

ORIGINAL ARTICLE

Food Allergy & Anaphylaxis

Immune components of early breastmilk: Association with maternal factors and with reported food allergy in childhood

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Abstract

Background: Breastmilk (BM) may participate in driving gut barrier function and immunity in the neonate. We analyzed immune and growth factor concentrations in early BM and their association with maternal/environmental characteristics and with food allergy (FA) in childhood.

Methods: One BM sample was collected in maternity from some mothers in the EDEN birth cohort ($n = 2002$ mother-child dyads). A random selection was performed among available samples (subcohort, $n = 272$), for which all deliveries were full-term, various maternal/environmental characteristics were recorded, and parents answered yearly the question "Has a medical doctor diagnosed a FA in your child?" (26 parent-reported FA cases). Only samples collected between day 2 and day 6 post-partum were considered for descriptive analysis ($n = 263$). Samples for all other FA cases available were added to the subcohort (46 additional cases; "casecohort" design). Fifty cytokines, antibodies, and growth factor concentrations were determined using multiplexed kits and analyzed using robust statistical procedures.

Results: BM components exhibited wide concentration ranges and global day-to-day variation. Different clusters of correlated factors appeared, with components from the main cluster related to maternal diet during pregnancy. Primiparity was positively associated with eleven other components, whereas other factors (eg, maternal atopy and smoking) were related to fewer components. Finally, the casecohort design highlighted a positive association between CXCL10, TNF β , and IL-2 concentrations and reported FA in childhood.

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Conclusion: Beyond the unique description of early BM composition, we show that immune information transmitted to the neonate is related to various maternal factors and identified components associated with FA diagnosis in childhood.

KEYWORDS

early breastmilk, EDEN mother-child cohort, food allergy, growth factors, immune factors

1 | INTRODUCTION

Breastfeeding has health benefits in various pathophysiologic conditions (eg, diarrhea episodes, respiratory infection, or malocclusion in children), but its effect on allergy prevention is still controversial.^{1,2} This may be explained by heterogeneity in breastfeeding duration, but also by interindividual variations in breastmilk (BM) composition. In this context, BM content in immunomodulatory components may be critical, notably during the neonatal period. Early BM allows the transfer of immunomodulatory nutrients, various growth and angiogenic factors, and immune factors such as immune cells, antibodies, and cytokines.³⁻¹⁰ All these components will modulate the growth, differentiation, maturation, function, and angiogenesis of the neonate organs, notably of the gastrointestinal tract and associated immune system. Immaturity of the neonatal digestive tract and the presence of antiproteases in BM may reduce the degradation of all these factors and allow their absorption by the neonate in an active form, notably in the first days of life when permeability is increased.^{11,12} Early BM may then be considered more as providing immune and trophic stimuli than as a nutritional source.¹³

Despite the potential role of early BM in neonate immunity and intestinal homeostasis, there are currently very few studies analyzing its composition in immune and growth factors, with only a limited number of components analyzed.^{10,14-21} The impact of the concentrations of early BM components on child atopy or allergy has also been poorly analyzed.^{10,22} Moreover, all these studies provide often conflicting results due to small sample size and to differences in the analytical tools used, in the day of collection considered and the way of sampling (foremilk vs hindmilk), and in the population analyzed (reviewed in Ref. ²³).

Our aims were then (a) to provide the deepest and broadest characterization to date of immune and growth factor concentrations in early BM and to assess their day-to-day variations and their correlations in a non-selected population, (b) to analyze the association between their concentrations and various maternal and environmental factors, and (c) to analyze their potential correlation with the offspring's health problems, focusing on the development of food allergy in childhood.

2 | MATERIALS AND METHODS

2.1 | Study population

The EDEN mother-child study is a prospective cohort for investigation of the pre- and post-natal determinants of child growth,

development, and health. The study is described in detail elsewhere.²³ In brief, 2002 pregnant women were recruited in two French university hospitals (Poitiers and Nancy) and the children's health was followed up for 5 years using yearly questionnaires. Data collected during pregnancy and at birth include socio-demographic variables, maternal dietary pattern during the last trimester of pregnancy, maternal smoking, gestational diabetes, parental anthropometric measurements, and newborn characteristics (gender, gestational age, birthweight).²⁴ Mothers were defined as atopic if they reported at least one medical diagnosis of allergic disease (asthma, eczema, allergic rhinitis, food allergy). Among other biologic samples collected, one BM sample was collected per mother during the maternity stay.

Maternal dietary patterns were previously identified by principal component analysis.²⁵ For each participant, a score was calculated for each identified pattern, a higher score indicating a higher adherence of the women to this pattern. By construction, scores are independent and standardized. In the present analysis, we considered the "Healthy pattern," characterized by high positive loading for vegetables, high-fat dairy products, pods and peas, fish and whole grain cereals, and the "Western pattern," characterized by high positive loading for snacks, cakes, processed meat, French fries, soda, sandwich, and chocolate.

"FA cases" in children correspond to doctor-diagnosed FA reported by the parents at least at one yearly follow-up within the first 5 years of life. In line with a recent cohort-based study,²⁶ FA cases were defined by a positive answer to the question "Has a medical doctor diagnosed a food allergy in your child?" Food-specific IgE measurement or food challenge was not included in the protocol. Corresponding data were censored by age at the last follow-up.

2.2 | Subcohort and casecohort design

2.2.1 | Subcohort

We first randomly selected 150 mother-child dyads (full-term) from each center (ie, Nancy and Poitiers) for which BM samples were initially collected. Because of BM samples still available, 139 samples from Poitiers and 133 samples from Nancy could be included. In this initial subcohort ($n = 272$), 26 children (9.5%) had a parent-reported diagnosis of FA. Randomized selection of early BM consisted of 3, 14, 112, 85, 40, 12, and 6 samples collected on days 1 (day of birth), 2, 3, 4, 5, 6, and days >6 (from days 8 to 63), respectively. Due to the low number of samples collected on day 1 and to the low number

and heterogeneity in collection dates after day 6, we excluded the corresponding samples from descriptive analyses (no FA cases). The subcohort used to describe the characterization of immune and growth factor concentrations in early BM contained 263 BM samples collected from day 2 to day 6.

2.2.2 | Casecohort design

All the other FA cases identified in the whole cohort, for which BM samples were available, were added to the subcohort. Forty-six additional FA cases were therefore added. More additional cases were from Nancy, due to the larger number of samples collected and still available in this center. The casecohort then contains 318 samples and includes 72 FA-reported cases.

Description of the mother and environmental and birth characteristics of the subcohort and casecohort is provided in Table S1.

2.3 | BM collection

The collection was performed by manual expression after feeding the newborn (hindmilk), at any time of day and anytime during the maternity stay, following a standardized protocol. The collection day was categorized as day 1 (day of birth) to days >6. Samples were collected in sterile tubes and kept at +4°C until their transfer to the laboratory where they were aliquoted and transferred to -80°C. The time between collection and freezing was recorded for all samples. Sixty percent of the samples were frozen within 24 hours, the third quartile for sample freezing at -80°C was 42 hours, but the maximal delay observed was 141 hours. However, no association between component concentrations and time between sampling and freezing was observed for all the measured factors (not shown).

2.4 | Sample pre-treatment for analysis

After thawing, samples were centrifuged (2000 x g, 20 minutes, +4°C). The clear defatted fraction was collected and immediately analyzed. In order to control the recovery and to assess the dilution to be used in the different assays, preliminary experiments were performed using a pool of colostrum from 3 mothers obtained commercially (CliniSciences, Nanterre, France). All samples from the cohort were then analyzed randomly and blindly.

