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Serum cytokine levels differ according to major cardiovascular risk factors in patients with lower limb atherosclerosis

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ABSTRACT

Different cardiovascular risk factors present a heterogenic manifestation of lower limb atherosclerosis. The molecular mechanisms behind this phenomenon remain unknown. We aimed to clarify this phenomenon by studying the association of major cardiovascular risk factors with the profile of serum cytokines in 226 consecutive patients with lower limb atherosclerosis treated at a department of Vascular Surgery during a one-year enrollment period. Increasing age independently associated with higher levels of IFN-γ inducible factors MIG, CTACK and IP-10 ($P < 0.001$ for all). Patients with chronic kidney disease had higher serum levels of MIF, IL-16 and SCF ($P = 0.001$ or less for all). Smoking and hypertension associated with IL-17 ($P = 0.037$ and 0.015, respectively). In addition, smoking associated with growth factors known to induce myeloid progenitor cell proliferation: GM-CSF ($P = 0.035$), PDGF ($P = 0.024$), bFGF ($P = 0.026$), and HGF ($P = 0.030$). Dyslipidemia also associated with myeloproliferative factors: MIB-1α ($P = 0.005$) and PDGF ($P = 0.01$). Type II diabetes associated with Th2 mediated inflammation: IL-5 ($P < 0.001$), IL-7 ($P = 0.004$) and IL-13 ($P = 0.015$). Major cardiovascular risk factors are associated with different circulating cytokines implicating different immunological pathology.

1. Introduction

A strong body of literature based on animal models and analyses of human atherosclerotic plaques have revealed the involvement of several cytokines, chemokines and growth factors in the pathology of atherosclerosis. This has led to the contemporary understanding of atherosclerosis as an inflammatory disease [1,2]. Lymphocyte mediated inflammation and macrophage polarization has been shown to be an essential part of the progression of atherosclerosis [1,3]. Cytokines, chemokines and growth factors are the key signaling molecules in this process and divided into Th1, Th2 and Th17 cell mediated pathways [4,5]. Molecular level differences in the way risk factors cause atherosclerosis have been elucidated in several animal models [1,6]. We postulate that in addition to certain generally accepted molecular mechanisms of atherosclerosis the molecular mechanisms of lower limb atherosclerosis varies depending on the risk factor leading to the disease, for example we hypothesis that age related peripheral artery disease (PAD) differs from those of smoking and dyslipidemia. Macroscopic signs of this phenomenon can be seen in the localization lower limb atherosclerosis, which is highly dependent of the underlying risk factor [7]. Thus, far no molecular level explanation has been presented to explain this phenomenon. Some human studies have detected that certain cytokines that are elevated in atherosclerotic patients may associate with a certain risk factors, but not with another, e.g. interferon induced chemokine IP-10 was shown to associated with aging but not with type II diabetes in a cohort of patients with coronary artery disease [8]. These are single observations from a screening of certain cytokines in atherosclerotic patient cohorts. According to our knowledge no systematic approach has been taken to map the molecular mechanisms of atherosclerotic risk factors in a well-defined atherosclerotic patient cohort. In this study we aim to investigate the serum cytokine and chemokine patterns of patient with lower limb atherosclerosis, and the association of these patterns with major atherosclerotic risk factors. To accomplish this 48 inflammatory cytokines, chemokines, and growth factors were studied in a recently presented cohort of 226 consecutive PAD patients treated at a department of vascular surgery [9].

2. Materials & methods

2.1. Patient cohort

The study patient cohort consists of the PURE ASO Study population

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as described recently [9]. The study was approved by the local Ethical Committee of the Hospital District of South-West Finland. By approval of the Ethical Committee, a register under the name *The Role of Purinergic Signaling in Atherosclerosis* (PURE ASO Registry) was formed and is held at the Department of Vascular Surgery, Turku University Hospital.

Shortly, constantly for one year, from February 2012 to March 2013, we collected samples from every consecutive patient requiring elective intervention for lower limb atherosclerosis at the Department of Vascular Surgery at Turku University Hospital, Finland, which serves a primary population of 360 000 inhabitants. Patients admitted for urgent treatment from the emergency unit and those with acute ischemia, vasculitis, major infection or major tissue loss were not included in the study. During our enrollment period, 227 suitable patients were screened. Only one patient declined, and 226 gave written informed consent. The following major cardiovascular risk factors were noted on a 0/1 basis as follows: hypertension, if systolic blood pressure > 140 mmHg as a mean of two measurements at rest and/or use of blood pressure lowering medication; dyslipidemia, if LDL cholesterol calculated using the Friedewald formula level was > 4.0 mmol/L or triglycerides were over 2.5 mmol/L and/or statin use; chronic kidney disease (CKD), if creatinine was > 115 μmol/L; diabetes, was defined as either completely insulin dependent or orally treated diabetics according to their medication; rheumatic/autoimmune disease, diagnosed by a rheumatologist as rheumatoid arthritis, inflammatory bowel disease, colitis ulcerosa, multiple sclerosis or systemic lupus erythematosus; smoking, if current smoking according to self reporting.

