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Cytokine atlas of the population-based cohort SHIP-TREND-0 – Associations with age, sex, and BMI

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ABSTRACT

Background: The characterization of physiological immune signatures in a population-based cohort is a prerequisite for identifying pathological immune signatures associated with inflammatory or autoimmune diseases.
Methods: Here, 47 plasma cytokines, chemokines, and growth factors were quantified with a bead-based multiplex-assay (Merck HCYTA-60 K) using a FLEXMAP 3D™ instrument in 1175 individuals of the Study of Health in Pomerania (SHIP; TREND cohort, 532 men and 643 women, age: 20 to 81, BMI: 17.7 to 53.6). Associations of cytokine concentrations with age, sex, BMI, season, and blood cell parameters (BCP) were examined by multivariate regression models.
Results: The physiological cytokine concentrations differed strongly between analytes, with median concentrations ranging from 0.6 to 7820 pg/mL. Many cytokine levels showed a large dynamic range within the study population. Higher levels of the pro-inflammatory cytokines and chemokines IL-6, IL-8, CXCL9, CXCL10, IL-12p40, CCL2, CCL4, CCL11, IL-27, FLT3LG, and TNFα were significantly associated with increasing age. The strongest age-associated effects were seen for CXCL9 ($\beta_{st} = 0.4$, $p < 0.001$) and CXLC10 ($\beta_{st} = 0.3$, $p < 0.001$). Significant sex differences were detected for CCL2, CCL3, CCL4, CCL11, CCL22, IL-12p40, IL-1RA, IL-18, IL-27, and TNFα levels among which CCL11 showed the strongest effect ($\beta_{st} = -0.24$, $p < 0.001$) with a lower level in women compared to men. Moreover, seven cytokines and chemokines, i.e. CCL4, CCL22, CXCL10, IL-1RA, IL-18, IL-6, and TNFα, displayed higher levels with increasing BMI. Among those, the strongest effect was seen for IL-1RA ($\beta_{st} = 0.19$, $p < 0.001$), CCL4 ($\beta_{st} = 0.16$, $p < 0.001$) and CXCL10 ($\beta_{st} = 0.14$, $p < 0.001$). Only CCL11 ($\beta_{st} = -0.17$, $p < 0.001$) decreased with increasing BMI. Subjects categorized as obese exhibited significantly elevated levels of CCL4, CCL22, CXCL10, and IL-1RA, while only CCL11 showed significantly reduced levels compared to normal weight. Certain cytokines such as IL-6, IL-18, or TNFα showed decreased significance levels after adjustment for blood cell components indicating blood cell components (BCPs) as potential confounders. We observed no significant non-linear seasonal effects for the investigated cytokines.
Conclusion: The generated cytokine atlas provides detailed information on cytokine variations in the general population and will provide a reference base for disease-related studies in the future. Furthermore, BCPs should be considered as potential confounders in association studies based on plasma cytokine levels.

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1. Introduction

Cytokines are small soluble proteins crucial for the communication between immune cells as well as between the immune system and other organ systems. They are secreted by a broad range of cells, including immune cells like lymphocytes and mononuclear phagocytic cells, as well as endothelial cells, fibroblasts, and epithelial cells [1]. Cytokines are extracellular key regulators of the immune response. They modulate the balance between inflammation and anti-inflammation, as well as between humoral and cell-based immune responses by regulating the maturation, proliferation, migration, effector functions, and responsiveness of particular cell populations. For instance, some cytokines initiate inflammation by mediating immune cell recruitment, extravasation and activation [2]. Cytokines often have redundant and pleiotropic effects. Moreover, they can act synergistically or antagonize each other. This complexity is further increased by the fact that the cytokine effect depends on the cellular source, target cell, and specific phase of the immune response during which it is presented [1]. Cytokines can be grouped by their effects on the immune response - e.g., acting on cells of the innate versus adaptive immune system, or by their pro- versus anti-inflammatory function [2]. Some cytokines can have both pro- and anti-inflammatory potential; which activity is observed depends on the immune cells present and their state of responsiveness to the cytokine [1].

Altered cytokine production is not only observed upon infection, but also in various non-infectious diseases that are characterized by an imbalanced immune response, including autoimmune diseases, allergy, chronic inflammatory diseases, tumors, transplant rejection, and pregnancy loss [3–6]. For instance, chronic inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease are characterized by an overactive proinflammatory immune response and the production of pro-inflammatory cytokines [7–9]. Additionally, cardiovascular diseases (CVD), including e.g. atherosclerosis, thrombosis, acute myocardial infarction (ischemic heart disease), hypertension, cardiac hypertrophy and fibrosis, heart failure (cardiac remodeling), various cardiomyopathies as well as ventricular arrhythmias, atrial fibrillation, but also metabolic diseases such as non-alcoholic fatty liver disease and type 2 diabetes have strong links to the immune system [10–12]. Furthermore, emerging evidence suggests that inflammatory processes and pro-inflammatory cytokines contribute significantly to blood-brain-barrier disruption and neurodegeneration, as observed in Alzheimer's disease or stroke [13]. Thus, cytokines influence a very broad range of diseases from different medical fields.

Epidemiological studies have mainly focused on investigating the role of cytokines in disease, while our knowledge about physiological cytokine levels in healthy individuals, as well as the natural variation of cytokines between sexes and with increasing age in the general population is still limited. Moreover, most studies focused on a limited set of pre-selected cytokine candidates in their analyses or meta-analyses [14,15]. Ter Horst et al. investigated the effect of host and environmental factors on the modulation of six cytokines in 500 healthy humans [14]. In particular age, gender, and seasonality were found to have strong influence on cytokine production capacity. Further, associations with BMI and related anthropometric traits were analyzed in a Brazilian sample of 743 individuals from the general population, but only for six cytokines, too [16]. The most frequently studied factors are the cytokines interleukin (IL) -1β, IL-6, and tumor necrosis factor-alpha (TNFα) as well as the acute phase protein C-reactive protein (CRP) [15,17]. Considering the huge variety of cytokines, their diverse effects on immune cells and host tissues as well as their intricate dependencies and interactions, a more comprehensive cytokine profiling of a general population cohort promises new insights into cytokine biology in health and disease.

Therefore, we conducted comprehensive cytokine profiling in individuals from the large population-based Study of Health in Pomerania (SHIP) from Northeast Germany. We quantified 47 blood plasma cytokines, chemokines, and growth factors in1175 SHIP-TREND-0 individuals, representative of the adult population in this region, and evaluated associations of cytokine concentrations with the basic phenotypes age, sex, and BMI. We also investigated potential seasonal effects, which are often observed in respiratory diseases. The generated cytokine atlas provides detailed information on cytokine variations in the general population and will provide a reference base for disease-related studies in the future.

2. Material & Methods

2.1. Ethics statement

The study has been conducted according to the recommendations of the Declaration of Helsinki. The study protocol of SHIP-TREND-0 was approved by the local ethics committee of the University of Greifswald (registration no. BB39/08) with all participants giving informed written consent.

2.2. Study population

The Study of Health in Pomerania (SHIP) is a population-based project conducted in Northeast Germany and the design and sampling methods for SHIP-TREND were previously described [18,19]. Briefly, SHIP-TREND is a longitudinal population-based cohort study assessing the prevalence and incidence of common diseases and their risk factors in 4420 subjects in the baseline sample. A subset of 1175 participants of the baseline examination SHIP-TREND-0, for whom EDTA plasma samples and data on genetic information, protein, microRNA and/or metabolites are available, were selected for cytokine profiling. The descriptive statistics of these study participants is provided in Table 1.

2.3. Descriptive statistics

Descriptive analyses include mean, standard deviation, median, and range for continuous variables such as age, BMI, and sampling day of the year (Table 1). To compare the distributions between males and females for continuous, non-normal distributed variables, a Mann-Whitney *U* test was performed. In addition, interquartile range is given (Table 1). For categorical variables such as sex, or BMI class numbers and percentages are presented. Significant differences between males and females for categorical variables were tested using the Chi^2-test.

2.4. Study protocol and assays for profiling of circulating plasma cytokines

Blood samples were collected from peripheral blood using antecubital venipuncture. Plasma was obtained by centrifugation at 2000 $\times g$ for 10 min at 4 °C, and aliquots were frozen at −80 °C. In total, 25 μL neat EDTA plasma of each individual sample without any additional freeze-thaw cycles was used for profiling of 47 cytokines using the "MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A – Immunology Multiplex Assay PC48" (HCYTA-60 K-PX48, Merck Millipore, Boston, MA, USA) by a bead-based multiplex assay. The following analytes were simultaneously measured: CD40 ligand (sCD40L), pro-epidermal growth factor (EGF), eotaxin (CCL11), fibroblast growth factor 2 (FGF-2), Fms-related tyrosine kinase 3-ligand (FLT-3 L), fractalkine (CX3CL1), granulocyte colony stimulating factor (CSF3, alias: G-CSF), granulocyte-macrophage colony stimulating factor (CSF2, alias: GM-CSF), C-X-C motif chemokine ligand 1 (CXCL1; alias: GRO-alpha), interferon-alpha 2 (IFNα2), interferon gamma (IFNγ), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-1 receptor antagonist protein (IL1RA), interleukin (IL) IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, interleukin-12 subunit beta p40 (IL-12p40), interleukin-12 p70 (IL-12p70), IL-13, IL-15, IL-17 A, IL-17E/interleukin-25 (IL-17E/IL-25), IL-17F, IL-18, IL-22, interleukin-17D (IL-27), C-X-C motif chemokine 10 (CXCL10, alias: IP-10), C-C motif chemokine 2 (CCL2, alias:

Table 1
Characterization of the SHIP-TREND-0 subsample.