2.5 | Cytokine and IgG assays

We selected BM components based on their potential role as growth factor and/or immune stimulating/regulating activity in the neonate. Chemokines (eotaxin (CCL11), fractalkine (CX3CL1), GRO (CXCL1), IL-8 (CXCL8), IP-10 (CXCL10), MCP-3 (CCL7), MDC (CCL22), MCP-1 (CCL2), MIP-1 α (CCL3), MIP-1 β (CCL4), RANTES (CCL5)), interleukins (IFN α 2, IFN γ , IL-10, IL-12p40, IL-12p70, IL-15, IL-17A, IL-1RA, IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-13, sCD40L, TNF α , TNF β), and growth factors (EGF, FGF-2, G-CSF, Flt3L, GM-CSF, PDGF-AA, PDGF-BB, TGF α , VEGF) were assayed using the Milliplex® MAP

panel kit from Merck Millipore (Human CytoMAG Premix 41-plex mix; Saint-Quentin-en-Yvelines, France). Samples were diluted 1:2 in the assay buffer provided, except for CCL2, EGF, CXCL1, and CXCL10 (dilution 1:100), which were analyzed separately. Minimum detectable concentrations ranged from 0.26 to 10 pg/mL for almost all components, with higher values only for CX3CL1 (22.7 pg/mL) and VEGF (26.3 pg/mL). Standards and quality controls (QC) provided and pooled commercial colostrum were included on each assay plate.

TGF β 1, TGF β 2, and TGF β 3 were assayed using the Bio-Plex Pro™ kit, following the supplier's recommendations (Bio-Rad, Marnes la Coquette, France). Latent TGF β s were first activated by acidic pre-treatment of samples (HCl 1 N, 10 minutes), before neutralization (1.2 N sodium hydroxide/0.5 M HEPES).

Total antibodies (IgM, IgA, IgG1, IgG2, IgG3, and IgG4) were assayed using commercial kits (Bio-Plex Pro™ Human Isotyping Panel assay, Bio-Rad) following the supplier's recommendations.

Data acquisitions were performed using Bio-Plex 200® apparatus and analyses using associated software (Bio-Plex Manager) (Bio-Rad). For CCL3, IL-5, IL-9, IL-1 β , and IL-3, signals of some samples were above the signals obtained with buffer, but below that obtained with the lowest standard point: Corresponding concentrations were extrapolated (the numbers of samples are specified in the corresponding tables).

2.6 | Statistical analysis

Descriptive statistics and associations of early BM components with maternal characteristics were conducted on the subcohort (263 non-selected samples). Associations between immune and growth factor concentrations and incidence of food allergy were estimated thanks to the casecohort design (ie, with additional FA cases, $n = 318$ samples including 72 FA-reported cases). All statistics procedures, including corrections for multiple testing,^{27,28} are described in details in online repository file.

3 | RESULTS

3.1 | Concentrations of the immune and growth components in early breastmilk

We first classified growth and immune components depending on the range of measured concentrations. As summarized in Table 1, the concentrations were the highest for antibodies, chemokines CXCL1, CXCL10, and CCL2, and the growth factor EGF. Other growth factors and chemokines and all the IL were found at lower concentrations (<50 pg/mL for most of the IL). We provide concentrations of all the BM components at each day of collection in supplementary files, depending on their physiologic role: antibodies (Table S2), growth factors (Table S3), chemokines (Table S4), T helper-related or regulatory cytokines (Table S5), and proinflammatory/proliferation cytokines (Table S6). Depending on the sampling date, 50% to 75% and 50% to 58% of the samples did not show detectable levels of

Concentration range	Factors
0.1-1 µg/mL	IgA and CXCL1
10-100 ng/mL	IgM, EGF, CXCL10, IgG2, CCL2
1-10 ng/mL	IgG1, TGF-β1, TGF-β2, CX3CL1, CXCL8, CCL22
50-1000 pg/mL	VEGF, IgG4, IgG3, TGF-β3, PDGF-AA, TGFα, IL-15, PDGF-BB, IL-1RA, CCL5, CCL11, sCD40L
1-50 pg/mL	CCL4, CCL7, FGF-2, IL-7, IL-6, CCL3, IFNα2, G-CSF, IL-1α, TNFα, IL-12p40, Flt3L, IL-5, IL-9, IL-10, IL-4, IFNγ, IL-1β, IL-13, GM-CSF, IL-17A, IL-12p70, IL-2, TNFβ, and IL-3

TABLE 1 Range of concentrations of the components analyzed in breastmilk collected from day 2 to day 6

CCL3 and IL-3, respectively. IL-12p70, IL-4, IL-5, and TNFβ were not detectable in a few samples (up to 5 out of 263), and IL-9 was not detectable in 16 samples. All the other components were detectable in all the samples.

3.2 | Overall day-to-day variations of the concentrations of the immune components and growth factors in early BM

Supervised multivariate statistical analysis evidenced a progressive change in the global concentrations of components in BM during the first days of life (Figure 1). TGFα, TGF-β1, TGF-β2, VEGF, CCL5, CCL11, IL-7, and sCD40L were the components exhibiting the strongest contribution to the model (Variable Importance in the Projection obtained from the PLS-DA model

(VIP) > 1), that is, for which concentrations changed over time. Additional comparisons were performed for each component, confirming the impact of the day of collection on the concentrations of TGFα, TGFβ1, TGFβ2, VEGF, CCL5, CCL11, and IL-7, and further evidencing significant day-to-day variations for IL-15 and IL-17 (Tables S2-S6). Further analysis, integrating correction for multiple testing, showed that TGFβ1 (Figure 2A) and VEGF (Figure 2B) concentrations globally increased over time, whereas TGFα (Figure 2C) concentrations showed opposite variations. CCL5 was the chemokine the most affected over time, globally decreasing as early as day 3 (Figure 2D). The only affected interleukin was IL-15, which slightly decreased over time (Figure 2E). In order to consider all the samples independently of their sampling date in the following analyses, we performed data adjustment on the day of collection.