2.2. Blood sampling and analysis of cytokines, chemokines and growth factors

The serum analytical methods and results have been described previously [10]. These cytokine results are the same as in Jalkanen et al. [10]. Shortly, all blood samples were drawn in the morning after at least 8 h of fasting. 9 mL of whole blood was collected in a serum sample tube and left to clot for three hours at room temperature while it was transported to the MediCity Research Laboratory, University of Turku. On arrival, it was centrifuged at 2000*g* for 10 min, after which the serum was extracted and stored at −70 °C until analyses. All samples were handled identically and major violations from this protocol lead to the exclusion of the sample. All analyses were performed at once with the same magnetic bead suspension array kit of Bio-Plex Pro Human Cytokine 21- and 27-plex panels. According to the manufacturers instructions the amount of beads, detection antibodies and streptavidin-phycoerythrin conjugate were used at half of their original concentration recommended in the manual. This approach does not jeopardize results and can be used in the analysis of large cohorts. Results were analyzed using the Bio-Plex 200 System and Bio-Plex Manager 6.0 software (Bio-Rad Laboratories). Cytokine levels outside detection limits were left blank and not extrapolated. Detection limits for each cytokine are presented in Supplement Table I. Bio-Plex Pro Human Cytokine 21- and 27-plex panels were chosen because together they formed an exhaustive repertoire of cytokines known to be involved in atherosclerosis.

2.3. Statistical analyses

Statistical analyses were performed in association with a professional statistical provider, 4Pharma Ltd. (Turku, Finland). All statistical analyses were performed using SAS Software for Windows (version 9.3). For descriptive statistics the study subjects were divided into four age groups: < 60 years, 60–69 years, 70–79 years and over 80 years olds. The prevalence of cardiovascular risk factors, medications, and other baseline characteristics are presented as percentages, and differences between age groups was examined using the Chi-square test.

For multivariable modeling of cytokine associations with major cardiovascular risk factors we included: hypertension, dyslipidemia, type I or type II diabetes, CKD, rheumatic disease, current smoking, sex and age groups. As a confounding factor we included the localization of significant atherosclerotic lesions. The presence of significant atherosclerotic lesions was determined on a YES/NO basis in the aortoiliac, femoropopliteal, and/or crural regions. A > 50% stenosis of the vessel diameter was the threshold of a clinically significant lesion. Subjects were also divided into having either intermittent claudication (IC) or critical limb ischemia (CLI), and this was incorporated in the multivariable modeling since the presence of CLI is known to affect cytokine levels [10]. An association of major cardiovascular risk factors with each cytokine was individually explored using a linear regression model for log-transformed values. Variables to be included in the model were selected using nonparametric methods. Variables with a test P value < 0.15 were entered into the model. Dichotomous variables were tested using the Wilcoxon rank-sum test, and variables in more than two categories were tested using the Kruskal–Wallis test. The full model for each cytokine was constructed by fitting all the variables selected using the *P* value criterion. In addition to the full model, a reduced model was constructed by removing variables that showed little or no association with the explored cytokine in the full model. This model was defined by removing the least significant variable from the model one by one until all remaining variables showed a *P* value < 0.15. Thus, in the end the most decisive variables affecting each cytokine independently were left in the model. Model fit was inspected visually using studentized residuals showing a reasonable fit for all models.

3. Results

3.1. Description of the patient cohort

The patient cohort has been described in detail originally in Jalkanen et al. [9]. It consisted of 226 participants of which 128 (56.6%) were male. All were of Caucasian origin. Overall mean age was 71.5 years (SD ± 11.44, 46–93 years) with a small tendency for males to be younger (males, 67.9 years [SD ± 11.3], and females, 72.3 years [SD ± 11.2]). Males were dominant in younger age groups ($P = 0.01$). A history of smoking was clearly more prevalent in younger age groups ($P < 0.001$), while hypertension and chronic kidney disease (CKD) were more prevalent in old age ($P = 0.002$ and $P = 0.01$, respectively). Critical limb ischemia (CLI) was also clearly more prevalent in older age groups ($P < 0.001$), see Table 1. Of the 35 rheumatic patients, only one was diagnosed with vasculitis, the clear majority had rheumatoid arthritis (20/35), by psoriasis (8/35), systemic lupus erythematosus (3/35), and inflammatory bowel disease (3/35). We also tested the association of disease localization with the defined risk factors to be sure that this cohorts coincides with previous findings concerning this phenomenon. Subjects were divided in three major localization groups aortoiliac, femoropopliteal and crural atherosclerosis according the arterial segment that was treated, i.e. the most disease-ridden segment. Younger age ($P < 0.001$), a history of smoking ($P < 0.001$), and dyslipidemia ($P = 0.01$) were more prevalent in those with proximal PAD. Diabetes ($P = 0.004$), chronic kidney disease (CKD) ($P < 0.001$), and rheumatic diseases ($P = 0.001$) were more prevalent in those with distal PAD (Table 2).

