Inflammatory Markers at Birth and Risk of Early-Onset Inflammatory Bowel Disease

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The development of inflammatory bowel disease (IBD) may be preceded by a long preclinical phase characterized by several disease-associated immunologic changes, as observed for other immune-mediated inflammatory diseases.[1] As an example, disease-associated changes in systemic inflammatory proteins have been observed up to a decade before a diagnosis of Crohn's disease (CD) or ulcerative colitis (UC).[2,3]

Despite increasing evidence of early life events being important for later risk of disease,[4,5] no studies have, to our knowledge, linked systemic inflammation at time of birth with later risk of IBD.

In a unique cohort of neonatal blood spots from individuals who developed IBD during childhood, we aimed to study how systemic inflammatory changes around birth are associated with development of (1) IBD before the age of 19 years and (2) very-early-onset (VEO) IBD diagnosed before the age of 6 years.

Our cohort included 464 individuals (CD: n = 243, 46% female; UC: n = 221, 50% female) with pediatric-onset IBD diagnosed at a median age of 11 years (interquartile range, 7–15 years) identified via the Danish National Patient Registry. In the cohort, a total of 35 individuals (3.8% of the cohort) had mothers with IBD, and 26 of these individuals developed IBD later in life. In the VEO subcohort, 4.5% had a mother with IBD, and 75% of these developed IBD later in life. For each IBD case, we identified a non-IBD control individual from the general population based on time of sampling, sex, and gestational age or birthweight (ie, when gestational age was not available). Dried blood spots on case and control individuals (n = 928) were identified in the Danish National Biobank, which stores blood spots collected 4–6 days after birth from Danish individuals since 1982, thus providing an opportunity for investigating disease-associated changes at birth. Nine cytokines were measured in the fg/mL range using a highly sensitive multiplex immunoassay (S-PLEX, MesoScale) (Supplementary Table 1). Six of the 9 cytokines were measurable in at least 20% of individuals (interleukin [IL] 1β, IL-4, IL-6, IL-17A, interferon gamma, and tumor necrosis factor α), and further analyses were based on these. We performed univariate and multivariate logistic regression to assess whether the level of an individual inflammatory marker, used as a continuous variable, was associated with future risk of IBD, CD, or UC. Analyses were repeated for the subsample of patients with VEO-IBD. Results are reported as odds ratios (ORs) with 95% confidence intervals (CIs). The measured concentration in fg/mL was divided by 100 such that the shown OR corresponds to the risk associated with an increase of 0.1 pg/mL (or 100 fg/mL). Correction for multiple testing by false discovery rate (FDR) was performed in all analyses described. *P* values and *Q* values (ie, FDR-corrected *P* values) lower than .05 were considered statistically significant.

In the overall cohort of pediatric-onset IBD, we found no statistically significant association between levels of individual neonatal inflammatory proteins and following risk of IBD (Figure 1*A*). This was also the case when studying CD and UC separately (Figure 1*B* and *C*). In a sensitivity analysis, cytokines were analyzed as categorical variables, with each marker divided into 4 groups based on concentration, which showed similar results (Supplementary Figure 1).

We then confined our analyses to the 88 patients in the pediatric cohort who were diagnosed with VEO-IBD. Of these, 36 had CD (28% female), 52 had UC (54% female), and their median age at diagnosis was 2 years (range, 0–5 years). We assessed neonatal inflammatory levels in the 88 VEO-IBD patients and their matched control individuals, using cytokine concentrations as a continuous variable and adjusting for gestational age, birth year, mode of delivery (vaginal or cesarean birth), and sex. We observed a statistically significant protective effect of IL-4 on later development of VEO-IBD (OR, 0.43; 95% CI, 0.19–0.83; $P = .02$). In contrast, increased IL-17A was marginally significantly associated with increased risk of VEO-IBD (OR, 1.17; 95% CI, 1.01–1.42; $P = .06$) (Figure 1*D*). The significance was lost after adjustment for multiple testing, likely due to low power.