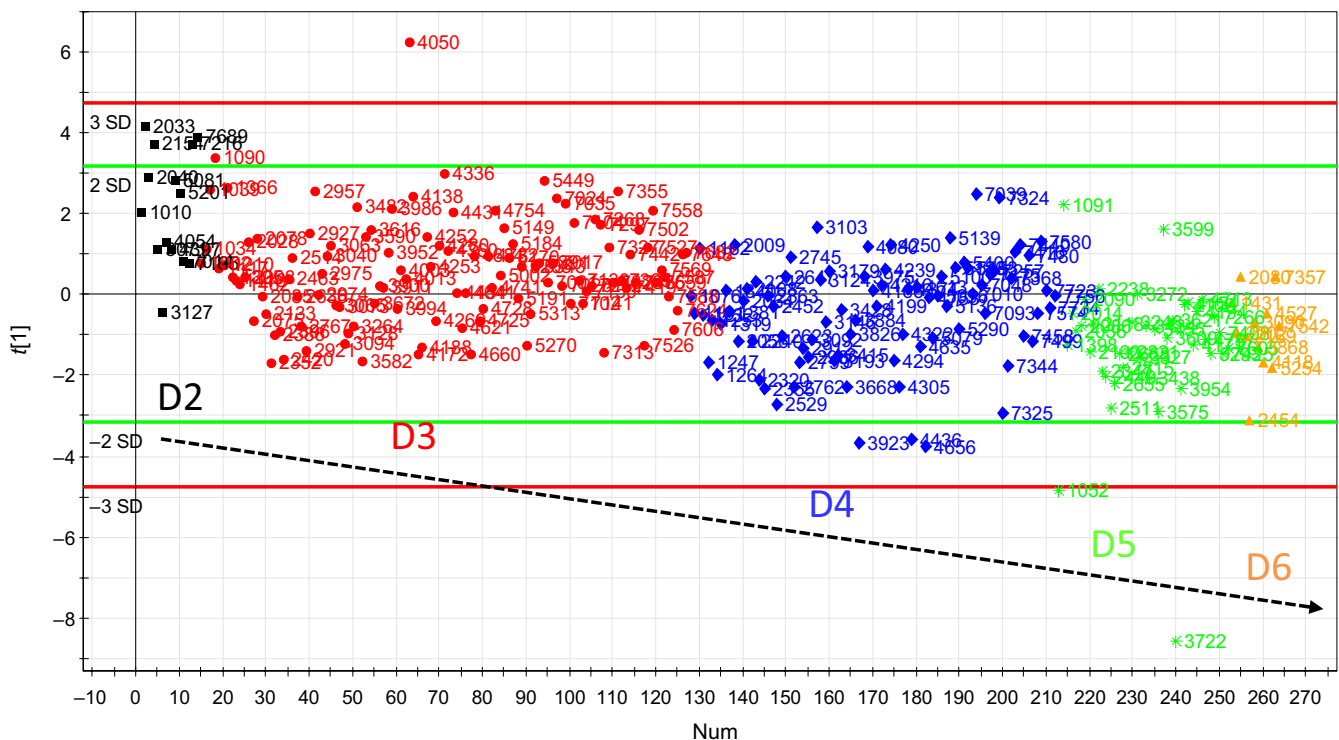


FIGURE 1 PLS-DA analysis of the samples based on the day of collection. A supervised analysis of the weighted centering data set was performed using PLS-DA, where dependent (Y) variable—that is, the variable used for classification, corresponds to the sampling date. Corresponding days of collection are represented using a color code. A total of 100 permutations were performed for model validation

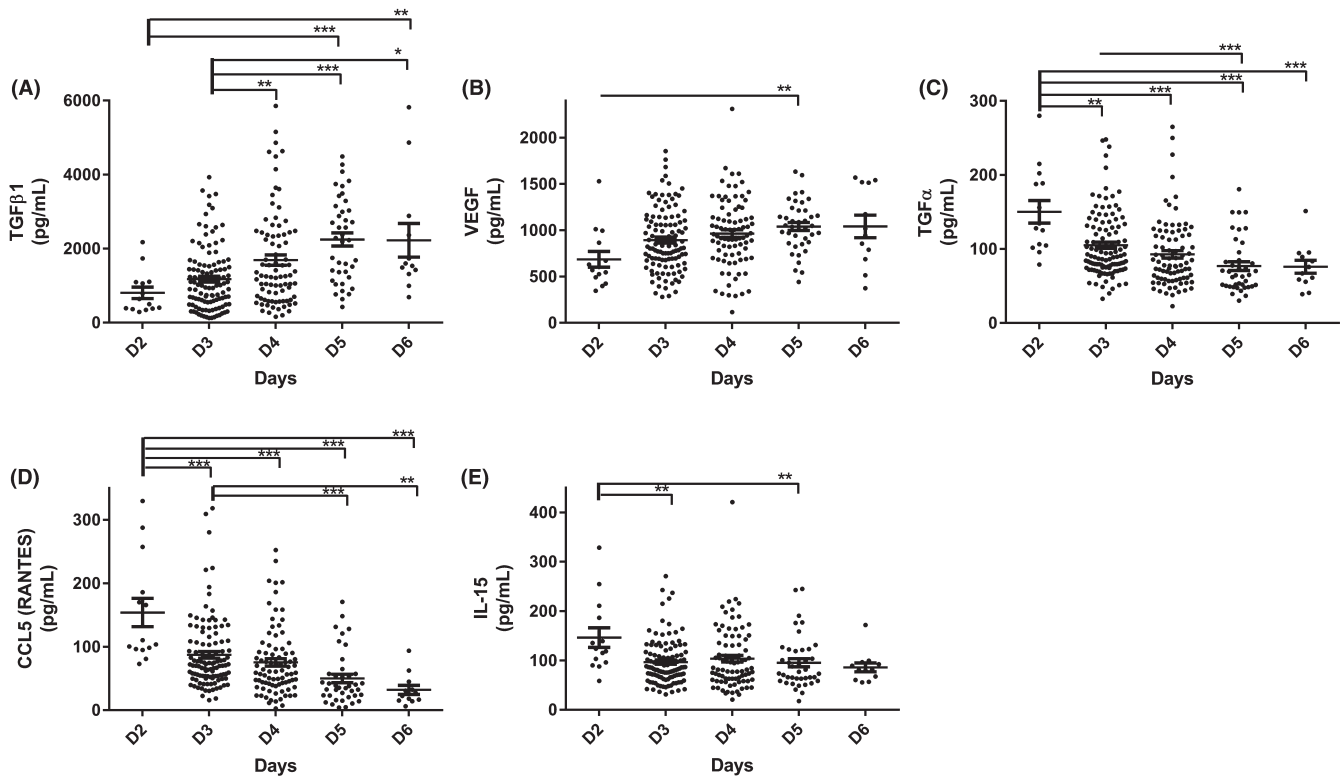


FIGURE 2 TGFβ1 (A), VEGF (B), TGFα (C), CCL5 (RANTES, D), and IL-15 (E) concentrations in breastmilk samples collected from day 2 to day 6. Concentrations are expressed in pg/mL. Each point represents an individual BM sample, and mean ± SEM is indicated for each sampling day. Bars indicate significant differences between specified sampling dates, using least square means and adjustment for multiple comparison test (Bonferroni test): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

3.3 | Clustering of the different growth and immune components of early BM

A hierarchical cluster analysis of all the components was first performed to analyze their correlations (Figure 3). A first main cluster of positively associated components was found, containing IFNα2, Flt3L, IL-12p40, GM-CSF, FGF-2, IL-12p70, sCD40L, IL-3, IL-2, and IL-4, and to a lesser extent L-1RA (cluster 1). Other components were positively associated, as seen in cluster 2 (CCL7, IgG1, IL-13, and IL-5 that also correlate with IL-2, CCL22, and PDGF-BB), cluster 3 (TGFβ1, TGFβ2, and to a lesser extent CCL11), cluster 4 (IL-1β, G-CSF, and IL-6), and cluster 5 (CCL4, CCL3, TNFα, and IL-10). There was also strong correlation between specific components, such as IgG2 and IgM. Conversely, VEGF was negatively associated with numerous components, notably IL-15, TGFα, CCL5, and IgA. IgA and TGFβ1-2 were also negatively associated.

3.4 | Association between growth/immune components in early BM and maternal, environmental, or birth-associated characteristics

Multiple linear regressions revealed relations between the concentrations of some early BM components and parameters such as maternal atopy, age at first pregnancy, BMI before pregnancy,

smoking during pregnancy, place of residence (Poitiers or Nancy), C-section, educational level, and primiparity (Table 2). However, these relations were far less numerous after applying a false discovery rate (FDR)-controlling procedure that takes into account multiple testing.²⁸ For example, the positive association between maternal atopy and concentrations of EGF and IL-4 was no longer significant after the FDR procedure. The most related factor was primiparity, which was associated with higher concentrations of all IgG isotypes and IgM, growth factors Flt3L and TGFα, chemokines CXCL8 and CX3CL1, Th1 cytokine IL-12p40, and proinflammatory cytokine IFNα2. The less related factors were a high BMI before pregnancy (Flt-3L), smoking during pregnancy (IgG2, IgM), and age at first pregnancy (TGFβ1, TGFβ2).