	Total	Men	Women	p-value[1]
Sex [n], (%)	1175 (100 %)	532 (45 %)	643 (55 %)	$p < 0.001$
Age				
mean ± SD (year)	51 ± 14	51 ± 14	51 ± 13	0.69
range (year)	20–81	22–81	20–81	
median, IQR (years)	52, 41–61.5	51, 41–61	52, 41–62	
BMI				
mean ± SD (kg/m^2)	28.3 ± 4.8	28.7 ± 4.0	27.9 ± 5.4	$p < 0.001$
range (kg/m^2)	17.7–53.6	17.7–43.9	18.5–53.6	
median, IQR (kg/m^2)	28.1, 24.8–31.4	28.9, 25.6–31.4	27.6, 23.8–31.4	
BMI class (%)				$p < 0.001$, Chi^2 = 32.7
Lean (BMI <20)	22 (1.9 %)	5 (0.9 %)	17 (2.6 %)	
Normal (20 ≥ BMI <25)	294 (25.0 %)	96 (18.0 %)	198 (30.8 %)	
Overweight (25 ≥ BMI <30)	408 (34.7)	212 (39.8 %)	196 (30.5 %)	
Obese (BMI ≥ 30)	451 (38.4)	219 (41.2 %)	232 (36.1 %)	
Smoking status				$p < 0.001$, Chi^2 = 45.8
Never	493 (41.9 %)	169 (31.8 %)	324 (50.4 %)	
Former	432 (36.8 %)	243 (45.6 %)	189 (29.4 %)	
Current	250 (21.3 %)	120 (22.6 %)	130 (20.2 %)	
Comorbidities				$p = 0.92$, Chi [2] = 0.63
Diabetes	4 (0.3 %)	3 (0.6 %)	1 (0.2 %)	
Hypertension	522 (44.4 %)	266 (50.0 %)	256 (39.8 %)	
Sampling time point (day of year)				
mean ± SD (in d)	164 ± 103	167 ± 106	162 ± 101	0.49
range (in d)	1–352	1–352	1–349	
median, IQR (in d)	159, 75–257	162, 75–263	158, 75–255	
Biobank storage time (in d)				
mean ± SD (in d)	4347 ± 380	4359 ± 379	4337 ± 381	0.10
range (in d)	3198–5294	3198–5291	3233–5294	
median, IQR (in d)	4340, 4098–4587	4381, 4113–4593	4308, 4078–4580	

[1] *p*-value continuous variables: non-parametric Mann-Whitney *U* Test, categorical variables: Chi-square.

MCP-1), C—C motif chemokine 7 (CCL7, alias: MCP-3), macrophage-colony stimulating factor 1 (CSF1, alias: M-CSF), C—C motif chemokine 22 (CCL22, alias: MDC), C-X-C motif chemokine 9 (CXCL9, alias: MIG), C—C motif chemokine 3 (CCL3, alias: MIP-1α), C-C motif chemokine 4 (CCL4, alias: MIP-1β), platelet-derived growth factor subunit A (PDGF-AA), platelet derived growth factor subunit B (PDGF-AB/BB), pro-transforming growth factor alpha (TGFα), tumor necrosis factor (TNFα), lymphotoxin-alpha (TNFβ), vascular endothelial growth factor (VEGF-A), hereafter referred to as analytes or cytokines. Standards and controls were measured in duplicates. The variance of each assay was investigated using technical replicates of an independent sample set ($N = 36$) and showed a paired coefficient of variation (CV) <20 % (see Supplemental methods). Because of the good reproducibility, individual plasma samples ($N = 1175$) were measured only once. The measurement of 47 cytokines was performed according to manufacturer's instructions and recorded with the Luminex FLEXMAP 3D (Luminex, Austin, TX, USA). Briefly, the Luminex system enables the simultaneous measurement of a pool of microspheres (magnetic beads), each one defined by a unique fluorescence emission spectrum. Each bead population is coated with capture antibodies against a single analyte. Samples were incubated for 16 h at 4 °C (overnight protocol) with the microsphere-bound-capture-antibody mix in a 96-well plate to bind the analytes present in the EDTA plasma. Next, a biotinylated detection antibody mix was added to detect the captured analytes on each microsphere. Finally, 25 μL streptavidin-phycoerythrin complex (Strep-PE) was added per well, which bound to the detection antibody. After washing steps, the plate was recorded in the FLEXMAP 3D system according to equipment settings (50 events per bead, sample size: 100 Ll, gate settings: 8000-15,000, reporter gain: low PMT (default), time out: 60 s).

2.5. Data analysis and pre-processing

The median fluorescence intensity (MFI) per analyte and individual sample, as extracted by the XPonent® Software 4.2.1441.0 of the FLEXMAP 3D system, was exported per panel run, each comprising 75 samples, and analyzed within the R package DrLumi [20]. The data preparation using the DrLumi pipeline included quality control routines for multiplex immunoassay data, addressing the background noise of the assay, fitting the dose-response curves, and estimating the limits of quantification (LOQ). Convergence for each analyte was achieved by using a four parametric logistic function (4PL), without background subtraction. Lower limit of quantification (LLOQ) and higher limit of quantification (HLOQ) were determined according to the CV method implemented in the DrLumi package (loq_cv, CV ≤ 20 %) for each analyte and per panel run. Minimum detectable concentration values of the overnight protocol were taken as reference for the lower level of detection (LOD). The detection limits of the assays, absolute number as well as percentage of values within quantification range (LLOQ / HLOQ), and missing values (< LOD) are provided per analyte (Supplemental Table 1 and Supplemental Fig. 1). The region of the curve considered to be the quantification range for each analyte with values above LOD and < LLOQ, or > HLOQ (extrapolated values) and values above LOD and within LLOQ and HLOQ (robust values), were used for the data analysis. In addition, sensitivity analyses were performed using only the robust quantification range for each analyte with measurement values above the LOD and within the LLOQ and HLOQ. The concentration of each analyte is given in pg/mL and summarized for all individuals, men and women, respectively (Supplemental Table 2).

2.6. Data analysis of associations between analyte levels and phenotypes

To identify associations between plasma analyte levels and the phenotypes of interest, linear regression models were fitted for each analyte separately. Stepwise linear regression models were performed on $\log_{10}$-transformed data ($\log_{10}(1+$ analyte concentration)) to ensure positivity and reduce skewness of the data. The phenotypes of interest included age, sex (assigned at birth), BMI, and season. The impact of season included as day within the year was implemented as a nonlinear function using the B-Spline bs() function in the R "splines" package (V4.2.2) [21]. Age and BMI were used as linear variables and sex as factor variable (numerically encoded as the number of X-chromosomes) in the model. Technical parameters as the storage time of plasma samples in the biobank ranging from 3198 to 5294 days (dt_biobank), number of batch (1–3, in year 2021–2022) and the order of measurements per day (panel_number_d) explained a high proportion of variance of the analytes (further details see Supplemental methods). To account for this variance introduced by technical parameters, linear regression was performed according to the model: Analyte(x) ~ age + as.factor (sex) + BMI + bs(day_year) + as.factor(panel_number_d) + storage_time + as.factor(batch), which treats the analyte as dependent variables and the phenotypes of interest and technical parameters as independent variables – for each analyte. The final full model included all four phenotypes of interest and technical parameters in the regression model. The aim of these models was to explain the association between analyte levels and basic phenotypes under consideration of

technical parameters in a multivariate model. Two additional sensitivity analyses were performed to further pin down the variation in cytokine levels in the general population.

1. In order to account for a potential impact of an individual's blood cell composition the following blood parameters were selected: leukocytes (Gpt/L), erythrocytes (Tpt/L), hemoglobin (mmol/L), hematocrit, mean erythrocyte volume (fl, mcv), mean hemoglobin content of the erythrocytes (fmol, mch), mean hemoglobin concentration of erythrocytes (mmol/L, mchc), erythrocyte distribution width (%, rdw), thrombocytes (Gpt/L) plt), mean platelet volume (fL, mpv), neutrophils (%), lymphocytes (%), monocytes (%), eosinophils (%), and basophils (%), hereafter referred as blood cell parameters (BCPs). Principal component (PC) analysis was performed on the full set of BCPs to reduce the number of variables in the model (for details see Supplemental Material). Analysis revealed that the first five principal components (PC1-PC5) explained more than ~70 % of variance on the BCPs and were thus included into the model for sensitivity analysis: Analyte(x) ~ age + as.factor (sex) + BMI + bs (day_year) + as.factor(panel_number_d) + storage_time + as.factor (batch) + PC1-PC5.
2. In a second step, we excluded all extrapolated values from the analyses and performed linear regression analysis according to the following equation: Analyte(x) ~ age + as.factor (sex) + BMI + bs (day_year) + as.factor(panel_number_d) + storage_time + as.factor (batch). This was done as an additional sensitivity analysis.

In a further step, SHIP-TREND-0 participants were classified as lean (BMI < 20), normal weight (20 ≥ BMI <25), overweight (25 ≥ BMI <30), and obese (BMI ≥ 30). In binary comparisons, each two groups were included in the regression model to assess the association with cytokine levels. Due to the low number of lean individuals ($N = 22$), we excluded the analysis of the lean group. This resulted in three different regression models (obese vs. normal weight, overweight vs. normal weight, and obese vs. overweight) for each analyte: Analyte(x) ~ age + as.factor(sex) + as.factor(BMI group) + PC1-PC5 + bs(day_year) + as. factor(panel_number_d) + storage_time + as.factor(batch).

The β estimates of the linear regression model represent the magnitude of the change in the dependent variable (Analyte (x)) for a one-unit-change in the independent variable (e.g. age). In addition, the standardized estimate β_{st} was calculated and allows for comparison of effect estimates between variables measured on different units by converting them to a common scale (z-score, according to the R package *lm.beta*). It represents the change in the dependent variable in terms of standard deviations for a one standard deviation change in the independent variable.

The statistical strength of an analyte-phenotype association was assessed by the Benjamini-Hochberg-corrected *p*-value (*p*BH - value) of the corresponding model coefficient corrected for all analytes in a given model. For non-linear season effects, the significance was assessed via a post-hoc Wald-test on the non-linear coefficients of the regression model. Cytokines with more than 80 % undetected, missing values (< lower LOD) – that is, CSF2 (GM-CSF), IL-17F, and CSF1 (M-CSF) - were excluded from the analyses, resulting in the analysis of 44 analytes. For all analyses, an adjusted *p*-value (*p*BH - value) < 0.05 was set as significance level.

In addition, a heatmap was generated using the *ComplexHeatmap* R package [22]. Based on robust and extrapolated values the lower 5th percentile per analyte was determined. From the range of the lower 5th percentile, values were randomly selected to fill the missing values gaps.

In order to get an overview on the correlation pattern among the different analytes, we calculated a pairwise correlation matrix including all 44 analytes used in the regression models. To achieve a maximum comparability with the regression analyses, input values were $\log_{10}$-transformed ($\log_{10}$(1+ analyte concentration)) and only valid or extrapolated measures were included. Pairwise Spearman correlation coefficients were calculated only including the pairwise complete observations resulting in different sample sizes for each correlation pair visualized using the *corrplot* R package [23]. For grouping and highlighting analyte patterns a heatmap was conducted, splitted by rows and columns using k-means clustering of the *ComplexHeatmap* R package [22].

