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Circulating soluble IL-6 receptor associates with plaque inflammation but not with atherosclerosis severity and cardiovascular risk

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ABSTRACT

Background: The residual cardiovascular risk in subjects receiving guideline-recommended therapy is related to persistent vascular inflammation and IL-6 represents a target for its treatment. IL-6 binds to receptors on leukocytes and hepatocytes and/or by forming complexes with soluble IL-6 receptors (sIL-6R) binding to gp130 which is present on all cells. Here we aimed to estimate the associations of these two pathways with risk of cardiovascular disease (CVD).

Methods: IL-6 and sIL-6R were analyzed using the proximity extension assay. Baseline plasma samples were obtained from participants in the prospective Malmö Diet and Cancer (MDC) study ($n = 4661$), the SUMMIT VIP study ($n = 1438$) and the Carotid Plaque Imaging Project (CPIP, $n = 285$). Incident clinical events were obtained through national registers. Plaques removed at surgery were analyzed by immunohistochemistry and biochemical methods.

Results: During 23.1 ± 7.0 years follow-up, 575 subjects in the MDC cohort suffered a first myocardial infarction. Subjects in the highest tertile of IL-6 had an increased risk compared to the lowest tertile (HR and 95% CI 2.60 [2.08–3.25]). High plasma IL-6 was also associated with more atherosclerosis, increased arterial stiffness, and impaired endothelial function in SUMMIT VIP, but IL-6 was only weakly associated with plaque inflammation in CPIP. sIL-6R showed no independent association with risk of myocardial infarction, atherosclerosis severity or vascular function, but was associated with plaque inflammation.

Conclusions: Our findings show that sIL-6R is a poor marker of CVD risk and associated vascular changes. However, the observation that sIL-6R reflects plaque inflammation highlights the complexity of the role of IL-6 in CVD.

There is accumulating evidence that the residual cardiovascular risk in subjects treated with therapies recommended by current guidelines is related to persistent vascular inflammation [1]. The CANTOS trial showed that treatment with interleukin (IL)-1β blocking antibody reduced the risk of ischemic cardiovascular events by 15% during a 48-months study period [2]. Even greater reductions of cardiovascular risk were observed in the LoDoCo2 and COLCOT trials that evaluated the effect of treatment with the anti-inflammatory drug colchicine [3–5]. An interesting observation in the CANTOS trial was that the protective effect of anti-IL-1β treatment was restricted to those subjects in whom a greater than median reduction of IL-6, the down-stream mediator of IL-1β, was observed [5]. Accordingly, IL-6 signaling is receiving increasing

Abbreviations: sIL-6R, soluble IL-6 receptor; MDC study, Malmö Diet and Cancer study; SUMMIT VIP study, SUrrogate markers for Micro- and Macro-vascular hard endpoints for Innovative diabetes Tools Vascular Imaging Prediction study; CPIP, Carotid Plaque Imaging Project; sgp130, soluble gp130; CRP, C-reactive protein; CCA, common carotid artery; IMT, intima media thickness; PECAM-1, platelet endothelial adhesion molecule-1 (also called CD31); TRAIL receptor-2, TNF-related apoptosis-inducing ligand receptor-2; PlGF, placental growth factor..

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attention as a possible target for intervention in subjects with cardiovascular disease (CVD) and signs of residual inflammation [6,7].

Activation of IL-6 signaling differs from that of many other cytokines. The IL-6/IL-6 receptor (IL-6R) complex lacks independent signaling capacity and must bind to another membrane protein, glycoprotein (gp) 130, to induce intracellular signaling [8]. Activation of IL-6 signaling through pre-existing membrane-bound receptors (classic signaling) only occurs in immune cells and in hepatocytes. For activation of other cells, IL-6 needs to form a complex with the soluble form of the IL-6R (sIL-6R). This complex will then activate cells through binding to gp130 expressed on the surface of cells (trans-signaling) [9]. Again, this is different from most other cytokines for which soluble receptors generally act as decoys inhibiting activation of cells. For IL-6 this function is instead served by soluble gp130 (sgp130) that can inhibit the binding of circulating IL-6/IL-6R complexes to membrane-bound gp130 [8]. There are two major mechanisms through which sIL-6R is generated. One involves cleavage of membrane-bound IL-6R by proteases such as ADAM10 and ADAM17. Cleavage by ADAM10 is constitutive while that of ADAM17 is induced by pro-inflammatory cytokines [10]. The second mechanism depends on alternative splicing of IL-6R mRNA resulting in generation of IL-6R lacking the transmembrane and cytosolic domains [11]. Studies in mice with cell-specific silencing of the IL-6R gene have suggested that around 30% of sIL-6R is released from hepatocytes and around 60% from hematopoietic cells [12]. More recently, a third way of activation of IL-6 signaling has been identified (trans-presentation) in which IL-6/IL-6R complexes on dendritic cells binds to gp130 on the surface of T cells [13].