The association between maternal diet ("healthy" vs "Western") during the last trimester of pregnancy and concentrations of early BM components was also assessed. Selected data from the sub-cohort ($n = 237$ out of 263; Table S1) were considered, corresponding to data for which maternal dietary scores were available. Concentrations of some immune and growth factors were associated with the dietary pattern, but this was no longer significant after the FDR procedure (Table 3). We then performed further analyses using only samples from Poitiers where significantly more pronounced differences in dietary patterns were seen. The concentrations of 8 factors were then shown to be negatively

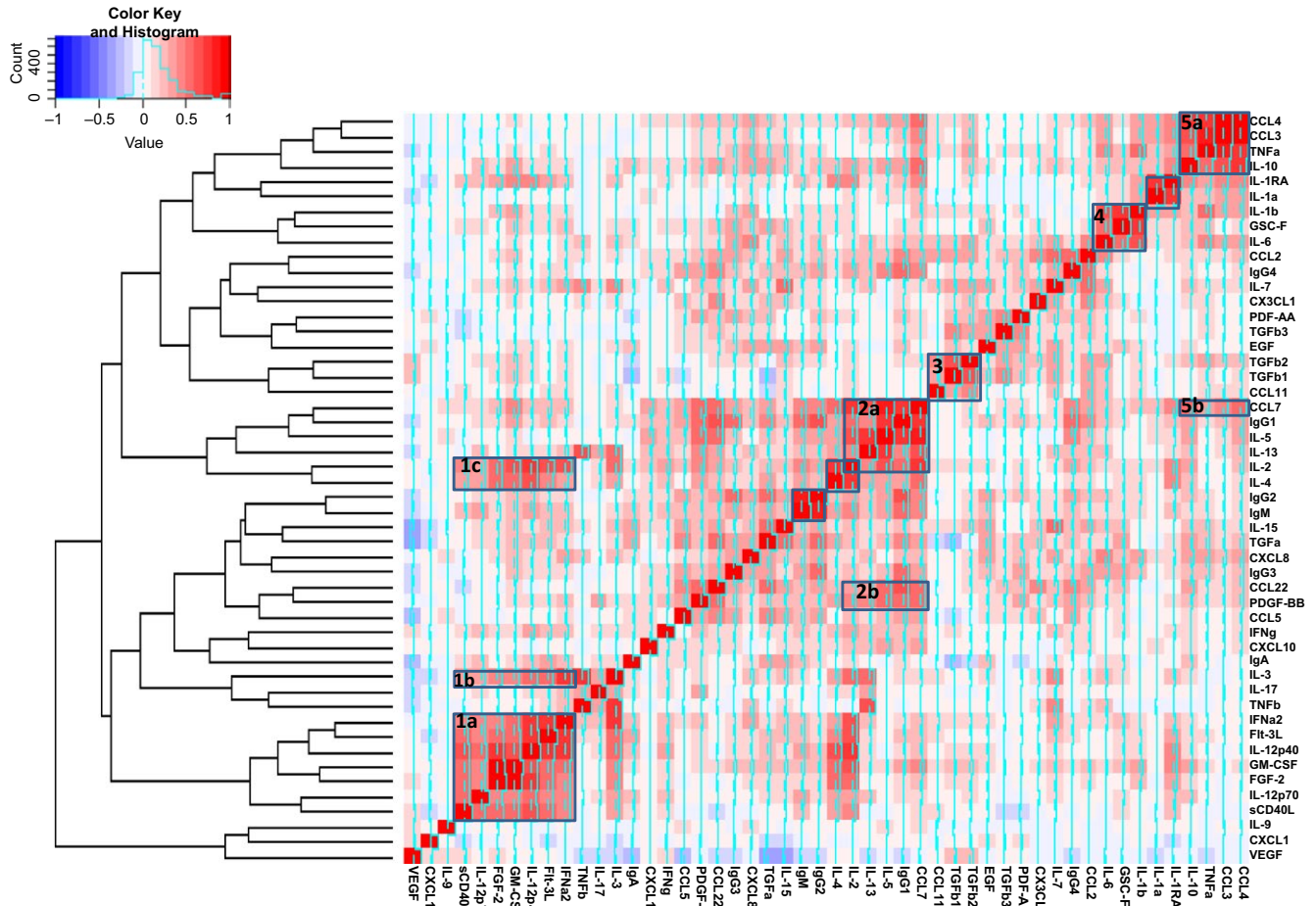


FIGURE 3 Correlation heatmap and cluster dendrogram for concentrations of the 50 components in early BM. Automatic hierarchical clustering was performed using Heatmap.2 algorithm (ggplot2 package from R) without modification of R script and with optimized inertia break between classes. Clusters were then defined visually from the heatmap generated. Heatmap of the distance matrix is shown with the color code corresponding to Spearman correlation values. Color coding corresponds to values between -1 (blue) and $+1$ (red), corresponding to negative and positive correlation, respectively

associated with the Western dietary pattern even after the FDR-controlling procedure (FGF-2, Flt3-L, IL-12p40, IL-12p70, sCD40L, IFN α 2, IL-3, IL-1RA, TNF β), all of them except one (TNF β) belonging to cluster 1.

Inversely, the most affected components in early BM were Flt-3L (high BMI, primiparity, diet), IgG2 (place of residence, smoking, primiparity), and IFN α 2 (living in Nancy, primiparity, diet).

3.5 | Associations between growth/immune component concentrations in early BM and food allergy in early childhood

Finally, thanks to the casecohort design, higher concentrations of G-CSF, IL 12p40, IL-1 β , CXCL10, TNF β , and IL-2 in early BM were first associated with parental reports of doctor-diagnosed FA, but this was still significant only for CXCL10, TNF β , and IL-2 after the FDR correction (Table 4). The association with CXCL10 was significant only among children with no maternal history of allergy (P for interaction = 0.0009).

4 | DISCUSSION

Up to 50 immune and growth factor components were detected in almost all the 263 early BM samples analyzed. Early BM should then be considered as a strong provider of immune and trophic stimuli. Data for around 30 of the factors analyzed were not available to date, and our study provides new reference values. Data obtained for other factors were globally in agreement with published data obtained in colostrum,^{8,16,19,20,29,30} although some discrepancies were observed, for example, for TGF β , IgG4, and CCL2 concentrations.^{15,20,29} These differences may stem from variations in methodologies and standards provided by different manufacturers that are not normalized using an international reference.

Besides providing the widest quantitative analysis of cytokines, growth factors, and antibodies in early BM, and although samples were not collected at different time points from the same mothers, the sample size was large enough to provide evidence of a global variation of immune and growth factor concentrations in BM in the first days of life. However, we observed that this relies on few components,

TABLE 2 Maternal, environmental, and birth characteristics significantly related to immune and growth factor concentrations in breastmilk collected between days 2 and 6

Characteristics	Condition	Impacted immune or growth factors	
		Positive association	Negative association
Maternal atopy		EGF* IL-4*	
BMI before pregnancy (ref=normal weight 18.5 < x < 25 kg/m ²)	x < 18.5 kg/m ² (underweight)	IgG1** IL-5*	
	25 < x < 30 kg/m ² (overweight)	Flt-3L*** IgM* IL-12p70*, IFN-α2*	
	x > 30 kg/m ² (obesity)	Flt-3L*** IgM* CXCL8*	
Place of residence ^a (recruitment center)	Nancy	IgA *** Flt-3L* IFNα2***IL-15**	
	Poitiers	IgG2*** PDGF-AA***	
C-section		CCL2* CCL22*	
Age at first pregnancy (by scale of 5 years)		TGF-β1** , TGF-β2** CCL2** IL-15*	IFNγ*
Smoking during pregnancy (ref=no smoking)			IgG2*** , IgM***
Educational level (ref=maternal high educational level)		CCL7* IL-17A*, IL-1β*, TNF-α*	IgG2*
Primiparity (ref=multiparity)		IgG1** , IgG2*** , IgG3** , IgG4*** , IgM** Flt-3L*** , TGFα** , PDGF-AA* CXCL8** , CX3CL1** IL-12p40** , IFN-α2* , IL-2*	

All maternal factors were considered simultaneously in a multiple linear regression for each immune or growth factor, previously adjusted for sampling day by the residual method. Positive correlation: higher concentrations associated with the factor analyzed. Factors impacted by the parameters considered were first identified using multiple linear regression testing *: 0.01 < P < 0.05, **: 0.001 < P < 0.01, ***P < 0.001. The factors still significant after the false discovery rate procedure (q-value cutoff of 0.1) are shown in bold characters.

