pregnancy outcomes (maternal pre-pregnancy weight, gestational age at delivery, birth order, and delivery mode) were collected by questionnaire. Maternal height, highest parental educational level (according to the International Standard Classification of Education), and smoking during pregnancy were assessed at the baseline visit. Details of the study design have been published previously (13,19).

Sample Collection

Plasma samples were collected at an infant age of 6 months. No blood was collected from children enrolled in Italy as ethical consent was not obtained in Italy. Efforts have been made to draw blood samples at 2 or more hours after the last feeding. The plasma samples were stored at $-80^{\circ}C$ except for Belgium where samples were stored at $-20^{\circ}C$. Urine samples were stored at $-70^{\circ}C$ and transported on dry ice to 1 central laboratory (The Children's Memorial Health Institute, Warsaw, Poland) for analysis of urinary C-peptide and creatinine.

Laboratory Procedures

Eighteen plasma amino acids (AA) were quantified by high performance liquid chromatography (HPLC) with the use of the Pico-Tag method (Waters Corporation, Milford, MA). Additionally, citrulline, ornithine, and proline AA were quantified by ion-pair liquid chromatography coupled to mass spectrometry detection as described previously (20). We furthermore quantified the sum of hexoses along with 146 polar lipids (free carnitine; acylcarnitines [Carn], $n = 40$; lysophosphatidylcholines [LPC], $n = 11$; phosphatidylcholines [PC], $n = 91$; and sphingomyelins [SM], $n = 14$). These metabolites were qualified and quantified with the Absolute IDQ p 150 kit (Biocrates Life Sciences AG, Innsbruck, Austria) the same way as the acylcarnitines analysis as described previously (12,20). As part of quality control, we calculated the coefficient of variation (CV) and excluded the analytes whose CV was greater than 20% from the statistical analyses. Variables of the insulin-like growth factor (IGF) axis (total IGF-I, free IGF-I, IGF-BP2, IGF-BP3) were measured with the use of immunoradiometric assay kits. Urine creatinine was analyzed with a kinetic assay based on the Jaffe reaction in an automated ADVIA 1650/Mega (Bayer Healthcare AG, Leverkusen, Germany). Urinary C-peptide was quantified with a radioimmunoassay kit (both from Diagnostic Systems Laboratories Inc, Webster, TX). For more details see (21).

Data Analysis

Metabotype clusters were identified using Bayesian agglomerative clustering. For graphical summary of the cluster solution, we plot a cluster dendrogram and the importance of the metabolites in a

TABLE 1. Baseline Characteristics of the Infants

	Infants (N = 154)
Gender	
Female	82 (53%)
Male	72 (47%)
Country	
Belgium	25 (16%)
Spain	41 (27%)
Germany	47 (31%)
Poland	41 (27%)
Age at blood withdrawal, months	6.0 ± 0.19
Parental educational level*	
No/low	6 (4%)
Middle	41 (27%)
High	107 (69%)
Nationality	
Study country	121 (79%)
One parent other country	26 (17%)
Both parents other country	7 (5%)
Age of mother at birth, years	30.8 ± 4.52
Pre-pregnancy BMI of mother, kg/m^2	22.4 ± 3.62
Smoking during pregnancy†	
No	143 (93%)
Yes	11 (7%)
Gestational age at delivery, weeks	
38	17 (11%)
39	29 (19%)
40	52 (34%)
41	38 (25%)
42	16 (11%)
Delivery mode	
Caesarean section	28 (18%)
Forceps or vacuum extraction	19 (12%)
Spontaneous	106 (69%)
Fully breast-fed up to 6 months of age	18 (12%)

Anthropometrics	Birth	6 months
Weight, kg	3.4 ± 0.38	7.5 ± 0.90
Length, cm	51.4 ± 2.92	67.0 ± 2.61
BMI, kg/m^2	12.8 ± 1.18	16.7 ± 1.55
BMI z score	−0.5 ± 1	−0.4 ± 1.06

Data are given as numbers (%) or mean ± SD
*Highest educational level of mother and father; low: <10 years; middle, 10–12 years; high, ≥12 years.
†Smoking up to the 12th week of gestation.

bar plot depicting the variable Bayes factors. As a sensitivity analysis we applied hierarchical cluster analysis using Ward's method coupled with the Euclidian distance metric. The metabolite cluster characteristics are described with respect to the clinical variables, the BMI trajectory over time, and the metabolite concentrations. If sample sizes in the metabolite clusters were small (<5 infants) or consisted of 1 child only, we focused on the biggest metabolite clusters to have enough sample size to perform statistical inference tests. Our primary focus lay on the investigation of the 3 biggest metabolite clusters, but we also tested for differences across the 5, 7, and 10 biggest metabolite clusters. If 2 clusters had equal sample size, we included both of them in the respective analysis. We report the clusterwise numbers (%) and median [inter quartile range (IQR)] of the clinical parameters. To test for differences across the metabolite clusters, we used Fisher's exact test for categorical variables and Kruskal-Wallis rank-sum test for continuous variables. If Fisher's exact test failed to calculate the exact P value, we used Monte-Carlo simulation with 10,000 replicates to simulate the P value. Taking advantage of the longitudinal study design, we plot the course of the median BMIs over time (birth until 6 years). At each time point we calculate the Kruskal-Wallis rank-sum test to test for differences in BMI across clusters. In addition, we pairwisely compared the metabolite concentrations across the metabolite clusters using 2-sample Wilcoxon tests. Results are represented in Manhattan plots. All reported P values are not adjusted for multiple testing but Bonferroni correction can easily be applied by multiplying the P values by the number of tests performed (the authors suggest to use the number of variables in one table, ie, the number of characteristics in Table 1 in this explorative analysis). All statistical analyses were performed using R (version 3.3.0, R Foundation for Statistical Computing, Vienna, Austria). Detailed information on the statistical analyses are provided in the supplement (Supplemental Digital Content, *http://links.lww.com/MPG/B506*).