IL-6 is well established as a marker of CVD risk both in the general population and in subjects with prevalent CVD [7,14]. Much less is known about the association of sIL-6R with CVD risk and if the association between elevated IL-6 and increased CVD risk is dependent on classical or trans-activation of IL-6 signaling. A better understanding of the respective roles of IL-6 and sIL-6R in CVD is important because available therapies target different types of IL-6 signaling [7]. In a population-based case-control study, Moreno Velásquez and coworkers found that subjects in the highest quartile of sIL-6R had 40% higher risk of myocardial infarction, while those with highest levels of sgp130 had a decreased risk [15]. In a subsequent population study, the same group also reported that subjects with high ratio of circulating IL-6/IL-6R to IL-6/IL-6R/gp130 complexes had an increased risk of CVD [16]. In the present study we used several different cohorts to analyze the associations of IL-6 and sIL-6R with risk of a first myocardial infarction, severity of atherosclerosis, vascular function and atherosclerotic plaque inflammation.

1. Methods

1.1. Study cohorts

The Malmö Diet and Cancer (MDC) study is a prospective population-based cohort ($n = 28,449$) study examining the association between diet and cancer [17]. Subjects born between 1926 and 1945 living in Malmö, Sweden were eligible for inclusion in the study. Between October 1991 and February 1994, every other participant was also invited to take part in a sub-study focusing on the epidemiology of carotid artery disease (MDC study cardiovascular cohort, $n = 6103$) [18]. Out of these, 5405 came to a second examination where fasting plasma samples were collected. We excluded 545 of these subjects from the present study due to incomplete clinical data, additionally 118 subjects were excluded because the analysis of their plasma samples did not pass the internal quality control for the biomarker analyses and 81 subjects because of previous myocardial infarction. The remaining 4661 subjects were followed from baseline examination until first myocardial infarction, emigration from Sweden, or death, up until December 31st, 2020, using the Swedish Hospital Discharge Register and the National Cause of Death Register. Myocardial infarction was defined as a fatal or non-fatal myocardial infarction based on the International Classification of Diseases 9th and 10th revisions (ICD-9 and ICD-10) codes 410 and I21. Diabetes at baseline was based on baseline interviews and data from national registries. Risk factors were determined as previously described [18]. For the occurrence of plaques (defined as a focal thickening of the intima-media complex >1.2 mm and with an area $\geq$ 10 mm^2) the bifurcation area of the right common carotid artery was scanned within a pre-defined section comprising 3 cm of the distal common carotid artery, the bifurcation, and 1 cm of the internal and external carotid artery.

The SUMMIT VIP baseline study investigation included 458 subjects with type 2 diabetes (T2D) and clinically manifest CVD, 527 subjects with T2D but without clinical signs of CVD, 245 subjects with CVD but without T2D, and 270 subjects with neither CVD nor T2D. Subjects were recruited from existing population cohorts and hospital registers at the university hospitals in Malmö (Sweden), Pisa (Italy), Dundee and Exeter (UK) between November 2010 and June 2013 as previously described [19,20]. We excluded 62 subjects from the present study due to lack of biomarker data.

The carotid plaque cohort (CPIP) consisted of 285 patients who underwent carotid endarterectomy between October 2005 and October 2010 at Skåne University Hospital [21]. Indications for carotid endarterectomy have been previously described [21]. The clinical information of the patients was registered. Analyses of plaque components by histological staining, immunohistochemistry, ELISA, PEA and biochemical assays were performed as previously reported [22,23].

All studies were approved by the respective Regional Ethical Review Boards and conducted in accordance with the Helsinki Declaration. All subjects gave written consent. The reporting of the studies is done in accordance with the STROBE guidelines.

1.2. Vascular assessments in the SUMMIT VIP cohort

The intima-media thickness (IMT) of the right and left common carotid artery (CCA) and the carotid bulbs were measured by ultrasound as previously described [21]. Endothelial function was measured using an EndoPat device (Itamar Medical, Caesarea Ind. Park, Israel) to estimate the endothelium-dependent post-ischemic hyperaemia in response to 5-min of upper arm arterial occlusion. Arterial stiffness was assessed by calculating the carotid-femoral pulse wave velocity (PWV) using a Sphygmocor device (Atcor Medical, Australia). Before the start of the study, staff at the participating centers completed a joint carotid ultrasound training program to minimize intra-observer variability resulting in an intra-observer variability of <5% in the CCA and < 10% in the carotid bulb. Detailed information about the methods used for vascular assessments, as well as data regarding intra- and inter-observer variability and calibration between centres, has been published previously [19]. The average of the left and right carotids were used to calculate the mean IMT in the CCA and the bulb. Where IMT data was available from one side only that was used as the mean value. The ankle brachial pressure index (ABPI) was calculated as the mean of the ratios between the highest systolic BP values from the right and left feet and arms.

1.3. Proximity extension assay

Plasma levels of IL-6, sIL-6R, placental growth factor (PlGF), TRAIL receptor-2, and PECAM-1 in the MDC cohort and IL-6 and sIL-6R in the CPIP cohort and the SUMMIT VIP study were analyzed by the Proximity Extension Assay (PEA) technique using the Proseek Multiplex CVD^{96x96} reagents kit (Olink Bioscience, Uppsala, Sweden) at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala. The CV for intra-assay variation (within-run) and inter-assay variation (between-run) were 6% and 12% for IL-6, 7% and 10% for sIL-6R, 12% and 13% for PlGF, 10% and 12% for TRAIL receptor-2, and 7% and 10% for PECAM-1, respectively and the analytical ranges 0.8–3125 pg/mL, 0.2–3125 pg/mL, 0.5–31,250 ng/mL, 0.2–7812 pg/mL, and 1.0–15,625 pg/mL,

respectively. All data are presented as log2 normalized protein expression values (NPX). General calibrator curves to calculate the approximate concentrations based on NPX as well as technical information about the assays are available on the Olink homepage (http://www.olink.com).