All computational analyses and data visualizations were performed using R (R version 4.2.2 "Innocent and Trusting", R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna: (2017), available online at: https://www.R-project.org/), RStudio (version 2023.06.1, RStudio: Integrated Development for RStudio R, PBC. Boston, MA: (2020). available online at: http://www.rstudio.com/), the *tidyverse* (version 1.3.2) [24], and the *ggstatsplot* R packages [25].

3. Results

In this study, we profiled cytokines in a sub-sample of the population-based cohort SHIP-TREND-0 including 532 men (45.3 %) and 643 women (54.7 %). The age of the participants ranged from 20 to 81 years and did not differ between men and women. The body mass index (BMI) ranged from 17.7 to 53.6 (kg/m^2) (Table 1). The BMI was significantly higher in men compared to women ($p < 0.001$) (Table 1). The mean storage time for biobank samples was 4347 days and did not differ significantly between men and women (Table 1).

3.1. Cytokine levels in the SHIP-TREND-0 population

A total of 47 plasma cytokines, chemokines, and growth factors were quantified using the MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A. Values above the LOD and LLOQ were defined as robust values; values above the LOD and below the LLOQ were calculated and designated as extrapolated, rather than being set as missing. Missing values (below LOD) were excluded from the subsequent analyses. The proportion of robust and extrapolated values per analyte is given in Supplemental Table 1. For the analytes PDGF-AB/BB, PDGF-AA, CCL22, CXCL9, CCL11, CCL2, CXCL10, IL-27, CCL4, IL-18, IL-5, and CX3CL1, IL-12p40, sCD40L, FLT3LG, TNFα, and VEGF-A robust values were obtained in >80 % in SHIP-TREND-0 individuals. A lower detection rate of 40 % to 80 % was observed for the analytes IL-1RA, IL-8, TNFβ, IL-7, FGF2, CXCL1, EGF, IL-15, CCL3, IFN-α2, and CCL7. In contrast, the analytes IL-2, IL-22, IFN-ɣ, IL-10, IL-17E/IL-25, IL-17 A, IL-9, IL-12p70, IL-4, IL-1β, IL-6, CSF3, TGFα, and IL-13 yielded robust values in only 10–40 % of all individuals. The analytes CSF2, IL-17F, and CSF1 were undetectable in more than 80 % of the individuals and were thus excluded from further analyses (Supplemental Fig. 1).

Next, the physiological range of all 44 remaining cytokines, chemokines, and growth factors was determined based on both robust and extrapolated values. Notably, the physiological concentration was highly analyte specific, with median cytokine concentrations ranging from 0.6 to 7820 pg/mL, spanning 4 orders of magnitude. The highest median concentration (conc. >1000 pg/mL) was observed for PDGF-AB/BB, followed by IL-27, and CXCL9 (Supplemental Table 1, Fig. 1).

The analytes PDGF-AA, CCL22, CCL2, CXCL10, and sCD40L were also detected at comparably high concentrations (median conc. > 100 pg/mL). Most analytes, however, showed a median concentration between 10 and 100 pg/mL, among those IL-12p40, IL-13, TNFα, IFN-ɣ, and CCL3. Moreover, our highly sensitive assay enabled the detection of a range of low abundant analytes, e.g. IL-1β, IL-5, IL-6, IL-8, IL-10, and IL-17 A, with median concentrations between 0.6 and 6 pg/mL (Supplemental Table 1, Figure1). Overall, cytokines tended to be analytes of low abundance, whereas chemokines and growth factors were often factors of high abundance.

Several cytokine and chemokine levels showed a large dynamic range in our population-based SHIP-TREND-0 cohort, spanning up to three orders of magnitude, based on robust values only. The

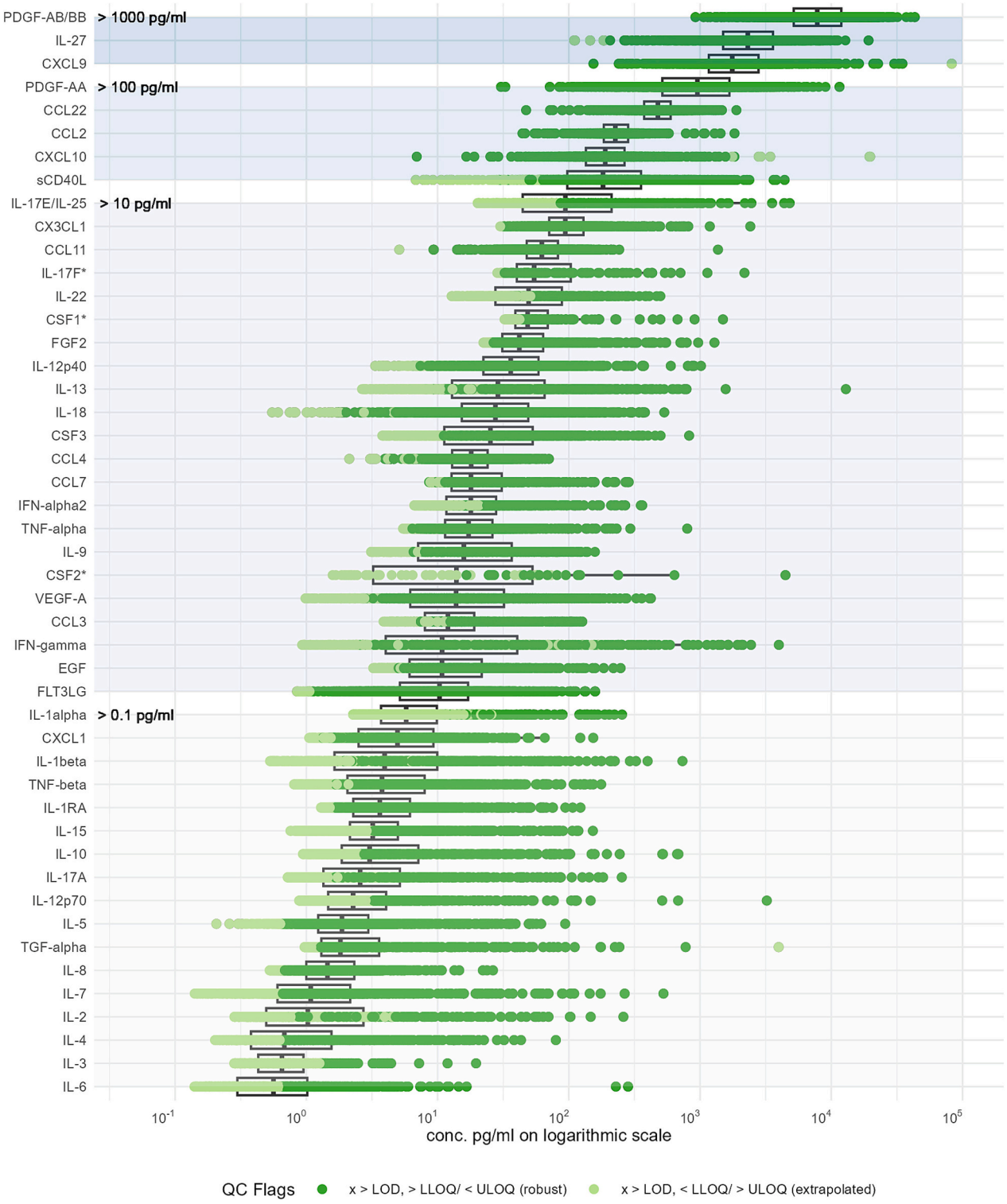


Fig. 1. Cytokine and chemokine levels strongly differ in their physiological range in the SHIP-TREND-0 population. Analytes (y-axis) are ranked by their median plasma concentration based on detected (robust and extrapolated) values on a $\log_{10}$ transformed scale (x-axis). The analytes marked with an asterisk were not detected in more than 80 % of the individuals and excluded from further analyses. Boxes indicate median with interquartile range. Cytokines and chemokines with high (> 1000 pg/mL), moderate (> 100 pg/mL, < 1000 pg/mL), low (> 10 pg/mL, < 100 pg/mL) and very low (> 0.1 pg/mL, < 10 pg/mL) levels are indicated.

interquartile range (IQR) depicts the spread of the data which is less pronounced for e.g. CCL11, CCL2, CCL22, or CCL4 as compared to INF-ɣ, IL-17E/IL-25, or IL-13 (Fig. 1). The highly individual and analyte-specific abundances along with their dynamic range were also visualized in a heatmap, showing the calculated concentration values ($\log_{10}$ scale) for all cytokines and all subjects (Supplemental Fig. 2). A group of cytokines, including PDGF-B, IL-27, CXCL9, and PDGF-A, exhibited high concentrations in the plasma of all SHIP-TREND-0 individuals. In contrast, other cytokines, including IL-13, IL-17E/IL-25, CSF3, CSF1, INF-ɣ, IL-22, CXCL1, IL-1β, IL-17 A, IL-9, and IL-10, exhibited a more pronounced inter-individual variability within the SHIP-TREND-0 population. To investigate correlations between analyte levels, Spearman

correlation was analyzed based on the adjusted concentration values per cytokine and pair, respectively. A hierarchical k-means clustering highlighted 5 clusters of cytokines with pronounced positive correlation (Fig. 2, Supplemental Figs. 3–4). PDGF-A, PDGF-B, VEGF-A (cluster 1), as well as IL-5, IL-9, IL-15, FLT3LG (cluster 2), IL-1RA, CCL3, IL-1α, IL-1β, IL-17 A (cluster 3), IL-2 and FGF2 (cluster 4) and IL-5, IL-9, IL-15, IL-1α, CCL3, and IL-1RA (cluster 5) were strongly correlated with each other.

To conclude, our sensitive Luminex® array enabled the generation of a cytokine atlas for the SHIP-TREND-0 population, covering 44 cytokines, chemokines, and growth factors. Physiological cytokine concentrations were strongly analyte-specific with median concentrations ranging between 0.6 and > 100 pg/mL. Individual cytokine and chemokine levels, however, were highly variable spanning several orders of magnitude.