RESULTS

Blood samples were available for 183 breast-fed infants. Out of these, 163 had valid measurements for all 168 metabolites. After outlier deletion, complete data were available for 154 infants. Their characteristics are described in Table 1. At blood withdrawal, 18 infants (12%) were fully breast-fed.

Using Bayesian agglomerative clustering of metabolite measurements, we identified 20 metabolite clusters whose size ranged from one infant to 39 infants. The cluster dendrogram is presented in the supplemental material (Supplemental Figure 1, Supplemental Digital Content, *http://links.lww.com/MPG/B506*). The Bayesian clustering method estimates the proportions of important metabolites and of the appearance of different cluster means for an important metabolite. The proportions were estimated to be 100% and 19%, respectively. Table 2 summarizes the clinical characteristics of the 4 biggest metabolite clusters (Cluster 1: N = 39; Cluster 4: N = 24, Cluster 2 & 3: N = 14). While the country of origin differed strongly between the metabolite cluster (unadjusted $P < 0.0001$), we could not observe a trend in grouping according to sex (unadjusted $P = 0.47$). There was a trend towards more parents with middle educational level in cluster 3 (unadjusted $P = 0.07$). The infants of the 4 biggest metabolite clusters furthermore differed in their birth length (unadjusted $P < 0.0001$) and height-for-gestational-age (unadjusted $P < 0.0001$), but not in their birth weight (unadjusted $P = 0.16$). Consequently, BMI and ponderal index building on these measurements differed as well (unadjusted $P = 0.001$ and unadjusted $P < 0.0001$, respectively). The difference in height was also observed at 6 months of age when blood was withdrawn (unadjusted $P < 0.0001$). In contrast to the BMI z score that was not different (unadjusted $P = 0.94$), the weight at 6 months was different (unadjusted $P = 0.012$). With respect to early weight gain, we did not observe cluster differences in weight change, neither from birth to 6 months nor from birth to 24 months (unadjusted $P = 0.3$ and unadjusted $P = 0.38$, respectively). Variables of the IGF axis, except for IGF-I total, all differed between the metabolite clusters (unadjusted $P < 0.025$). Apart from maternal pre-pregnancy BMI (unadjusted $P = 0.017$), all other pre- or perinatal characteristics such as delivery mode did not differ between the metabolite clusters. Same observation was made for weight gain. A more detailed view of these associations is given in the Supplementary Figure 5 (Supplemental Digital Content, *http://links.lww.com/MPG/B506*). Except for the maternal age (metabolite cluster 7 (N = 12) consisted of comparatively young mothers with a median age of 25.6 years), results were similar when also looking at other big cluster and comparing the 5, 7, or 10 biggest metabolite clusters (Supplemental Table 2, Supplemental Digital Content, *http://links.lww.com/MPG/B506*).

