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Biological Determinants of and Reference Values for Plasma Interleukin-8, Monocyte Chemoattractant Protein-1, Epidermal Growth Factor, and Vascular Endothelial Growth Factor: Results from the STANISLAS Cohort

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Background: **Interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) are known to be involved in various diseases related to inflammation, vascular remodeling, or growth deregulation. In addition, increases in plasma concentrations of these cytokines appear to provide useful diagnostic and prognostic information. We therefore investigated which factors most strongly influence the biological variations of plasma IL-8, MCP-1, EGF, and VEGF concentrations.**

Methods: **We used the Evidence® biochip array analyzer to quantify plasma IL-8, MCP-1, EGF, and VEGF concentrations in a subsample of 304 children (age range, 4–17 years) and 540 adults (age range, 18–55 years) from the STANISLAS family study. We also calculated reference intervals for the 4 cytokines.**

Results: **We found the following associations with plasma marker concentrations: Age, neutrophil count, and glucose concentration were positively associated with IL-8 concentrations in children and adults, as were smoking and platelet count in adults. MCP-1 concentrations were associated with age and smoking in both children and adults, monocyte count in children, and sex and hematocrit in adults. EGF concentrations were associated with platelet count in children and monocyte count and glucose in adults. VEGF concentrations were associated with age in children and adults and platelet count and alanine aminotransferase activity in adults.**

Conclusion: **Our results for IL-8, MCP-1, EGF, and VEGF may be useful for interpretation of patients' laboratory results and for understanding the regulation of concentrations of these cytokines in physiologic conditions.**

The inflammation process is known to be involved in many diseases. Cytokines, which are effectors of inflammation, are found in high concentrations in blood and at sites of inflammation. Chemokines and growth factors are 2 types of cytokines that are involved in inflammation.

The chemokines interleukin-8 (IL-8)[3] and monocyte chemoattractant protein-1 (MCP-1) belong to the CXC and the CC chemokine subfamilies, respectively. In vitro and in vivo at sites of inflammation, IL-8 attracts neutrophils and MCP-1 attracts monocytes *(1, 2)*. These 2 chemokines also play important roles in atherosclerosis pathogenesis *(3, 4)* and autoimmune and inflammatory diseases *(5, 6)*. Blood concentrations of IL-8 are increased in patients with autoimmune diseases *(7)*, and MCP-1 concentrations are increased in patients with Alzheimer disease or myocardial infarction *(8, 9)*.

Epidermal growth factor (EGF) and vascular endothe-

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[3] Nonstandard abbreviations: IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; AST, aspartate aminotransferase; and ALT, alanine aminotransferase.

lial growth factor (VEGF) are chemoattractants for monocytes and therefore are involved in physiologic and pathologic tissue growth and vascular remodeling as well as inflammation *(10–13)*. Several studies reported possible involvement of EGF in atherosclerosis and many cancers *(12, 14)*. Moreover, VEGF may be involved in arteriosclerosis *(15)* and, because it stimulates adult neurogenesis, in neurodegenerative disorders *(16)*. VEGF concentrations are also increased in several kinds of cancers *(17)*.

Because IL-8, MCP-1, EGF, and VEGF may be used as diagnostic biochemical markers, the main factors influencing the biological variation of these markers and adequate reference values must be established. Most of the available data were derived from case–control studies or from patients undergoing drug therapies, and little is known about what factors influence the biological variation of these markers in a physiologic state *(18–20)*. In addition, reference intervals have not been established. We therefore investigated what factors were most related to the biological variation of plasma IL-8, MCP-1, EGF, and VEGF concentrations and determined reference values for these cytokines in samples from apparently healthy participants in the STANISLAS family study.

