Serum Cytokine Tumor Necrosis Factor-Alpha and Interleukin-6 Associated with the severity of Coronary Artery Disease: Indicators of an Active Inflammatory Burden?

Israel Gotsman MD[1], Ayala Stabholz DMD[2], David Planer MD[1], Thea Pugatsch PhD[1], Ludmila Lapidus MD[1], Yelena Novikov MD[1], Siham Masrawa BSc[1], Aubrey Soskolne DMD[2] and Chaim Lotan MD[1]

[1]Heart Institute, Department of Cardiology, and [2]Department of Periodontology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel

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Abstract

Background: Atherosclerosis is a chronic inflammatory process resulting in coronary artery disease.

Objectives: To determine the relationship between inflammatory markers and the angiographic severity of CAD.

Methods: We measured inflammatory markers in sequential patients undergoing coronary angiography. This included C-reactive protein, fibrinogen, serum cytokines (interleukin-1 beta, IL-1 receptor antagonist, IL-6, IL-8, IL-10) and tumor necrosis factor-alpha), all measured by high sensitivity enzyme-linked immunoabsorbent assay.

Results: There was a significant correlation between TNFα and the severity of CAD as assessed by the number of obstructed coronary vessels and the Gensini severity score, which is based on the proximity and severity of the lesions. Patients had more coronary vessel disease (> 70% stenosis) with increasing tertiles of serum TNFα; the mean number of vessels affected was 1.15, 1.33, and 2.00 respectively ($P < 0.001$). IL-6 correlated with the Gensini severity score and coronary vessel disease (> 70% stenosis). A weaker correlation was present with IL-1 receptor antagonist. A significant correlation was not found with the other inflammatory markers. After adjustment for major risk factors, multivariate analyses showed that significant independent predictors of CAD vessel disease were TNFα ($P < 0.05$) and combined levels of TNFα and IL-6 ($P < 0.05$). IL-6 levels were independently predictive of Gensini coronary score ($P < 0.05$).

Conclusion: TNFα and IL-6 are significant predictors of the severity of coronary artery disease. This association is likely an indicator of the chronic inflammatory burden and an important marker of increased atherosclerosis risk.

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Inflammation plays a key role in the initiation and propagation of atherosclerosis [1]. Inflammatory markers such as C-reactive protein and other cytokines are elevated in the acute phase of a coronary event [2], presumably due to activation of the inflammatory cascade, and are predictive of future events [3]. Risk factors for the development of atherosclerosis have been identified, but several inflammatory response markers may also be important in identifying patients at risk.

Several pro-inflammatory cytokines, including interleukin-1, IL-6, IL-8, tumor necrosis factor-alpha, as well as anti-inflammatory cytokines IL-1 receptor antagonist and IL-10, have been identified as part of the inflammatory process of atherosclerosis. Serum levels may be elevated in patients with coronary artery disease. We therefore evaluated the relationship between the level of inflammatory cytokines and the extent and severity of CAD.

We compared the association of specified inflammation markers and cytokines to the extent and severity of CAD in a sequential cohort of patients undergoing diagnostic coronary angiography due to stable angina or acute coronary syndromes. The relationship was evaluated within the entire cohort and in each group separately.

Patients and Methods

We recruited 201 sequential patients undergoing diagnostic coronary angiography due to stable CAD or ACS within a 6 month study period. All patients gave informed consent according to the guidelines and approval of the Hebrew University-Hadassah Hospital Institutional Committee for Human Studies

The data recorded included age, gender, smoking status, hypercholesteremia, diabetes, hypertension, family history of CAD, body mass index, the clinical presentation of the patient and the presence of stable CAD (stable angina pectoris) or ACS (unstable angina or myocardial infarction).

Hypertension was defined as blood pressure over 140/90 mmHg, as measured on several occasions, or the use of antihypertensive treatment; diabetes as a fasting plasma glucose over 126 mg/dl or the use of glucose-lowering treatment; hyperlipidemia as low density lipoprotein levels over 130 mg/dl or high density lipoprotein < 35 mg/dl; triglycerides > 200 mg/dl or the use of lipid-lowering therapy.

CAD = coronary artery disease
IL = interleukin
TNFα = tumor necrosis factor-alpha
ACS = acute coronary syndrome

Angiographic assessment of CAD severity

The anatomic severity of the coronary atherosclerosis was based on high quality cine-angiograms. The number of lesions and arteries involved and the severity of the individual lesions were measured and recorded. An estimation of the atherosclerotic burden was based on the number of coronary vessels involved (non-obstructive, single, double or triple-vessel disease with stenosis > 50% or 70%). We also used an index based on the Gensini Coronary Artery Severity Score that takes into account the number of lesions, severity of obstruction and anatomic location [4]. The analysis was undertaken by an independent experienced angiographer blinded to the patient's inflammatory and cytokine profile.