TABLE 2. Characteristics of the Children in the 4 Biggest Metabolite Clusters

	N	Cluster 1 (N = 39)	Cluster 4 (N = 24)	Cluster 2 (N = 14)	Cluster 3 (N = 14)	P
Gender						
Female	91	19 (49%)	11 (46%)	7 (50%)	10 (71%)	0.47
Male		20 (51%)	13 (54%)	7 (50%)	4 (29%)	
Country						
Belgium	91	10 (26%)	0 (0%)	3 (21%)	3 (21%)	<0.0001
Germany		9 (23%)	9 (38%)	4 (29%)	4 (29%)	
Poland		2 (5%)	14 (58%)	3 (21%)	0 (0%)	
Spain		18 (46%)	1 (4%)	4 (29%)	7 (50%)	
Age at blood withdrawal, 6 months	91	6.0 [0.20]	6.0 [0.11]	6.0 [0.10]	6.0 [0.11]	0.49
Fully breast-fed up to 6 months of age	90	2 (5%)	5 (21%)	1 (7%)	3 (23%)	0.12
Socioeconomic parameters						
Parental educational level*						
No/low	91	2 (5%)	0 (0%)	0 (0%)	1 (7%)	0.07
Middle		9 (23%)	3 (12%)	2 (14%)	7 (50%)	
High		28 (72%)	21 (88%)	12 (86%)	6 (43%)	
Maternal and pregnancy parameters						
Age of mother, years	91	31.2 [5.86]	30.9 [5.14]	30.9 [5.63]	31.4 [8.02]	0.92
Pre-pregnancy BMI of mother, kg/m^2	89	22.1 [5.19]	19.9 [4.14]	21.3 [2.20]	23.9 [2.71]	0.017
Smoking during pregnancy†	91	6 (15%)	0 (0%)	1 (7%)	0 (0%)	0.1
Gestational age at delivery, weeks						
38	90	3 (8%)	6 (25%)	2 (14%)	1 (7%)	0.009
39		12 (32%)	0 (0%)	3 (21%)	2 (14%)	
40		15 (39%)	13 (54%)	3 (21%)	5 (36%)	
41		7 (18%)	3 (12%)	6 (43%)	3 (21%)	
42		1 (3%)	2 (8%)	0 (0%)	3 (21%)	
Parity						
1	91	23 (59%)	14 (58%)	7 (50%)	9 (64%)	0.11
2		15 (38%)	5 (21%)	3 (21%)	4 (29%)	
3		1 (3%)	5 (21%)	4 (29%)	1 (7%)	
Delivery mode						
Caesarean section	91	10 (26%)	5 (21%)	2 (14%)	3 (21%)	0.65
Forceps or vacuum extraction		5 (13%)	3 (12%)	0 (0%)	3 (21%)	
Spontaneous		24 (62%)	16 (67%)	12 (86%)	8 (57%)	
Birth anthropometrics						
Birth weight, g	91	3320 [477.50]	3540 [807.50]	3410 [285.00]	3340 [667.50]	0.16
Birth weight (categorical)‡						
1st quartile	91	12 (31%)	5 (21%)	2 (14%)	4 (29%)	0.42
2nd quartile		7 (18%)	4 (17%)	2 (14%)	2 (14%)	
3rd quartile		12 (31%)	3 (12%)	6 (43%)	3 (21%)	
4th quartile		8 (21%)	12 (50%)	4 (29%)	5 (36%)	
Weight-for-gestational-age percentiles	90	0.4 [0.39]	0.4 [0.54]	0.5 [0.31]	0.4 [0.49]	0.17
Length at birth, cm	91	50.0 [3.00]	54.0 [3.25]	50.0 [4.50]	49.5 [2.75]	<0.0001
Height-for-gestational-age percentiles	90	0.1 [0.33]	0.8 [0.28]	0.2 [0.54]	0.2 [0.37]	<0.0001
BMI at birth, kg/m^2	91	13.1 [1.30]	12.1 [1.65]	13.4 [1.34]	12.4 [1.95]	0.001
Ponderal index at birth, kg/m^3	91	26.4 [2.48]	22.8 [3.53]	26.5 [4.61]	25.8 [4.44]	<0.0001
Anthropometrics at blood withdrawal (6 months)						
Weight at 6 months, kg	91	7.3 [1.00]	8.0 [0.58]	7.3 [1.18]	7.0 [0.64]	0.012
Height at 6 months, cm	91	66.5 [2.65]	68.7 [1.35]	66.8 [4.38]	65.5 [2.03]	<0.0001
BMI z-score at 6 months, kg/m^2	91	−0.4 [1.29]	−0.4 [1.25]	−0.3 [1.20]	−0.4 [1.18]	0.94
Growth parameters						
Weight gain 0–6 months, kg	90	4.2 [1.25]	3.9 [1.11]	3.5 [1.64]	3.8 [1.20]	0.3
Change in weight-for-age z score 0–6 months	90	0.2 [1.46]	0.0 [1.17]	−0.3 [1.14]	−0.0 [1.25]	0.28
Weight gain 0–24 months, kg	76	8.9 [2.00]	8.6 [1.29]	8.5 [1.41]	8.3 [1.55]	0.38
Change in weight-for-age z score 0–24 months	76	0.7 [1.02]	0.2 [1.19]	0.3 [1.39]	0.3 [1.32]	0.19
Variables of the IGF axis, measured at 6 months						
IGF-I total, ng/mL	74	28.4 [40.2]	19.6 [23.9]	28.9 [23.8]	8.4 [20.3]	0.2
IGF-I free, ng/mL	83	0.3 [0.32]	0.4 [0.26]	0.4 [0.28]	0.2 [0.15]	0.003
IGF-BP2, ng/mL	82	1405 [793]	1270 [502]	1190 [383]	2072.5 [1105]	0.025
IGF-BP3, ng/mL	83	2773.5 [696]	2732.5 [589]	2712.5 [551]	2228 [1155]	0.017
C-peptide:creatinine ratio, ng/mg	53	84.4 [147.1]	44.0 [52.2]	170.0 [243]	117.3 [52.1]	0.013
Anthropometrics at 6 years						
Weight at 6 years, kg	61	21.1 [4.16]	20.5 [2.61]	24.1 [5.29]	21.9 [3.42]	0.42
Height at 6 years, cm	61	118.5 [7.53]	118.2 [2.55]	121.2 [5.90]	114.2 [4.80]	0.12
BMI z score at 6 years	61	−0.1 [1.65]	−0.6 [1.15]	0.6 [1.44]	0.7 [1.39]	0.07
BMI categories at 6 years§						
Underweight	61	3 (10%)	5 (36%)	1 (14%)	0 (0%)	0.08
Normal weight		22 (71%)	9 (64%)	3 (43%)	6 (67%)	
Overweight		4 (13%)	0 (0%)	3 (43%)	2 (22%)	
Obese		2 (6%)	0 (0%)	0 (0%)	1 (11%)	

Metabolite clusters were built using Bayesian agglomerative clustering based on the metabolomics data. Data are given as numbers (%) or median [IQR]. Cluster differences were tested for significance using Fisher's exact test (categorical variables) or Kruskal-Wallis rank-sum test (continuous variables)

*Highest educational level of mother and father; low: <10 years; middle, 10–12 years; high, ≥12 years.