No relation was observed with the child's characteristics such as gender or with the mother's educational level.

^aPoitiers is considered as a more rural region than Nancy: in 2009, the population density of Poitiers vs Nancy regions was 68 vs 100 people/km², and the utilized agricultural area was 1736 vs 1135 thousands of hectares, respectively (French National Institute of Statistics and Economic Studies (Insee); Agricultural land use: regional comparisons. https://www.insee.fr/fr/themes/tableau.asp?reg_id=99&ref_id=t_3603 R (Accessed 2 October 2012, Document in French).

TABLE 3 Impact of maternal dietary habits during the last trimester of pregnancy on BM component concentrations

	Selected subcohort (n = 237)		Poitiers (n = 122)	
	Positive association	Negative association	Positive association	Negative association
"Healthy" dietary pattern	IL-4* IL-10*		VEGF**	
"Western" dietary pattern		sCD40L*, IL1RA* IFNα2*	CCL22*	FGF-2** , Flt3-L** IL-12p40**, IL-12p70*, IL-4*, sCD40L*** , IL-1RA** , IFNα2*** , TNFβ** , IL-2*, IL-3**

Selected data from the subcohort (n = 115 from Nancy and n = 122 from Poitiers), for which maternal dietary practices were available, were used in a multiple linear regression model analyzing each immune or growth factor, previously adjusted for sampling day by the residual method. Positive correlation: higher concentrations associated with the analyzed factor. Components impacted by the parameters considered were first identified using multiple linear regression testing *: 0.01 < P < 0.05, **: 0.001 < P < 0.01, ***P < 0.001. The component concentrations still significantly affected after the false discovery rate procedure (P value cutoff of 0.1) are shown in bold characters.

TABLE 4 Immune and growth components positively related to higher risk of food allergy in early childhood

	P value before FDR	P value after FDR	HR [95% CI]
G-CSF (/10)	0.0109	0.1176	1.07 [1.02;1.12]
IL-12p40 (/10)	0.0245	0.2039	1.08 [1.01;1.15]
IL-1 β	0.0118	0.1176	1.01 [1.00;1.02]
CXCL10 (IP10) (/10000)	0.0000	0.0001	1.02 [1.01;1.04]
TNFβ	0.0003	0.0005	1.13 [1.06;1.21]
IL-2	0.0000	0.0008	1.06 [1.03;1.09]

Prentice-weighted Cox regressions, adjusted for maternal atopy, sibling atopy (no sibling, siblings without atopy, at least one sibling with atopy), maternal age, BMI before pregnancy, center, C-section, smoking, educational level, and child gender.

The factors still significant after the false discovery rate procedure (q-value cutoff of 0.1) are shown in bold characters.

suggesting that the passage from colostrum to transition milk (days 3-4) impacted the immune composition of BM only modestly.

However, we performed data adjustment that allowed integration of all samples into further analysis, independently of these temporal variations. We analyzed the inter-relationships between concentrations of early BM components and found several clusters of positively or negatively correlated components. Some of them were quite surprising, for example, the negative correlation between IgA and TGF β 1/2, which could reflect a compensation phenomenon to speed up IgA production in neonates when low levels of maternal IgA are provided. We also assessed the association of some components with maternal, environmental, or birth-associated parameters (Table 2). We clearly observed that the concentrations of components from the core cluster 1 were negatively associated with a "Western" dietary pattern during the last trimester of pregnancy. Immune information transmitted to the neonate is then associated with the eating habits of the mother and probably more globally with her associated lifestyle. Altogether, this may partially explain the impact of a Western lifestyle on overall health, participating in maladaptation of individuals to their environment from early life and predisposing them to "modern" diseases. Primiparity was also positively related to numerous component concentrations in BM. This can be placed in the context of the birth-order effect on disease risk. As an example, primiparity was associated with a higher cow's milk allergy prevalence in a case-control study nested in the Finnish population.³¹ This birth-order effect may rely on different in utero programming of the child's immune system, as evidenced by a reduced anti-inflammatory profile of cord blood T cells from first-born infants following polyclonal activation.³² In continuity with this observation, we now suggest that early BM composition in growth and immune components would not be the same for the first-born and for her/his subsequent brothers/sisters, which should be further confirmed in samples obtained from the same brothers and sisters.

Some studies show an association of maternal atopy with concentrations of IL-4,¹⁶ EGF,¹⁴ CXCL8, and CCL5³⁰ or IgA³³ in early BM.

Although our first analysis showed a positive association of maternal atopy with IL-4 and EGF concentrations, these associations were no longer significant after FDR correction, thus confirming results obtained for IL-4 in other studies.^{22,34} We also confirmed the absence of an association between maternal atopy and IgA^{8,10,35} or TGF β ^{8,21,22,34} concentrations. As observed when comparing colostrum from France, Brazil, and Australia,³⁶ but in contrast to the data obtained on colostrum from UK, Russia, and Italy²⁰ or from farm vs non-farm mothers,¹⁷ we did not find any association between TGF β concentrations and place of residence (Poitiers being considered more rural than Nancy). Finally, we did not confirm the association between TNF α and IL-6 and C-section,³⁷ nor the lower IL-1 β concentrations in smoking mothers.¹⁹

At birth, immune immaturity and dysregulation may favor an inappropriate immune response to food antigens and then FA development. However, we did not find any deficiency in regulatory components in the early BM ingested by children with future suspected FA. Furthermore, we neither confirmed previous results underlying the critical role of components such as TGF β , IgA, IFN γ , IL-12, or IL-4^{35,38-40} nor found any association with other components, such as those recently evidenced in mature milk (IL-1 β , IL-6, IL-10).⁴¹ Instead, our casecohort design demonstrated a positive association between CXCL10, TNF β , and IL-2 concentrations in early BM and the development of FA in childhood. These factors, revealed through robust statistical analysis, have never been assessed with regard to the development of FA, probably as not directly involved in Th/Treg orientation or in gut barrier permeability. Notably, we observed that high concentrations of CXCL10 were highly related to FA development in children of non-atopic mothers. CXCL10 has already been detected in colostrum, transition, and mature milk in a Japanese cohort and has been suggested to be involved in T-cell recruitment within the gastrointestinal mucosa of the neonate, notably of Th1 cells, which would have a beneficial effect in Th1/Th2 cell equilibrium in the neonatal period.⁴² However, we observed the opposite, that is, a higher concentration of CXCL10 was associated with FA. Interestingly, CXCL10/CXCR3 signaling axis is overactivated in the small intestine of active celiac patients, participating in lymphocytic recruitment, suggesting an inflammatory role of this chemokine in the intestine.⁴³ It is also noteworthy that CXCL10 has an antibacterial role⁴⁴: A negative effect of high concentrations of CXCL10 on microbiota shaping/implantation therefore warrants further attention.