Materials and Methods

PARTICIPANTS AND DATA COLLECTION

This work was carried out on a subsample of 304 children (age range, 4–17 years) and 540 adults (age range, 18–55 years) of the STANISLAS family study *(21)*. Participants were of French origin, were free from acute or serious diseases, and were not being treated with lipid-lowering, antihypertensive, antiinflammatory, or antidiabetic drugs. Additional exclusion criteria were aspartate aminotransferase (AST), alanine aminotransferase (ALT), or γ-glutamyl transferase activities >200 U/L; orosomucoid or haptoglobin concentrations >3 g/L; C-reactive protein >30 mg/L; cholesterol or triglyceride concentrations >10 mmol/L; or a glucose concentration >8 mmol/L. Each participant or participant's parent or legal guardian gave written informed consent for participation, and the study was approved by the local ethics committee of Nancy (France).

Data were collected by use of questionnaires that included questions about lifestyle, such as tobacco, alcohol, and drug consumption, and personal medical history. In addition, physical examinations and functional tests were performed, and basic blood constituents were measured as described previously *(22)*.

BLOOD SAMPLES AND ANALYTICAL METHODS

Venous blood samples were collected by venipuncture after an overnight fast. Sodium EDTA-plasma was separated by centrifugation at 2000*g* for 15 min at 4 °C and stored at −196 °C in liquid nitrogen until analysis.

The analytes of interest were quantified by Randox, Ltd. (Crumlin, UK) with a biochip array analyzer, the Evidence®. The biochip used consists of a 9 × 9 mm substrate on which discrete test regions have been constructed. The binding ligands (antibodies) are attached to predefined sites on the chemically modified surface of the biochip. After a simple ELISA procedure, each spot is imaged to capture chemiluminescent signals generated at each spot on the array. The light signal is captured by a charge-coupled device camera as part of an imaging station and converted by image-processing software to provide results compared with calibration curves for each location on the biochip *(23)*.

The minimum detectable concentrations, defined as the lowest concentrations that could be differentiated from 0 (2 SD above 0), were 0.56, 19.3, 0.9, and 9.7 ng/L for IL-8, MCP-1, EGF, and VEGF (variant 165), respectively. The intraassay imprecision (as CV) was calculated from 1 run before analyzing the samples. The interassay imprecision (as CV) was calculated from the data for 2 controls, run over the 5 days of sample analysis (n = 30). Intraassay imprecision was 8.4%–11%, and interassay imprecision was 8.9%–13%.

STATISTICAL ANALYSES

We performed statistical analyses with the SAS software package, Ver. 8.01 (SAS Institute). Because we could not obtain all results for IL-8, MCP-1, EGF, and VEGF (17.3%, 0.6%, 5.1%, and 9.5% of participants, respectively, had concentrations below the detection limit), undetectable values were set at 0.56, 19.3, 0.9, and 9.7 ng/L, respectively. The distributions of the concentrations of IL-8, MCP-1, EGF, and VEGF exhibited a long-tailed positive skewness and kurtosis. Log_{10} transformation removed most of the skewness and kurtosis, leaving a nearly gaussian distribution verified by normal probability plots. Before statistical analyses, concentrations were adjusted for the effect of between-run variation.

Differences in mean plasma concentrations of IL-8, MCP-1, EGF, and VEGF according to age (4–9, 10–14, and 15–17 years for children and 18–34, 35–44, and 45–55 years for adults) and sex were tested with the SAS GLM procedure by use of the Tukey–Kramer test.

Stepwise multiple regression analysis was carried out in the overall sample to select significant covariates of marker values adjusted for age and sex as well as lifestyle factors and related biological variables (oral contraceptive use, tobacco use, alcohol consumption, and basic blood constituents such as lipid and glucose concentrations, enzyme activities, and blood cell counts). Regression coefficients were then computed for the overall sample and for children and adults separately. Because persons within a family are not independent, multiple regressions were based on the estimating equation (EE) technique using the SAS GENMOD procedure with a repeated statement. For all analyses, statistical significance was taken at $P \leq 0.05$; results with $P \leq 0.10$ were discussed.