Markers of inflammation

Blood was drawn from patients prior to angiography and aliquots were sent for assessment of inflammatory markers while the remaining samples were frozen and stored at -4°C until used for cytokine analysis. We measured erythrocyte sedimentation rate, white blood cell count, platelets and fibrinogen. CRP was measured using a standard kit (COBAS Integra, Roche Diagnostic Systems, Basel, Switzerland). Serum cytokine levels were measured at the end of the study in a selection of the patients, taken in sequential order from both the stable and acute patients, representing a random sample. IL-1, IL-1RA, TNFα, IL-6, IL8 and IL-10 were measured in triplicate using highly specific enzyme-linked immunosorbent assay assay kits (R&D Inc. Minneapolis, USA). Serum cytokine levels were available for 119 patients.

Statistical analysis

As the cytokine data were skewed, non-parametric tests were used to analyze data and are presented as median and inter-quartile range. Normally distributed data are presented as mean ± SEM. Data were compared and analyzed statistically using the independent sample Mann-Whitney U Test for the continuous variables. Correlations between cytokine levels and CAD were assessed by calculation of Spearman's rank correlation coefficients. Comparison of the difference in mean vessel disease or severity index between different tertiles of cytokines was done using the Kruskal-Wallis Test with post hoc multiple comparison using Dunnett's test. Multivariant linear regression analysis was performed on the whole cohort in order to define independent predictors of the severity of CAD. Skewed variables were log-transformed prior to the analysis. A P value of less than 0.05 was considered to indicate a statistically significant difference. All analyses were done with the SPSS statistical package version 11.5 (SPSS Inc, Chicago, IL).

Results

Clinical characteristics and inflammatory markers

We recruited 201 sequential patients for this study. Table 1 presents the clinical characteristics of the sample and the distribution of their risk factors and selected cytokines.

Table 1. Clinical characteristics of the patients

	Patients (N=201)
Age (yrs) (mean ± SD) (range)	58 ± 10 (35–88)
Gender (male)	175 pts. (87%)
All smokers (past and present)	125 (62%)
Present smoker	51 (25%)
Hyperlipidemia	127 (63%)
Family history	88 (44%)
Hypertension	95 (47%)
Diabetes	52 (26%)
BMI (mean ± SD)	27 ± 4.4
S/P myocardial Infarction	71 (35 %)
S/P coronary bypass surgery	24 (12 %)
Stable/acute coronary syndrome	101/100 pts
Myocardial infarction/unstable angina	74/26
Vessel disease (stenosis > 70%)	
Non-obstructive	41 (19%)
Single vessel	66 (31%)
Double vessel	46 (21%)
Triple vessel	44 (20%)
CRP (mg/L) (normal value < 0.5)	0.5 (0.2–1.6) (158)
TNFα (pg/ml) (normal value < 20)	6.7 (4.3–8.8) (119)
IL-6 (pg/ml) (normal value < 10)	7.5 (0–14.3) (119)

Cytokine data are given as median (interquartile range); separate parentheses denote the sample size.

All inflammatory markers and cytokines were elevated in patients with acute coronary syndromes as compared to stable patients (data not shown). This difference was significant in all markers except TNFα, IL-8 and IL-10.

Correlation between CAD and inflammatory markers

There was a significant correlation between the severity of obstructive coronary disease and the cytokine TNFα and IL-6 and to a lesser degree with IL-1RA in the whole cohort of patients [Table 2A]. Patients with increasing serum TNFα levels had significantly more coronary arteries with > 70% stenosis. Tertiles of serum TNFα levels strengthened the correlation. The three tertiles were associated with a mean of 1.15 ± 0.16, 1.33 ± 0.17 and 2.00 ± 0.15 vessels involved per patient ($P < 0.001$) [Figure

Table 2A. Spearman's correlation between IL-6, TNFα and coronary vessel disease in all patients and with TNFα separately in the subgroups of patients with stable and acute coronary disease

	All patients (n=119)				Stable patients (n=74)		Acute coronary syndromes (n=45)	
	IL-6	Tertiles of IL-6	TNFα	Tertiles of TNFα	TNFα	Tertiles of TNFα	TNFα	Tertiles of TNFα
Vessel disease (> 70%)	0.22*	0.26**	0.30**	0.32***	0.26*	0.29*	0.37*	0.36*
Vessel disease (> 50%)	0.13	0.16	0.24**	0.30***	0.23*	0.30**	0.27	0.28*
Gensini Severity Index	0.28**	0.33***	0.23*	0.27**	0.26*	0.30**	0.15	0.20