1.4. Statistical analyses

IL-6 and sIL-6R were selected a priori from the OLINK CVD 1 panel to study the interaction of these factors in assessment of risk for development of myocardial infarction. Analysis of skewness and kurtosis were used to test for normality. Differences between means of normally distributed continuous variables were assessed with independent sample *t*-tests and between skewed variables with the Mann-Whitney *U* test. χ^2 test was used for categorical variables. Correlation coefficients between continuous variables were calculated using the Spearman Rank test. The relation between marker tertiles and a first myocardial infarction during follow-up was assessed by Kaplan Meier survival curves and differences assessed by Log rank test. Cox proportional hazards regression models were used to assess the hazard ratio (HR), and 95% confidence interval (CI) of the first event in relation to marker tertiles and their ratios. The time variables used in Kaplan Meier curves and the Cox regressions models were time from the baseline investigation to first clinical event or end of follow up which was 2020-12-31. IBM SPSS Statistics 27 was used for statistical analyses.

2. Results

The MDC cohort study involved 4661 men ($n = 1831$) and women ($n = 2830$) with an age-interval from 46 to 68 years. During a mean follow-up period 23.1 ± 7.0 years, 575 subjects suffered a first myocardial infarction. Subjects with incident myocardial infarction had more cardiovascular risk factors at baseline including higher levels of CRP. They also had higher baseline levels of IL-6 and sIL-6R (Table 1). The distribution of IL-6 and IL-6R NPX levels are shown in Fig. 1A and B. Calibrator curves on the Olink website estimate that the ranges of these NPX values correspond approximately to 10 to 10^4 pg/mL for IL-6 and 10^3 to 10^4 pg/mL for sIL-6R. There was a correlation between IL-6 and sIL-6R (Fig. 1C), but this association was primarily seen in those in the lowest tertile of IL-6 (Spearman rank correlations coefficients $r = 0.35$, $p < 0.001$ in the 1st tertile versus $r = 0.06$, $p = 0.03$ in the 2nd tertile and $r = -0.01$, n.s. in the 3rd tertile). Kaplan Meier survival curves plotting the association between tertiles of IL-6 and incidence of myocardial infarction confirmed that elevated IL-6 increases risk, whereas only a weak association with risk of myocardial infarction was observed for sIL-6R (Fig. 2A and B). To investigate the importance of the IL-6 to sIL-6R ratio we split values into below and above median, forming groups with low IL-6/low sIL-6R, high IL-6/low sIL-6R, low IL-6/high sIL-6R and high IL-6/high sIL-6R. Only groups with IL-6 above median were at an increased of myocardial infarction (Fig. 2C). Moreover, when both IL-6 and sIL-6R tertiles were entered into a Cox proportional hazard

Table 1
Baseline clinical characteristics of study subjects with and without incident myocardial infarction in the MDC cohort.

	No MI ($n = 4086$)	Incident MI ($n = 575$)	*P*
Age (years)	57.2 ± 6.0	59.4 ± 5.5	<0.001
Sex (n (%) males)	1509 (38.9)	322 (56.0)	<0.001
Current smoking (n (%))	862 (21.1)	144 (25.1)	0.031
Diabetes (n (%))	146 (3.6)	46 (8.0)	<0.001
BMI (kg/m^2)	25.5 ± 3.9	26.3 ± 4.1	<0.001
fB-glucose (mmol/L)	5.08 ± 1.19	5.45 ± 1.79	<0.001
LDL (mmol/L)	4.15 ± 0.98	4.31 ± 0.92	<0.001
HDL (mmol/L)	1.41 ± 0.37	1.28 ± 0.35	<0.001
TG (mmol/L)	1.13 (0.85–1.55)	1.28 (0.94–1.73)	<0.001
Systolic BP (mm Hg)	140 ± 19	148 ± 20	<0.001
Diastolic BP (mm Hg)	86 ± 9	89 ± 10	<0.001
CRP (mg/L)	1.30 (0.60–2.70)	1.60 (0.80–3.30)	<0.001
IL-6 (NPX)	4.36 ± 1.03	4.60 ± 0.96	<0.001
sIL-6R (NPX)	8.75 ± 0.46	8.80 ± 0.47	0.012

Variables with normal distribution are shown as mean and standard deviation and statistical differences between groups calculated by Student's *t*-test. Variables with skewed distribution are shown as median and interquartile range and statistical differences between groups calculated by Mann-Whitney U test. Statistical differences between categorical variables were analyzed by χ^2 test. BMI; body mass index; fB-glucose; fasting blood glucose, LDL; low-density lipoproteins; HDL; high-density lipoproteins; TG; triglyceride; CRP; C-reactive protein.