To our knowledge, we present the first study investigating systemic inflammatory markers around birth and later risk of IBD. In a unique cohort of 464 pediatric IBD cases, we measured levels of interferon gamma, tumor necrosis factor α, IL-1β, IL-4, IL-6, and IL-17A a few days after birth. In the overall pediatric patient cohort diagnosed with IBD at a median age of 11 years, neonatal levels of cytokines were not associated with later disease occurrence. However, in the subcohort of 88 patients diagnosed with VEO-IBD before age 6 years, we observed a statistically significant association between increased levels of IL-4 and reduced risk of IBD. Increased IL-17A levels were, contrarily, borderline statistically significantly associated with an increased risk of IBD. IL-4, a cytokine generally associated with the T helper (Th) type 2 immune response, has been shown to be implicated in wound healing and tissue repair.[6] The specific role of the Th2 response in IBD is still elusive[7]; however, IL-4–treated human macrophages have been observed to promote epithelial wound repair in vitro, and the transfer of such cells to an acute colitis mouse model reduced disease severity.[8] Thus, a decrease in IL-4 in VEO-IBD patients might indicate a

Abbreviations used in this paper: **CD, Crohn's disease; CI, confidence interval; FDR, false discovery rate; IBD, inflammatory bowel disease; IL, interleukin; OR, odds ratio; Th, T helper; UC, ulcerative colitis; VEO, very early onset.**

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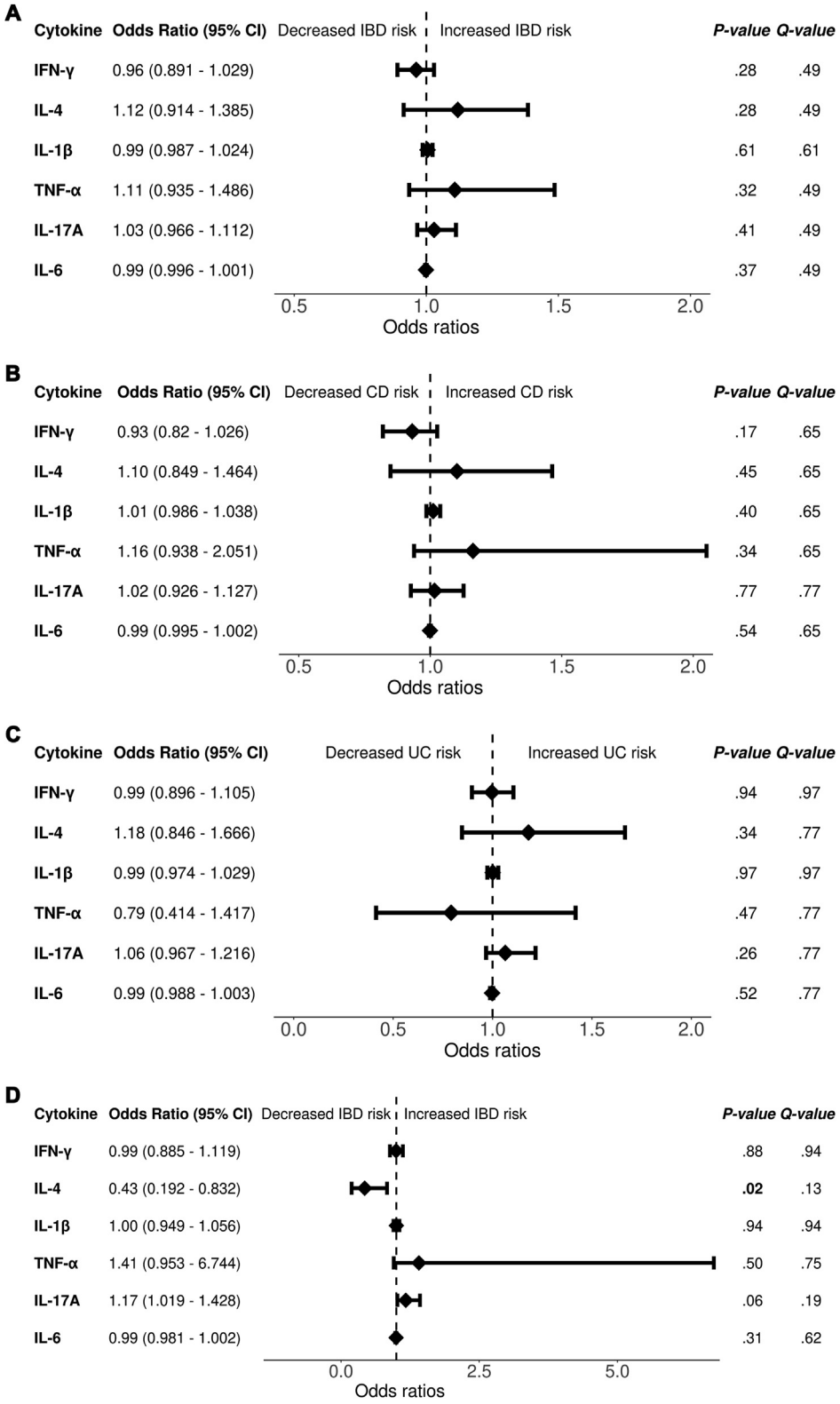