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

CRP = C-reactive protein
IL-1RA = IL-1 receptor antagonist

Table 2B. Independent predictors of coronary disease by multivariate linear regression analysis (n=119)

Predictor*	Standardized coefficient α	*P*
Vessel disease (> 70%)		
Age	0.24	0.03
Hyperlipidemia	0.20	0.03
Tertiles of TNFα	0.26	0.02
Tertiles of TNFα x IL-6	0.25	0.04
Vessel disease (> 50%)		
Age	0.20	0.05
Hyperlipidemia	0.30	0.002
Tertiles of TNFα	0.25	0.01
Gensini Severity Index		
Age	0.19	0.07
Hyperlipidemia	0.23	0.02
Tertiles of IL-6	0.22	0.03

* Variables included in the model were: age, gender, hypertension, hyperlipidemia, diabetes, current or prior smoker, BMI, family history of CAD and tertiles of TNFα, tertiles of IL-6 or combined tertiles of IL-6 and TNFα.

1A]. Post hoc analysis demonstrated a significant difference between the lowest and highest tertiles of TNFα ($P < 0.001$). This significant correlation was evident also when comparing the number of vessels involved with a stenosis > 50% and with the Gensini coronary severity score [Table 2A]. Separate analysis of the correlations in the subgroups of patients with stable angina and acute coronary syndromes also demonstrated a significant correlation between TNFα and CAD [Table 2A]. In the stable group, increasing tertiles of serum TNFα levels had significantly more coronary arteries with > 70% stenosis. The three tertiles were associated with a mean of 1.04 ± 0.23, 1.33 ± 0.24 and 1.69 ± 0.21 vessels involved per patient ($P < 0.05$) [Figure 1B]. Post hoc analysis demonstrated a significant difference between the lowest and highest tertiles of TNFα ($P < 0.05$).

IL-6 serum levels also correlated with the severity of CAD within the entire cohort. As with TNFα, this correlation was evident when compared to the Gensini coronary severity score and with the number of obstructed vessels > 70%. A stronger correlation was evident when the patients were divided into tertiles of serum IL-6. There was a higher Gensini coronary severity score with increasing IL-6 tertiles: mean score 47 ± 6.9, 53 ± 10 and 90 ± 8.5 ($P < 0.001$) for first, second and third tertiles, respectively. Post hoc analysis demonstrated a difference between the lowest and highest tertiles of IL-6 ($P < 0.001$). There was more obstructive vessel disease (> 70%) with increasing IL-6 tertiles: mean vessel disease 1.30 ± 0.16, 1.44 ± 0.16 and 2.00 ± 0.15 respectively ($P < 0.001$) [Figure 1A]. There was a significant difference between the lowest and highest tertiles of IL-6 ($P < 0.001$). We did not find a significant correlation when analyzing the subgroups of ACS and stable patients separately.

A weaker, yet evident, correlation with IL-1RA in the whole cohort became significant by comparing tertiles of the cytokine levels to obstructive vessel disease (> 70%) ($r = 0.24$, $P < 0.05$) (n=86). There was more obstructive vessel disease (> 70%) with increasing IL-1RA tertiles: 1.14 ± 0.20, 1.52 ± 0.19 and 1.74 ± 0.19, respectively (n=40, $P < 0.05$).

Combining IL-6 and TNFα

Combining tertiles of TNFα with tertiles of IL-6 demonstrated a strong association with CAD. A correlation in the whole cohort between tertiles of the combination of TNFα and IL-6 was evident with all three parameters of CAD that we analyzed: vessel disease > 70% ($r = 0.39$, $P < 0.001$); Gensini severity score ($r = 0.34$, $P < 0.001$); vessel disease > 50% ($r = 0.28$, $P < 0.01$). This correlation was evident also when analyzing separately the subset of stable patients (n=74). There was a correlation evident with all three parameters of CAD: vessel disease > 70% ($r = 0.3$, $P < 0.01$); Gensini severity score ($r = 0.3$, $P < 0.01$); vessel disease > 50% ($r = 0.33$, $P < 0.01$). This correlation was not present when analyzing the ACS subgroup.

There was no significant correlation, neither in the whole cohort nor in the patient subsets, between coronary disease and the other inflammatory markers: ESR, WBC, platelets, fibrinogen, CRP or the other cytokines evaluated (IL-1, IL-8 and IL-10).