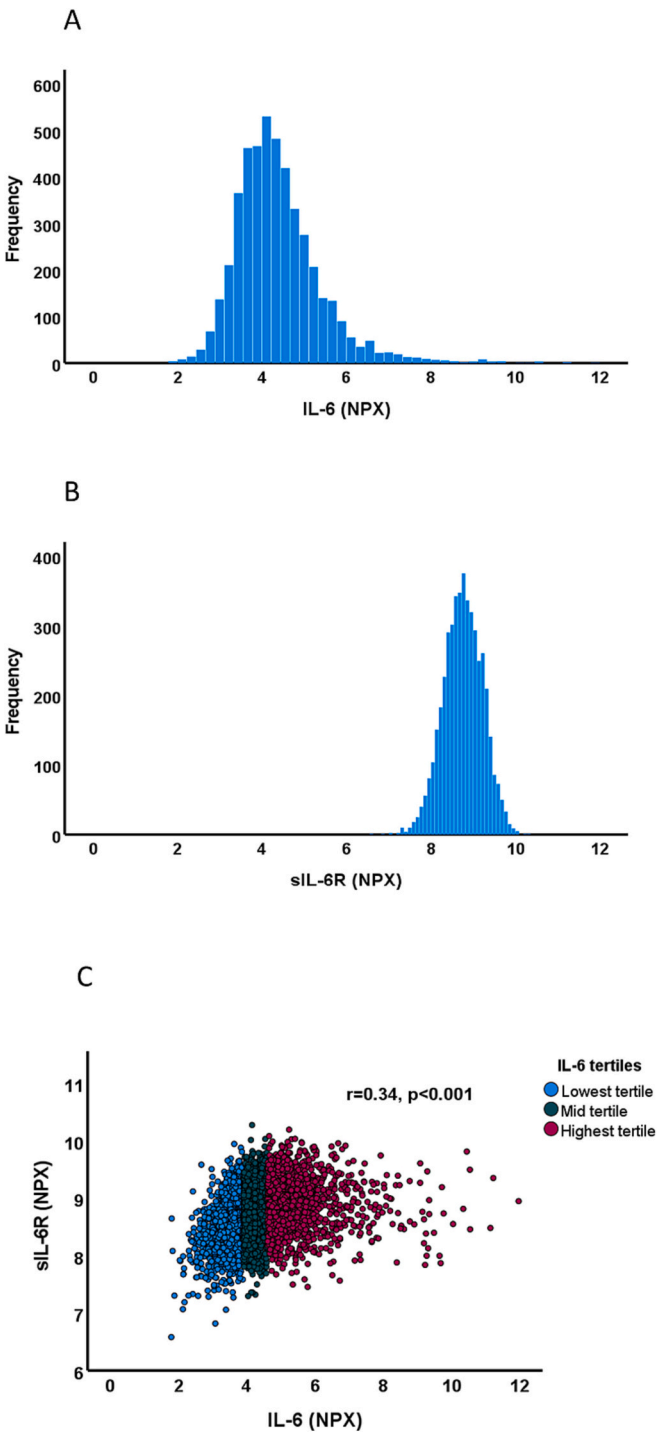

Fig. 1. *Distributions of IL-6 and sIL-6R in the MDC cohort.* Distributions of (A) IL-6 and (B) sIL-6R NPX values in the MDC cohort. (C) Dot plot showing the correlation (Spearman rank correlation coefficient) between IL-6 and sIL-6R.

