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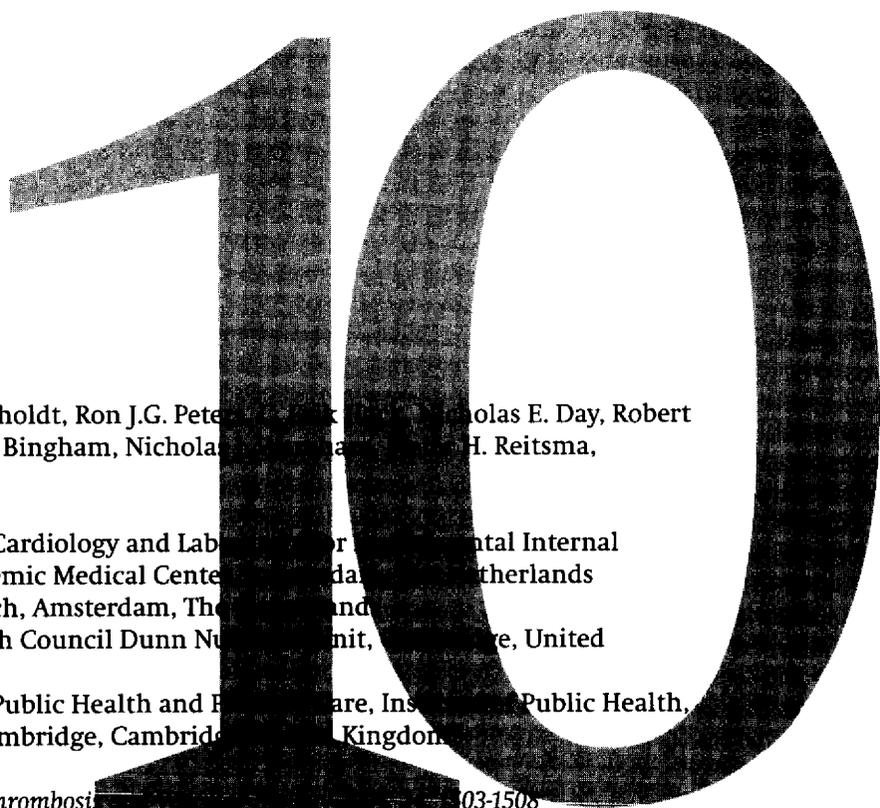
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IL-8 plasma concentrations and the risk of future coronary artery disease in apparently healthy men and women; the EPIC-Norfolk prospective population study



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Abstract

Background

Interleukin-8 (IL-8) has been implicated as a risk factor for future coronary artery disease (CAD).

Methods and Results

A nested case-control study was performed in the prospective EPIC-Norfolk population study. We measured baseline IL-8 concentrations among 785 apparently healthy individuals who developed fatal or non-fatal CAD during follow-up, and 1570 matched controls. Baseline IL-8 concentrations were higher in cases than in matched controls (3.5 pg/ml versus 3.1 pg/ml, $p=0.001$). The risk of future CAD increased with increasing quartiles of IL-8 (p linearity < 0.0001). Among individuals in the highest IL-8 quartile, the unadjusted odds ratio for future CAD was 1.72 (95%CI = 1.34-2.21, $p < 0.0001$). The odds ratio for future CAD was still significant after adjustment for traditional risk factors (OR = 1.58, 95%CI = 1.19-2.09, $p = 0.002$), and after additional adjustment for C-reactive protein and white cell count (OR = 1.77, 95%CI = 1.21-2.60, $p = 0.001$).

Conclusions

We conclude that among apparently healthy men and women, elevated levels of IL-8 are associated with an increased risk of future CAD. These prospective data support a role for IL-8 in the development of CAD events.