†Smoking up to the 12th week of gestation.

‡First quartile, 2600–2998 g; second quartile, 3050–3280 g; third quartile, 3310–3550 g; fourth quartile, 3575–4290 g.

§Defined according to the International Obesity Task Force criteria (16,17).

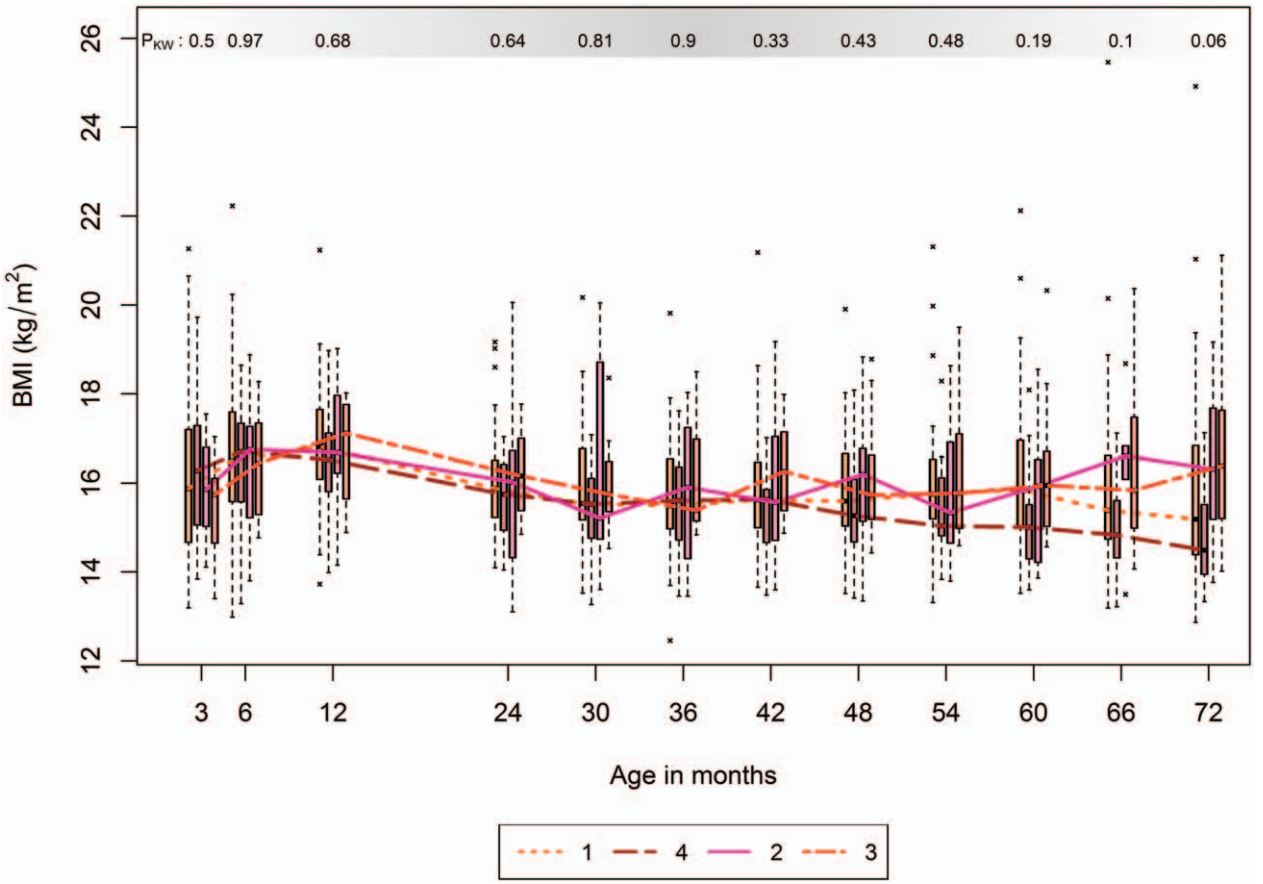

FIGURE 1. Boxplots of the BMI trajectories in the 4 biggest metabolite cluster of infants (Cluster sizes at month 6 are Cluster 1: N = 39; Cluster 4: N = 24; Cluster 2 and 3: N = 14). For each time point, we report the *P* value P_{KW} of the Kruskal-Wallis rank-sum test that tests for cross-sectional differences in BMI across the clusters.

The cluster differences in BMI *z* score at 6 years of age marginally missed the significance threshold (unadjusted $P = 0.07$). Figure 1 summarizes the BMI values over time: The older the children get, the more clearly the median BMI of the cluster separate. Cluster 4 has consistently the lowest median BMI from the age of 3 $^{1}/_{2}$ years on.

Figure 2 displays the respective importance of a metabolite in the clustering process. Except for acylcarnitine C8:1 (log (Bayes Factor $B_\delta) < 0$), all metabolites were found to be important. Especially the phospholipids with 40 or more Carbon atoms had high Bayes factors. The type of binding of the fatty acid to the PC, acyl/acyl or acyl/ether, did not make a difference. Supplemental Fig. 7 & 8 (Supplemental Digital Content, *http://links.lww.com/MPG/B506*) summarize the metabolite concentrations in the metabolite clusters in more detail and the results match those of the variable importance plot (Fig. 2).