Reference values were calculated by use of a nonparametric method. In children and adults, partitioning criteria for separation of subgroups according to age (4–12,

13–17, 18–39, and 40–55 years) and sex were adopted from Harris and Boyd *(24)* and Lahti et al. *(25)*. The partition criteria were applied to the $\log_{10}$ distributions.

Results

The characteristics of the population are summarized in Table 1. Plasma IL-8 and MCP-1 concentrations were significantly higher in men than in women and children (both $P \leq 0.001$). Plasma VEGF concentrations were significantly higher in adults of both sexes than in children ($P \leq 0.001$), whereas plasma EGF concentrations were not significantly different among the 4 groups classified by sex and age.

AGE AND SEX VARIATIONS

Plasma IL-8, MCP-1, EGF, and VEGF concentrations according to age and sex groups are shown in Fig. 1. Two-way ANOVA showed that IL-8 and VEGF concentrations decreased significantly with age in children ($P = 0.009$ and $P = 0.048$, respectively), that concentrations of IL-8, MCP-1, and VEGF significantly increased with age in adults ($P = 0.002$, $P \leq 0.001$, and $P = 0.005$, respectively), and that men had significantly higher concentrations of IL-8 and MCP-1 than women ($P = 0.01$ and $P \leq 0.001$, respectively). EGF concentrations did not differ significantly according to age and sex groups. In addition, we found no interaction between age and sex for the 4 plasma cytokine concentrations.

OTHER DETERMINANTS IN MULTIPLE REGRESSION ANALYSIS AND REFERENCE INTERVALS

Multiple regression analysis of all sample results from children and adults indicated that plasma IL-8 concentrations were positively associated with smoking, glucose concentration, and platelet counts and negatively associated with neutrophil count ($P = 0.06$, $P = 0.005$, $P \leq 0.001$,

Table 1. Characteristics of the population sample.