Background

Inflammation plays a key role in the initiation and progression of atherosclerosis.¹ For clinical purposes, C-reactive protein (CRP) is gradually gaining acceptance as the most useful inflammatory plasma marker.² However, the inflammatory processes that underlie atherosclerosis are mediated by a multitude of cytokines, and are unlikely to be reflected by CRP levels alone. Prospective evidence on other cytokines in apparently healthy individuals is limited, and exists only for interleukin-6,^{3,6} and macrophage inhibitory cytokine-1.⁷

Interleukin-8 (IL-8) is a proinflammatory cytokine that is produced by various cell types involved in atherosclerosis, including endothelial cells,⁸ peripheral blood monocytes,⁹ and vascular smooth muscle cells.¹⁰ The role of IL-8 in atherosclerosis may be through its chemoattractant and mitogenic effects on vascular smooth muscle cells.¹¹ In addition, IL-8 plays an important role in the immigration of monocytes into the subendothelial space, which is a crucial process in the early stages of atherosclerosis.¹² Evidence from murine atherosclerosis models suggests that initial leukocyte adhesion to the endothelium is mediated via KC, the murine equivalent of IL-8, while subsequent interaction between monocyte chemoattractant protein-1 (MCP-1) and its receptor CCR1 is essential for transendothelial diapedesis and recruitment into the subendothelial space.¹³ Experimental atherosclerosis can be largely prevented by eliminating the genetic expression of IL-8, MCP-1, or their leukocyte receptors.¹⁴⁻¹⁶ IL-8 plasma levels are higher in patients with unstable coronary artery disease (CAD) than in healthy controls¹⁷⁻²⁰ but these measurements were all obtained after an acute coronary syndrome which may have affected these levels substantially. No prospective evidence exists on the relationship between IL-8 levels in individuals free of symptomatic cardiovascular disease, and the risk of future CAD.

It was our objective to determine whether elevated plasma concentrations of IL-8 in apparently healthy individuals were associated with an increased risk of future CAD. In addition, we investigated whether this relationship was modified by other cardiovascular risk factors.

Methods

Study design

We performed a nested case-control study among participants of the EPIC-Norfolk study, a prospective population study of 30,466 men and women aged between 45 and 79 years, resident in Norfolk, UK, who completed a baseline questionnaire survey, and of whom 25,663 attended a clinic visit.²¹ Participants were recruited from age-sex registers of general practices in Norfolk as part of a nine-country collaborative study (EPIC, European Prospective Investigation into Cancer and Nutrition) designed to investigate dietary and other determinants of cancer. Additional data were obtained to enable the assessment of determinants of other diseases.

Table 1. Baseline characteristics

	Controls	Cases	P
Men, n	1028	514	
Age, y	64.3 ± 7.8	64.3 ± 7.8	Matched
Smoking			< 0.0001
- Never	304 (30.0)	120 (23.5)	
- Past	608 (60.0)	306 (59.9)	
- Current	102 (10.1)	85 (16.6)	
Body mass index, kg/m ²	26.4 ± 3.1	27.2 ± 3.4	< 0.0001
Total cholesterol, mmol/l	6.06 ± 1.10	6.27 ± 1.11	< 0.0001
LDL cholesterol, mmol/l	3.96 ± 0.98	4.11 ± 0.94	0.007
HDL cholesterol, mmol/l	1.24 ± 0.33	1.15 ± 0.29	< 0.0001
Triglycerides, mmol/l	1.7 (1.2 - 2.4)	1.9 (1.4 - 2.8)	< 0.0001
Systolic blood pressure, mmHg	140.5 ± 17.8	146.0 ± 18.6	< 0.0001
Diastolic blood pressure, mmHg	84.6 ± 11.1	87.2 ± 12.0	< 0.0001
Diabetes	31 (3.0)	39 (7.6)	0.001
CRP, pg/ml	2.92 ± 5.09	3.92 ± 5.62	< 0.0001
IL-8, mg/l	3.1 (28 / 24 / 23 / 25)	3.5 (23 / 24 / 23 / 31)	0.001
Women, n	541	271	
Age, y	66.2 ± 7.0	66.3 ± 7.0	Matched
Smoking			< 0.0001
- Never	319 (59.4)	118 (44.2)	
- Past	179 (33.3)	112 (41.9)	
- Current	39 (7.3)	37 (13.9)	
Body mass index, kg/m ²	26.4 ± 4.1	27.4 ± 4.5	< 0.0001
Total cholesterol, mmol/l	6.69 ± 1.14	6.95 ± 1.36	0.006
LDL cholesterol, mmol/l	4.31 ± 1.06	4.48 ± 1.15	0.09
HDL cholesterol, mmol/l	1.57 ± 0.42	1.46 ± 0.42	0.001
Triglycerides, mmol/l	1.6 (1.1 - 2.2)	1.9 (1.4 - 2.8)	< 0.0001
Systolic blood pressure, mmHg	139.9 ± 19.9	143.2 ± 19.8	0.02
Diastolic blood pressure, mmHg	82.4 ± 11.5	84.9 ± 12.2	0.003
Diabetes	8 (1.6)	17 (6.3)	0.02
CRP, mg/l	3.3 ± 4.8	4.7 ± 6.0	< 0.0001
IL-8, pg/ml	3.0 (28 / 24 / 25 / 23)	3.5 (24 / 23 / 26 / 27)	0.2