DISCUSSION

To our knowledge, this exploratory study is the first to investigate whether metabolic profiles of 6 months old infants could be clustered to identify subgroups of children with specific characteristics, for example, with higher risk for later childhood obesity. Using an agglomerative, unsupervised data mining technique to identify groups of breast-fed infants aged 6 months with similar metabolite profiles, we identified 20 metabolite clusters. This considerable variation of metabolic patterns indicates that breast-fed infants are not metabolically homogeneous. Restricted by the small sample size in some metabolite clusters, we focused on the biggest clusters in order to draw statistical conclusions. The observed association of birth length with metabolic state at 6 months reflects an apparent lasting impact of prenatal metabolism and growth, whereas metabolic clusters at age 6 months have only limited predictive value for obesity risk at early school age.

Metabolic Characteristics

The pairwise comparisons of the metabolite concentrations between the metabolite clusters and the Bayes factors from the Bayesian clustering point in the same direction. Above all, polar lipids (LPC, PC, SM) and not the Carn or AA differ and play an important role in the clustering process. The main sources of PC and SM are lipoproteins. The SM to PC ratio is lower in high-density (HDL) than in low-density lipoproteins (LDL) (22), but we have not measured the LDL or HDL concentrations. In contrast, LPC species are largely derived from cleavage of PC largely by the action of lecithin cholesterol acyltransferase (23). This enzyme catalyzes the transfer of fatty acids from position *sn*-2 of PC to free cholesterol—a process that results in the formation of LPC and cholesterol ester which are incorporated in HDL. In those clusters where all SM and PC are elevated one could thus speculate on more lipoproteins in the blood and hence more transport of fat in those children. One could also consider an involvement of parameters of the IGF-axis since

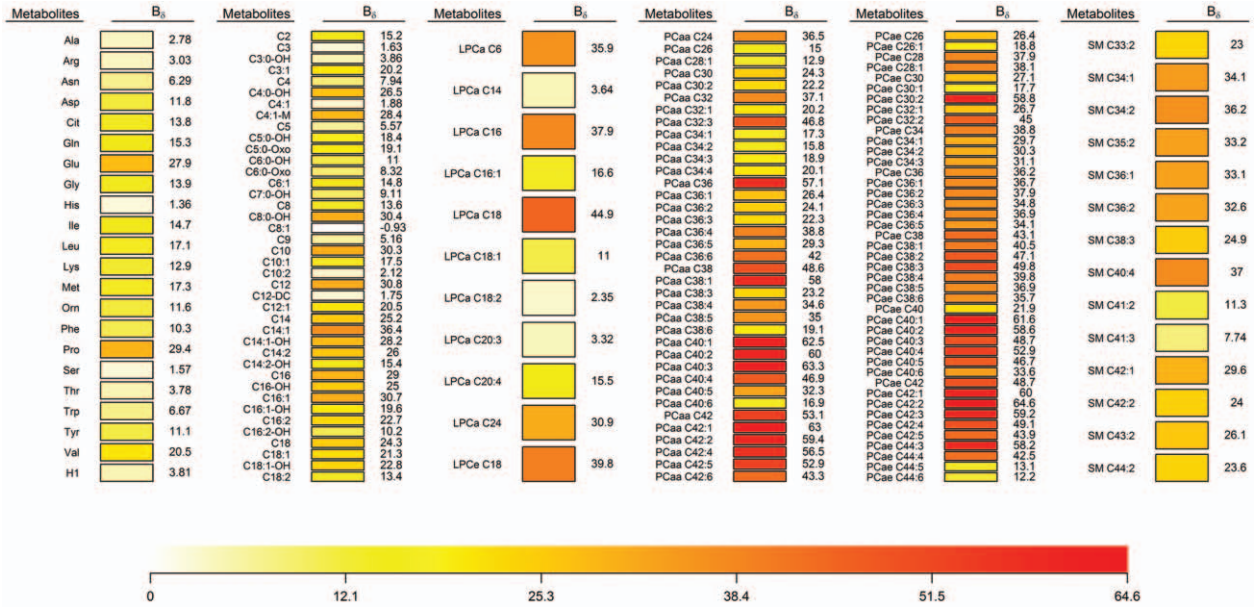

FIGURE 2. Results from the Bayesian agglomerative clustering. Represented is the logarithm of the Bayes factors B_δ indicating the variable importance for each metabolite in the clustering process. A high Bayes factor indicates a high importance (red).

IGF-1 and IGFBP-3 were found to be cross-sectionally associated to LDL and HDL (24). Furthermore, IGF is supposed to be a key hormone in the pathophysiology of metabolic syndrome (25).