	Children		Adults		
	Boys	Girls	Men	Women	P^a
n	159	145	267	273	
Age,[b] years	13.9 (2.9)[f]	13.7 (2.7)[f]	37.6 (11.9)[g]	36.0 (11.2)[g]	≤0.001
BMI,[b,c] kg/m^2	19.3 (2.7)[f]	19.7 (2.7)[f]	24.7 (3.6)[g]	23.3 (4.1)[h]	≤0.001
DBP,[b] mmHg	59.8 (10.4)[f]	60.1 (10.5)[f]	74.0 (10.4)[g]	69.5 (10.2)[h]	≤0.001
SBP,[b] mmHg	117.1 (10.5)[f]	114.3 (10.3)[g]	125.9 (12.3)[h]	119.2 (12.5)[f]	≤0.001
Tobacco consumption (smokers),[d,e] cigarettes/day	5.7 (3.1–10.1)[f]	8.9 (6.1–13.0)[g]	11.1 (4.6–26.9)[h]	9.4 (4.5–19.4)[g]	≤0.01
Moderate smokers (≤10 cigarettes/day), n (%)	17 (10.6)	4 (2.7)	43 (16.1)	41 (15.0)	
Heavy smokers (>10 cigarettes/day), n (%)	1 (0.6)	1 (0.7)	46 (17.2)	20 (7.3)	
Alcohol consumption (drinkers),[d,e] g/day	6.7 (3.4–13.2)[f]	5.2 (3.6–7.5)[g]	15.5 (5.6–43.0)[h]	7.2 (2.9–17.9)[f]	≤0.001
Oral contraceptives, n (%)		7 (4.8)		90 (32.9)	
Hormone replacement therapy, n (%)				32 (11.7)	
Total cholesterol,[b] mmol/L	4.26 (0.66)[f]	4.60 (0.91)[g]	5.44 (1.14)[h]	5.35 (0.91)[i]	≤0.001
Triglycerides,[d,e] mmol/L	0.76 (0.51–1.15)[f]	0.82 (0.50–1.32)[f]	1.13 (0.67–1.90)[g]	0.93 (0.61–1.42)[h]	≤0.001
Glucose,[b] mmol/L	4.77 (0.48)[f]	4.75 (0.46)[f]	5.06 (0.52)[g]	4.79 (0.46)[h]	≤0.001
ALT activity,[d,e] U/L	16.6 (12.1–22.7)[f]	14.9 (10.4–21.4)[f]	26.4 (15.8–44.0)[g]	15.6 (10.6–23.0)	≤0.001
AST activity,[d,e] U/L	23.9 (18.4–31.1)[f]	21.3 (16.6–27.4)[g]	23.9 (18.1–31.4)[f]	18.2 (14.5–22.9)[h]	≤0.001
Leukocyte count,[b] 10^9/L	6.52 (1.59)	6.78 (1.52)	6.70 (1.77)	6.96 (1.70)	NS
Lymphocyte count,[b] 10^9/L	2.36 (0.62)[f]	2.39 (0.61)[f]	2.05 (0.53)[g]	2.07 (0.61)[g]	≤0.001
Monocyte count,[b] 10^9/L	0.61 (0.19)[f]	0.59 (0.16)[f,g]	0.57 (0.14)[f,g]	0.55 (0.15)[g]	0.002
Eosinophil count,[d,e] 10^9/L	0.26 (0.05–0.50)[f]	0.19 (0.06–0.33)[g]	0.18 (0.07–0.29)[g]	0.15 (0.05–0.27)[g]	≤0.001
Platelet count,[b] 10^9/L	248.6 (54.9)[f,g]	261.5 (54.6)[g]	238.7 (50.4)[f]	249.4 (53.6)[f,g]	≤0.001
Erythrocyte count,[b] 10^9/L	4.95 (0.35)[f]	4.59 (0.35)[g]	5.00 (0.31)[h]	4.43 (0.33)[i]	≤0.001
Hematocrit[b]	0.43 (0.03)[f]	0.40 (0.03)[g]	0.45 (0.02)[h]	0.40 (0.03)[g]	≤0.001
IL-8,[d,e] ng/L	1.28 (0.63–2.58)[f]	1.24 (0.63–2.42)[f]	1.59 (0.78–3.22)[g]	1.35 (0.64–2.83)[f]	0.002
MCP-1,[d,e] ng/L	78.9 (53.7–115.8)[f]	71.8 (50.7–101.6)[f]	95.7 (68.2–134.2)[g]	77.5 (52.7–113.9)[f]	≤0.001
EGF,[d,e] ng/L	10.6 (4.3–26.0)	10.1 (4.21–24.2)	11.5 (4.32–30.8)	11.3 (4.14–30.7)	NS
VEGF,[d,e] ng/L	21.1 (11.1–40.1)[f]	23.6 (11.9–47.2)[f]	26.4 (13.1–53.5)[g]	28.9 (14.5–57.5)[g]	≤0.001

[a] Global ANOVA across the 4 groups.
[b] Arithmetic mean (SD).
[c] BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; NS, not significant.
[d] Geometric mean (range of 1 SD).
[e] Statistical tests performed on $\log_{10}$-transformed values.
[f–i] Means not sharing a common superscript are significantly different (Tukey–Kramer test): $P \leq 0.05$.