Data are presented as mean ± SD, median (quartile distribution in percentages), or number (%). P-values are for mixed effect model on continuous variables, and for conditional logistic regression on dichotomous variables. Triglyceride and IL-8 concentrations were log-transformed before analysis, but untransformed medians are presented in the table.

The design and methods of the study have been described in detail.²¹ In short, eligible participants were recruited by post. At the baseline survey between 1993 and 1997, participants completed a detailed health and lifestyle questionnaire. Blood was taken by venepuncture into plain and citrate bottles. Blood samples for assay were processed for assay at the Department of Clinical Biochemistry, University of Cambridge, or storage at -80°C . All individuals have been flagged for death certification at the UK Office of National Statistics, with vital status ascertained for the entire cohort. In addition, participants admitted to hospital were identified using their unique National Health Service number by data linkage with ENCORE (East Norfolk Health Authority database), which identifies all hospital contacts throughout England and Wales for Norfolk residents. Participants were identified as having CAD during follow-up if they had a hospital admission and/or died with CAD as underlying cause. CAD was defined as codes 410-414 according to the International Classification of Diseases 9th revision. We report results with follow-up up to January 2003, an average of about 6 years. The study was approved by the Norwich District Health Authority Ethics Committee and all participants gave signed informed consent.

Participants

For the present analysis, we only considered individuals who did not report a history of heart attack or stroke at the baseline clinic visit. Cases were 785 individuals who developed a fatal or non-fatal CAD during follow-up. Controls were study participants who remained free of CAD during follow-up. Two controls were matched to each case by sex, age (within 5 years), general practice, and date of visit (within 3 months).

Biochemical analyses

Serum levels of total cholesterol, HDL cholesterol, and triglycerides were measured on fresh samples with the RA 1000 (Bayer Diagnostics, Basingstoke, UK), and LDL cholesterol levels were calculated with the Friedewald formula.²² From 1994, full blood count was additionally measured on fresh EDTA samples using a Coulter Counter and this measure was available on approximately 60% of the cohort. In 2003, plasma samples for cases and controls were retrieved from frozen storage, thawed, and the plasma concentration of IL-8 was measured by use of a validated cytometric bead array kit (BD Biosciences Pharmingen, San Diego, Ca, <http://www.bdbeurope.com/temp/497527.pdf>) with slight modifications to extent the detection range in the lower part of the distribution. The specificity of the antibody pair was screened using recombinant protein, and no cross-reactivity or background detection was observed. The inter-assay and intra-assay variability were both 4% in the appropriate range. The lower detection limit was 2.0 pg/ml, the upper detection limit was 4000 pg/ml. CRP levels were measured with a sandwich-type ELISA in which polyclonal rabbit anti-CRP antibodies were used as catching antibodies and biotinylated