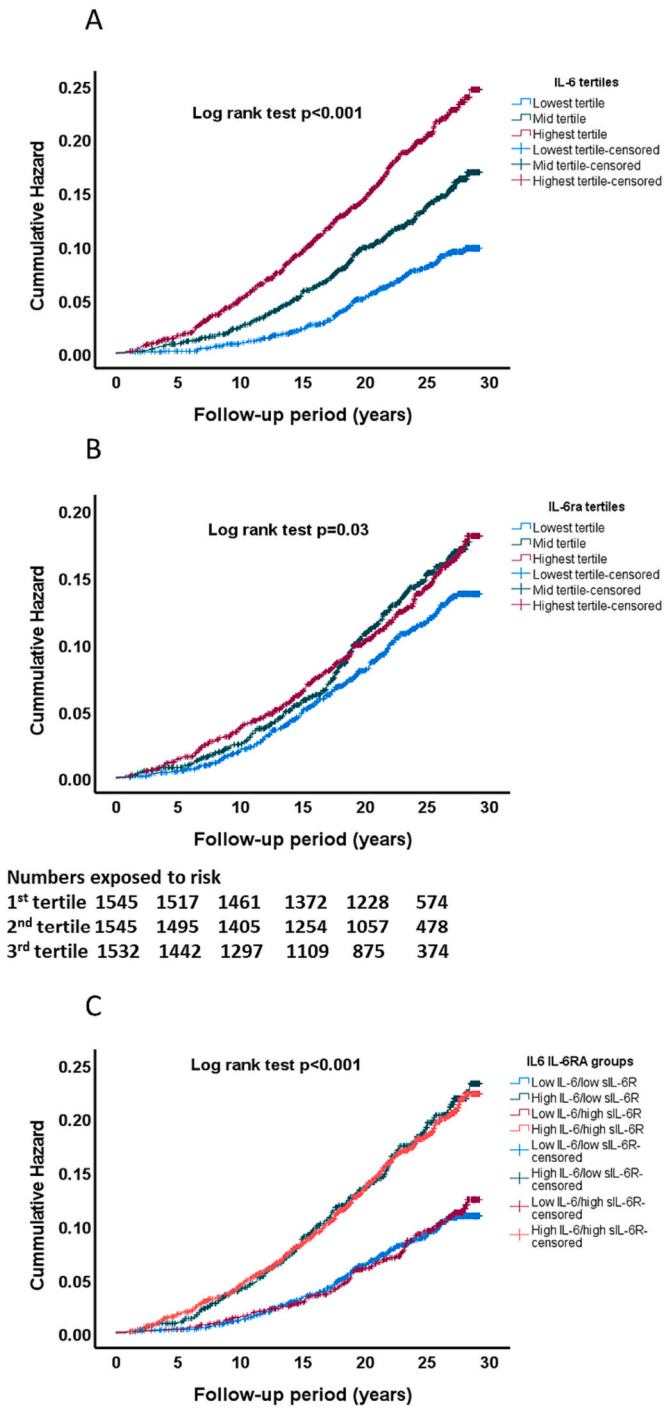

Fig. 2. *Kaplan-Meier survival curves of the associations IL-6 and sIL-6R with risk of myocardial infarction in the MDC cohort.* Kaplan-Meier curves demonstrating the association between (A) IL-6 and (B) sIL-6R tertiles with risk of myocardial infarction. (C) Associations between IL-6/sIL-6R ratios and risk of myocardial infarction. Low indicates a NPX value below median and high above median. Significance of differences were calculated using the Log rank test.

regression model simultaneously only IL-6 remained significantly associated with risk of myocardial infarction (HR and 95% CI 1.72 [1.37–2.16] for 2nd tertile and 2.60 [2.08–3.25] for 3rd tertile compared to the first tertile). When also age, sex, diabetes, current smoking, LDL and HDL cholesterol, and systolic blood pressure were included in the model only the 3rd tertile of IL-6 remained independently associated with risk of myocardial infarction (HR and 95% CI 1.46 [1.10–1.96]). Using IL-6 and sIL-6R normalized Z scores as continues variables in the Cox regression models to analyze increase in risk per standard deviation increase in IL-6 and sIL-6R similar observations were made. When both IL-6 and IL-6R were included simultaneously in the model only IL-6 remained significantly associated with risk of myocardial infarction (HR and 95% CI 1.20 [1.12–1.29]). In the fully adjusted model, the hazard ratio per standard deviation increase in IL-6 was 1.11 (1.2–1.21).

In the MDC cohort, IL-6 was higher in males (4.49 ± 1.02 versus 4.33 ± 1.03 NPX in females, $p < 0.001$), current smokers (4.73 ± 1.05 versus 4.30 ± 1.00 NPX in non-smokers) and in subjects with diabetes (4.82 ± 1.03 versus 4.37 ± 1.03 NPX in subjects without diabetes, $p < 0.001$ for all). Also, sIL-6R was higher in males (8.78 ± 0.46 versus 8.74 ± 0.46 NPX in females, $p = 0.001$) and in subjects with diabetes (8.89 ± 0.44 versus 8.75 ± 0.46 NPX in subjects without diabetes, $p < 0.001$), whereas there was no significant difference between current smokers and non-smokers (8.75 ± 0.46 versus 8.76 ± 0.46 NPX). Both IL-6 and sIL-6R also demonstrated association with other risk factors, but only IL-6 correlated with CRP (Table 2). Both IL-6 and sIL-6R correlated with biomarkers that have been associated with increased endothelial metabolic stress including platelet endothelial adhesion molecule-1 (PECAM-1), TNF-related apoptosis-inducing ligand (TRAIL) receptor-2 and placental growth factor (PlGF; Table 2) [24,25]. Experimental studies have identified hematopoietic cells as a major source of sIL-6R [12] and that release from apoptotic neutrophils is of particular importance [8]. Although some weak associations were observed between sIL-6R and blood leukocytes they did not reach the same level of statistical significance as those between IL-6 and blood leukocytes (Table 2). However, as noted above there was a highly significant association between sIL-6R and the apoptosis marker TRAIL-R2.

2.1. Associations of IL-6 and sIL-6R with markers of atherosclerosis and vascular function

We next investigated the association of IL-6 and sIL-6R with atherosclerosis as assessed by ultrasound of the carotid artery in the baseline investigation of the MDC cohort. Subjects with a carotid plaque (defined as a focal thickening of the intima-media complex >1.2 mm and with an area ≥ 10 mm^2, $n = 1501$) had higher IL-6 than those with no plaque (4.49 ± 1.05 versus 4.32 ± 1.00 NPX, $p < 0.001$), while there

Table 2
Spearman rank correlation coefficients between IL-6, sIL-6R, risk factors and biomarkers of endothelial stress in the MDC cohort.