Perinatal and Early Life Factors

We observed large cluster differences with regard to length at birth and at 6 months of age. In contrast, the infants' birth weight was not associated with metabolic clusters, and less closely at 6 months of age when compared to length. Since the late 1980s, birth weight has been discussed as a surrogate measure of intrauterine growth and development (26,27), and it has been reproducibly and independently linked to increased overweight risk (3). Although high birth length has been linked to higher adult height (28,29), higher BMI in childhood (30), an increased risk of overweight in adulthood (31), and a higher central body fat distribution (32), it received less attention compared to high birth weight which was more closely associated to later BMI (33). Our results suggest a greater consideration of birth length as a marker for the influence of programming factors on the metabolism. But measurement error in the infants' length or the country effect may artificially increase the findings on infant height. Further studies are needed to detangle potential mechanisms of action. Several variables of the IGF-axis measured at 6 months of age differed between metabolite clusters. The IGF axis regulates early growth and was also shown to influence adipose tissue differentiation and early adipogenesis (34,35). Especially striking in these variables was metabolite cluster 3 associated with low IGF-1 free and IGF-BP3 levels along with high levels of IGF-BP2. IGF-1 has a higher affinity for the IGF-BP3 than for IGF-BP2 (36).

BMI Trajectories

We investigated the BMI evolution from birth until early school age. We could show that approximately from the age of 4 years onwards, differences in median BMI across the clusters became apparent. Children of metabolite cluster 3 are rather small at birth and have a high BMI during childhood. Vice versa, a high median birth weight together with a low BMI at 6 years was observed for metabolite clusters 4 and 7. Caution is, however, needed when interpreting these anthropometric data at 6 years because the sample size in the clusters is relatively small ($n \geq 9$). Hence, although metabolomics data seem to contain information on the infant's state that predict later growth, further investigations with a larger sample size are needed.

Country of Residence

Although metabolite concentrations were aligned country-wise before clustering, infants still clustered together by country. Up to 58% of one cluster belonged to the same country. As the removal of the country effect was done in a univariate setting, this finding may be explained by the different (multivariate) composition of the metabolome. Country is a possible confounder in our context: the BMI in childhood and also birth height differ between the countries. For instance, next to maternal diet also birth weight (37,38), maternal age, or complementary feeding practice differ by country (39). A recent very large study, the INTERGROWTH 21st project, however, found that fetal growth and newborn length are similar across geographical settings where there are no maternal nutritional or environmental constraints on growth (40). Due to our very low sample size in the clusters, we were not able to detangle the effects of these different factors or the effects of other possible confounders which may all influence the metabolome at 6 months of age. Nevertheless, no variable perfectly separated by the clusters. For instance, all clusters were a mix of infants of different countries where certain countries of residence dominated in the clusters. The key question thus is: What is it that makes these children similar in these children? Why do clusters only differ in birth length and not birth weight which are both different across countries? The look at the IGF-values may give us a first idea: While we have not observed any country differences in the IGF-1 concentrations or their binding proteins, the metabolite cluster had different values. Sample size was too low for multivariate regression models, we cannot draw conclusions on which factor was driving the results.

Statistical Considerations

We applied Bayesian agglomerative clustering to find groupings in the infants based on their metabolomics profile. This approach is highly suitable for low-sample-size-high-dimensional data (41) where it is commonly difficult to provide reasonable statistical models (42,43). In contrast to the hierarchical cluster approach where the user has to decide and calculate other metrics such as the silhouette width in order to decide for the optimal grouping, the optimal grouping is returned by the Bayesian clustering procedure. Despite the advantages of the Bayesian clustering, its application revealed some difficulties: the optimal grouping proposed a cluster solution with a large number of clusters ($k = 20$) resulting in very low sample sizes in certain clusters. Therefore, a full characterization of all these clusters was not possible and we had to focus on the biggest clusters with a sample size large enough to allow for meaningful conclusions.

The reason why we chose Bayesian clustering is that it is useful for high-dimensional continuous data (41). This is in contrast to distance-based hierarchical clustering techniques which may fail in high-dimensional settings (42,43). Since metabolomics data are ''small high-dimensional'' datasets when compared to, for example, genetic data, we performed hierarchical agglomerative clustering as sensitivity and compared the results of both clustering techniques. Using hierarchical agglomerative clustering we identified 2 clusters. The average silhouette width, a measure reflecting the consistency of a cluster, was 0.17 which suggests that the clusters may not be well separated and the underlying structure in our metabolomics data was likely ''blurry'' (44,45). The bootstrapped Jaccard mean similarity values of the 2 clusters were 0.6 and 0.71 and therefore also indicated only a moderate stability of the 2 clusters (46). In contrast to the Jaccard measure, the silhouette width is calculated based on distances. This measure may be inappropriate for a high dimensional dataset where the observations are sparse (42). In fact, this is also a great drawback of the hierarchical clustering. When comparing the results of both, the Bayesian and the hierarchical clustering, we, however, found that the clusters could clearly be allocated to each other. To our knowledge, and especially with respect to the unique nature of metabolomics data, there are no studies on the validity of clustering methods yet. Taken together, the properties of the 2 clusters found by the hierarchical cluster approach were rather poor, but, on the other hand, very well coincided with the cluster solution found by the Bayesian approach.