Table 2. Distribution of cardiovascular risk factors by sex and IL-8 quartile

IL-8 quartile	1	2	3	4	P
Quartile range, pg/ml	< 2.1	2.2 - 3.2	3.3 - 4.8	> 4.8	
Men					
Age	62.9 ± 7.8	63.8 ± 8.0	65.2 ± 7.7	65.2 ± 7.6	< 0.0001
Smoking					0.2
- Never	131 (33.1)	97 (26.2)	95 (25.9)	101 (25.8)	
- Past	223 (56.3)	226 (61.1)	230 (62.7)	235 (59.9)	
- Current	41 (10.4)	47 (12.7)	42 (11.4)	56 (14.3)	
Body mass index	26.8 ± 3.7	26.7 ± 3.1	26.7 ± 3.0	26.5 ± 3.0	0.5
LDL cholesterol	4.02 ± 0.95	4.00 ± 0.92	4.06 ± 0.97	3.97 ± 1.02	0.6
HDL cholesterol	1.20 ± 0.30	1.24 ± 0.35	1.24 ± 0.35	1.19 ± 0.29	0.8
Triglycerides	2.05 ± 1.09	2.02 ± 1.13	2.04 ± 1.03	2.09 ± 1.17	0.6
Systolic blood pressure	141.5 ± 18.5	141.8 ± 18.7	143.3 ± 17.6	142.7 ± 18.2	0.2
Diabetes	9 (2.3)	24 (6.4)	17 (4.5)	20 (5.1)	0.2
CRP	3.10 ± 5.66	3.38 ± 6.11	2.98 ± 3.74	3.55 ± 5.34	0.9
Women					
Age	65.7 ± 7.0	65.5 ± 7.4	66.8 ± 6.8	67.1 ± 6.7	0.1
Smoking					0.9
- Never	124 (55.6)	99 (51.3)	110 (51.9)	104 (59.1)	
- Past	84 (37.7)	74 (38.3)	78 (36.8)	55 (31.3)	
- Current	15 (6.7)	20 (10.4)	24 (11.3)	17 (9.7)	
Body mass index	26.3 ± 4.2	27.2 ± 4.6	26.7 ± 4.1	27.0 ± 4.0	0.8
LDL cholesterol	4.31 ± 1.04	4.37 ± 1.05	4.48 ± 1.11	4.31 ± 1.15	0.4
HDL cholesterol	1.54 ± 0.44	1.52 ± 0.43	1.52 ± 0.39	1.54 ± 0.45	0.4
Triglycerides	1.84 ± 0.89	2.04 ± 1.25	2.06 ± 1.24	1.84 ± 0.99	0.7
Systolic blood pressure	139.1 ± 18.3	142.2 ± 21.5	142.5 ± 20.0	140.4 ± 20.0	1.0
Diabetes	6 (2.7)	5 (2.6)	8 (3.7)	6 (3.4)	0.5
CRP	3.26 ± 5.28	3.88 ± 5.28	3.99 ± 5.47	3.88 ± 5.04	0.4

Distribution of characteristics by sex-specific IL-8 quartiles. Values are mean ± SD or number (%).

monoclonal antibodies against CRP (CLB anti-CRP-2) as the detecting antibody.²³ Results were related to a standard consisting of commercially available CRP (Behringwerke AG, Marburg, Germany), and expressed as mg/l. The lower detection limit was 0.1 mg/l. Samples were analyzed in random order to avoid systemic bias. Researchers and laboratory personnel had no access to identifiable information, and could identify samples by number only.