3.2. Association of plasma cytokine levels with age, BMI, sex, and season

To investigate associations of the 44 analytes with the phenotypes age, sex, BMI, as well as non-linear seasonal effects, linear regression models were fitted for each analyte separately in a stepwise manner. We also accounted for technical variables and blood cell components in these regression models (see section 2.5, Supplemental Tables 3–5). The full models including age, sex, BMI, seasonal effects, and technical covariates were able to explain between 0.1 % and 38.3 % of variance given by the adj.R^2 of the models. Looking at the models with at least one significant association, we saw that in most cases cytokines had associations with more than one predictor (Fig. 3).

A total of 11 cytokines were significantly associated with age (*p*BH - value <0.05) after adjustment for sex, BMI, season, and technical covariates (Fig. 3 model I, Table 2). Older probands had significantly higher levels of the CCL11, CCL2, CCL4, CXCL10, CXCL9, FLT3LG, IL-12p40, IL-27, IL-6, IL-8, and TNFα. Three cytokines were exclusively affected by age (CXCL9, FLT3LG, IL-8). Comparing the age associated analytes, CXCL9 showed the highest standardized effect size with age ($\beta_{stCXCL9} = 0.38$) (Supplemental Fig. 5). A standardized β of 0.38 (Table 2) represents the by far strongest effect on a cytokine for all significant models. The impact of age on CXCL9 levels was approximately twofold higher in comparison to CCL11, CCL2, or CCL4 levels. Further adjustment for blood cell parameter (BCP)-derived PCs (sensitivity analysis, see section 2.5 and Supplemental methods) did not change the significance for the above-mentioned cytokines except for TNFα and IL-6 (Fig. 2, model II, Supplemental Table 6).

Next, we analyzed the association of cytokines with sex. After adjustment for age, BMI, season, and technical covariates, 10 analytes were significantly associated with sex (Fig. 3 model I, Table 2). Females presented with significantly lower levels of CCL11, CCL2, CCL4, IL-18, and TNFα, but harbored higher levels of CCL22, CCL3, IL12p40, IL-1RA, and IL-27. Comparing the sex-associated analytes, CCL11 showed the highest negative effect size ($\beta_{stCCL11} = -0.24$). Assuming other

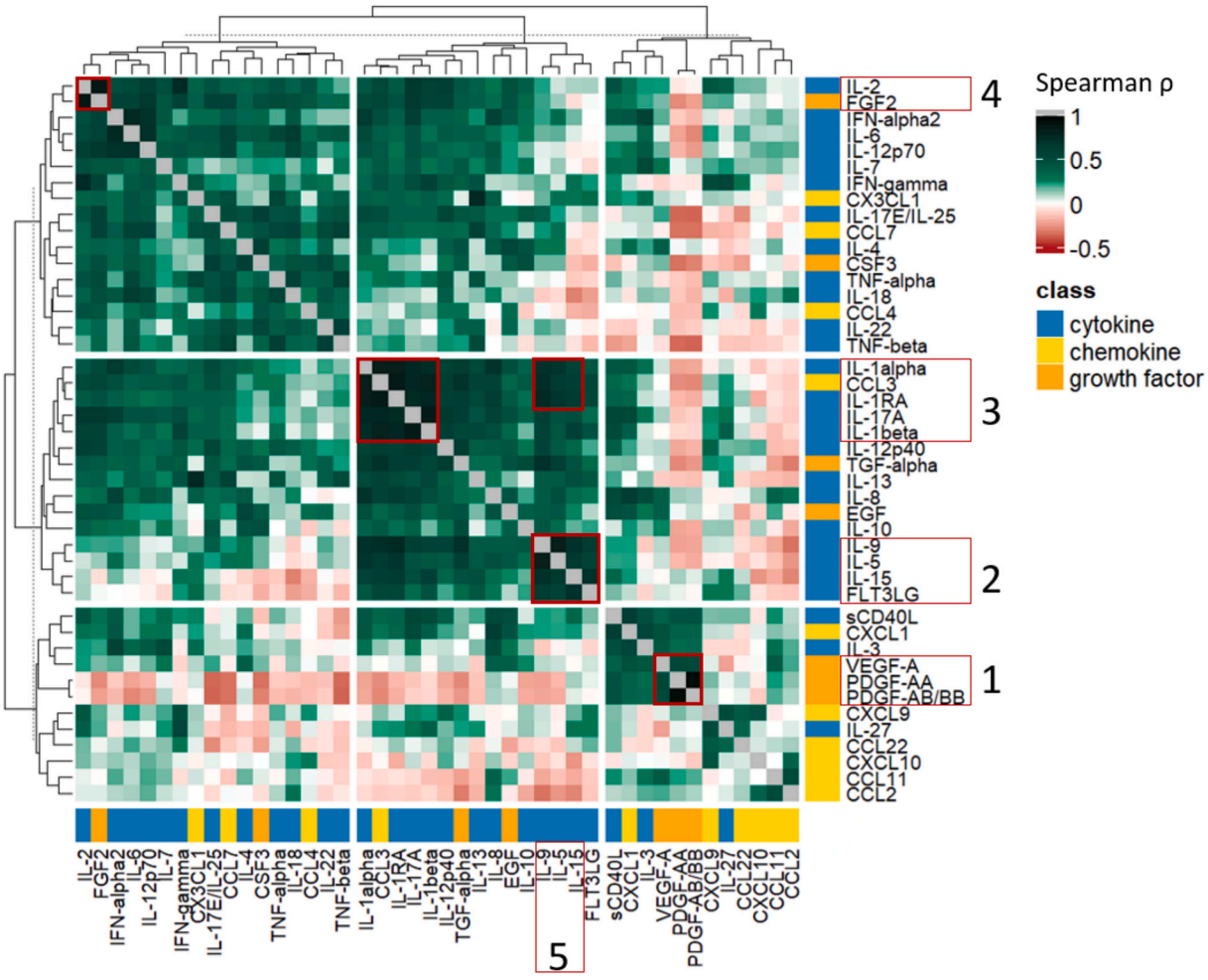


Fig. 2. Strong correlation among small clusters of cytokines, chemokines, and growth factors. A heatmap visualizes Spearman correlation coefficients, split by k-means cluster analysis for the correlating analytes on the x- and y-axis simultaneously to highlight the patterns. Five cluster of analytes were highlighted by red rectangles. The class of each analyte and the direction of the spearman correlation coefficient ρ is color-coded. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

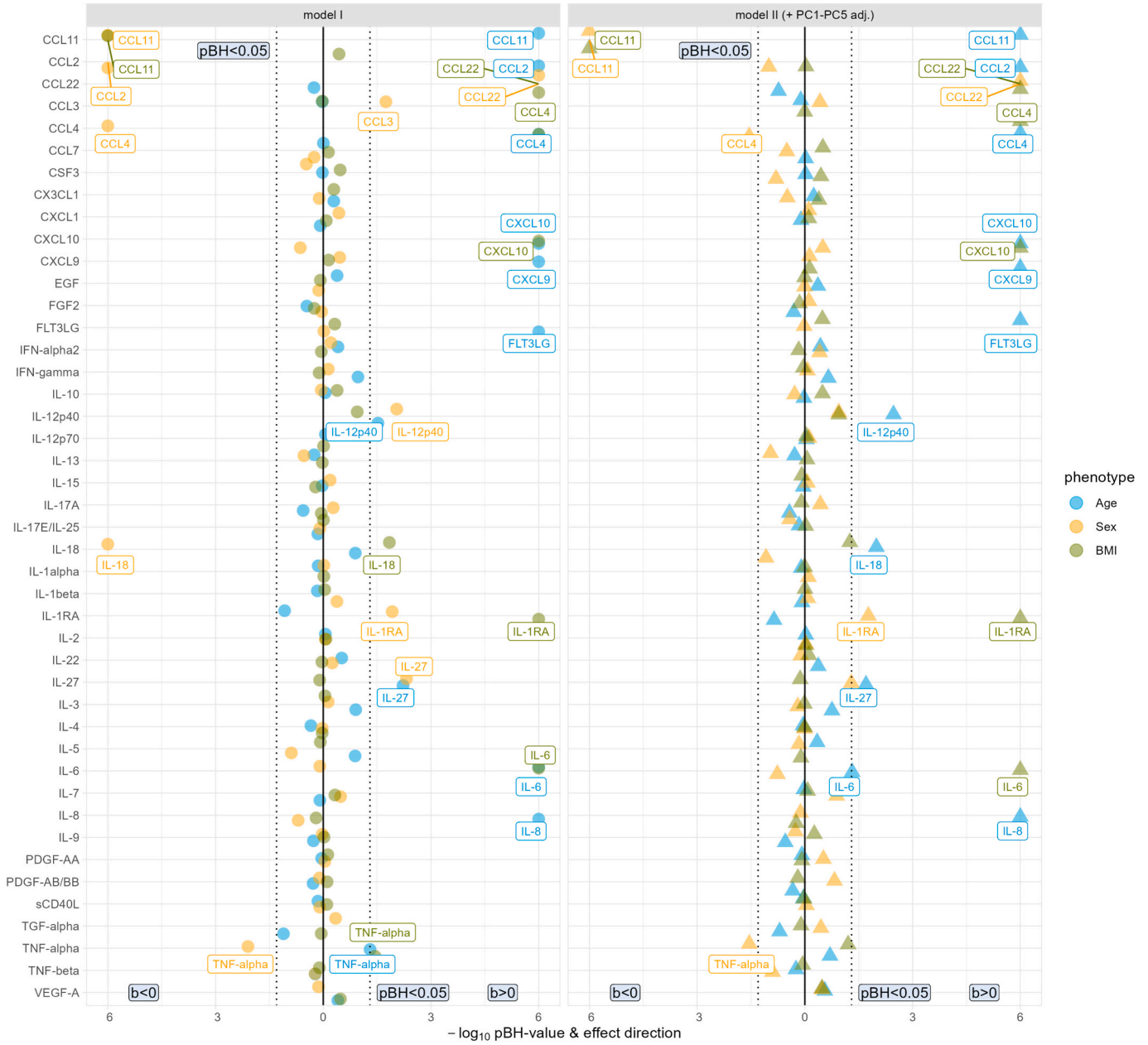


Fig. 3. Plasma cytokine and chemokine levels are associated with age, sex, and BMI. In total, 44 cytokines were analyzed in the SHIP-TREND-0 sample. Age-, sex-, and BMI- associated cytokines were determined before (model I) and after adjustment for the first five principal components (PCs) accounting for blood cell parameters (model II, indicated by triangles). The x-axis displays the level of significance ($-\log_{10}$ *p*-value after Benjamini-Hochberg correction, pBH), along with the effect directions, indicated by β estimate (b > 0: positive, b < 0: negative association). For age and BMI, a positive association (β > 0) indicates an increasing analyte level with older age or higher BMI, while a negative association (β < 0) indicates a decreasing analyte level with older age or higher BMI. Sex has been numerically encoded as the number of X-chromosomes. Hence, positive association (β > 0) indicates an analyte with higher levels in women while negative association (β < 0) indicates an analyte with higher levels in men. Dotted lines indicate the pBH < 0.05. Cytokines that exhibited a significant association with age (blue), sex (yellow), or BMI (green) were labeled accordingly. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

variables were held constant, effects for woman were approximately 0.25 standard deviations below the CCL11 levels for men. (Supplemental Fig. 5, Supplemental Table 2). Further adjustment for BCP-derived PCs (sensitivity analysis) reduced the significance for several cytokines as CCL4, CCL2, CCL3, IL-12p40, IL-27, and IL-18 (Supplemental Table 7). Only CCL3 was exclusively affected by sex.