3.2. The association of circulating cytokines with cardiovascular risk factors

The measurable values of MCP-3, IFN-α2, LIF, IL-1α, IL-3, IL-15, and TNF-β were mostly below a detectable limit, and did not provide a solid basis for further statistical analyses. The values of RANTES and IL-12p40 were mostly over the detectable range and they too were not included in further analyses. In general, cytokine values were very scattered and tailing to the higher end of the range. Despite logarithmic transformation two distinct outlier subjects with persistent exponential

Table 1
Baseline characteristics of patient cohort divided according to defined age groups.

	All	< 60	60–69	70–79	> 80	*P* value*
N	226	24	75	69	59	
Male/female	57%/43%	62%/38%	68%/32%	58%/42%	39%/61%	0.01
IC/CLI	46%/54%	58%/42%	62%/38%	38%/62%	32%/68%	< 0.001
History of smoking	58%	75%	77%	62%	24%	< 0.001
Hypertension	74%	50%	70%	78%	83%	0.002
Dyslipidemia	32%	38%	39%	22%	32%	0.13
Diabetes	35%	42%	41%	38%	22%	0.09
CKD	24%	13%	15%	26%	37%	0.01
Rheumatic disease	16%	8%	10%	25%	17%	0.06

Abbreviations: intermittent claudication (IC), critical limb ischemia (CLI), chronic kidney disease (CKD).
* Chi-square test across age groups.

Table 2
The association of disease localization with risk factors.

	Aortoiliac	Femoropopliteal	Crural	*P* value*
Localization	26%	44%	30%	N/A
Male/female	58%/42%	59%/41%	52%/48%	NS
Mean age	66 (SD, 9.2)	71 (SD, 9.5)	77 (SD, 11.9)	< 0.001**
History of smoking	85%	67%	22%	< 0.001
Hypertension	62%	78%	79%	NS
Dyslipidemia	42%	33%	19%	0.01
Diabetes	25%	31%	49%	0.004
CKD	10%	17%	46%	< 0.001
Rheumatic disease	7%	13%	28%	0.001

* Chi-square test across age groups.
** Kruskal-Wallis test across groups.

values throughout the measured cytokines were identified and excluded from the final multivariate analyses. Both had rheumatoid arthritis, the other with accompanying vasculitis.

Table 3 illustrates the raw values of measured cytokines, and the behavior of cytokines across age groups by non-parametric univariate analyses. According to univariate analysis TNFα, GM-CSF and PDGF were significantly higher with younger age, while IP-10, CTACK, MIG and SCF were very strongly associated with increasing age ($P < 0.001$ for all) (Table 3). According to multivariate modeling a strong association of IP-10, CTACK, MIG and SCF ($P < 0.001$ for all) remained with increasing age (Fig. 1). All of these chemokines are induced by IFN-γ. Active smoking was independently associated with elevated levels of IL-17 (13% increase; $P = 0.037$; 95% CI, 1–27% increase) and MIF (27% increase; $P < 0.001$; 95% CI, 11–45% increase) when compared to non-smokers in multivariate analyses, but a predominant feature of smoking was an association with elevated levels of several growth factors: GM-CSF (10% increase; $P = 0.035$; 95% CI, 1–19% increase), PDGF (24% increase; $P = 0.004$; 95% CI, 7–43% increase), FGF (9% increase; $P = 0.026$; 95% CI, 1–17% increase) and HGF (12% increase; $P = 0.03$; 95% CI, 1–24% increase), see Fig. 2. Hypertension had somewhat similar associations, as did smoking. The presence of hypertension as a diagnosis in medical charts was independently associated with elevated levels of IL-17 (17% increase; $P = 0.015$; 95% CI, 3–32% increase), MIP-1α (18% increase; $P = 0.003$; 95% CI, 6–31% increase) and FGF (9% increase; $P = 0.03$; 95% CI, 1–17% increase) (Fig. 2). Dyslipidemia as a diagnosis in medical charts had its own independent associations, and was linked with elevated levels MIP-1β (16% increase; $P = 0.005$; 95% CI, 5–29% increase), PDGF (22% increase; $P = 0.007$; 95% CI, 6–41% increase) and TRAIL (24% increase; $P = 0.003$; 95% CI, 8–43% increase) (Fig. 2). During initial univariate modeling diabetic subjects were first divided into type I and type II diabetics. However, the cytokine profile of insulin treated type II diabetics was more similar to that of type I diabetics (data not shown), thus for the final multivariate model diabetics were divided into insulin or orally treated disease forms. Surprisingly, according to multivariate modeling insulin treated diabetes was only associated with lower levels of MIF (23% decrease; $P = 0.002$; 95% CI, 10–35% decrease) and CTACK (12% decrease; $P = 0.046$; 95% CI, 0–23% decrease). While on the other hand, type II diabetes with only per oral medical therapy was associated with lower levels of IP-10 (18% decrease; $P = 0.042$; 95% CI, 1–33% decrease), but increased levels of IL-5 (52% increase; $P < 0.001$; 95% CI, 20–92% increase), IL-7 (31% increase; $P = 0.004$; 95% CI, 9–57% increase), IL-13 (29% increase; $P = 0.015$; 95% CI, 5–57% increase), and SDF-1α (53% increase; $P = 0.003$; 95% CI, 16–103% increase), see Fig. 2. Chronic Kidney Disease (CKD) is a well-known cardiovascular risk factor causing sclerosis of small vessels. In this study it was associated with elevated levels of several cytokines and chemokines. CKD was associated with elevated levels of IL-2Rα (19% increase; $P = 0.033$; 95% CI, 1–39% increase), IL-16 (35% increase; $P < 0.001$; 95% CI, 13–60% increase), IL-18 (19% increase; $P = 0.035$; 95% CI, 1–41% increase), MIF (32% increase; $P < 0.001$; 95% CI, 12–55% increase), MIG (30% increase; $P = 0.014$; 95% CI, 6–61% increase), M-CSF (51% increase; $P = 0.019$; 95% CI, 7–212% increase), SCF (45% increase; $P < 0.001$; 95% CI, 28–65% increase), and (β-NGF (20% increase; $P = 0.007$; 95% CI, 5–36% increase), but lower levels of bFGF (9% decrease; $P = 0.026$; 95% CI, 1–17% decrease), see Fig. 2.