Statistical analysis

Baseline characteristics were compared between cases and controls taking into account the matching between them. A mixed effect model was

used for continuous variables, and conditional logistic regression was used for dichotomous variables. Because triglyceride and IL-8 levels had a skewed distribution, values were log-transformed before statistical analysis but in the tables we show untransformed medians and corresponding interquartile range (triglycerides) or the distribution across quartiles (IL-8). Mean risk factor levels by IL-8 quartile were calculated in order to determine relationships between IL-8 and traditional cardiovascular risk factors. Conditional logistic regression analysis was used to calculate odds ratios (OR) and corresponding 95% confidence intervals (95%CI) as an estimate of the relative risk of incident CAD. IL-8 concentrations were analysed as categorical variables after division into quartiles. The lowest quartile was used as reference category. OR's were adjusted for the following cardiovascular risk factors: age, sex, systolic blood pressure, total cholesterol, HDL cholesterol, body mass index (BMI), smoking (never, past, current), diabetes and hormone replacement therapy. OR's were also estimated after additional adjustment for CRP, and for both CRP and white cell count. The interaction between sex and IL-8 was calculated to assess the validity of pooling sexes. Statistical analyses were performed using SPSS software (version 10.1, Chicago, Illinois). A p-value less than 0.05 was considered significant.

Results

From the total number of 785 cases, 196 (25.0%) died with CHD as underlying cause, and 589 (75.0%) were non-fatal CAD events. Owing to matching, age was comparable between cases and controls. As expected, both women and men who developed CAD during follow-up were more likely than controls to smoke and have diabetes (table 1). Total cholesterol levels, systolic and diastolic blood pressure, BMI, white cell count and CRP were significantly higher in cases than controls, and HDL cholesterol levels were significantly lower in cases than controls. Among men, median IL-8 concentrations were higher in cases (3.5 pg/ml, quartile distribution 23%, 24%, 23%, 31%) than in controls (3.1 pg/ml, quartile distribution 28%, 24%, 23%, 25%, $p = 0.001$). Among women, the IL-8 concentrations in cases (3.5 pg/ml, quartile distribution 24%, 23%, 26%, 27%) and controls (3.0 pg/ml, 28%, 24%, 25%, 23%) were significantly different ($p = 0.2$). Baseline IL-8 levels were not significantly different between people with fatal and non-fatal CAD. Table 2 shows the distribution of cardiovascular risk factors by sex and IL-8 quartile. Among men, a linear relationship was observed between IL-8 quartile and age (p for linearity < 0.0001). Among women, age also increased per IL-8 quartile but linearity was not statistically significant ($p = 0.1$). No linear relationship was observed between IL-8 quartiles and any of the other cardiovascular risk factors.

Table 3 shows the unadjusted and adjusted ORs for future CAD by IL-8 quartile. For both the unadjusted and adjusted ORs, the interaction between sex and IL-8 was not statistically significant, and data for men and women were therefore pooled, though sex specific ORs are also shown.

Table 3. Odds ratios for future CAD events by IL-8 quartile and sex

IL-8 quartile	1	2	3	4	P
Quartile range, pg/ml	< 2.1	2.1 - 3.2	3.2 - 4.8	> 4.8	
Men and women					
OR unadjusted	1.00	1.27 (0.99 - 1.65)	1.45 (1.13 - 1.88)	1.72 (1.34 - 2.21)	< 0.0001
OR adjusted (1)	1.00	1.21 (0.91 - 1.61)	1.31 (0.98 - 1.75)	1.58 (1.19 - 2.09)	0.002
OR adjusted (2)	1.00	1.20 (0.90 - 1.60)	1.30 (0.97 - 1.74)	1.56 (1.18 - 2.07)	0.003
OR adjusted (3)	1.00	1.60 (1.09 - 2.34)	1.49 (1.01 - 2.22)	1.77 (1.21 - 2.60)	0.01
Men					
OR unadjusted	1.00	1.30 (0.94 - 1.81)	1.49 (1.07 - 2.07)	1.78 (1.31 - 2.43)	0.001
OR adjusted (1)	1.00	1.31 (0.91 - 1.88)	1.48 (1.02 - 2.15)	1.62 (1.14 - 2.30)	0.01
OR adjusted (2)	1.00	1.29 (0.89 - 1.85)	1.44 (0.99 - 2.10)	1.58 (1.11 - 2.25)	0.02
OR adjusted (3)	1.00	1.93 (1.15 - 3.22)	2.08 (1.20 - 3.59)	1.74 (1.05 - 2.91)	0.04
Women					
OR unadjusted	1.00	1.22 (0.80 - 1.86)	1.38 (0.92 - 2.09)	1.60 (1.05 - 2.45)	0.03
OR adjusted (1)	1.00	1.13 (0.71 - 1.82)	1.12 (0.70 - 1.80)	1.58 (0.97 - 2.57)	0.1
OR adjusted (2)	1.00	1.16 (0.72 - 1.86)	1.13 (0.70 - 1.83)	1.60 (0.98 - 2.62)	0.1
OR adjusted (3)	1.00	1.53 (0.83 - 2.81)	1.12 (0.61 - 2.06)	2.05 (1.11 - 3.80)	0.1