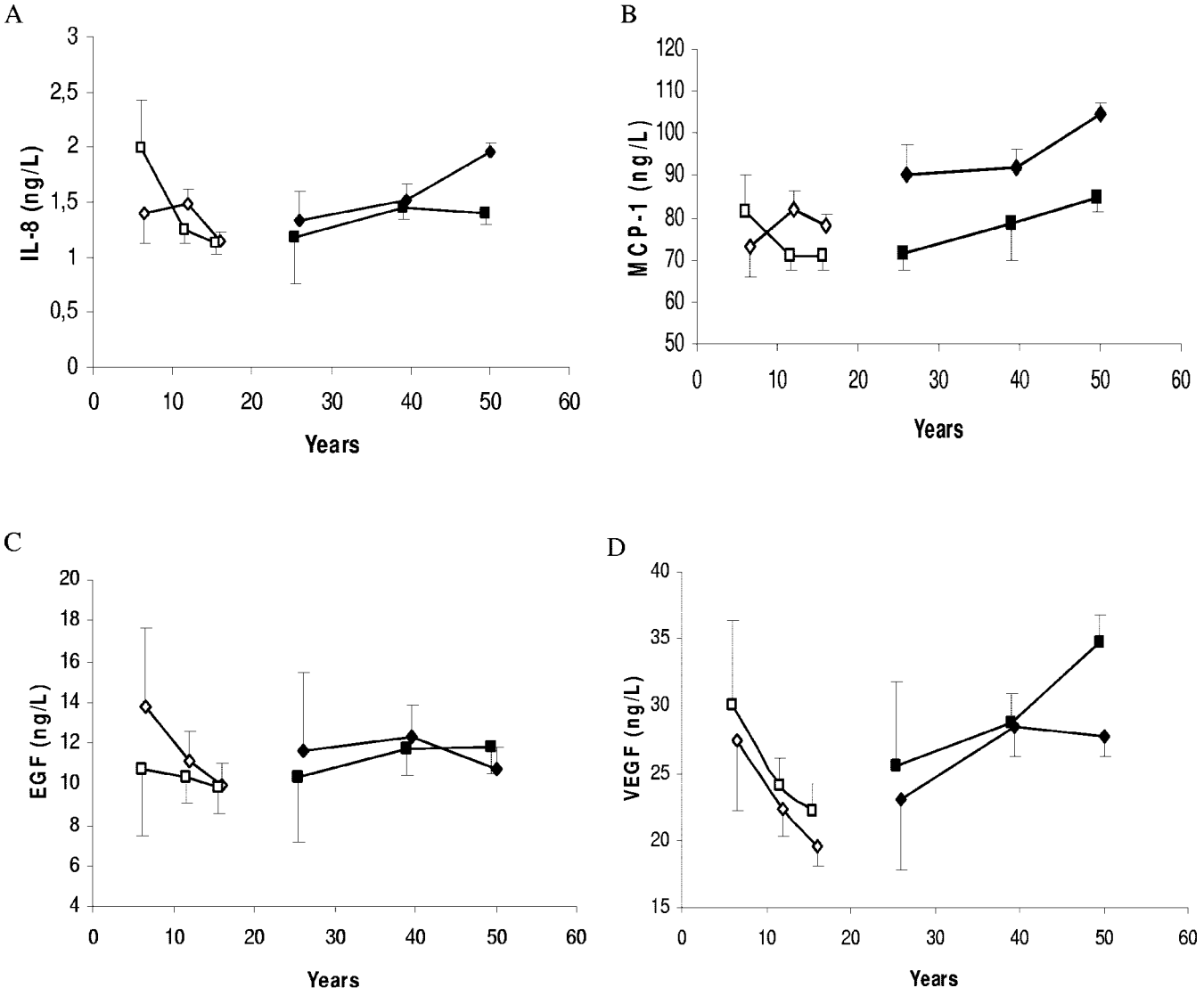

Fig. 1. Geometric means (SE; *error bars*) of plasma IL-8 (*A*), MCP-1 (*B*), EGF (*C*), and VEGF (*D*) concentrations according to age and sex groups. ◆, men; ■, women; ◇, boys; □, girls. Results of two-way ANOVA by age and sex were as follows: for IL-8 (*A*), in children, *P* (age) = 0.009; *P* (sex) and *P* (age × sex interaction), not significant; in adults, *P* (age) = 0.002; *P* (sex) = 0.011; and *P* (age × sex interaction), not significant; for MCP-1 (*B*), in children, *P* for age, sex, and (age × sex interaction), all not significant; in adults, *P* (age) and *P* (sex) ≤0.001; *P* (age × sex interaction), not significant; for EGF (*C*) in both children and adults, *P* (age), *P* (sex), and *P* (age × sex interaction), all not significant; for VEGF (*D*), in children, *P* (age) = 0.047; *P* (sex) and *P* (age× sex interaction), not significant; in adults, *P* (age) = 0.005; *P* (sex) = 0.063; and *P* (age × sex interaction), not significant.

and $P \leq 0.001$, respectively). In adults, these 4 associations remained significant, whereas in children, significant relationships were found only with glucose concentration and neutrophil count (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue3/).

