Inflammatory Cytokines and Risk of Coronary Heart Disease: New Prospective Study and Updated Meta-Analysis

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Short title: Inflammatory cytokines and CHD risk
Word count: abstract 250, main text 3842
3 tables, 3 figures, 10 supplementary tables and figures, 49 references

Abstract

Aims Because low-grade inflammation may play a role in the pathogenesis of coronary heart disease (CHD), and pro-inflammatory cytokines govern inflammatory cascades, this study aimed to assess the associations of several pro-inflammatory cytokines and CHD risk in a new prospective study, including meta-analysis of prospective studies.

Methods and Results Interleukin-6 (IL-6), interleukin-18 (IL-18), matrix metalloproteinase-9 (MMP-9), soluble CD40 ligand (sCD40L), and tumour necrosis factor–alpha (TNF-α) were measured at baseline in a case-cohort study of 1514 participants and 833 incident CHD events within population-based prospective cohorts at the Danish Research Centre for Prevention and Health. Age- and sex-adjusted hazard ratios (HR) for CHD per 1-SD higher log-transformed baseline levels were: 1.37 (95% CI, 1.21-1.54) for IL-6, 1.26 (1.11-1.44) for IL-18, 1.30 (1.16-1.46) for MMP-9, 1.01 (0.89-1.15) for sCD40L, and 1.13 (1.01-1.27) for TNF-α. Multivariable adjustment for conventional vascular risk factors attenuated the HRs to: 1.26 (1.08-1.46) for IL-6, 1.12 (0.95-1.31) for IL-18, 1.21 (1.05-1.39) for MMP-9, 0.93 (0.78-1.11) for sCD40L, and 1.14 (1.00-1.31) for TNF-α. In meta-analysis of up to 29 population-based prospective studies, adjusted relative risks for non-fatal MI or CHD death per 1-SD higher levels were: 1.25 (1.19-1.32) for IL-6; 1.13 (1.05-1.20) for IL-18; 1.07 (0.97-1.19) for MMP-9; 1.07 (0.95-1.21) for sCD40L and 1.17 (1.09-1.25) for TNF-α.

Conclusions Several different pro-inflammatory cytokines are each associated with CHD risk independent of conventional risk factors and in an approximately log-linear manner. The findings lend support to the inflammation hypothesis in vascular disease, but further studies are needed to assess causality.

Keywords Inflammation, CHD, Cytokines, Risk factors, Meta-analysis

Introduction

As inflammatory processes may play an important role in the pathogenesis of vascular disease,[1, 2] there is interest in the relevance of circulating markers of inflammation to coronary heart disease (CHD). Previous epidemiological studies have mainly reported on associations between "downstream" markers of inflammation (eg C-reactive protein [CRP] and fibrinogen) with the risk of incident CHD.[3, 4] However, human genetic evidence has reduced the likelihood that these liver-derived factors are causally relevant.[5-7] By contrast, "upstream" markers of inflammation, such as pro-inflammatory cytokines, may be more likely to be directly etiologically relevant to CHD because they govern inflammation cascades.[8] Most epidemiological evidence on the relevance of such markers has been obtained for interleukin-6 (IL-6).[9] Previous work has suggested that long-term soluble IL-6 levels are associated with CHD risk about as strongly as some major established risk factors,[9] and a causal role for IL-6 signaling in CHD has been supported by human genetic evidence.[10, 11] These findings have intensified interest in recently launched large-scale phase 3 clinical trials of various anti-inflammatory agents in the secondary prevention of cardiovascular disease (CVD).[12, 13]

However, in contrast with IL-6, other further upstream markers of the inflammatory response have been less well studied (**eTable 1**). These include factors which are involved in vascular inflammation and which are produced mainly by cells of the innate immune system, such as interleukin-18 (IL-18)[14] and matrix metalloproteinase-9 (MMP-9),[15] and factors that are also produced by cells of the adaptive immune system, such as soluble CD40 ligand (sCD40L),[16] and tumour necrosis factor–alpha (TNF-α).[17] So far, direct comparison between the markers has been difficult, because the individual markers have not been measured in the same studies or participants. Furthermore, there is little information about the extent to which these analytes fluctuate within individuals over time, as such data are essential to the interpretation of epidemiological studies with an etiological motivation.

We report new findings on the association of the above five inflammatory markers and incident CHD outcomes based on data from population-based prospective cohorts at the Danish Research Centre for Prevention and Health (RCPH),[18] comprising a total of 1514 participants aged 30-70 years at baseline and an average of follow-up of 12 years. Furthermore, we used data from serial measurements of these cytokines from two other prospective studies (Reykjavik[9] and British Regional Heart Study[16, 17, 19, 20]) to assess long-term within person variability. Finally, to contextualise our findings, we also report an updated systematic review and meta-analyses of the association of these cytokines and non-fatal MI or CHD death in population-based prospective studies.