3.3. The association of circulating cytokines with disease localization

Crural disease per se was clearly associated with increased levels of IL-5 (40% increase; 95% CI, 21–62%; $P < 0.001$;) and IL-1ra (10% increase; 95% CI, 1–19%; $P = 0.024$) indicating both Th1 and Th2 mechanisms. Large vessel aortoiliac disease had an opposing finding an independently showed negative association with IL-7 (18% reduction; 95% CI, 5–27%; $P = 0.007$) and IFN-γ (10% reduction; 95% CI, 2–17%; $P = 0.013$) (Fig. 3). Multivariate modeling further revealed that the presence of clinically significant aortoiliac and femoropopliteal atherosclerosis was associated with increased levels of CTACK (a 13% increase; 95% CI, 3–24%; $P = 0.009$, and a 15% increase; 95% CI, 3–28%; $P = 0.012$; respectively) and SCGF-β (a 14% increase; 95% CI, 2–28%; $P = 0.027$, and a 13% increase; 95% CI, 3% decrease–52% increase; P = 0.084, respectively) indicating they have a role proximal but not distal atherosclerosis (Fig. 3). In addition, atherosclerosis of the femoropopliteal segment was independently associated with increased G-CSF (13% increase; 95% CI, 4–24%; $P = 0.006$) and FGF (9% increase; 95% CI, 1–18%; $P = 0.034$) levels (Fig. 3). Indicating that growth factors and Th17 cytokines are more predominant for in femoropopliteal atherosclerosis rather than Th1 or Th2 cytokines.

4. Discussion

A wide range of cytokines, chemokines and growth factors are present during the formation of atherosclerotic plaques [1,2]. The present study demonstrates that circulating cytokine levels are associated with defined cardiovascular risk factors (Fig. 4). Increasing age

Table 3
Cytokine values as median pg/mL (range) by age groups.