	IL-6	*P*	sIL-6R	*P*
Risk factors				
Age	0.20	<0.001	0.08	<0.001
BMI	0.23	<0.001	0.09	<0.001
fB-glucose	0.21	<0.001	0.09	<0.001
LDL	0.04	<0.05	0.08	<0.001
HDL	−0.25	<0.001	−0.10	<0.001
Triglycerides	0.23	<0.001	0.14	<0.001
Systolic BP	0.20	<0.001	0.11	<0.001
CRP	0.48	<0.001	0.03	n.s.
Biomarkers of endothelial stress				
PECAM-1	0.27	<0.001	0.51	<0.001
TRAIL receptor-2	0.50	<0.001	0.46	<0.001
PlGF	0.47	<0.001	0.57	<0.001
Blood cells				
Total leukocytes	0.26	<0.001	0.04	0.035
Monocytes	0.17	<0.001	0.06	<0.001
Lymphocytes	0.13	<0.001	0.03	n.s.
Neutrophils	0.25	<0.001	0.03	0.046
Erythrocytes	0.11	<0.001	0.08	<0.001

BMI; body mass index; fB-glucose; fasting blood glucose, LDL; low-density lipoproteins; HDL; high-density lipoproteins; systolic BP; systolic blood pressure, CRP; C-reactive protein, PECAM-1; platelet endothelial adhesion molecule-1 (also called CD31), TRAIL receptor-2; TNF-related apoptosis-inducing ligand receptor-2, PlGF; placental growth factor.

was no significant difference in sIL-6R (8.75 ± 0.46 versus 8.75 ± 0.47 NPX). To further explore the associations of IL-6 and sIL-6R with markers of atherosclerosis and vascular function we analyzed data from the SUMMIT VIP (SUrrogate markers for Micro- and Macro-vascular hard endpoints for Innovative diabetes Tools Vascular Imaging Prediction) study. IL-6 was higher in subjects with a history of CVD (5.48 ± 0.99 versus 5.15 ± 0.98 NPX, $p < 0.001$) as well as in those with diabetes (5.42 ± 0.99 versus 5.10 ± 0.98 NPX, $p < 0.001$), whereas there were no statistical differences in sIL-6R (6.69 ± 0.46 versus 6.66 ± 0.44 NPX and 6.68 ± 0.44 versus 6.67 ± 0.45 NPX). There were significant associations between IL-6 and markers of atherosclerosis (common carotid artery and carotid bulb intima media thickness, and ankle brachial pressure index), increased arterial stiffness (pulse wave velocity) and impaired endothelial function (reactive hyperemia index) (Table 3). Except for an association with the ankle brachial pressure index there were no significant associations between sIL-6R and markers of atherosclerosis and vascular function (Table 3).

2.2. Associations of IL-6 and sIL-6R with atherosclerotic plaque markers of inflammation and fibrosis

We finally investigated how circulating IL-6 and sIL-6R related to plaque inflammation and factors of importance for maintaining the stability of plaques. For this purpose, we used plasma and plaques from patients participating in the Carotid Plaque Imaging Project (CPIP). Blood samples were taken the day before carotid surgery and the removed plaque analyzed by several different technologies. We used immunohistochemistry to assess the presence of smooth muscle cells (α-actin) and macrophages (CD68) in plaque sections. Plaque homogenates were used to analyze the content of pro-inflammatory cytokines, fibrous proteins, and apoptotic activity (expression of activated caspase 3). There was an association between plasma and plaque levels of IL-6, but otherwise circulating IL-6 did not reflect the plaque inflammation, content of fibrous proteins or apoptotic activity (Table 4). In contrast, sIL-6R correlated significantly with plaque staining for macrophages (CD68) as well as with the plaque content of tumor necrosis factor (TNF)-α, CX3CL1 (also known as fractalkine) and interferon (INF)-γ. Interestingly, high plasma sIL-6R was also associated with several factors known to promote plaque stability including more smooth muscle cells (α-actin), elastin and collagen as well as with less apoptosis (decreased levels activated caspase-3, Table 4).

3. Discussion

In line with previously published studies [14,26], we found that high levels of IL-6 are associated with an increased risk of a first myocardial infarction. We also confirm previous observations of associations between high IL-6 and more advanced atherosclerosis [27], increased arterial stiffness [28] and impaired endothelial function [29]. If IL-6 transactivation is involved in these associations it could be expected that also sIL-6R would be associated with CVD risk as previously reported by Moreno Velásquez and coworkers [15]. However, although we found a weak association between sIL-6R and the risk of a first myocardial infarction, this did not remain significant when adjusting for IL-6. Neither did the ratio of IL-6 to sIL-6R appear to be of importance since subjects with IL-6 above median were at increased risk independently of their level of sIL-6R and likewise subjects with below median IL-6 were at a lower risk independent on their level of sIL-6R. Moreover, although sIL-6R showed a weak inverse correlation with the ABPI, there were no associations with carotid IMT, pulse wave velocity or endothelial function. Collectively, these observations imply that IL-6 is a marker of CVD risk and the severity of vascular pathologies, whilst sIL-6R is not. One possible explanation for this could be that classical IL-6 activation, but not transactivation, is involved in CVD processes. Unfortunately, this possibility cannot be properly tested in the present study because the analytical technique used cannot differentiate between free IL-6 and IL-6 bound to sIL-6R. Based on calculation of intramolecular affinities it was originally assumed that most circulating IL-6 was in a complex with sIL-6R [30], but more recent experimental data have shown that only a minor fraction of IL-6 is bound to sIL-6R [9]. This is in agreement with our observation that the level of IL-6 provides a better reflection of CVD risk than that of sIL-6R and may imply that classical IL-6 activation plays a more important role in cardiovascular disease than transactivation (Fig. 3).