Strengths and Limitations

Another limitation arose due to the small size of some metabolite clusters: We had to restrict our analyses to the largest clusters. Separate case reports on the children forming a cluster on their own, thus not matching metabolite profiles of other infants, may be highly interesting as well. Another limitation is that blood samples in 6 months old infants cannot be obtained after an overnight fast, and in spite of aiming at standardized conditions, variation in postprandial states had to be accepted. Furthermore, only 12% of the infants were fully breast-fed at blood withdrawal. While this reflects realistic data since most infants in Europe receive complementary food before the age of 6 months (47), this may add additional variation to our data. Similarly induced by the study design, we only included infants born with a birthweight appropriate for gestational age and thus cannot draw any conclusions of the potential impact of very low or high birthweights.

Strengths of our study are the unique design with longitudinal follow-up and the statistical approaches undertaken. To our knowledge, we are the first to investigate metabotypes in healthy, breast-fed infants. The multicenter study set up, although imposing certain limitations, adds greater validity, and generalizability to our findings. Another strength of our work certainly is represented by the high-quality liquid chromatography-tandem mass spectrometry metabolite measurements. The metabolites measured cover a wide range of metabolic processes, among others protein, fatty acid, and lipoprotein metabolism. Thus, the clusters are formed based on information on many metabolic pathways.

CONCLUSIONS

Already at the very young age of 6 months, metabolomics data contain valuable information on the child's metabolism that may reflect lasting adaptations of structure, physiology, and metabolism to early life factors that may affect long-term health outcomes. Breast-fed infants are not metabolically homogeneous and show a considerably variation in metabolic state. Grouping infants according to metabotypes may offer opportunities for developing more targeted and personalized intervention strategies.

Acknowledgments: We are grateful to Wolfgang Peissner and Ulrike Rulands (Division of Metabolic and Nutritional Medicine, Dr von Hauner Children's Hospital, University of Munich, Munich, Germany), who analyzed the plasma.

REFERENCES

1. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* 1992;35:595–601.
2. Osmond C, Barker DJ, Winter PD, et al. Early growth and death from cardiovascular disease in women. *BMJ* 1993;307:1519–24.
3. Schellong K, Schulz S, Harder T, et al. Birth weight and long-term overweight risk: systematic review and a meta-analysis including 643,902 persons from 66 studies and 26 countries globally. *PLoS One* 2012;7:e47776.
4. Agosti M, Tandoi F, Morlacchi L, et al. Nutritional and metabolic programming during the first thousand days of life. *Pediatr Med Chir* 2017;39:157.
5. Feinberg AP. The key role of epigenetics in human disease prevention and mitigation. *N Engl J Med* 2018;378:1323–34.
6. Nicholson JK. Global systems biology, personalized medicine and molecular epidemiology. *Mol Syst Biol* 2006;2:52.
7. Gavaghan CL, Holmes E, Lenz E, et al. An NMR-based metabonomic approach to investigate the biochemical consequences of genetic strain differences: application to the C57BL10J and Alpk: ApfCD mouse. *FEBS Lett* 2000;484:169–74.
8. Brennan L. 7 - Metabotyping: moving towards personalised nutrition. In: Sébédio J-L, Brennan L, eds. *Metabolomics as a Tool in Nutrition Research.* Cambridge, United Kingdom: Woodhead Publishing; 2015:137–144.
9. O'Donovan CB, Walsh MC, Nugent AP, et al. Use of metabotyping for the delivery of personalised nutrition. *Mol Nutr Food Res* 2015;59:377–85.
10. O'Sullivan A, Gibney MJ, Connor AO, et al. Biochemical and metabolomic phenotyping in the identification of a vitamin D responsive metabotype for markers of the metabolic syndrome. *Mol Nutr Food Res* 2011;55:679–90.
11. Arenz S, Ruckerl R, Koletzko B, et al. Breast-feeding and childhood obesity—a systematic review. *Int J Obes Relat Metab Disord* 2004;28:1247–56.
12. Kirchberg FF, Harder U, Weber M, et al. Dietary protein intake affects amino acid and acylcarnitine metabolism in infants aged 6 months. *J Clin Endocrinol Metab* 2015;100:149–58.
13. Koletzko B, von Kries R, Closa R, et al. Lower protein in infant formula is associated with lower weight up to age 2 y: a randomized clinical trial. *Am J Clin Nutr* 2009;89:1836–45.
14. Voigt M, Rochow N, Schneider KTM, et al. New percentiles for single pregnancy newborn anthropometry: results of the German Perinatal Survey in 2007-2011 with participation of all 16 German states. *Z Geburtshilfe Neonatol* 2014;218:210–7.
15. Peneau S, Rouchaud A, Rolland-Cachera MF, et al. Body size and growth from birth to 2 years and risk of overweight at 7-9 years. *Int J Pediatr Obes* 2011;6:e162–9.