	< 60	60–69	70–79	> 80	*P* value*
IL-1b	5.97 (3.65–97)	4.99 (3.43–6.33)	5.49 (3.75–9.04)	5.92 (3.42–352)	NS
IL-1ra	143 (85–4524)	101 (74–128)	122 (84–209)	123 (60–13 k)	< 0.05
IL-2	46.6 (16.9–455)	30.6 (19.1–38.7)	32.4 (19.0–91.3)	32.1 (18.0–3309)	NS
IL-2Rα	143 (70.5–1119)	102 (37.2–399)	133 (35.9–494)	183 (42.0–1531)	< 0.01
IL-4	7.78 (5.52–17.3)	6.79 (4.74–7.95)	7.09 (5.98–9.71)	7.09 (5.78–36.9)	NS
IL-5	3.98 (1.71–105)	2.48 (1.39–5.74)	2.48 (1.61–5.70)	2.75 (1.43–59.1)	NS
IL-6	19.0 (9.7–303)	15.1 (9.7–24.2)	19.2 (12.5–2073)	22.9 (12.5–2073)	NS
IL-7	22.8 (12.9–197)	10.9 (3.5–21.0)	14.9 (9.9–40.1)	17.0 (6.8–528)	< 0.05
IL-8	63.0 (34.6–111)	47.4 (24.5–171)	64.6 (42.2–279)	59.5 (29.5–109)	NS
IL-9	66.8 (38.3–103)	59.3 (39.9–95.5)	57.9 (34.3–176)	60.1 (31.9–158)	NS
IL-10	23.9 (11.5–372)	7.19 (1.47–30.3)	14.0 (4.4–144)	8.78 (3.98–1149)	NS
IL-12	173 (97–1510)	101 (32–223)	134 (54–414)	98 (29–1976)	NS
IL-13	12.5 (6.9–214)	7.58 (4.07–29.9)	8.91 (4.36–15.8)	8.57 (2.67–269)	NS
IL-16	130 (100–870)	128 (59.5–367)	131 (54.4–399)	195 (114–676)	< 0.01
IL-17	141 (73–227)	116 (58–229)	112 (43–303)	128 (43–690)	NS
IL-18	110 (93.5–408)	69.6 (34.3–259)	60.3 (24.3–290)	80.7 (8.74–331)	< 0.05
Eotaxin	201 (133–2257)	152 (99–277)	153 (71–318)	167 (77–9563)	NS
TNF-a	90.5 (67.4–1242)	74.6 (55.2–107)	86.1 (54.3–165)	87.0 (57.0–4631)	< 0.05
IFN-g	224 (141–2503)	188 (122–289)	236 (149–371)	238 (187–7235)	NS
IP-10	424 (88–1992)	814 (286–1803)	943 (486–2389)	1162 (631–3021)	< 0.001
CTACK	1466 (966–2494)	1833 (996–3351)	2046 (1319–3446)	2361 (1131–3210)	< 0.001
MCP-1	40.1 (16.3–212)	26.4 (9.82–58.7)	33.7 (6.41–74.0)	34.9 (9.82–788)	NS
MIP-1a	10.7 (6.67–19.1)	9.67 (3.88–21.0)	11.0 (4.08–27.1)	11.5 (5.80–52.5)	NS
MIP-1b	255 (131–583)	272 (195–470)	239 (128–515)	268 (137–553)	NS
MIF	121 (62.5–304)	136 (74.3–311)	144 (55.7–333)	196 (74.3–1848)	NS
MIG	1215 (304–2872)	1347 (544–14.7 k)	2330 (1121–5332)	4003 (1683–9813)	< 0.001
M-CSF	15.8 (8.50–63.2)	12.7 (0.25–104)	14.5 (0.71–139)	16.5 (4.46–204)	NS
GM-CSF	47.3 (32.5–357)	35.5 (23.6–53.9)	36.0 (18.5–75.6)	37.4 (18.5–823)	< 0.05
G-CSF	323 (163–1127)	244 (163–323)	259 (180–530)	268 (163–1863)	NS
PDGF	2967 (1470–5467)	1951 (172–4670)	1934 (1116–3502)	1833 (467–4387)	< 0.01
FGFb	176 (106–261)	156 (109–227)	152 (100–319)	170 (90–536)	NS
HGF	1196 (642–1497)	849 (502–1661)	864 (466–3144)	946 (637–2729)	NS
VEGF	200 (122–290)	126 (46.5–291)	153 (53.6–329)	124 (30.5–312)	NS
SCF	98.7 (40.9–359)	118 (49.0–867)	145 (57.1–235)	227 (109–396)	< 0.001
SCGF-b	22.1 k (5.7 k–38.5 k)	16.7 k (10.1 k–33.1 k)	17.5 k (0.9 k–29.0 k)	18.4 k (0.6 k–30.7 k)	NS
SDF-1a	70.0 (26.4–568)	54.6 (14.8–114)	72.3 (14.8–232)	75.2 (12.1–568)	< 0.01
b-NGF	2.58 (1.21–27.5)	1.75 (0.94–2.85)	2.03 (1.21–7.59)	2.30 (1.21–46.3)	< 0.05
GROa	74.0 (27.8–171)	63.6 (20.4–158)	80.1 (20.4–274)	88.1 (14.9–203)	NS
TRAIL	189 (57.6–1139)	164 (62.8–379)	135 (46.7–409)	137 (32.1–1073)	NS

* Kruskal-Wallis test across groups.