Table 3
Spearman rank correlation coefficients between IL-6, and sIL-6R, and markers of atherosclerosis and vascular function in the SUMMIT VIP cohort.

	IL-6	*P*	sIL-6R	*P*
CCA mean IMT	0.12	<0.001	0.00	n.s.
CCA max IMT	0.13	<0.001	0.01	n.s.
Carotid bulb mean IMT	0.19	<0.001	0.09	n.s.
Carotid bulb max IMT	0.18	<0.05	0.08	n.s.
Ankle brachial pressure index	−0.17	<0.001	−0.10	0.003
Pulse wave velocity	0.27	<0.001	0.14	n.s.
Reactive hyperemia index	−0.11	<0.001	0.11	n.s.

CCA; common carotid artery. IMT: intima media thickness, n.s.; not significant.

Table 4
Spearman rank correlation coefficients between circulating IL-6 and sIL-6R and carotid plaque content of cytokines, connective tissue components and apoptotic activity.

	IL-6	*P*	sIL-6R	*P*
Pro-inflammatory factors				
CD68 (% stained area, n = 285)	0.05	n.s.	0.12	0.04
IL-1β (pg/g plaque tissue, *n* = 194)	0.00	n.s.	−0.02	n.s.
IL-6 (pg/g plaque tissue, *n* = 195)	0.20	0.006	0.00	n.s.
TNF-α (pg/g plaque tissue, n = 195)	0.00	n.s.	0.18	0.015
CCL2 (pg/g plaque tissue, n = 195)	0.07	n.s.	−0.05	n.s.
CX3CL1 (pg/g plaque tissue, n = 195)	0.04	n.s.	0.19	0.008
INF-γ (pg/g plaque tissue, n = 194)	−0.07	n.s.	0.17	0.02
Connective tissue components				
α-actin (% stained area, n = 285)	−0.10	n.s.	0.14	0.03
Elastin (mg/g plaque tissue, *n* = 205)	−0.01	n.s.	0.23	0.001
Collagen (mg/g plaque tissue, *n* = 216)	0.04	n.s.	0.14	0.042
Apoptosis				
Activated caspase-3 (ng/g plaque tissue, n = 194)	−0.07	n.s.	−0.31	<0.001

Plasma IL-6 and sIL-6R were measured by PEA while plaque cytokines were measured by ELISA. n.s.; not significant.

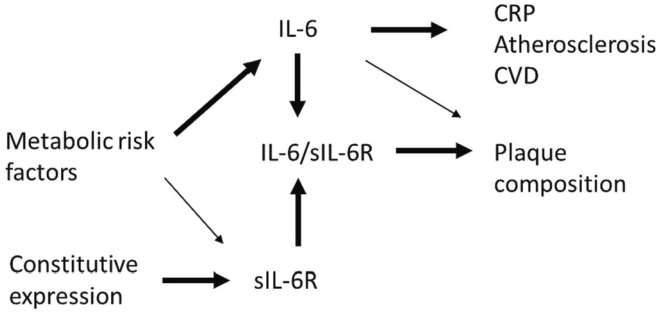

Fig. 3. *IL-6 and sIL-6R in CVD.* Metabolic risk factors activate the expression of IL-6 in various cell types and to some extent also the expression of sIL-6R. The latter is likely to be caused by inflammatory activation of immune cells and hepatocytes. sIL-6R is also constitutively expressed by the same cell types. IL-6 contributes to inflammation, atherosclerosis, and increased CVD risk by activation of membrane-bound IL-6R on immune cells and/or by forming complexes with sIL-6R that induces inflammation by binding gp130 on other cells. The magnitude of this response is primarily dependent on the circulating level of IL-6 rather than that of sIL-6. Despite this, sIL-6R appears critical for the effects of IL-6 on atherosclerotic plaque inflammation and stability.

The factors regulating the release of sIL-6R in humans remain to be fully characterized. Experimental studies have identified the liver and hematopoietic cells as the major source of sIL-6R [12], and that inflammation and apoptosis are the most important factors responsible for activation of the release [8]. While the release of sIL-6R induced by proinflammatory cytokines is mediated by the protease ADAM17 [31], there is also a slow constitutive release mediated by ADAM10 [32]. CRP is a well-established marker of activation of hepatocyte inflammatory responses [33]. We observed associations between metabolic factors known to induce hepatocyte stress (as assessed by CRP) and sIL-6R, but these were not as strong as for IL-6 itself. Moreover, we found no association between CRP and sIL-6R suggesting that activated hepatocytes are not likely a major source of sIL-6R in humans. However, as mentioned above there is also a constitutive release of sIL-6R. In accordance, we observed less variation in sIL-6R (ten-fold) than in IL-6 (thousand-fold) levels. As there were weak associations between sIL-6R and the number of leukocytes in the blood it is likely that most of the constitutively expressed sIL-6R originates from the liver and not from leukocytes.