Figure 1. Results from multivariate logistic regression analysis aimed to investigate associations between (*A*) increased cytokine concentration with later early onset of IBD, (*B*) increased cytokine concentration with later early onset of CD, (*C*) increased cytokine concentration with later early onset of UC, and (*D*) increased cytokine concentration with later VEO IBD. The measured concentration for each cytokine was used as a continuous variable in the analysis. The measured concentration in fg/mL was divided by 100 such that the shown ORs correspond to the risk associated with an increase of 0.1 pg/mL (or 100 fg/mL). Each subplot represents a separate multivariate analysis with individuals included corresponding to the specific disease type and their matched control individuals: (*A*) IBD, n = 928 (cases = 464); (*B*) CD, n = 486 (cases = 243); (*C*) UC, n = 442 (cases = 221); and (*D*) VEO IBD, n = 176 (cases = 88). Analysis was adjusted for gestational age, birth year, mode of delivery (vaginal vs cesarean birth), and sex. *P* values within each analysis were adjusted for multiple testing by FDR correction (*Q* value). IFN, interferon; TNF, tumor necrosis factor.

reduced potential for homeostatic tissue repair. IL-17A, on the other hand, is a well-known proinflammatory cytokine, generally produced by Th17 and type 3 innate lymphoid cells (ILC3), which has previously been associated with IBD as part of the IL-23/IL-17 axis and has been observed to be upregulated in active IBD patients.[9,10]

The strength of this study is access to a unique population-based resource of neonatal blood spots from the

Danish National Biobank, which, in the Danish PREDICT project, is linked to nationwide health information from a health care system that covers the entire population. This enables unique insights into disease development. Limitations include variations in measured concentrations due to varying sample storage time, difference in hematocrit levels, and differences in handling of samples over time. To mitigate this risk, we matched case and control individuals on time of sampling and adjusted for the year of birth, that is, year of sampling. Also, likely due to a lack of statistical power, significance disappeared after adjustment for multiple testing in the VEO-IBD analysis.

In conclusion, in a unique sample of neonatal blood spots from 464 individuals who later developed IBD, we measured cytokine concentrations a few days after birth. Whereas neonatal cytokine levels were not associated with pediatric IBD in the overall cohort, we observed decreased levels of IL-4 and increased levels of IL-17A a few days after birth in patients who developed IBD before the age of 6 years. The observation of inflammatory changes already at birth in patients who develop IBD at an early age is novel and may shed light on disease occurrence and pathophysiology.

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Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2024.07.007.

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Tine Jess, MD, PhD, DMSc (Conceptualization: Equal; Funding acquisition: Lead; Supervision: Lead; Writing – review & editing: Equal)

Conflicts of interest
The authors disclose no conflicts.