Afterwards, we investigated whether the BMI was associated with plasma cytokine and chemokine levels. A total of eight analytes were significantly associated with BMI, following an adjustment for sex, age, season, and technical covariates (Fig. 3 model I, Table 2). While CCL22, CCL4, CXCL10, IL18, IL-1RA, IL-6, and TNFα were positively associated with an increasing BMI, CCL11 levels decreased with increasing BMI. Comparing significantly associated analytes, BMI had strong effects on IL-1RA ($\beta_{stIL\text{-}1RA} = 0.19$) and CCL11 ($\beta_{stCCL11} = -0.17$) levels, as indicated by the standardized β estimate. All BMI-associated cytokines also showed associations with either age or sex, demonstrating the strong intercorrelations with both parameters, as BMI is also influenced by sex and age. Sensitivity analysis adding BCP-derived PCs to the model did not alter the aforementioned list of significant analytes except for IL-18 and TNFα (Fig. 3 model II, Supplemental Table 8). For all association analyses, we observed similar association results when the analysis was based on robust values only (Supplemental Tables 6–8).

Table 2
Cytokines significantly associated with age, sex, or BMI[1].

		Age						Sex: Women vs. Men						BMI							
systematic name	common name	β	SE	lower ci	upper ci	pBH	adj.R^2	βst	β	SE	lower ci	upper ci	pBH	adj.R^2	βst	β	SE	lower ci	upper ci	pBH	adj.R^2	βst
CCL11	Eotaxin	**0.003**	**0.000**	**0.002**	**0.004**	**< 0.001**	**0.12**	**0.20**	**−0.089**	**0.010**	**−0.110**	**−0.069**	**< 0.001**	**0.12**	**−0.24**	**−0.007**	**0.001**	**−0.009**	**−0.004**	**< 0.001**	**0.12**	**−0.17**
CCL2	MCP-1	**0.002**	**0.000**	**0.002**	**0.003**	**< 0.001**	**0.09**	**0.19**	**−0.050**	**0.009**	**−0.068**	**−0.032**	**< 0.001**	**0.09**	**−0.16**	0.001	0.001	−0.001	0.003	0.363	0.09	
CCL22	MDC	0.000	0.000	−0.001	0.000	0.552	0.11		**0.053**	**0.010**	**0.034**	**0.072**	**< 0.001**	**0.11**	**0.15**	**0.005**	**0.001**	**0.002**	**0.007**	**< 0.001**	**0.11**	**0.13**
CCL3	MIP-1α	0.000	0.001	−0.002	0.001	0.939	0.00		**0.064**	**0.022**	**0.021**	**0.107**	**0.018**	**0.00**	**0.11**	0.000	0.002	−0.005	0.004	0.939	0.00	
CCL4	MIP-1β	**0.003**	**0.000**	**0.002**	**0.003**	**< 0.001**	**0.16**	**0.19**	**−0.054**	**0.010**	**−0.074**	**−0.034**	**< 0.001**	**0.16**	**−0.14**	**0.006**	**0.001**	**0.004**	**0.009**	**< 0.001**	**0.16**	**0.16**
CXCL10	IP-10	**0.005**	**0.001**	**0.004**	**0.006**	**< 0.001**	**0.10**	**0.26**	−0.024	0.015	−0.053	0.006	0.230	0.10		**0.008**	**0.002**	**0.005**	**0.011**	**< 0.001**	**0.10**	**0.14**
CXCL9	MIG	**0.009**	**0.001**	**0.008**	**0.010**	**< 0.001**	**0.34**	**0.38**	0.021	0.015	−0.008	0.051	0.348	0.34		0.001	0.002	−0.003	0.004	0.708	0.34	
FLT3LG	FLT-3 L	**0.004**	**0.001**	**0.002**	**0.005**	**< 0.001**	**0.12**	**0.15**	0.003	0.021	−0.038	0.043	0.962	0.12		0.003	0.002	−0.002	0.007	0.478	0.12	
IL-12p40	IL-12p40	**0.002**	**0.001**	**0.000**	**0.003**	**0.030**	**0.07**	**0.07**	**0.056**	**0.019**	**0.019**	**0.094**	**0.009**	**0.07**	**0.08**	0.004	0.002	0.000	0.008	0.113	0.07	
IL-18	IL-18	0.001	0.001	0.000	0.003	0.128	0.38		**−0.098**	**0.020**	**−0.136**	**−0.059**	**< 0.001**	**0.38**	**−0.12**	**0.006**	**0.002**	**0.002**	**0.010**	**0.014**	**0.38**	**0.07**
IL-1RA	IL-1RA	−0.001	0.001	−0.003	0.000	0.084	0.05		**0.054**	**0.018**	**0.018**	**0.090**	**0.012**	**0.05**	**0.09**	**0.012**	**0.002**	**0.008**	**0.016**	**< 0.001**	**0.05**	**0.19**
IL-27	IL-27	**0.002**	**0.001**	**0.001**	**0.003**	**0.006**	**0.31**	**0.08**	**0.047**	**0.015**	**0.018**	**0.076**	**0.005**	**0.31**	**0.08**	0.000	0.002	−0.004	0.003	0.795	0.31	
IL-6	IL-6	**0.002**	**0.001**	**0.001**	**0.003**	**< 0.001**	**0.07**	**0.12**	−0.011	0.013	−0.037	0.015	0.802	0.07		**0.006**	**0.001**	**0.003**	**0.009**	**< 0.001**	**0.07**	**0.14**
IL-8	IL-8	**0.002**	**0.000**	**0.001**	**0.003**	**< 0.001**	**0.07**	**0.14**	−0.018	0.011	−0.039	0.003	0.200	0.07		−0.001	0.001	−0.003	0.002	0.632	0.07	
TNFα	TNFα	**0.001**	**0.001**	**0.000**	**0.002**	**0.050**	**0.05**	**0.07**	**−0.048**	**0.016**	**−0.078**	**−0.017**	**0.008**	**0.05**	**−0.09**	**0.004**	**0.002**	**0.001**	**0.008**	**0.036**	**0.05**	**0.08**

[1] Results of the model I according to the formula: Analyte(x) ~ age + as.factor(sex) + BMI + bs(day_year) + as.factor(panel_number_d) + storage_time + as.factor(batch), respectively. For age and BMI, a positive association (β > 0) indicates an increasing analyte level with older age (years), higher BMI (kg/m^2), while a negative association (β < 0) indicates a decreasing analyte level with older age, higher BMI. Sex has been numerically encoded as the number of X-chromosomes (1 = male, 2 = female). Hence, positive association (β > 0) indicates an analyte with higher levels in women while a negative association (β < 0) indicates an analyte with higher levels in men. Abbreviations: β – effect size - corresponds to change in ($\log_{10}$(1+ analyte conc.)) transformed concentration (pg/mL) per unit change in age (year), or BMI (kg/m^2); SE – standard error; pBH – adjusted p-value after Benjamini-Hochberg multiple test correction; ci – confidence interval; adj.R^2: fraction of variance explained by the model adjusted for the number of covariates, β_{st} - standardized estimate - allows for comparison between variables measured on different units by converting them to a common scale; bold - significant results (pBH <0.05).

Finally, we tested whether the season of sampling influenced plasma cytokine and chemokine levels. However, after adjusting for the effects of age, sex, and BMI, we did not observe any cytokines that were significantly associated with season. The sensitivity analysis, based on either only robust values or including the BCP PC1–5 did not change this result.

To directly compare the effects of cytokines and chemokines on age, sex, and BMI, pBH-values and standardized β coefficients were plotted side by side (Fig. 4). Age, sex, and BMI were each associated with changes in the pro-inflammatory chemokines CCL11, CCL4, and TNFα. Further pro-inflammatory cytokines IL-6 and CXCL10 were associated with both age and BMI. The overlap between age and sex was restricted to IL-12p40, IL-27, and CCL2. The analytes IL-1RA, IL-18, and CCL22 were associated with both sex and BMI (Fig. 4). Highly significant cytokine associations were obtained for age (CCL11, CCL2, CCL4, CXCL10, CXCL9, FLT3LG, IL-6, IL-8), sex (CCL11, CCL2, CCL22, CCL4, IL-18) and BMI (CCL11, CCL22, CCL4, CXCL10, IL-1RA, IL-6). However, according to the standardized β coefficients, the largest effect sizes were observed for CXCL9, followed by CXCL10 and CCL11 (all with age) (Fig. 4).

To conclude, linear regression models demonstrate that a total of 15 cytokines and chemokines are significantly associated with age, sex, or BMI, with a pronounced overlap between the groups reflecting biological interactions between predictor variables. The standardized effect sizes β differ for the individual analytes and between the respective phenotypes. The most pronounced effects were seen for age followed by moderate effects for sex and BMI.

3.3. Cytokines and chemokines with different levels in BMI subgroups

Next, we investigated the association of cytokine and chemokine levels with BMI in more depth, performing group-wise comparisons of obese vs. normal weight, obese vs. overweight, and overweight vs. normal weight subjects. While cytokine and chemokine levels did not differ between normal weight and overweight subjects (except for CCL3), obese subjects showed an analyte pattern that differed strongly from both these groups. In detail, obese subjects (N = 451) presented with increased levels of CCL4, CCL22, CXCL10, IL-1RA, and lower levels of CCL11 as compared to normal weight subjects (N = 294) (Fig. 5).