Correlations between inflammatory markers and cytokines

We examined correlations between the inflammatory markers and cytokines. There was a significant correlation between IL-6 and

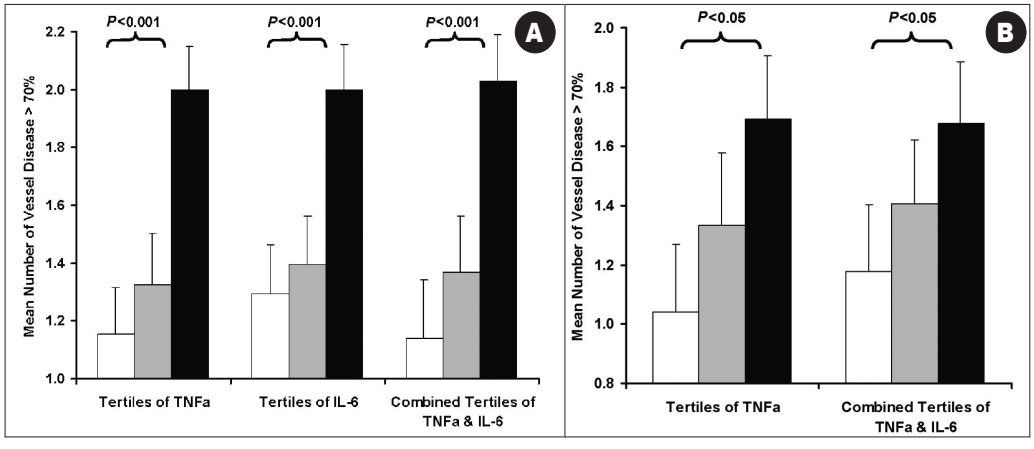

Figure 1. Association between inflammatory cytokines and CAD, as measured by mean number of obstructed coronary vessel disease > 70% in the whole cohort (n=119) **[A]** and in the subset of patients with stable angina (n=74) **[B]**.

ESR = erythrocyte sedimentation rate
WBC = white blood cells

TNFα (r = 0.24, P < 0.001) and between IL-6 and CRP (r = 0.30, P < 0.001) in the entire cohort and the ACS subset. There were no significant correlations within the stable subset. There was no correlation in any subset between TNFα and CRP. Interestingly, CRP significantly correlated with the inflammatory markers ESR (r = 0.32, P < 0.01), fibrinogen (r = 0.33, P < 0.01) and platelet count (r = 0.29, P < 0.01) in the stable group.

Predictors of coronary disease:

Univariate analysis including risk factors and inflammatory markers in the whole cohort (n=119) demonstrated that age (r = 0.31, P < 0.0001), hyperlipidemia (r = 0.25, P < 0.001), hypertension (r = 0.15, P < 0.01), TNFα (r = 0.29, P < 0.001) and IL-6 (r = 0.22, P < 0.01) significantly correlated with severe coronary disease (stenosis > 70%). Multivariate linear regression analysis for the prediction of the number of arteries involved (stenosis > 70%), examining age, gender, hypertension, hyperlipidemia, diabetes, current or prior smoker, BMI, family history of CAD, and either tertiles of TNFα or tertiles of IL-6, showed that independent predictors were age, hyperlipidemia and tertiles of TNFα [Table 2B]. Independent predictors of the number of arteries involved (stenosis > 50%) were age, hyperlipidemia and tertiles of TNFα. Independent predictors of the Gensini severity score were hyperlipidemia, tertiles of IL-6 and age. Analysis of the combined parameter of TNFα and IL-6 demonstrated that the TNFα and IL-6 tertile combination was an independent predictor of vessel disease > 70% [Table 2B].

Discussion

The aim of our study was to analyze several inflammation markers and to determine their association with CAD severity. To this end, we found that the inflammatory markers TNFα and IL-6 significantly correlated with the severity of coronary disease, as assessed by the number of vessels involved or the Gensini coronary severity score. In multivariate analysis, TNFα independently predicted overall atherosclerotic burden, as manifested by the number of obstructed vessels (> 50% or >70%), and IL-6 independently predicted the Gensini coronary severity score. The correlation was also significant when analyzing only the subset of patients with stable CAD. In this group, TNFα significantly correlated with the number of vessels involved. These data strongly support the hypothesis that the inflammatory cytokines are surrogate markers of low grade inflammatory burden, present in patients with active atherosclerotic coronary disease (including stable patients) and may serve as a marker of increased risk for significant CAD.