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Data Availability
Study material is under the governance of the Danish National Biobank and will therefore not be made freely available, and analytical methods are based on a commercially available kit and disclosed publicly available R packages.

Supplementary Methods

Study Cohort

The study cohort was established via the Danish National Patient Registry and the Danish National Biobank.

We included 464 individuals with predominantly pediatric-onset IBD, born during 1997–2018, and identified via the Danish National Patient Registry using International Classification of Diseases, 8th Revision codes (56301, 56302, 56308, 56309, 56319, 56904) and International Classification of Diseases, 10th Revision codes (DK50, DK51). To qualify for the cohort, case patients should have either 2 hospital/outpatient visits within 2 years or 2 inpatient contacts and have lived in Denmark in the 2 years before diagnosis.

One healthy control individual was matched to each IBD case patient based on time of sampling, sex, and gestational age or birthweight (when information about gestational age was not available). Information about gestational age, birthday, mode of delivery (vaginal or cesarean birth) and maternal IBD status was collected from the Danish National Patient Register, the National Birth Register, and the Central Person Register.

For all IBD case and control individuals, dried blood spots (DBSs) were identified in the Danish National Biobank. All DBSs had been sampled 4–6 days after birth as part of the routine screening program of all neonates born in Denmark since 1982 and were stored at −20°C.

Measurement of Inflammatory Markers

Two 3.2-mm disks were punched from the DBS into microtiter wells using a DBS Puncher instrument (PerkinElmer), and 130-μL extraction buffer (1× phosphate-buffered saline [Gibco], containing protease inhibitor [Roche cOmplete]) was added. Plates were sealed and incubated for 1 hour at room temperature with shaking (450 revolutions/min). The protein extract solution was transferred to 0.5-mL Matrix tubes (Thermo Scientific) and stored at −80°C until use.

Samples were analyzed for the levels of cytokines interferon gamma (IFN-γ), IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, and tumor necrosis factor α (TNF-α) at the fg/mL range using S-PLEX proinflammatory panel 1 kit (no. K15396S, MesoScale Diagnostics) per the supplier's instructions. All washing steps were performed on the Biotek Model 405 LS plate washer using the settings recommended by the supplier. Controls were made in-house from part of the calibrator solution in one batch and aliquoted into portions appropriate for one plate and stored at −20°C until use. Calibrator standards were made fresh for each plate. The concentrations of analytes were calculated from the calibrator curves of each plate using 4-parameter logistic regression in the Meso Scale Discovery Workbench software.

Analytical Characterization

Intra-assay variation was calculated from 16 replicates of high controls (calibrator 4) on the same plate. Inter-assay variation was calculated from 2 replicates of high and low controls (calibrators 4 and 3) on each plate, 13 plates in total. Lower limits of detection (LLODs) were calculated as 2.5 standard deviations from 26 repeated measurements of the 0 standard. Outliers within the repeated measurements were identified using the Tukey method (also known as the Tukey fence method) and were removed before the calculation of the LLOD. The upper detection limits (ULODs) were defined as the highest calibrator concentrations. Concentrations for measurements below the LLOD were defined as the LLOD for the given analyte divided by 2; similarly, concentrations above the ULOD were defined as the ULOD for the given analyte times 2 (Supplementary Table 1).

Statistical Analyses

All statistical analyses were performed in R Studio (Posit Software) using the following packages: tidyverse, gt, gtsummary, metafor, patchwork, DescTools, and ggpubr. In all analyses described, P values and Q values (FDR-corrected P values) lower than .05 were deemed to be statistically significant.

Differences between CD patients, UC patients, and healthy control individuals in the distribution of sex was estimated using the Pearson chi-square test, and difference in age at diagnosis (in years) and gestational age (in days) were estimated using the Kruskal-Wallis rank sum test.