Odds ratios for the risk of CAD for males, females, and for sexes combined, adjusted for sex. (1) Adjustment for age, systolic blood pressure, LDL cholesterol, HDL cholesterol, BMI (continuous variables), smoking, diabetes, and hormone replacement therapy (2) Adjustment for variables above and in addition CRP (3) Adjustment for variables above and in addition CRP and white cell count. P-value for χ^2 linear trend with 1 degree of freedom.

Plasma concentrations of IL-8 were strongly related to the risk of future CAD, such that individuals in the highest IL-8 quartile had an unadjusted OR of 1.72 (95%CI = 1.34-2.21), compared to those in the lowest IL-8 quartile (p for linearity < 0.0001). The relationship between IL-8 and risk of CAD was not substantially changed upon adjustment for traditional cardiovascular risk factors (OR for the highest IL-8 quartile = 1.58, 95%CI = 1.19-2.09, p = 0.002) or upon adjustment for traditional risk factors and CRP levels (OR = 1.56, 95%CI = 1.18-2.07, p = 0.002). Interestingly, additional adjustment for white cell count had two effects on the relationship between IL-8 levels and future CAD. First, the CAD risk estimates per IL-8 quartile became higher. Second, the shape of the relationship appeared to change from a linear relationship to one with a threshold such that the risk of future CAD was significantly higher in the second IL-8 quartile compared to the lowest reference quartile (OR = 1.60, 95%CI = 1.09-2.34, p = 0.01) and did not increase further in the third and fourth IL-8 quartile. The assumption that the relationship was more compatible with a threshold model than with a linear one, was underlined by the observation that the fit of this threshold model did not differ significantly from a model where IL-8 quartiles were

entered as categorical variables (data not shown). The fully adjusted risk of CAD appeared to be increased in all individuals with an IL-8 concentration above the lower detection limit of 2.0 pg/ml.

Plasma concentrations of IL-1B, IL-6, IL-10, IL-12 and TNF- α were also determined in all samples. However, for these respective cytokines, 60.8%, 96.9%, 77.9%, 82.5%, and 90.4% of the samples had cytokine concentrations below the lower detection limit of 2.0 pg/ml. Thus, our assay was not sensitive enough to detect plasma concentrations in healthy individuals, and we were not able to give a reliable estimate of the predictive value of these cytokines in our cohort.

Discussion

The present study provides evidence that elevated plasma levels of IL-8 are associated with an increased risk of incident CAD in apparently healthy individuals, such that people in the highest IL-8 quartile had a fully adjusted OR of 1.77 (95%CI = 1.21 - 2.60) compared to those in the lowest quartile ($p = 0.001$). This relationship was independent of traditional cardiovascular risk factors and also independent of CRP levels.