IL-2 is a T-cell proliferative cytokine whose effects are concentration-dependent. Low doses of IL-2 have been associated with the preferential induction of Treg cells and have been successfully used to control experimental FA.⁴⁵ We can therefore hypothesize that the positive association between IL-2 concentrations and FA may stem from incorrect maturation/function of the Treg compartment to the benefit of Th cells in the presence of too high IL-2 concentrations, thus favoring immune dysregulation leading to health disorder such as FA.

Finally, TNF β is a proinflammatory cytokine that acts as an auto-crine mitogenic factor for B cells and has a role in lymphocyte homing and development of lymph nodes and Peyer's patches, and in

CD40- and IL-4-induced B-cell proliferation and IgE synthesis.⁴⁶ The latter study suggested that the role of TNF β in the induction of allergic reaction and of atopic state would have to be considered, a hypothesis that our present results also highlight. The positive association between CXCL10, IL-2, and TNF β concentrations and FA development clearly need further validation in other cohorts, and the mechanisms involved merit further investigations in experimental models.

We did not assess other immunologic components such as the cellular and complement systems, nor factors such as human milk oligosaccharides or microbiota, which may also greatly impact the gut barrier and immunity in neonates. We are aware of the other limitations of our study, the most important being that no longitudinal sampling was performed and that FA cases rely on parental reporting of a medical diagnosis of FA, that is, no laboratory tests or food challenges were additionally performed. Our results then need to be confirmed by further studies with repeated BM collection throughout the first week of life and a validated method to confirm food allergies. However, we should highlight the breadth of the analysis performed (50 immune and growth factors) and the number of samples analyzed, which yield the widest analysis hitherto of immune and growth factors in early BM. Moreover, those samples were obtained in a mother-child cohort also collecting a comprehensive set of data on the maternal environment and habits, and on the child. Although we focus here on FA in childhood, all the data we generated will be available in the cohort to be related to other child health outcomes and global development.

In conclusion, thanks to samples and information collected in the EDEN mother-child cohort, we show that immune and growth factor concentrations in early BM, and thus potentially the immune, trophic, and growth information transmitted from the mother to the neonate, are related to maternal and environmental factors, notably dietary habits and parity. Resulting interindividual variations in early BM composition may already explain controversies regarding the effect of breastfeeding on immune-associated pathologies in offspring. Moreover, we show that parental reporting of medical diagnosis of FA in childhood is positively associated with concentrations of CXCL10, TNF β , and IL-2 in early BM. The role of these factors in FA should be further studied.

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CONFLICT OF INTEREST

No conflict of interest to declare.

AUTHOR CONTRIBUTION

MB performed all the assays and analyzed all the data; BLG designed the subcohort and casecohort and supervised all the statistical

analyses that were performed with AF; FC, FF, and CJ participated in the conception of the work and analysis of the results, MAC and BH provided all the breastmilk samples and related information and participated in the statistical analysis; KAP designed and supervised the whole study and wrote the manuscript. All authors participated in manuscript writing and/or revised the manuscript and approved the final version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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1 Supplementary tables

2 Suppl table 1: Sample characteristics.

	EDEN cohort	Sub-cohort	Additional cases
N	2002	272	46
Center			
Poitiers	48% (968)	51% (139)	13% (6)
Nancy	42% (1034)	49% (133)	87% (40)
Child's gender			
Boy	53% (1000)	48% (130)	54% (25)
Girl	47% (903)	52% (142)	46% (21)
Maternal educational level			
No diploma	27% (549)	25% (67)	28% (13)
High school diploma	17% (340)	14% (39)	20% (9)
2-year university degree	21% (414)	22% (60)	26% (12)
At least a 3-year university degree	35% (699)	39% (106)	26% (12)
Maternal smoking during pregnancy			
Nonsmoker	76% (1518)	77% (206)	74% (34)
Smoker	24% (484)	23% (61)	26% (12)
Maternal BMI before pregnancy			
<18.5 kg/m ²	8% (161)	9% (25)	4% (2)
18.5 – 24.9 kg/m ²	67% (1345)	70% (191)	83% (38)
25.0 – 29.9 kg/m ²	16% (330)	15% (42)	9% (4)
≥ 30.0 kg/m ²	8% (166)	5% (14)	4% (2)
Maternal history of allergy			
None	71% (1416)	72% (195)	67% (31)
At least one	29% (586)	28% (77)	33% (15)
Cesarean birth	16% (299)	16 % (44)	17% (8)
Primiparous women	42% (848)	43% (117)	50% (23)

		Day (number of samples)					p values
		D2 (14)	D3 (112)	D4 (85)	D5 (40)	D6 (12)	
IgG1	Median	7026	6534	7295	6379	5235	0.46
	Mean	9023	9338	9150	7101	6074	
	Range	3479-29611	2002-100395	2709-37278	3236-18815	2996-14883	
IgG2	Median	20979	24644	23708	20938	19571	0.281
	Mean	29989	28017	28715	22197	25022	
	Range	14093-89490	5679-104921	10049-111187	9057-50763	7737-65053	
IgG3	Median	491	467	477	370	354	0.142
	Mean	592	636	542	434	486	
	Range	156-1679	129-3162	106-1910	143-1045	118-1822	
IgG4	Median	536	542	662	759	419	0.236
	Mean	759	807	1061	1138	608	
	Range	69.6-2686	61.2-7714	34.7-8509	74.8-5608	109-1985	
IgA	Median	928532	351544	380411	636009	467553	0.739
	Mean	1175125	967766	860374	810482	618777	
	Range	176349-3520574	106877-6991813	92307-5952680	110846-3530574	88702-1604821	
IgM	Median	52178	55376	47201	47178	42351	0.265
	Mean	75050	102711	78597	63564	63164	
	Range	11401-228116	6048-711740	2384-844146	10292-229927	15284-158072	

Suppl. table 2: Total antibody concentrations (pg/mL) in early BM samples depending on the day of collection. The number of samples per sampling date is given in brackets. Comparison of the means obtained at the different sampling dates was performed using analysis of variance (general linear model- (GLM) procedure in SAS software) and corresponding p values are provided.

		Day (number of samples)					p values
		D2 (14)	D3 (112)	D4 (85)	D5 (40)	D6 (12)	
EGF	Median	38274	32196	38819	38940	42206	0.752
	Mean	38363	39397	40686	40933	50409	
	Range	1089-78920	8008-357167	12284-122387	18638-95692	27366-112275	
FGF-2	Median	41.56	43.05	44.07	43.95	40.07	0.324
	Mean	43.17	45.09	50.67	56.81	38.87	
	Range	28.71-57.92	20.48-114.4	27.39-208	21.92-533	20.48-48.7	
Flt3L	Median	14.24	13.03	13.81	13.73	12.72	0.485
	Mean	14.23	14.43	15.56	15.66	12.32	
	Range	6.1-24.33	6.49-38.91	5.7-74.22	9.18-45.62	3.78-19.03	
TGF- α	Median	131.4	90.61	78.15	64.79	71.49	<0.0001
	Mean	150.5a	105.2b	92.94bc	77.04c	76.11bc	
	Range	79.15-280.1	32.94-248	22.9-265	30.37-180.9	39.07-151.3	
G-CSF	Median	39.75	27.21	25.21	20.16	17.15	0.131
	Mean	46.02	32.75	33.69	25.52	52.60	
	Range	17.87-122	7.03-119.7	7.57-223	7.57-118.5	8.97-424	
GM-CSF	Median	4.72	4.81	5.36	5.34	4.08	0.399
	Mean	5.53	5.35	6.40	7.08	4.96	
	Range	3.07-9.21	1.93-21.65	2.06-34.4	2.44-75.65	2.72-11	
PDGF-AA	Median	181.4	201.5	192.3	151.9	246.4	0.137
	Mean	184.1	228.7	235.7	175	261.2	
	Range	34.11-319	39.62-820	41.69-904	33.25-482	76.17-471	
PDGF-BB	Median	99.35	59.51	70.14	57.93	81.59	0.212
	Mean	161	92.03	115.5	84.99	82.44	
	Range	9.81-557	2.26-1146	6.75-786	4.08-428	32.25-147.5	
VEGF	Median	588	818	945	1044	982	0.007
	Mean	687a	895ab	966abc	1042bc	1043abc	
	Range	346-1530	281-1857	116-2313.3	443-1636	374-1571	
TGF- 1	Median	572	1007	1258	2259	1568	<0.0001
	Mean	808ab	1178a	1694bc	2249c	2229c	
	Range	293-2180	129-3930	165-5859	425-4494	690-5822	
TGF- 2	Median	1390	2048	2658	3038	2795	0.01
	Mean	1490a	2828a	4215a	5019a	3703a	
	Range	486-3219	79.2-16182	277-38099	502-30225	1058-10850	
TGF- 3	Median	352	347	276	302	268	0.307
	Mean	469	416	442	397	744	
	Range	57.2-1556	44.1-1813	51-2340	58-1580	101-5821	