Before statistical modeling, cytokine concentrations in fg/mL were divided by 100, such that the shown calculated ORs correspond to the risk associated with an increase of 0.1 pg/mL (or 100 fg/mL). We then performed both univariate and multivariate logistic regression to estimate the association between cytokine concentrations and later IBD, CD, or UC development. In the multivariate model, all 6 cytokines were included as fixed explanatory variables, and the model was adjusted for gestational age (in days), year of birth (in years), mode of delivery (vaginal or cesarean birth), and sex. ORs and 95% CIs were calculated, and P values were adjusted for multiple testing by FDR correction. Furthermore, as a sensitivity analysis, the multivariate analysis was performed as described, but with cytokines grouped into 4 groups corresponding to increasing levels of cytokine abundance. For sparsely abundant cytokines (see Supplementary Table 1: IL-4, IL-17a, IFN-γ, and TNF-α) group 1 comprised cytokines that were measured below the LLOD. The remaining groups (groups 2–4) were then grouped by percentiles (P1 = 33.33%, P2 = 66.66%). In contrast, highly abundant cytokines (IL-1β and IL-6) were grouped into quartiles based on cytokine concentrations.

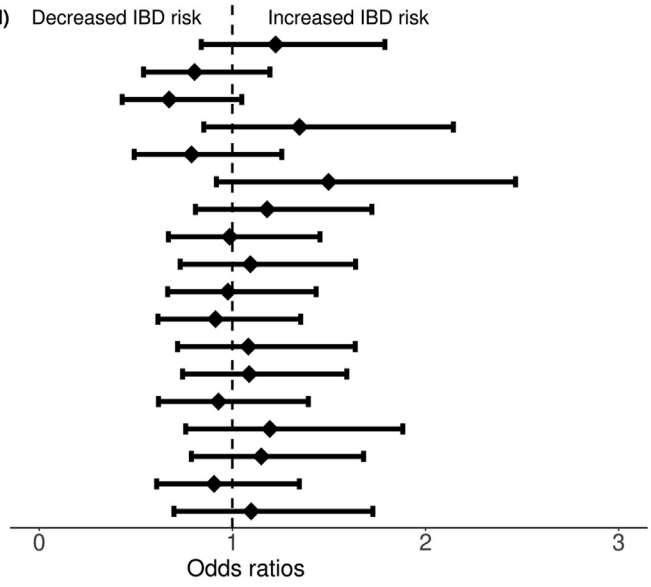
Exploratory analyses were performed to investigate associations between the measured cytokine levels and VEO cases of IBD. VEO IBD was defined as individuals diagnosed with IBD before the age of 6 years. The subcohort was analyzed by univariate and multivariate logistic regression using cytokine concentrations in fg/mL as a continuous variable. The analyses were performed as described for the full cohort.

Ethics

The present project has been approved by the Regional Ethical Committee of the Capital Region of Denmark and by the Scientific Board of the Danish National Biobank.