and CKD were strongly associated with MIG and SCF, suggesting similarity in the inflammatory mechanisms associated with these central risk factors of atherosclerosis. Significantly increased levels of MIG, SCF, IP-10 and CTACK were detected with aging. These paracrine factors are all induced by IFN-γ [3], indicating that Th1 related inflammatory mechanisms prevail in both aging and CKD. Both of these risk factor are known to associate with distal lower limb atherosclerosis [7], as also shown in this cohort. A supporting observation is that proximal atherosclerosis (aortoiliac disease) had a negative association with IFN-γ. Elevated levels of IFN-γ itself were not, however, associated with aging, but this can be a result of the strong presence of CLI in association of aging and the stronger association of IFN-γ with CLI [10]. IL-18 behaved in an opposite way in response with aging, although it too has been suggested to be an important mediator of atherosclerosis through stimulating the release of IFN-γ in apolipoprotein E-knockout mice [11]. The expression of IL-18 is known to deteriorate with age [12]. It could be hypothesized that the progression of IFN-γ/Th1 related atherosclerosis requires downstream chemokines such MIG, SCF, and IP-10 in aging while in younger patients with lower limb atherosclerosis or in rodent models the IFN-γ/Th1 mechanism is mediated via IL-18 expression. CKD had very diverse associations with several cytokines, chemokines and growth factors, which may help to explain its devastating impact on the vascular wall. In addition, to similar molecular mechanisms relating to aging CKD was associated with elevated levels of IL-2Rα and IL-16 emphasizing its diverse Th1 related activity [13]. CKD also had similar associations, as did smoking via MIF, and smoking and hypertension via M-CSF. The Th17 elevation seen in this study with smoking and hypertension has been shown to be a pro-inflammatory response of reactive oxygen species (ROS) leading to the induction of pro-inflammatory cytokines such as G-CSF and expression of myeloid progenitor cells [14,15]. In this study smoking especially shows affinity to several growth factors inducing myeloid progenitor cell proliferation implicating similar effects as dyslipidemia [16]. Both of these risk factors have a strong tendency to cause proximal lower limb atherosclerosis from the aorta to the popliteal level, but not to the below the knee arteries [7]. Smoking and hypertension were also associated with elevated levels of bFGF. However, somewhat surprisingly CKD was associated with lower levels of bFGF, although it showed affinity with other myeloproliferative mechanisms. Previous studies have shown that bFGF is essential in vascular injury responses and atherogenesis, but is not essential in developed plaques and end-stage disease [17,18]. It could be postulated that in terms of bFGF expression smoking and hypertension exhibit active injury while CKD reflects a later state of atherosclerosis in the context of this patient cohort. This interpretation is supported by the fact that CKD has been associated the more severe disease of PAD than smoking or hypertension [19]. Insulin treated diabetics in this patient population represent clearly hyperglycemic subjects while orally treated diabetics represent those with metabolic disorder and insulin resistance. Surprisingly, insulin treated diabetes, which is notorious for diffuse distal atherosclerosis [20], as is CKD [19], showed no independent associations to elevated levels of cytokines. On the contrary insulin treated diabetes was associated with lower levels of MIF and CTACK opposite to aging. Orally treated diabetes was also associated with lower levels of IP-10. These findings imply that diabetes

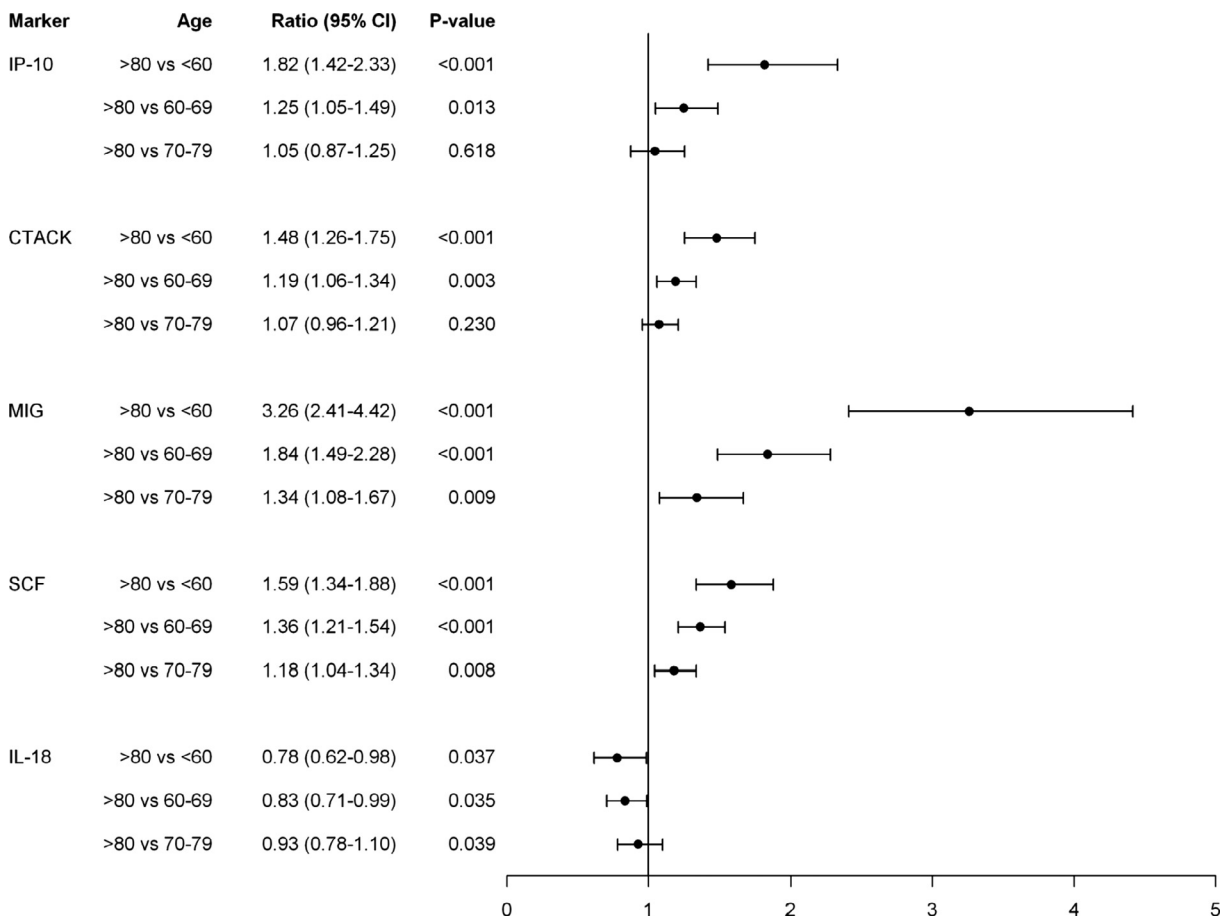

Fig. 1. Forest plot illustrating the essential results of multivariate analysis and the effect of age on circulating levels of cytokines, chemokines and growth factors in patients with clinically symptomatic lower limb atherosclerosis.