Suppl. table 3: Growth factor concentrations (pg/mL) in early BM samples depending on the day of collection. Comparison of the means obtained at the different sampling dates was performed using analysis of variance (GLM procedure in SAS software) and corresponding p values are provided. Differences between sampling dates, evidenced when $p < 0.05$, were further assessed using least square means and adjustment for multiple comparison tests using the Bonferroni test. Differences between sampling dates are indicated using letters (a, b or c): the use of the same letter means that no statistical difference between sampling dates was found.

		Day (number of samples)					p values
		D2 (14)	D3 (112)	D4 (85)	D5 (40)	D6 (12)	
CCL11 (Eotaxin)	Median	47.27	60.28	70.65	65.24	65.01	0.049
	Mean	46.45a	73.39a	90.18a	85.92a	66.55a	
	Range	20.41-93.91	8.10-399	9.27-333	14.74-259	42.61-109.2	
CX3CL1 (Fractalkine)	Median	3765	1849	1675	1285	3082	0.075
	Mean	3654	2426	2370	1853	3062	
	Range	507-7653	152-9804	164-9163	290-6585	696-6664	
CXCL1 (GRO)	Median	252473	287523	309452	320913	330963	0.924
	Mean	323490	342493	329479	311490	362949	
	Range	119604-1333093	49562-2307771	50426-1092232	150837-479763	251106-698461	
CXCL8 (IL-8)	Median	528	739	727	599	618	0.929
	Mean	1577	1458	1457	1323	1156	
	Range	85.8-5130	40.83-6809	65.81-7168	46.39-5635	161.5-4736	
CCL7 (MCP-3)	Median	22.51	20.48	21.47	21.13	17.04	0.772
	Mean	27.38	46.29	34.65	32.26	24.04	
	Range	12.7-66.99	9.39-1080	11.26-173.8	13.19-216	11.8-60.71	
CCL22 (MDC)	Median	1592	1054	925	748	937	0.210
	Mean	1861	1226	1234	1102	1073	
	Range	431-3847	30.47-8767	19.11-5404	19.11-4844	62.23-3024	
CXCL10 (IP-10)	Median	10605	14991	16714	13828	12271	0.631
	Mean	13517	28482	19189	15913	12698	
	Range	6673-32799	2577-887491	3748-77574	3236-42366	3495-26252	
CCL2 (MCP-1)	Median	4602	9172	13670	13282	14260	0.247
	Mean	19841	13719	24219	22236	18312	
	Range	2262-171932	1045-81629	1447-347641	1226-240063	3332-46887	
CCL3 (MIP-1~F)	Median	0	0	0	0	0.84	0.58
	Mean	20.23	42.71	17.70	8.52	5.76	
	Range (neg) [extrap]	0-99.57 (8)	0-1890 (72)[1]	0-645 (55)[1]	0-194.3 (30)	0-24.56 (6)	
CCL4 (MIP-1 F)	Median	21.23	21.82	23.36	21.89	17.63	0.596
	Mean	39.09	56.40	37.14	35.08	23.36	
	Range	4.17-207	4.97-1151	3.08-444	6.02-213	10.28-62.11	
CCL5 (RANTES)	Median	107.14	71.44	60.80	36.71	25.13	<0.0001
	Mean	153.98a	87.43b	75.44bc	50.08c	32.13c	
	Range	73.2-330	15.86-318	2.88-253	4.24-170.7	6.28-93.83	

Suppl. table 4: Chemokine concentrations (pg/mL) in early BM samples depending on the day of collection. For MIP1 α , the number of samples with undetectable concentrations is indicated in brackets; values in square brackets correspond to the number of samples for which concentrations were extrapolated beyond the standard range. Comparison of the means obtained at the different sampling dates was performed using analysis of variance (GLM procedure in SAS software) and corresponding p values are provided. Differences between sampling dates, evidenced when p<0.05, were further assessed as for suppl table 3.

			Day (number of samples)					p values
			D2 (14)	D3 (112)	D4 (85)	D5 (40)	D6 (12)	
Th1 cytokines	IFN γ	Median	6.20	5.81	5.79	5.75	5.57	0.508
		Mean	7.40	6.89	8.94	6.11	5.83	
		Range	3.28-20.71	2.62-47.69	2.9-145.5	2.51-10.73	4.06-8.53	
	IL-12p40	Median	13.03	15.96	15.72	16.36	14.20	0.378
		Mean	18.06	19.30	26.05	23.66	13.71	
		Range	9.25-46.32	1.31-131.3	5.94-333	8.19-222	8.45-18.65	
	IL-12p70	Median	3.53	2.95	3.28	2.98	2.53	0.284
		Mean	3.37	3.30	3.87	4.88	2.54	
		Range (neg)	0.95-6.04	0-9.48 (2)	0.52-45.51	0-46.47 (1)	0-5.43 (2)	
Th2 cytokines	IL-4	Median	3.71	4.44	4.46	4.61	3.80	0.867
		Mean	6.08	7.65	7.72	7.98	3.42	
		Range (neg)	0.52-21.4	0-101.9 (1)	0.52-91.7	0.98-100.6	1.49-4.99	
	IL-5	Median	1.04	0.91	1.01	0.92	0.94	0.851
		Mean	1.22	14.37	1.24	1.11	1.84	
		Range (neg) [extrap]	0.65-2.52	0.02-1457 [13]	0-6.37 (2) [12]	0-4.05 (1) [12]	0.02-12.35 [2]	
	IL-13	Median	2.13	2.65	3.36	3.52	1.97	0.956
		Mean	3.12	5.65	4.91	4.84	2.52	
		Range	0.77-10.51	0.02-238	0.02-93.2	0.81-43.66	0.77-6.53	
	IL-9	Median	0.45	0.44	0.53	0.52	0.55	0.79
		Mean	0.53	13.06	0.72	0.75	0.52	
		Range (neg) [extrap]	0-1.98 (3) [6]	0-1215 (7) [58]	0-7.3 (3) [40]	0-6.44 (2) [19]	0-1.06 (1) [5]	
Th17 cytokines	IL-17A	Median	2.29	1.79	1.80	1.73	1.31	0.015
		Mean	11.00a	3.57a	3.26a	2.49a	1.41a	
		Range (neg) [extrap]	0.67-81.98	0.36-85.14	0.44-43.52	0.38-11.38 [1]	0.61-2.88	
Regulatory cytokines/factors	IL-10	Median	3.08	4.23	3.70	4.37	2.50	0.328
		Mean	13.98	8.71	6.42	9.66	3.44	
		Range	0.78-123.6	0.37-140.3	1-53.31	0.27-100.9	1.74-8.54	
	sCD40L	Median	41.16	57.80	75.50	73.78	76.36	0.151
		Mean	61.84	69.92	90.12	97.25	87.87	
		Range	19.25-181.2	15.71-361	11.83-688	15.03-555	25.21-169.8	
	IL-1RA	Median	44.39	48.66	49.93	47.62	43.05	0.86
		Mean	83.35	77.72	86.16	80.53	46.94	
		Range	24.67-444	24.64-1050	20.09-851	20.48-854	25.41-99.66	