Similarly, obese (N = 451) subjects had higher levels of CCL4, CCL22, IL1RA, and IL-12p40 as well as FLT3LG compared to overweight (N = 408) individuals (Table 3, Fig. 5). Again, CCL11 levels were significantly lower in obese compared to overweight individuals. Box plots of these cytokines and chemokines, however, illustrate that the interindividual differences are much stronger than the differences between the BMI groups (Fig. 6).

A sensitivity analysis based on extrapolated and robust values adjusted for BCP-derived PCs (model II), or robust analyte values only (model III, IV) largely confirmed our findings (Supplemental Fig. 6).

4. Discussion

In the present study, we quantified a total of 44 blood plasma analytes including 28 predominantly pro-inflammatory cytokines (lymphokines, interferons, colony stimulating factors), ten chemokines, and six growth factors in 1175 SHIP-TREND-0 individuals representing the adult general population. Moreover, we evaluated associations with age, sex, BMI, and season, while accounting for the effects of blood cell composition and technical variation. This comprehensive analysis provided an overview of the normal cytokine variation in the general population, which will be helpful in evaluating the results of future longitudinal studies on the onset of common diseases.

Our data revealed that the physiological concentrations were highly variable between analytes with median cytokine concentrations ranging from 0.6 to 7820 pg/mL, spanning 4 orders of magnitude. This underscores the importance of establishing physiological reference levels

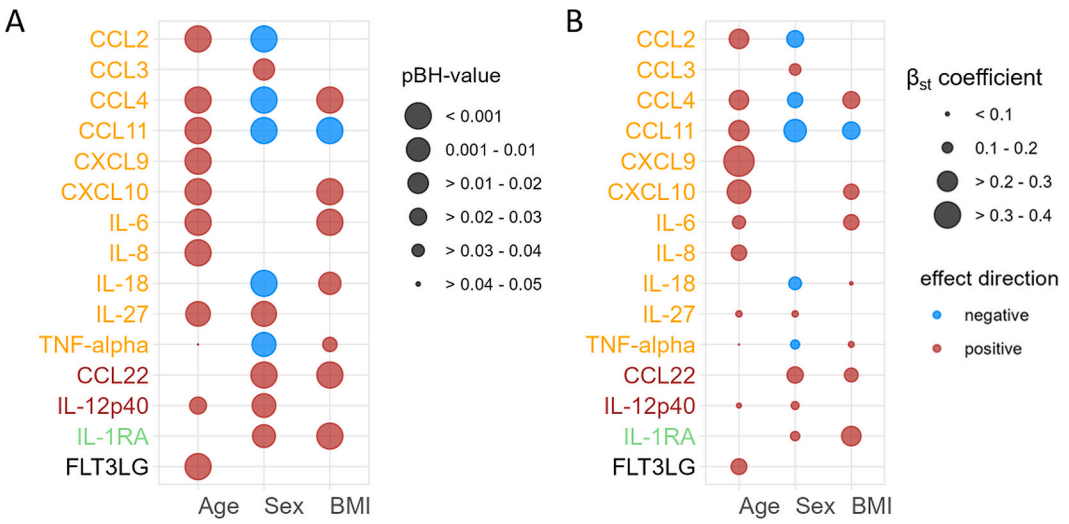


Fig. 4. Pronounced overlap between age, sex, and/or BMI-associated plasma cytokines and chemokines in the SHIP-TREND-0 population. A) Significance and B) standardized β coefficients are given per cytokine associated with increasing age, increasing BMI, and/or sex (women vs. men) according to model I, respectively. Effect direction (negative/positive) of the association is color-coded. Analytes are color-coded according to their pro-inflammatory (orange), anti-inflammatory (green), pro- and anti-inflammatory (brown), or unknown (black) role (according to Borish & Steinke et al., 2003, Hunter & Jones, 2015) [1,26]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

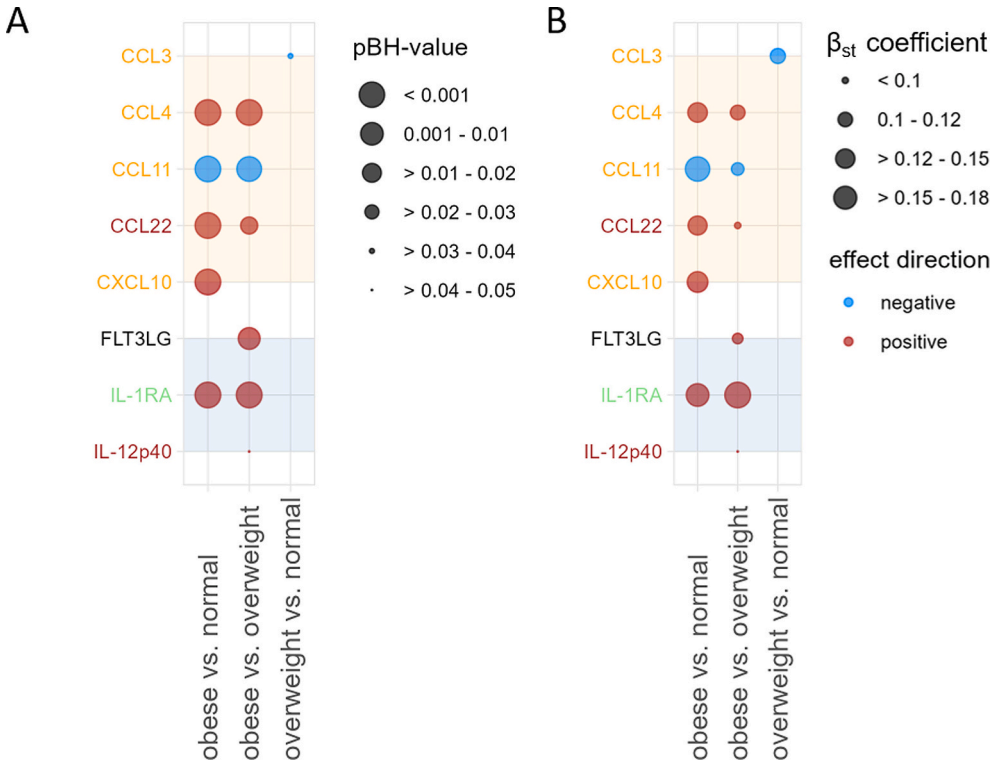


Fig. 5. Obese subjects have distinct cytokine and chemokine levels compared to overweight and normal subjects in the SHIP-TREND-0 population. A) Significance and B) standardized β coefficient (β_{st}) are given per cytokine, according to model I, respectively. Effect direction (negative/ positive) of the association is color-coded. Analytes are color-coded according to their pro-inflammatory (orange), anti-inflammatory (green), pro- and anti-inflammatory (brown), or unknown (black) role (according to Borish & Steinke et al., 2003, Hunter & Jones, 2015) [1,26]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for each analyte in population-based or disease cohorts. The interindividual variability of the individual cytokines, chemokines, and growth factors was also very pronounced, spanning up to three orders of magnitude, based on robust values only. Further studies are needed to investigate host genetic and environmental factors that drive this diversity.

Interestingly, our analyses also revealed several clusters of cytokines and chemokines that were positively correlated with each other. While some of these associations are well described in the literature, others require further studies to clarify common pathways and causalities. For example, PDGF-A, —B and VEGF-A (cluster 1) are all endothelial growth factors, that promote endothelial cell proliferation, cell survival, cell

Table 3
Differences in plasma cytokines and chemokines in obese vs. normal weight, obese vs. overweight, and overweight vs. normal weight individuals, respectively[1].

		obese vs. normal weight (ref.)						obese vs. overweight (ref.)						overweight vs. normal weight (ref.)						
systematic name	common name	β	lower ci	upper ci	SE	βst	pBH	adj.R^2	β	lower ci	upper ci	SE	βst	pBH	adj.R^2	β	lower ci	upper ci	SE	βst	pBH	adj.R^2
CCL11	Eotaxin	**−0.072**	**−0.100**	**−0.043**	**0.014**	**−0.195**	**< 0.001**	**0.125**	**−0.041**	**−0.066**	**−0.016**	**0.013**	**−0.111**	**0.003**	**0.120**	−0.028	−0.057	0.000	0.014	−0.076	0.141	0.126
CCL22	MDC	**0.053**	**0.025**	**0.080**	**0.014**	**0.149**	**< 0.001**	**0.102**	**0.031**	**0.008**	**0.055**	**0.012**	**0.090**	**0.024**	**0.106**	0.018	−0.008	0.045	0.013	0.052	0.261	0.117
CCL3	MIP-1alpha	−0.017	−0.080	0.046	0.032	−0.028	0.709	0.007	0.054	0	0.109	0.028	0.090	0.208	−0.003	**−0.073**	**−0.129**	**−0.017**	**0.028**	**−0.124**	**0.040**	**0.031**
CCL4	MIP-1beta	**0.059**	**0.030**	**0.087**	**0.015**	**0.152**	**< 0.001**	**0.167**	**0.045**	**0.02**	**0.07**	**0.013**	**0.121**	**< 0.001**	**0.134**	0.017	−0.013	0.047	0.015	0.043	0.396	0.118
CXCL10	IP-10	**0.087**	**0.045**	**0.128**	**0.021**	**0.161**	**< 0.001**	**0.117**	0.045	0.008	0.081	0.019	0.085	0.072	0.068	0.048	0.006	0.089	0.021	0.088	0.067	0.091
FLT3LG	FLT-3 L	0.001	−0.059	0.062	0.031	0.002	0.972	0.109	**0.073**	**0.023**	**0.124**	**0.026**	**0.102**	**0.012**	0.110	−0.058	−0.114	−0.002	0.029	−0.079	0.106	0.142
IL-12p40	IL-12p40	0.044	−0.012	0.099	0.028	0.063	0.207	0.064	**0.056**	**0.01**	**0.103**	**0.024**	**0.086**	**0.041**	**0.085**	−0.025	−0.079	0.028	0.027	−0.037	0.554	0.060
IL-1RA	IL-1RA	**0.108**	**0.055**	**0.161**	**0.027**	**0.178**	**< 0.001**	**0.033**	**0.124**	**0.079**	**0.169**	**0.023**	**0.207**	**< 0.001**	**0.084**	−0.014	−0.063	0.034	0.025	−0.025	0.749	0.013