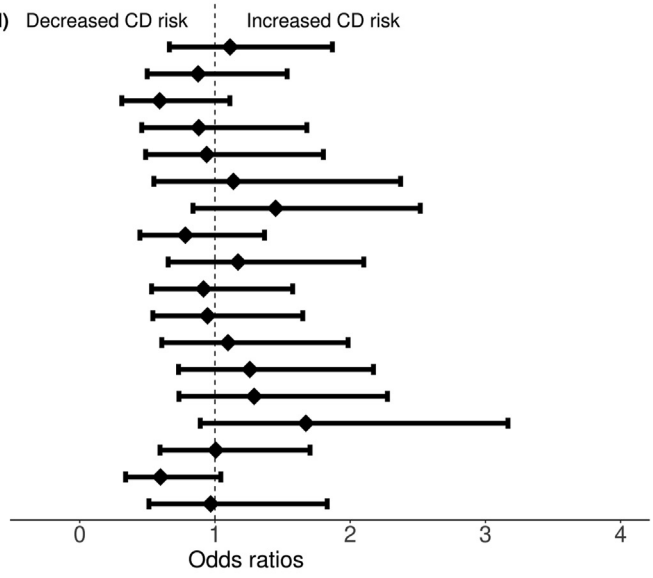
A

Cytokine [Group]	Odds Ratio (95% CI)	*P-value*	*Q-value*
IFN-γ [2]	1.22 (0.84 - 1.79)	.30	.81
IFN-γ [3]	0.80 (0.54 - 1.19)	.27	.81
IFN-γ [4]	0.67 (0.43 - 1.05)	.08	.81
IL-17A [2]	1.35 (0.85 - 2.14)	.21	.81
IL-17A [3]	0.79 (0.49 - 1.26)	.32	.81
IL-17A [4]	1.50 (0.92 - 2.47)	.11	.81
IL-1β [2]	1.18 (0.81 - 1.72)	.39	.81
IL-1β [3]	0.99 (0.67 - 1.45)	.95	.95
IL-1β [4]	1.09 (0.73 - 1.64)	.66	.81
IL-4 [2]	0.98 (0.67 - 1.43)	.90	.95
IL-4 [3]	0.91 (0.61 - 1.35)	.65	.81
IL-4 [4]	1.08 (0.72 - 1.64)	.70	.81
IL-6 [2]	1.09 (0.74 - 1.59)	.66	.81
IL-6 [3]	0.93 (0.62 - 1.39)	.72	.81
IL-6 [4]	1.19 (0.76 - 1.88)	.43	.81
TNF-α [2]	1.15 (0.79 - 1.68)	.48	.81
TNF-α [3]	0.91 (0.61 - 1.35)	.61	.81
TNF-α [4]	1.10 (0.70 - 1.73)	.70	.81



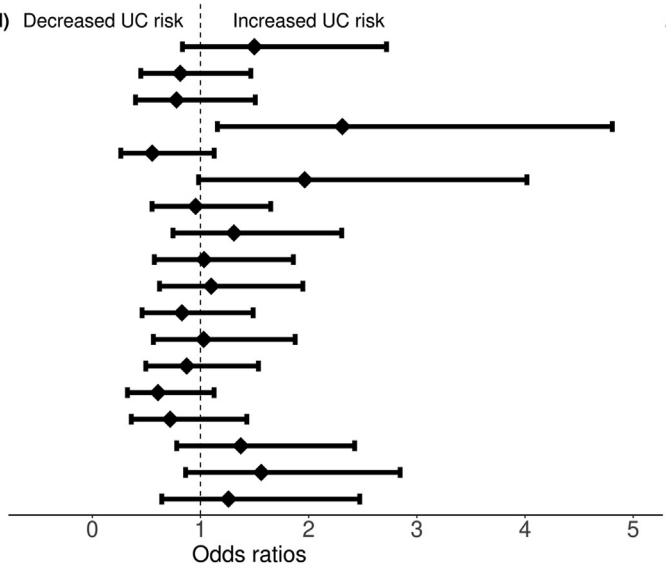
B

Cytokine [Group]	Odds Ratio (95% CI)	*P-value*	*Q-value*
IFN-γ [2]	1.11 (0.66 - 1.87)	.73	.94
IFN-γ [3]	0.88 (0.50 - 1.53)	.61	.94
IFN-γ [4]	0.59 (0.31 - 1.11)	.09	.57
IL-17A [2]	0.88 (0.46 - 1.68)	.69	.94
IL-17A [3]	0.94 (0.49 - 1.80)	.85	.94
IL-17A [4]	1.14 (0.55 - 2.37)	.75	.94
IL-1β [2]	1.45 (0.84 - 2.52)	.19	.84
IL-1β [3]	0.78 (0.45 - 1.37)	.39	.94
IL-1β [4]	1.17 (0.65 - 2.10)	.61	.94
IL-4 [2]	0.92 (0.53 - 1.57)	.74	.94
IL-4 [3]	0.94 (0.54 - 1.65)	.86	.94
IL-4 [4]	1.10 (0.61 - 1.98)	.72	.94
IL-6 [2]	1.26 (0.73 - 2.17)	.38	.94
IL-6 [3]	1.29 (0.73 - 2.28)	.35	.94
IL-6 [4]	1.67 (0.89 - 3.17)	.09	.57
TNF-α [2]	1.01 (0.59 - 1.70)	.97	.97
TNF-α [3]	0.60 (0.34 - 1.04)	.07	.57
TNF-α [4]	0.97 (0.51 - 1.83)	.89	.94