Recruitment of peripheral blood monocytes into the arterial wall is one of the earliest steps in atherosclerosis. The vascular wall itself orchestrates this process by modulating the expression of a wide variety of cytokines that attract leukocytes, enable rolling leukocytes to adhere to the endothelium, and facilitate trans-endothelial immigration into the subendothelial space.²⁴ Endothelial cells can be stimulated to express cytokines, such as IL-8 and MCP-1, by triggers such as high glucose,²⁵ modified LDL cholesterol,²⁶ homocysteine,²⁷ and nitric oxide.²⁸ If triggered by oxidized LDL cholesterol (oxLDL), macrophages in the subendothelial space can also be a source of IL-8.²⁹⁻³¹ In addition to its role in the initiation and progression of atherosclerosis, IL-8 may also increase the risk of cardiovascular events by destabilizing existing atherosclerotic plaque. Macrophage-derived foam cells in atherosclerotic plaque produce matrix metalloproteinases, which mediate extracellular matrix degradation and consequent plaque destabilization.^{24,30} This process is contained by the simultaneous release of tissue inhibitors of metalloproteinases (TIMP's). TIMP expression is down-regulated by oxLDL in a dose-dependent manner, and this process is mediated via IL-8. Additional evidence for this mechanism is provided by the observation that IL-8 plasma levels were found to be higher in patients with unstable CAD than in patients with stable CAD and controls.^{17,20}

The fact that the predictive value of IL-8 plasma levels is statistically independent from traditional cardiovascular risk factors is in contrast with other inflammatory risk factors such as CRP levels and IL-6 levels.⁶ Interestingly, the relationship between IL-8 levels and CAD risk was also independent of CRP concentration. However, this relationship was substantially affected by adjustment for white cell count, another inflammation marker implicated in cardiovascular disease independent

of classical risk factors.³² Interestingly, adjustment for white cell count resulted in, first, a stronger predictive value of IL-8 levels for CAD risk and, second, an apparent change in the shape of this relationship from a linear one to one with a threshold. These changes imply a biological relationship between IL-8 levels and white cell count which is potentially explained by IL-8 expression in leukocytes or vice versa, by the effect of IL-8 on leukocyte recruitment into the vessel wall. However, mean levels of white cell count, the number of observations and the white cell differentiation (into granulocytes, monocytes and lymphocytes) did not differ between IL-8 quartiles. It must be kept in mind that in each IL-8 quartile and in each adjustment model, the 95% confidence intervals before and after adjustment for white cell count overlapped, and thus that the apparent changes upon adjustment may have been chance findings. The fully adjusted risk of CAD appeared to be increased in all individuals with an IL-8 concentration above the lower detection limit of 2.0 pg/ml. However, this threshold value must be interpreted with caution because other IL-8 assays may yield different absolute IL-8 concentrations, especially when performed in other patient populations. In addition, we were not able to discern whether values below 2.0 pg/ml were more appropriate threshold values because 2.0 pg/ml was the lowest detectable concentration. Despite the evidence for a biological relationship between IL-8 levels and white cell count, IL-8 levels also had relationship with CAD risk that was statistically independent of white cell count. This observation suggests that IL-8 may have an effect on the risk of CAD which is not related to white cell recruitment, possibly through a destabilizing effect on atherosclerotic plaque through down-regulation of TIMP expression.^{24,30}

A number of issues have to be taken into account when interpreting the results of the present study. Plasma levels of IL-8 were determined in a single sample that was obtained at a non-uniform time of the day. Diurnal variation and variation over time could have affected the IL-8 concentrations. In addition, we cannot rule out that sample storage at -80°C for 6-10 years may have affected the detection of IL-8. However, both these limitations would lead to increased random measurement error, which leads to an underestimation of any relationship and therefore do not negate our findings. While the current data are unable to establish whether the relationship between IL-8 and CAD is causal, it is unlikely that differences in IL-8 plasma levels occurred as a consequence of cardiovascular events because individuals with symptomatic cardiovascular disease were excluded from our analysis. However, we cannot exclude the possibility that IL-8 concentration is a marker of advanced subclinical atherosclerosis. Of note, IL-8 concentrations were not related to other modifiable cardiovascular risk factors, and consistently, the OR for future CAD was not affected by adjustment for these risk factors.

We conclude that elevated plasma concentrations of IL-8 are associated with an increased risk of future CAD in apparently healthy individuals. This relationship appears to be stronger in men than in women and is

independent of traditional cardiovascular risk factors and CRP. These prospective data support a role for IL-8 in the development of symptomatic coronary artery disease.

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