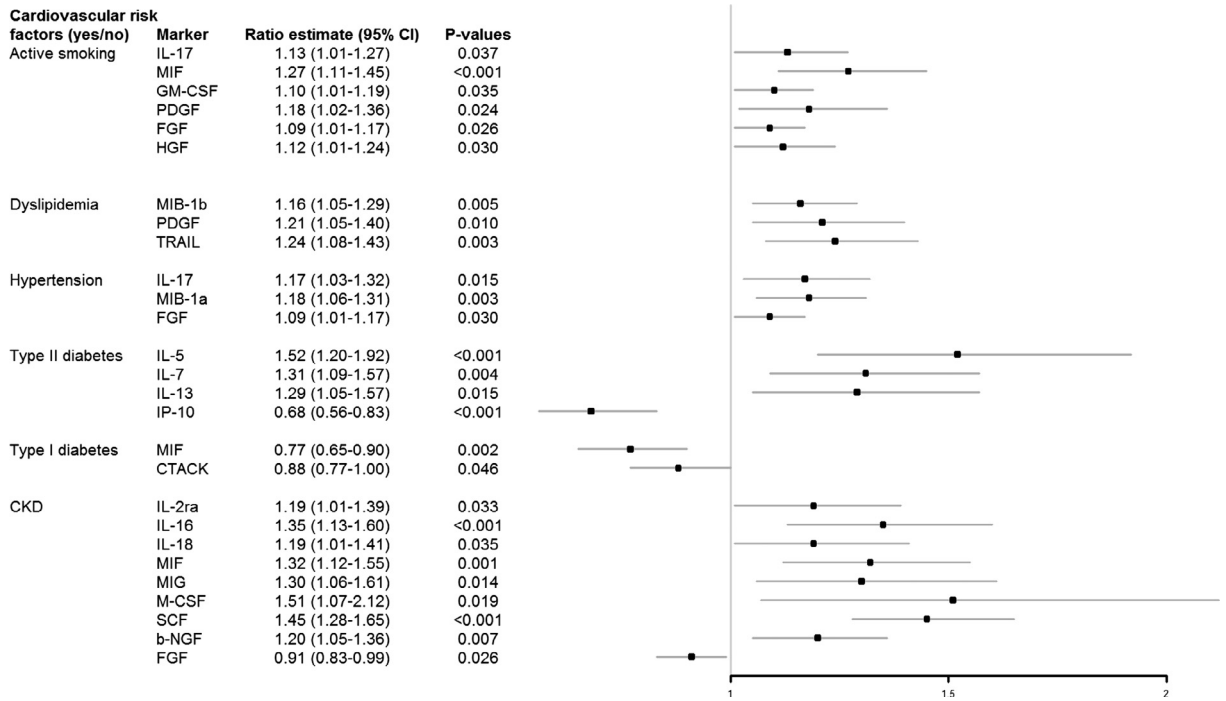

Fig. 2. Forest plot illustrating the essential results of multivariate analysis and the effect of cardiovascular risk factors on the levels of cytokines, chemokines and growth factors in patients with clinically symptomatic lower limb atherosclerosis.

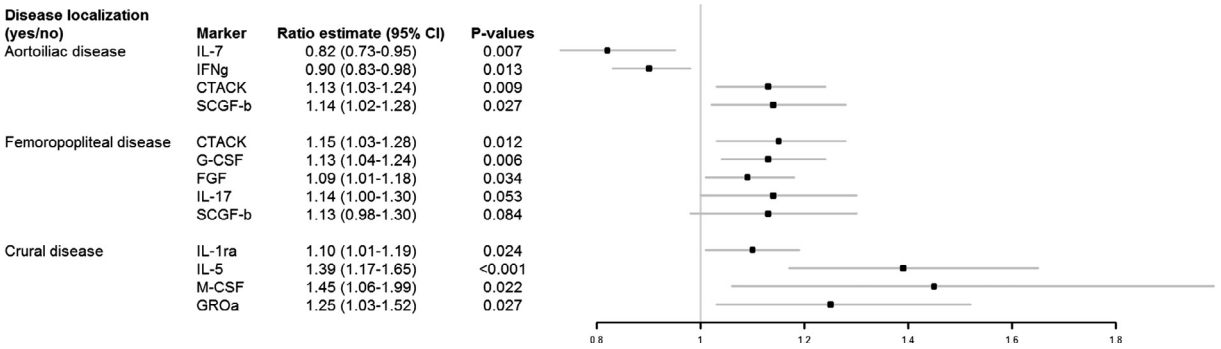

Fig. 3. Forest plot showing the independent associations of cytokines, chemokines and growth factors with the distinct localizations of atherosclerosis.