16. Cole TJ, Bellizzi MC, Flegal KM, et al. Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ* 2000;320:1240–3.
17. de Onis M, Onyango AW, Borghi E, et al. Development of a WHO growth reference for school-aged children and adolescents. *Bull World Health Organ* 2007;85:660–7.
18. de Onis M, Onyango AW, Van den Broeck J, et al. Measurement and standardization protocols for anthropometry used in the construction of a new international growth reference. *Food Nutr Bull* 2004; 25(1 suppl):S27–36.
19. Weber M, Grote V, Closa-Monasterolo R, et al. Lower protein content in infant formula reduces BMI and obesity risk at school age: follow-up of a randomized trial. *Am J Clin Nutr* 2014;99:1041–51.
20. Harder U, Koletzko B, Peissner W. Quantification of 22 plasma amino acids combining derivatization and ion-pair LC-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci* 2011;879:495–504.
21. Socha P, Grote V, Gruszfeld D, et al. Milk protein intake, the metabolic-endocrine response, and growth in infancy: data from a randomized clinical trial. *Am J Clin Nutr* 2011;94(6 suppl):1776S–S1784.
22. Subbaiah PV, Liu M. Role of sphingomyelin in the regulation of cholesterol esterification in the plasma lipoproteins. Inhibition of lecithin-cholesterol acyltransferase reaction. *J Biol Chem* 1993;268: 20156–63.
23. Aoki J, Taira A, Takanezawa Y, et al. Serum lysophosphatidic acid is produced through diverse phospholipase pathways. *J Biol Chem* 2002;277:48737–44.
24. Eggert ML, Wallaschofski H, Grotevendt A, et al. Cross-sectional and longitudinal relation of IGF1 and IGF-binding protein 3 with lipid metabolism. *Eur J Endocrinol* 2014;171:9–19.
25. Aguirre GA, De Ita JR, de la Garza RG, et al. Insulin-like growth factor-1 deficiency and metabolic syndrome. *J Transl Med* 2016;14:3.
26. Godfrey KM, Barker DJ. Fetal nutrition and adult disease. *Am J Clin Nutr* 2000;71(5 suppl):S1344–52.
27. Barker DJ. Fetal origins of coronary heart disease. *BMJ* 1995;311:171–4.
28. Leger J, Levy-Marchal C, Bloch J, et al. Reduced final height and indications for insulin resistance in 20 year olds born small for gestational age: regional cohort study. *BMJ* 1997;315:341–7.
29. Eide MG, Oyen N, Skjaerven R, et al. Size at birth and gestational age as predictors of adult height and weight. *Epidemiology* 2005;16:175–81.
30. Araujo CL, Hallal PC, Nader GA, et al. Effect of birth size and proportionality on BMI and skinfold thickness in early adolescence: prospective birth cohort study. *Eur J Clin Nutr* 2009;63:634–9.
31. Lundgren EM, Cnattingius S, Jonsson B, et al. Prediction of adult height and risk of overweight in females born small-for-gestational-age. *Paediatr Perinat Epidemiol* 2003;17:156–63.
32. Novotny R, Vijayadeva V, Grove J, et al. Birth size and later central obesity among adolescent girls of Asian, White, and Mixed ethnicities. *Hawaii J Med Public Health* 2013;72:50–5.
33. Pietilainen KH, Kaprio J, Rasanen M, et al. Tracking of body size from birth to late adolescence: contributions of birth length, birth weight, duration of gestation, parents' body size, and twinship. *Am J Epidemiol* 2001;154:21–9.
34. Hoppe C, Udam TR, Lauritzen L, et al. Animal protein intake, serum insulin-like growth factor I, and growth in healthy 2.5-y-old Danish children. *Am J Clin Nutr* 2004;80:447–52.
35. Grohmann M, Sabin M, Holly J, et al. Characterization of differentiated subcutaneous and visceral adipose tissue from children: the influences of TNF-alpha and IGF-I. *J Lipid Res* 2005;46:93–103.
36. Oh Y, Müller HL, Lee DY, et al. Characterization of the affinities of insulin-like growth factor (IGF)-binding proteins 1-4 for IGF-I, IGF-II, IGF-I/insulin hybrid, and IGF-I analogs. *Endocrinology* 1993;132: 1337–44.
37. OECD Infant health: Low birth weight. OECD Publishing; 2012.
38. OECD Health at a Glance 2003. OECD Publishing; 2003.
39. Damianidi L, Gruszfeld D, Verduci E, et al. Protein intakes and their nutritional sources during the first 2 years of life: secondary data evaluation from the European Childhood Obesity Project. *Eur J Clin Nutr* 2016;70:1291–7.
40. Villar J, Papageorghiou AT, Pang R, et al. The likeness of fetal growth and newborn size across non-isolated populations in the INTERGROWTH-21st Project: the Fetal Growth Longitudinal Study and Newborn Cross-Sectional Study. *Lancet Diabetes Endocrinol* 2014;2:781–92.
41. Nia VP, Davison AC. High-dimensional Bayesian clustering with variable selection. *The R Package bclust* 2012;47:22.
42. Steinbach M, Ertöz L, Kumar V. The challenges of clustering high dimensional data. In: Wille LT, ed. *New Directions in Statistical Physics: Econophysics, Bioinformatics, and Pattern Recognition.* Berlin, Heidelberg: Springer; 2004:273–309.
43. Bellman R. Adaptive Control Processes—A Guided Tour.Princeton University Press; 1961.
44. Struyf A, Hubert M, Rousseeuw P. Clustering in an object-oriented environment. *J Stat Softw* 1997;001:1–30.
45. Rousseeuw PJ. Silhouettes: A graphical aid to the interpretation and validation of cluster analysis. *J Comput Appl Mathematics* 1987;20:53–65.
46. Hennig C. Package 'fpc'. CRAN; 2015.
47. Schiess S, Grote V, Scaglioni S, et al. Introduction of complementary feeding in 5 European countries. *J Pediatr Gastroenterol Nutr* 2010;50:92–8.