In the whole sample group, plasma MCP-1 concentrations were positively associated with hematocrit and with tobacco consumption (in a dose-dependent manner) and negatively associated with monocyte count ($P \leq 0.001$, $P = 0.04$, and $P = 0.006$, respectively). In children there was no significant association with hematocrit, and in adults there was no significant association with monocyte count (see Table 1 in the online Data Supplement).

In the whole sample group, plasma EGF concentrations were positively associated with monocyte and platelet counts and glucose concentration ($P = 0.009$, $P \leq 0.001$, and $P = 0.005$, respectively). In children there was a significant association only with platelet count, whereas in adults the association with platelet count did not remain significant (see Table 1 in the online Data Supplement).

Plasma VEGF concentrations were positively associated with oral contraceptive use, platelet count, and ALT activity in the whole sample group ($P = 0.017$, $P \leq 0.001$, and $P \leq 0.001$, respectively). In adults, only platelet count and ALT activity remained significantly associated with VEGF concentration. There was only a trend of associa-

tion between oral contraceptive use and VEGF concentration in both children and adults ($P \leq 0.10$; see Table 1 in the online Data Supplement).

Geometric means and reference intervals for plasma IL-8, MCP-1, EGF, and VEGF concentrations, partitioned according to age/sex criteria provided by the Harris and Boyd method *(24)*, are shown in Table 2.

Discussion

The concept of reference values was launched by Gräsbeck and Saris in 1969 *(26)*, after which 6 IFCC guidelines for establishing biological reference intervals were published between 1987 and 1991 *(27)*. Our results, obtained by use of a new fully automated biochip analyzer, provide the first available data regarding the highly associated factors and reference intervals for plasma IL-8, MCP-1, EGF, and VEGF concentrations in individuals from a large, apparently healthy cohort. Because these 4 cytokines are involved in numerous diseases related to inflammation, vascular remodeling, or growth regulation *(3–6, 12, 14–16)* and their concentrations in blood have been found to be increased in several diseases *(7–9, 17)*, reference intervals for healthy individuals must be determined to provide comparison data for the interpretation of patient laboratory results.

Our finding that MCP-1 increased significantly with age in adults is in accordance with previous reports *(18–20)*. Moreover, for middle-aged participants, samples from men had higher MCP-1 concentrations than samples from women. On the other hand, a study of elderly persons *(19)* did not show a significant difference between men and women, probably because of a decrease in circulating sex steroid hormones with age. Treatment of postmenopausal women with 17β-estradiol could decrease plasma MCP-1 concentrations *(28)*. In accordance with the data of Svoboda et al. *(29)*, we did not find a significant relationship of plasma EGF concentration with age in children or adults. In addition, EGF concentrations did not differ significantly between men and women in the 2 age groups. IL-8 and VEGF concentrations decreased significantly with age in children and increased significantly with age in adults. In addition, there was a trend for higher VEGF concentrations in women than in men.

Several environmental and biological factors have been investigated as potential determinants of plasma IL-8, MCP-1, EGF, and VEGF concentrations. In our sample, tobacco consumption was independently and significantly associated with high IL-8 and MCP-1 concentrations. This result is consistent with other reported data. Indeed, in vitro studies showed that chemokines released by cultured cells were significantly increased in response to smoke *(30, 31)* and smokeless tobacco extracts *(32)*. Deo et al. *(20)* found a positive correlation between smoking and plasma MCP-1 concentrations in adults in a large probability-based population. On the other hand, Boekholdt et al. *(33)* found no association between plasma IL-8 concentrations and smoking in apparently healthy individuals.