Increased circulating levels of pro-inflammatory cytokines (IL-6 and TNFα), soluble adhesion molecules and cytokine-responsive acute-phase proteins, notably CRP, characterize a chronic low level inflammatory process. Several of these plasma markers of inflammation are significantly elevated in acute coronary syndromes and have been found to predict future cardiovascular risk [3]. IL-6 is a key pro-inflammatory and immune-stimulatory cytokine that activates acute-phase proteins. IL-6 enhances atherosclerotic lesion development [6] and induces endothelial dysfunction [7]. A positive correlation was found between CRP, fibrinogen, and IL-6 and severity of coronary and peripheral atherosclerotic disease independent of usual risk factors [8]. IL-6 and CRP were associated with the severity of atherosclerosis as measured non-invasively in peripheral arteries [9]. In patients with CAD, the coexistence of peripheral artery disease was associated with a greater inflammatory status evident by increased levels of IL-6 and CRP [10]. In the Edinburgh Artery Study, IL-6 was the strongest independent predictor for peripheral artery disease and its progression [11]. Recently, IL-6 has been shown to be associated with intracranial large artery atherosclerosis disease evident on magnetic resonance imaging [12].

TNFα is also a central pro-inflammatory cytokine involved in the propagation of atherosclerosis. TNFα is secreted in the vascular wall by endothelial smooth muscle cells and by monocytes/macrophages and is a powerful inducer of local inflammation [1]. It increases permeability of the endothelial cell barrier [13], promotes the expression of leukocyte adhesion molecules via nuclear factor-kappa B [14] and increases the uptake of macrophages in atherosclerotic lesions [15] thus directly promoting atherosclerosis. TNFα was strongly associated with early atherosclerosis measured by common carotid intima media thickness [16]. Carotid atherosclerosis was also associated with TNF receptor levels but not with serum TNFα levels [17]. A recent study analyzing patients with ACS did not find a correlation between atherosclerotic burden and IL-6 or TNFα [18]. However, this study was limited to patients with ACS only. As pro-inflammatory cytokines are acutely elevated during acute coronary events, this elevation may increase the cytokine levels beyond the angiographic atherosclerotic burden, therefore the association of the cytokines with the severity of CAD may be negated. Indeed in our study, the most significant associations were evident in stable patients and the associations were weak (TNFα) or not present (IL-6) when analyzing only patients with ACS.

These markers are not only associated with atherosclerosis, they have significant prognostic value. In a recent large clinical study, TNFα and IL-6 were strongly predictive of the presence of clinical and subclinical cardiovascular disease [19] and were also predictive of future cardiac events. The combination of TNFα, IL-6 and CRP as a composite summary indicator of inflammation showed a strong association with incident cardiovascular events [20]. IL-6 has been shown to be independently predictive of the development of myocardial infarction and TNFα has been shown to be an independent predictor of increased risk of recurrent coronary events including cardiovascular death after myocardial infarction [21]. In the elderly, IL-6 has been shown to be associated with increased atherosclerosis as manifested by clinical cardiovascular disease, [22] and IL-6 was a predictor of increased mortality in older women with cardiovascular disease [23].

We did not find a correlation between CRP and the severity of CAD. This is consistent with previous findings [20,24] that although CRP is an important predictor of the inflammatory status

BMI = body mass index

and has been shown to strongly correlate with the probability of cardiac events and has prognostic importance, it may not correlate with the overall severity of CAD. A possible explanation for this discrepancy is that CRP may be a marker of increased risk for CAD but may not be sensitive enough to discriminate between the severities of existing CAD. TNFα and IL-6 are upstream cytokines that activate CRP and hypothetically may be more sensitive in discriminating the severity of CAD that is present. Neither was there a correlation with the other pro- or anti-inflammatory cytokines except IL-1RA that had a weak association. Although IL-1RA has been shown to be elevated in patients with unstable coronary syndromes [25], the association with the severity of CAD has not been shown before. The association was weak and its clinical significance has yet to be determined.

The main limitation of this study was the relatively small sample size of patients, which may cause errors in the correlations due to other factors not taken into account in the statistical analysis. However, the correlations found were statistically significant and have strong plausibility in theory, concurring with several recent studies. Another limitation is the fact that this study included patients with acute coronary syndromes. As cytokines are elevated in acute myocardial infarction due to myocardial necrosis, this may contaminate the data. However, the correlation with TNFα was still valid in the subset of patients with stable angina only.

In conclusion, the inflammatory markers TNFα and IL-6 were independently associated with the severity of coronary disease in a group of stable and unstable coronary patients. This association may be an indicator of chronic inflammatory burden and may serve as a marker of increased risk for significant coronary disease.

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Correspondence: Dr. I. Gotsman, Heart Institute, Hadassah University Hospital, P.O. Box 12000, Jerusalem 91120, Israel.
Phone: 972-2-6776564
Fax: 972-2-6411028
email: igotsman@bezeqint.net