The observations discussed above indicate that it is the expression of IL-6 rather than that of sIL-6R that is of importance for CVD risk. Against this background, it was unexpected to find that, apart from the expression of IL-6 in atherosclerotic plaques, sIL-6R was a better marker of plaque inflammation than IL-6. This is, on the other hand, in line with studies in LDL receptor-deficient mice demonstrating that selectively blocking IL-6 transactivation using a fusion protein of natural gp130 and IgG1-Fc reduces vascular inflammation and development of atherosclerosis [34], as well as with other studies demonstrating that inhibition of IL-6 transactivation reduces organ inflammation in animal models of autoimmune diseases [8]. Moreover, studies using Mendelian randomization have shown that genetic variations in the IL-6R gene correlates with cardiovascular risk [35–37],but these have paradoxically shown that genetic variations associated with increased levels of sIL-6R are associated with decreased CRP and lower cardiovascular risk. Interestingly, our data provide support for a role of IL-6 transactivation in plaque inflammation despite the lack of association between sIL-6R and cardiovascular risk. One possible explanation for this apparent contradiction is that there was also an association between sIL-6R and the expression of plaque-stabilizing factors such as decreased apoptotic activity and an increased content of fibrous protein (Fig. 3). Activation of circulating leukocytes through transactivation represents one possible link to increased plaque inflammation. This would lead to migration of activated leukocytes into the intima. Activated leukocytes and cytokines, such as CX3CL1 and IFN-γ, are crucial for the removal of apoptotic cells (efferocytosis) in atherosclerosis and efficient efferocytosis is required for the resolution of inflammation and fibrotic tissue repair [38]. This could potentially explain the inverse association between sIL-6R and activated caspase-3 (marker of apoptotic cells), as well as the positive associations between sIL-6R, α-actin, collagen, CD68, CX3CL1 and IFN-γ, that were identified in the present study. However, our findings highlights the complex role of IL-6 signaling in atherosclerosis revealed in experimental studies demonstrating that while administration of exogenous IL-6 enhances the formation of early plaques in apo E deficient mice [39], genetic deletion of IL-6 has the same effect [40].

A strength of the present study is that the Swedish national registers provide complete and quality assessed data regarding incident clinical events. However, there are some limitations with the present study that need to be considered. The PEA methodology provides arbitrary units instead of the SI units used clinically which makes direct clinical translation of the present observations difficult. We did not analyze plasma levels of sgp130. This protein binds to circulating IL-6/sIL-6R complexes thus blocking transactivation by inhibiting the binding of IL-6/sIL-6R complexes to gp130 expressed on the surface of cells [41]. In a prospective population cohort including 60-year-old men and women Ziegler and coworkers [16] found that subjects with a high IL-6/sIL-6R to IL-6/sIL-6R/sgp130 ratio had a higher risk of future CVD events providing evidence for a role of IL-6 transactivation in CVD. Interestingly, they found that up to the 75th percentile of sgp130 there was an association with increased risk of CVD events, whereas higher values were associated with a lower risk. Hence, we cannot exclude that the level of sgp130 may have had an influence on the lack of associations of sIL-6R and cardiovascular risk found in our study. Another limitation is that IL-6 and sIL-6R were only determined at the baseline investigation and we lack data on changes in plasma levels during the follow-up period. Moreover, we did not have data on changes in medication during follow-up.

4. Conclusions

Our findings confirm previous observations that IL-6 is a marker of CVD risk, severity of atherosclerosis, arterial stiffness, and endothelial function whilst sIL-6R is not. Although this argues against a role for IL-6 transactivation in CVD we also found that sIL-6R demonstrated stronger activation with atherosclerotic plaque inflammation than IL-6. The possibility that IL-6 transactivation could promote plaque inflammation without affecting CVD risk appears counterintuitive. However, the observations that high circulating levels of sIL-6R also are associated with plaques that have less apoptosis and more stabilizing fibrous components indicate, in agreement with earlier mouse studies, a complex role of IL-6 in CVD.

CRediT authorship contribution statement

Andreas Edsfeldt: Conceptualization, Formal analysis, Investigation, Resources, Writing – review & editing. **Isabel Gonçalves:** Investigation, Resources, Writing – review & editing. **Isa Vigren:** Formal analysis, Writing – review & editing. **Anja Jovanović:** Formal analysis, Writing – review & editing. **Gunnar Engström:** Investigation, Resources, Writing – review & editing. **Angela C. Shore:** Investigation, Resources, Writing – review & editing. **Andrea Natali:** Investigation, Resources, Writing – review & editing. **Faisel Khan:** Investigation, Resources, Writing – review & editing. **Jan Nilsson:** Conceptualization, Formal analysis, Investigation, Resources, Funding acquisition, Writing – original draft.

Declaration of Competing Interest

The authors have no conflict of interest to disclose.

Data availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions.

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