The positive significant relationships of glucose concentration with IL-8 and EGF concentrations are in agreement with the finding that EGF exerts full insulin-like effects on glucose transport in human fat cells *(34)*. In addition, in vitro studies showed that glucose dramatically stimulated IL-8 promoter activity in cultured cells through several aligned carbohydrate response elements (also known as E-boxes) and activator protein-1 elements *(35, 36)*.

Blood cell counts (monocytes, neutrophils, and platelets) were significantly associated with cytokine concentrations. We found, however, that IL-8 and MCP-1 [already known to be powerful chemoattractants of neutrophils and monocytes, respectively *(1)*] were nega-

Table 2. Geometric means and reference intervals for plasma IL-8, MCP-1, EGF, and VEGF concentrations by age and sex.

Analyte	Age group	Sex	n	Geometric mean (1 SD range), ng/L	Reference interval,[a] ng/L
IL-8	4–12 years	Males	41	1.43 (0.83–2.46)	0.57–4.58
	4–12 years	Females	31	1.71 (0.91–3.22)	0.56–5.33
	13–17 years	Males + Females	232	1.20 (0.60–2.42)	0.56–5.54
	18–39 years	Males + Females	216	1.21 (0.67–2.18)	0.56–3.80
	40–55 years	Males + Females	324	1.64 (0.77–3.49)	0.56–7.52
MCP-1	4–17 years	Males + Females	304	75.2 (52.3–108.2)	32.7–146.6
	18–55 years	Males	267	95.7 (68.2–134.2)	43.4–156.4
	18–55 years	Females	273	77.5 (52.7–113.9)	29.2–138.5
EGF	4–17 years	Males + Females	304	10.6 (4.4–25.4)	1.0–41.5
	18–55 years	Males + Females	540	11.3 (4.4–28.9)	0.9–47.4
VEGF	4–12 years	Males + Females	72	26.3 (13.0–53.2)	9.7–130.9
	13–17 years	Males + Females	232	21.1 (11.2–39.8)	9.7–75.6
	18–39 years	Males + Females	216	25.0 (13.3–46.8)	9.7–83.4
	40–55 years	Males	169	27.7 (13.9–55.6)	9.7–141.6
	40–55 years	Females	155	31.9 (16.2–62.7)	9.7–147.4

[a] Lower limit is the 2.5th nonparametric percentile; upper limit is the 97.5th nonparametric percentile.

tively associated with neutrophil and monocyte counts, respectively. This effect could be the consequence of a negative feedback control of production of these chemokines by inflammatory cells under physiologic conditions. Hematocrit was significantly and positively associated with MCP-1 concentration in adults, probably because of the binding of erythrocytes by chemokines via promiscuous receptors *(37, 38)*. These results suggest a mechanism by which circulating concentrations of these chemokines are regulated and may indicate a role for erythrocytes as regulators of inflammatory processes.

Finally, oral contraceptive use and ALT activity were significantly and positively associated with plasma VEGF concentrations. These 2 associations have not been reported in the literature, and we can give no definite explanation for them.

Our study was based on a random subsample of the overall STANISLAS population. Thus, conclusions drawn from this subsample should be valid for males and females 4–55 years of age living in the east of France. Comparisons of our results with those of other studies should take into account the characteristics of our subsample. Moreover, because partition criteria were adopted from Harris and Boyd *(24)* and because some subgroups of children contained fewer than the 120 participants recommended in the IFCC guidelines and by Reed et al. *(39)* to obtain reliable two-sided 90% confidence intervals for the 2.5th or 97.5th percentiles, reference intervals for these cytokines should be supplemented by further studies of young children and older adults and on other samples of populations with different genetic backgrounds and lifestyles.

In conclusion, we provide reference intervals for plasma IL-8, MCP-1, EGF, and VEGF concentrations stratified by age and sex, with estimation of their main variation factors. These data could be useful in the clinical interpretation of measurements of these cytokines.

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