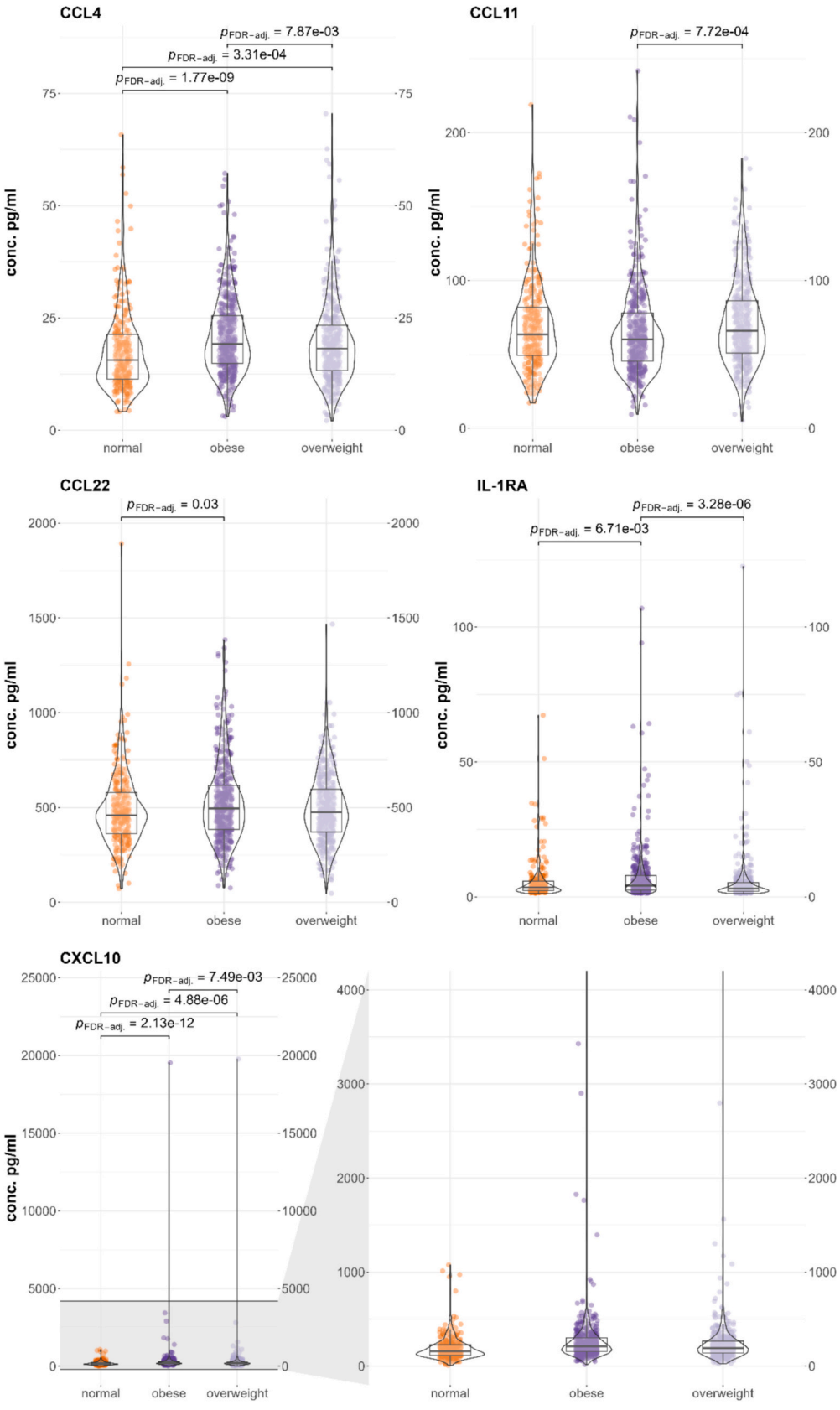
[1] Results of the model according to the formula: Analyte(x) ~ age + as.factor(sex) + as.factor(BMI group) + PC1-PC5 + bs(day_year) + as.factor(panel_number) + storage_time + as.factor(batch), respectively. Here, vs. indicates the reference (ref.) as the group of normal weighted individuals or overweight individuals, respectively. Abbreviations: β – estimate, SE – standard error, β_{st} – standardized β coefficient, pBH – adjusted p-value after Benjamini-Hochberg multiple test correction, ci – confidence interval., adj.R^2: fraction of variance explained by the model adjusted for the number of covariates, bold - significant results (pBH <0.05).

migration, and angiogenesis. They are stored in platelets and get released upon platelet activation, which explains the strong correlation between them [27]. In contrast, the strong association between the T cell mitogen IL-2 and the fibroblast growth factor (FGF-2) (cluster 4) seems surprising as there is no direct canonical signaling pathway linking these two factors. Clusters 2 and 5 contain cytokines that are primarily associated with type 2 immunity, i.e. IL-5 and IL-9 [28]. Both cytokines are released by activated Th2 cells and innate lymphoid cells type 2 (ILC2) in the context of allergic inflammation, helminth infections, but also wound healing, and tissue remodeling [29]. IL-9 can enhance mast cell expression of several cytokines, including IL-5 [28]. Further research, however, is required to elucidate the interactions between IL-5/IL-9 and IL-15, which is a critical factor for the survival, proliferation, and activation of NK cells and memory T cells [30], and FLT3LG, a growth factor for hematopoietic stem cells. Clusters 3 and 5 both contain several members of the IL-1 family, i.e. IL-1α, IL-1β, and IL-1RA. Although expressed and activated by different mechanisms, IL-1α and IL-1β share the same receptor, IL-1R1, and therefore have similar, pronounced pro-inflammatory effects [31] They initiate the early recruitment of immune cells, but also induce fever, vasodilation and hypotension. IL-1α, IL-1β, IL-1RA are tightly connected with CCL3 and IL-17 A (cluster 3) in the regulation of acute and chronic inflammatory responses. IL-1α and IL-1β promote the production of CCL3, which in turn recruits granulocytes to the site of infection. In conjunction with IL-23, IL-1 β induces the activation of ILC3 and maturation of Th17 cells, both of which release IL-17 A as their key effector cytokine [32,33]. The IL-1 receptor antagonist IL-1RA prevents the binding of IL-1α or IL-1β to their receptor [34] and thus serves to dampen this inflammatory cascade and restore balance. In consequence, the cluster 3 cytokines are tightly connected in the regulation of acute and chronic inflammatory responses. In contrast, the relationship between IL-1α, IL-1β, and IL-1RA and the type 2 cytokines IL-5 and IL-9 (cluster 5) is less obvious and requires further investigation. In the near future, the interpretation of cytokine networks such as the one presented here might be facilitated by further large-scale cytokine profiling studies in national cohorts using e.g. proximity extension assays (Olink, Somalogic) as well as by single cell RNAseq data.

To better understand and integrate our findings at the biological level, we thoroughly analyzed associations of the chemokines and cytokines with age, sex, BMI, and season and compared the results with recently published findings from studies of large population-based cohorts as well as smaller disease groups.

It is known, that aging is associated with changes in the levels of various cytokines, leading to a chronic low grade inflammation known as “inflammaging” – a consequence of immunosenescence [11,35,36]. In our SHIP-TREND-0 population, we observed higher plasma levels of predominantly pro-inflammatory cytokines and chemokines such as CCL11, CCL2, CCL4, CXCL9, CXCL10, FLT3LG, IL-27, IL-6, IL-8, IL-12p40, and TNFα with increasing age, which is largely in agreement with recently published or reviewed data [37–44]. For instance, IL-6, and tumor necrosis factor-α (TNFα) are well-known pro-inflammatory cytokines associated with aging [11]. High levels of these cytokines in older individuals are linked to increased risk of morbidity and mortality, frailty, sarcopenia, cardiovascular events, and neurodegenerative disorders such as Alzheimer’s disease [11,35,36,45]. CXCL9 was identified as a major contributor to the inflammatory clock “iAge” score, which is related to systemic age-related inflammation [43]. Other reported age-associated cytokines (members of the IL-1 family, IL-17 A, IL-17F, IL-12p70, IL-18, and anti-inflammatory cytokines as IL-1RA, and IL-10) did not match our observations or were excluded (IL-17F) from analysis due to high rate of missing values [11,37,46]. The pattern of cytokines in our SHIP-TREND-0 study population might differ from others due to differences in the population structure (ethnicity), in the proportion of underlying comorbidities (our subsample included e.g. only four cases of diabetes, and 522 cases of hypertension) or assay sensitivity.

We observed a strong overlap between significant age- and BMI-associated chemokines e.g. CCL11, CCL4, CXCL10, and cytokines as IL-6



(caption on next page)

Fig. 6. Median plasma concentrations of the significantly altered cytokines and chemokines in normal, overweight, and/or obese individuals. Analyte concentrations are plotted on a linear scale. The graph depicts median, IQR, min/max and distribution of values by violin plot for each analyte. Statistically significant pairwise differences are depicted (posthoc Dunn test, p_{FDR} – Benjamini-Hochberg corrected p-value). Significant alterations are most pronounced for CCL4 concentrations between normal and overweight BMI and overweight and obese BMI, respectively. Similarly, median IL-22, IL-1RA and CXCL10 plasma levels are significantly higher in obese vs. normal subjects. CCL11 shows a significant lower median level in obese vs. overweight individuals. The area containing most values for CXCL10 has been enlarged to clearly visualize the differences between the groups (lower right corner). A sensitivity analysis based on robust values only did not change the results. (Supplemental Fig. 6, model III).

and TNFα. The dysregulation of the cytokine network and its homeostasis, particularly the increased secretion of cytokines by adipose tissue, is one of the main causes of chronic inflammation at older age. Hence, the effect of age might be mixed with weight gain in older age, accompanied by an increase in adipose tissue which in turn contributes to the production of cytokines and chemokines [47]. Our findings on age-associated proinflammatory cytokines/ chemokines in plasma resemble the senescence-associated secretory phenotype (SASP) signature, which is induced by a variety of cellular stresses [48]. We observed an increase with age and BMI for proinflammatory SASP chemokines/ cytokines (IL-6, CCL4, CXCL10) in the circulation, which individually and/or cumulatively promote persistent inflammation. Notably, the expression of SASP chemokine CCL11, also described as an "Accelerated Brain-Aging Chemokine" (ABAC), increased only with aging. CCL11 is associated with reduced neurogenesis, neuroinflammation, and neurodegeneration, among others [41]. Our study population shows an overlap to SASP-derived signature, which may mediate further aging and age-related diseases [49].