is not IFN-γ/Th1 related as is for example aging and CKD. Instead, type II diabetes marked with metabolic disorder and insulin resistance showed clear association with Th2 related mechanisms and adaptive immunity (IL-5, IL-7 and IL-13). The literature on Th1 and Th2 mechanisms in relation to type II diabetes related vasculopathy is extensive. Adipose tissue expresses Th2 immunity and a M2 macrophage lineage by nature, which is considered anti-inflammatory. An imbalance towards pro-inflammatory Th1 responses and polarization to M1 macrophages has been considered a hallmark of type II diabetes related vasculopathy. However, this concept has been seriously questioned [21,22]. Prior work has shown that the resident Th2/M2 surrounding is a more significant source of inflammation rather than Th1 recruitment and that resident M2 macrophages are a prominent source of Th2 driven inflammation [23,24], just as our findings indicate. Hyperinsulinemia or insulin treatment, on the other hand, is known to posses anti-inflammatory properties [22,25], which could explain why insulin treated patients seen here did not show any affinity to the wide range of tested cytokines. Hyperglycemic vasculopathy with hyperinsulinemia is considered to be a result of vascular smooth muscle cell (VSCM) proliferation and inflammation via reactive oxygen species (ROS) and angiotensin II signaling [26], and not necessarily a result of the mechanisms studied in this setting.

The present observations help to explain the heterogenic appearance of PAD patients and disease manifestation in association with classical cardiovascular risk factors. These observations are now gathered into Fig. 4. Several cytokines have pleiotropic effects, thus, to some extent the role of each distinct cytokine, chemokine or growth factor can be questioned. The used statistical method does raise a problem of multiple testing and false significances. The cytokine associations and implications to different immunological pathology need further validation, especially at the level of the vascular wall. Our aim is not to state that any one cytokine is decisive in disease development and adherent to a specific cardiovascular risk factor. Instead, we merely wish to point out that the analysis of circulating cytokine profiles in subjects with lower limb atherosclerosis suggests that there may be meaningful molecular differences in the way each cardiovascular risk factor causes atherosclerosis.

5. Conclusion

According the present findings both smoking and CKD have diverse inflammatory and myeloproliferative characteristics, which lead to severe disease progression. Aging is associated with a Th1 driven atherosclerotic disease induced by IFN-γ, while adipose tissue and insulin

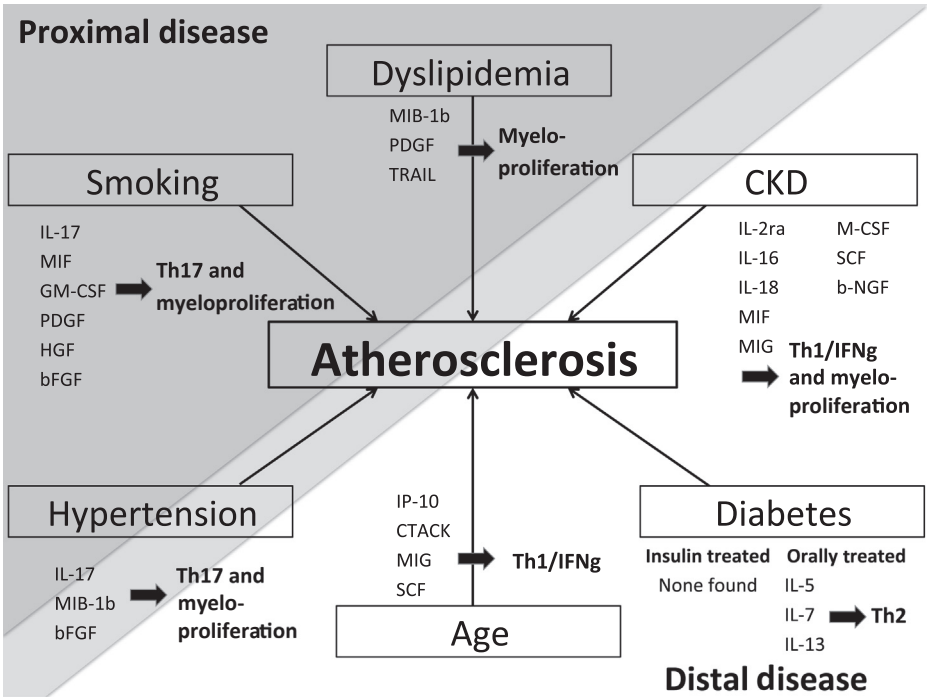

Fig. 4. Summary of results: Cardiovascular risk factors and their association to cytokines, chemokines, growth factors, and the signature pathways leading to atherosclerosis.

resistance is associated with a Th2 inflammatory cytokines.

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Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2018.11.001.

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