C

Cytokine [Group]	Odds Ratio (95% CI)	*P-value*	*Q-value*
IFN-γ [2]	1.50 (0.83 - 2.72)	.18	.54
IFN-γ [3]	0.81 (0.45 - 1.46)	.50	.74
IFN-γ [4]	0.78 (0.40 - 1.51)	.48	.74
IL-17A [2]	2.31 (1.16 - 4.81)	.02	.36
IL-17A [3]	0.55 (0.26 - 1.13)	.11	.49
IL-17A [4]	1.96 (0.98 - 4.02)	.06	.49
IL-1β [2]	0.95 (0.55 - 1.65)	.87	.93
IL-1β [3]	1.31 (0.75 - 2.31)	.36	.71
IL-1β [4]	1.03 (0.57 - 1.86)	.92	.93
IL-4 [2]	1.10 (0.62 - 1.95)	.75	.90
IL-4 [3]	0.83 (0.46 - 1.49)	.54	.74
IL-4 [4]	1.03 (0.56 - 1.87)	.93	.93
IL-6 [2]	0.87 (0.49 - 1.54)	.64	.83
IL-6 [3]	0.61 (0.33 - 1.13)	.12	.49
IL-6 [4]	0.72 (0.36 - 1.43)	.34	.71
TNF-α [2]	1.37 (0.78 - 2.42)	.27	.70
TNF-α [3]	1.56 (0.86 - 2.85)	.14	.49
TNF-α [4]	1.26 (0.64 - 2.47)	.50	.74



Supplementary Figure 1. Forest plots visualizing multivariate logistic regression analysis aimed to investigate associations between increased cytokine concentration groups with later early onset of (*A*) IBD, (*B*) CD, and (*C*) UC. In cytokine group analysis, samples were divided into 4 groups (1–4) for each cytokine corresponding to increasing concentrations of the specific cytokine (1, lowest; 4, highest). For sparsely abundant cytokines (see Supplementary Table 1, IL-4, IL-17a, IFN-γ, and TNF-α), group 1 comprised cytokines measured below the LLOD. The remaining groups (groups 2–4) were then grouped by percentiles (P1 = 33.33%, P2 = 66.66%). In contrast, highly abundant cytokines (IL-1β and IL-6) were grouped into quartiles based on cytokine concentrations. Group 1 was used as the reference group, and the model was adjusted for sex, birth year, mode of delivery (vaginal or cesarean birth) and gestational age. Each forest plot represents a separate multivariate analysis with individuals included corresponding to the specific disease type and their matched control individuals: (*A*) IBD, n = 928 (cases = 464), (*B*) CD, n = 486 (cases = 243), and (*C*) UC, n = 442 (cases = 221). *P* values within each analysis were adjusted for multiple testing by FDR correction (*Q* value).

Supplementary Table 1. Analytical Parameters for the Performed Multiplex Immunoassay

	Working range, *fg/mL*		Assay CV, %		
Analyte	Low	High	Intra-assay	Inter-assay	In detection range, %[a]
IL-1β	105.0	159,000	7.3	13.5	95.1
IL-2	15.4	85,800	6.0	16.9	10.6
IL-4	24.8	55,300	10.8	10.9	78.3
IL-6	19.1	39,500	12.6	15.2	95.0
IL-10	37.1	148,000	7.6	20.9	17.4
IL-12p70	231.0	484,000	7.0	12.2	5.4
IL-17A	87.4	210,000	15.4	17.6	29.3
IFN-γ	14.0	28,800	6.4	14.0	55.7
TNF-α	20.7	53,400	6.2	13.2	70.7

CV, coefficient of variation.
[a]Sample with a signal within the working range for the given analyte was considered to be within detection range.