In order to test whether the changes in the cytokine pattern with age are influenced by changes in the quantity of cytokine-releasing circulating blood cells, we adjusted our model for the BCP-derived first five principal components. The significant association of the cytokines IL-6, and TNFα disappeared, which fits to the fact that these molecules are primarily produced by macrophages [1]. However, since only few age-associated cytokines were affected by adjustment for BCP, it can be concluded that blood cell parameters exert only a limited effect on age-dependent circulating plasma cytokine levels. This finding underscores the importance of considering the activation state of cells and additional cellular cytokine sources, such as lymphoid organs, fibroblasts, adipose tissue, endothelial cells, or hepatocytes, in future research [1].

The strength of the immune response is also related to sex [50]. In our analysis, we investigated sex (assigned at birth)-associated chemokines and cytokines in our SHIP-TREND-0 sample. Women presented with higher levels of CCL3, CCL22, IL-1RA, IL-12p40, and IL-27, but lower levels of CCL11, CCL2, CCL4, IL-18, and TNFα. Rather few studies exist on plasma circulating cytokine level stratified for sex [16]. In a cohort suffering from coronary heart disease circulating IL-18 levels were determined and found to be lower in women compared to men [11]. Our data suggests that IL-18 levels are generally slightly lower in women - independent from underlying disease or health status. Our findings are further supported by lower female CCL11 levels determined in age-matched women compared with men in allergic and healthy control groups [40]. In contrast, our study did not confirm a previous report indicating higher levels of sex-associated cytokines, including IL-1β, IL-6, and IL-7 in men compared to women [51]. An explanation for the different results might be the lower number of individuals ($N = 104$), younger mean age and simple statistical models used in the study of Bernardi et al [51] In the present study, we used multivariate regression models jointly adjusting for age, BMI, season, and technical variation and found circulating cytokines and chemokines with significant different levels between men ($N = 532$) and women ($N = 643$). Several factors might contribute to this observation. It is known, that the functioning of the immune system is influenced by sex chromosome genes, hormones such as estrogens, progesterone, and androgens as well as environmental factors [50,52]. Further, nutrition and the composition of the microbiome contribute and alter the development and functioning of the immune system differently in males and females [50]. Moreover, men and women display differential susceptibility to e.g. autoimmune diseases or infectious diseases [50,52,53]. Hence, stratified analysis for sex should be considered in future research.

Finally, obesity is a major public health problem and increased adiposity is often assessed using BMI. In the SHIP-TREND-0 population, pro-inflammatory cytokines were found to be altered with increasing BMI and are in line with the current literature. In detail, the pro-inflammatory cytokines CCL4, CCL22, CXCL10, IL-6, IL-18, TNFα, and the anti-inflammatory IL-1RA were positively, while CCL11 was negatively associated with BMI. These findings are consistent with the results reported by the Finnish cross-sectional population studies YFS and FINRISK [54], except for CCL22, which was not included in the Finish cytokine panels, and TNFα, which did not reach significance in their study. A recently published sex-stratified analysis of a Brazilian population cohort ($N = 743$) also described IL-6 as positively associated with BMI in both men and women and TNFα only in men [16]. In contrast to our study, the predominant anti-inflammatory cytokines IL-10, and pro-inflammatory IL-12p70, IL-13, IL-7, IL-8, as well as CCL2, and VEGF-A showed a significant positive association with BMI in the YFS and FINRISK studies [54]. In the Brazilian cohort, IL-12p70, IL-10, IL-8, and IL-1β showed a positive association with BMI [16], a finding that could not be replicated in the SHIP-TREND-0 population. Besides ethnic or genetic differences, underlying comorbidities may also contribute to the discrepancy.

In addition to the linear regression analysis between cytokine levels and BMI, SHIP-TREND-0 individuals were grouped in normal weighted, overweight, and obese to identify differences in cytokine levels between the different BMI severity stages. The full model analysis again showed that the pro-inflammatory chemokines CXCL10, CCL4, the pro/anti-inflammatory CCL22 and the anti-inflammatory IL-1RA were significantly higher in obese compared to normal individuals. Of those, circulating CCL22 was also reported to be correlated with increasing BMI and elevated in morbidly obese patients compared with controls [55]. Furthermore, in line with our results, the same group observed an elevated level of CXCL10 - also known as interferon-γ inducible protein 10 (IP-10) – and linked those cytokines to impaired adipose tissue angiogenesis in obese individuals [56]. Elevated IL-1RA levels as observed in our study, were previously reported to be elevated in obese individuals, and were suggested as a biomarker of adipose tissue dysfunction in obesity-associated metabolic alterations [57]. Hence, potential biomarkers of obesity and adipose tissue dysfunction were replicated in our subsample of the general population-based study SHIP-TREND-0.

Interestingly, our study revealed significantly lower CCL11 levels in obese compared to normal weighted SHIP-TREND-0 individuals. In line with this result, our linear regression approach also revealed a significant negative association with BMI, and a similar effect was also reported by the YFS and FINRISK cohort study [54]. In contrast, CCL11 levels were described as elevated in a small sample of obese compared ($N = 40$) to normal patients ($N = 13$) [58]. A further study among healthy Saudi Arabian subjects revealed that CCL11 levels were elevated in obese women ($N = 16$), but remained within the normal range in obese men ($N = 15$) [59]. In our study, all phenotypes contribute to the CCL11 level and underline the need for age- and sex-matched investigation in larger cohorts and investigation of sex and age interactions. A further reason for opposite directed or missing BMI-associated cytokines might also be the variability in the metabolic health status among individuals presenting with obesity; some may be metabolically healthy, while others may have developed the metabolic syndrome, a cluster

including insulin resistance, hypertension, dyslipidemia, and increased risk of cardiovascular disease and type 2 diabetes [60]. Our population-based study included individuals with hypertension ($N = 522$) but only few with known diabetes (N = 4).

The comparison of obese versus overweight individuals confirmed altered levels of aforementioned cytokines and revealed in addition IL-12p40 and FLT3LG with significantly higher levels in obese individuals. In line with our results, IL-12p40 circulating plasma level was found to be elevated in a Mexican study comparing obese and overweight individuals with normal controls [61]. All of those aforementioned chemokines and cytokines were observed and described in the context of obesity, diabetes, or vascular reactivity [55,56,62–67].

Interestingly, in our study FMS-Like tyrosine kinase 3 ligand (FLT3LG) was found to be elevated in obese compared to overweight individuals only. However, both IL-12p40 and FLT3LG did not show a significant association with BMI in the linear regression approach and this might point to non-linear relations with increasing BMI. Here, stages of obesity –marked by BMI - are linked with increased levels of pro-inflammatory cytokines in a linear association.

4.1. Study limitations

The assessment of plasma cytokines is challenging given that these factors are typically present at very low levels in blood samples and, hence, require highly sensitive technologies for their detection. In this study, we employed the highly sensitive MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A – Immunology Multiplex Assay PC48 with a minimal detectable concentration of 1–10 pg/mL for most analytes and 0.1–1 pg/mL for a range of cytokines (Supplemental Table 1). Hence, our study is very sensitive, but nevertheless, several analytes could only be detected in less than 40 % of the samples. The plasma levels of CSF1, CSF2, and IL-17F were below the detection levels in more than 80 % of individuals, and thus excluded from the subsequent analyses. The low detection rate for CSF2 was also noted in the FINRISK studies. The authors similarly excluded cytokines with >90 % missing values from the analysis (CSF2, INF-α 2, LIF, IL-1 α, IL-3, IL-15, IL-12p40) [54].

This study did not measure analytes in duplicates, but assay accuracy was addressed in a parallel approach for a smaller sample (paired CV < 20 %, Supplemental methods). To assess this limitation and ensure the robustness of the results, we performed an additional sensitivity analysis excluding the extrapolated values for all 1175 individuals, which largely confirmed the previously obtained results.

Our analyses focused on circulating cytokines and associations with age, sex, BMI, and season, respectively. The influence of comorbidities or medication on the results was not analyzed in this study. This could be a topic for further investigation in future studies. Significant non-linear seasonal effects were not detected by our approach and might be addressed by monitoring individuals in a longitudinal approach with several time points over the year.

4.2. Outlook

As the BMI is influenced by several factors, a follow-up study using other BMI-related anthropometric measures such as body fat or waist-to-hip ratio is planned. In addition to extensive characterization of individuals through questionnaires and medical examinations, information on non-heritable factors, data from magnetic resonance imaging (MRI), genetic data as well as other omics data such as transcriptomics of blood cells, plasma proteomics and plasma-circulating miRNA are available for individuals in the SHIP-TREND-0 population and may provide further valuable insights into cytokine variability, cytokine associations across different biological domains and regulatory mechanisms. Moreover, such comprehensive data might be of value in validating cytokine-related analyses in other cohorts. In addition, circulating cytokine levels from the follow-up SHIP-TREND examination (SHIP-TREND-1) were measured in parallel for a subset of individuals, allowing for longitudinal analysis.

5. Conclusion

In the present association study, we generate for the first time a comprehensive cytokine atlas for the general population SHIP-TREND-0. Average cytokine levels strongly differed between analytes, and individual cytokine levels were highly variable. We demonstrate that plasma cytokine profiles in the general population are associated with sex, age, and BMI. Further, our population-based study confirmed and extended pro-inflammatory cytokines associated with BMI pointing to changes in cytokine levels associated with different stages of obesity, which might help to recognize the onset of pathological changes as a basis for subsequent studies of population-relevant diseases. In addition, for some cytokines blood cell parameters should be considered as potential confounders in association studies.

Data statement

The research data for this article is available upon reasonable request.

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CRediT authorship contribution statement

Sabine Ameling: Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Sandra Van der Auwera:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Silva Holtfreter:** Writing – review & editing, Validation, Investigation. **Anja Wiechert:** Writing – review & editing, Investigation, Data curation. **Stephan Michalik:** Writing – review & editing, Validation, Methodology. **Nele Friedrich:** Writing – review & editing. **Elke Hammer:** Writing – review & editing. **Henry Völzke:** Writing – review & editing, Funding acquisition. **Matthias Nauck:** Writing – review & editing, Resources. **Hans J. Grabe:** Writing – review & editing, Resources, Funding acquisition. **Barbara M. Bröker:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Uwe Völker:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2025.156896.

Data availability

The authors do not have permission to share data.

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