Suppl table 5: Th/Treg cytokine concentrations (pg/mL) in early BM samples depending on the day of collection. For IL-12p70, IL-4, IL-5, IL-9, IL-17A, the number of samples with undetectable concentrations is indicated in brackets; values in square brackets correspond to numbers of samples for which concentrations were extrapolated beyond the standard range. Comparison of the means obtained at the different sampling dates was performed using analysis of variance (GLM procedure in SAS software) and corresponding p values are provided. No difference between sampling dates was evidenced (p>0.05), except for IL17A, but no difference between sampling dates was further evidenced using least square means and adjustment for multiple comparison tests (Bonferroni test).

			Day (number of samples)					p values
			D2 (14)	D3 (112)	D4 (85)	D5 (40)	D6 (12)	
Pro-inflammatory cytokines	IFN- γ	Median	32.77	31.38	32.06	32.29	29.46	0.344
		Mean	34.98	33.46	38.72	38.23	30.94	
		Range	17.78-73.82	11.87-136.3	9.33-189.4	13.1-136.1	16.4-44.87	
	IL-1 β	Median	18.62	23.41	25.14	21.60	24.03	0.084
		Mean	47.23	25.95	28.49	21.41	25.05	
		Range	6.83-399	5.34-144.5	6.5-151	7.55-35.57	5.12-66.22	
	IL-1	Median	2.73	2.49	2.19	2.32	1.53	0.053
		Mean	4.59	6.54	4.91	4.83	22.27	
		Range [extrap]	0.87-20.08	0.61-105.5	0.45-42.1 [2]	0.8-88.26	0.69-248	
	IL-6	Median	15.94	22.78	31.15	30.38	20.07	0.307
		Mean	20.66	43.55	50.29	39.71	62.15	
		Range	3.63-71.99	1.85-330	3.37-260.3	3.49-136.3	10.39-481	
	TNF- α	Median	15.60	13.21	12.65	11.98	11.18	0.788
		Mean	18.07	22.51	21.74	15.03	18.12	
		Range	8.05-40.27	5.10-310	5.05-287	1.31-37.39	6.72-82.71	
	TNF	Median	1.54	1.27	1.54	1.60	1.56	0.438
		Mean	1.85	1.76	2.61	2.20	1.53	
		Range (neg)	0.65-4.4	0-13.52 (1)	0-41.94 (1)	0.72-11.90	0.59-2.49	
Proliferation cytokines	IL-2	Median	1.71	1.91	2.31	2.00	1.73	0.769
		Mean	2.11	2.73	2.93	2.94	1.61	
		Range	0.68-5.89	0.42-44.35	0.58-26.59	0.87-26.69	0.84-2.58	
	IL-15	Median	121.46	85.95	84.70	75.50	81.11	0.016
		Mean	146.45a	96.69b	103.82ab	95.42b	86.08ab	
		Range	58.5-329	31.22-271	20.69-421	17.93-245	55.81-171.8	
	IL-3	Median	0.00	0.00	0.00	0.00	0.05	0.948
		Mean	0.25	0.28	0.27	0.33	0.19	
		Range (neg) [extrap]	0-0.84 (8) [2]	0-3.27 (59) [28]	0-3.89 (49) [18]	0-4.38 (21) [10]	0-0.61 (6) [5]	
	IL-7	Median	42.82	39.80	44.76	47.61	68.99	0.017
		Mean	47.56a	43.67a	58.71a	62.04a	71.44a	
		Range	20.85-131	5.07-190	7.04-341	7.96-219	26.05-169.8	

Suppl table 6: Pro-inflammatory and proliferation cytokine concentrations (pg/mL) in early BM samples depending on the day of collection. For IL-1 β , TNF β and IL-3, the numbers of samples with undetectable concentrations are indicated in brackets; values in square brackets correspond to the numbers of samples for which concentrations were extrapolated beyond the standard range. Comparison of the means obtained at the different sampling dates was performed using analysis of variance (GLM procedure in SAS software) and corresponding p values are provided. Differences between sampling dates, evidenced when $p < 0.05$, were further assessed using least square means and adjustment for multiple comparison tests using the Bonferroni test (see suppl table 3).

Statistical analysis

Descriptive statistics and associations of early BM components with maternal characteristics were conducted on the **sub-cohort** (263 non-selected samples). Firstly, a principal component analysis (PCA) was performed to visualize the whole data set: no outlier could be identified, and no classification of the samples was found (not shown). Secondly, the overall day-to-day variation in BM factor concentrations was examined considering all measured values by performing a multivariate supervised analysis to cluster the samples depending on the time of collection. To this end, a partial least square discriminant analysis (PLS-DA, SIMCA-P, Umetrics, Umea, Sweden version 11) was performed, after having mean centered the variables (*i.e.*, immune factors) and scaled them to unit variance. Then, for each specific parameter, analysis of variance was performed to compare the means obtained at the different sampling dates (GLM procedure in SAS software, version 9.3; SAS Institute, Cary, NC, USA). Differences between sampling dates were then assessed comparing all sampling dates together using least square means and adjustment for multiple comparison tests (Bonferroni test, SAS software). Thirdly, to examine the association between maternal or child characteristics and immune/growth factor concentrations independently of sampling day, a linear regression was conducted on sampling day, considered as a categorical variable (D2, D3, D4, D5, D6), for each BM factor and the residual of this regression was used in further analyses. The Heatmap.2 algorithm (ggplot 2 package in R software, version 3.3.3) was used to generate the heatmap of correlation and draw dendrograms showing a hierarchical clustering between all factors. Then, for each residual of BM factors, a multiple linear regression was conducted to assess the association with maternal (age, BMI before pregnancy, number of pregnancies, atopy), environmental (education level, smoking during pregnancy, recruitment center, dietary pattern) or birth (C-section, child's gender) characteristics. For each immune/growth factor, the interaction between the recruitment center and other maternal characteristics was tested. To deal with the problem of multiple comparisons when studying large numbers of biological factors, we used the false discovery rate (FDR) procedure using the Benjamini and Hochberg method ¹(SAS, PROC Multtest, FDR option) with q-value cut-off of 0.10 ². Analyses were conducted using SAS software version 9.4 (SAS Institute, Inc., Cary, NC).

Associations between immune/growth factor concentrations and incidence of food allergy were estimated thanks to the **case-cohort design** (*i.e.* with additional FA cases, n=318 samples including 72 FA-reported cases). We used Prentice-weighted Cox regression, with age of FA report in months as the underlying timescale and additionally adjusting for parental history of allergy, sibling history of allergy, maternal age at delivery, maternal pre-pregnancy BMI, maternal smoking during pregnancy,

maternal education level, C-section birth, and the child's gender. The interaction between each immune/growth factor and recruitment center or maternal history of allergy was tested. To deal with multiple testing issues, we also used the FDR procedure (SAS, PROC Multtest). Analyses were conducted using SAS software version 9.4 (SAS Institute, Inc., Cary, NC).

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2. Streiner DL. Best (but oft-forgotten) practices: the multiple problems of multiplicity-whether and how to correct for many statistical tests. *AmJClinNutr*. 2015;102(4):721-728.