Methods

Participants

We measured circulating levels of IL-6, IL-18, MMP-9, sCD40L, and TNF-α in a case-cohort study nested within population-based prospective cohorts at the Danish Research Centre for Prevention and Health (RCPH). Details of the RCPH cohorts have been described previously.[18] By 2001 the study comprised 8,314 participants from 5 cohorts in the south-western part of Copenhagen county (**eTable 2**): (i) 70 year olds from the 1914 birth-cohort (70yr1914, n=992) (ii) 45 year olds from the 1936 birth-cohort (45yr1935, n=804) (iii) 5-year re-examination of the first Dan-MONICA cohort (GenMon, n=2987) (iv) the second Dan-MONICA cohort (MONICAII, n=1504) and (v) the third Dan-MONICA cohort (MONICAIII, n=2027). Standardised methods for follow up and risk factor assessment have been maintained in these 5 cohorts.[18] Our case-cohort study of inflammatory cytokines included all incident CHD cases (defined using ICD8 codes 410-414 or ICD10 codes I20-I25) recorded as of 31st December 2006, plus sub-cohorts of randomly sampled participants within each cohort (7% in each of 45yr1936, MONICAII, and MONICAIII; 9% in GenMon; and 28% in 70yr1914; reflecting the proportion of CHD cases within each cohort) for a total of 1514 participants aged 30-70 years (**eTable 2**). In total there were 833 incident CHD cases (comprising 320 non-fatal MI, 195 CHD deaths, and 318 angina - **eTable 2**). The study complies with the Declaration of Helsinki and was approved by the ethical committee of the county of Copenhagen.

Biochemical measurements

Blood samples were drawn after 8-12 hours of fasting and stored at -20 °C before measurements of pro-inflammatory cytokines in November 2008 (mean storage duration = 22 years). Assays of IL-6,[9] IL-18,[17] MMP-9,[15] sCD40L,[16] and TNF-α[17] were performed centrally at the University of Glasgow laboratory using high-sensitivity ELISAs (R & D systems, Abingdon, UK) as previously reported.[9, 15-17] Intra- and inter-assay coefficients of variation were 7.0 and 8.0% (IL-6); 5.6 and 10.4% (IL-18); 4.4 and 10.4% (MMP-9); 2.9 and 14.9% (sCD40L); and 8.4 and 12.5% (TNF-α) respectively.[9, 15, 17] Participants from the MONICA II cohort were excluded from the analyses of sCD40L due to technical pre-analytical issues related to sample handling (previous thawing and freezing) that led to erroneous low values of the sCD40L assay. CRP measurements were later done in another laboratory (MORGAM) using serum samples that remained available in three of the RCPH cohorts (55% of participants in the GenMon cohort and all participants in the MONICAII and MONICAIII cohorts). Measurements of other biochemical factors involved standard methods as described previously.[21]

Outcomes

Assessment of the cardiovascular endpoints was based on data from the Danish National Patient Registry (DNPR)[22] and the Danish Register of Causes of Death (DRCD).[23] Individuals who had died or emigrated were identified through the Central Population Registry of Statistics Denmark. Linkage between population surveys and national registries are made possible due to a unique individual ten digit code. The primary endpoint in the design of our study was pre-specified as the composite of all CHD outcomes (including non-fatal MI, fatal CHD, and angina) in order to enhance statistical power. The composite of non-fatal MI and fatal CHD was analysed as a secondary endpoint. Participants with pre-existing CHD at baseline were excluded from all analyses to avoid reverse association bias. While specific validation of endpoints against coding in the DNPR and DRCD national registers was not conducted in the current study, previous validation studies of the same registers have found excellent completeness and validity of recorded diagnosis of myocardial infarction (positive predictive value [PPV] = 82% overall, and 92% if associated with a ward diagnosis) although less so for diagnosis of unstable angina pectoris (PPV = 28% overall).[24, 25]

Statistical analyses

As none of the inflammatory cytokines studied were normally distributed, natural logarithm ($\log_e$) transformed values of each cytokine were used for all analyses. Partial correlation coefficients (adjusted for cohort, age, and sex) were calculated to assess the correlation between inflammatory markers and continuous vascular risk factors, whereas mean differences between groups were calculated for categorical factors. Analyses of the associations between inflammatory cytokines and CHD outcomes involved weighted Cox-regression modelling, using the Barlow weighting method to account for the case-cohort study design.[26, 27]. Shapes of association were assessed by plotting hazard ratios (HRs) estimated within quintiles of each cytokine relative to the bottom quintile against the mean cytokine level in each quintile. 95% confidence intervals (CI) were estimated from variances attributed to the groups to reflect the amount of information within each group (including the reference category).[28] To assess the independence of association between each cytokine and CHD, we calculated HRs per 1-SD higher baseline levels with progressive adjustment of baseline levels of potential confounders. The use of 1-SD higher HRs also facilitated direct comparison of the strength of association across the 5 cytokines. All analyses were stratified by cohort and a 2-sided p-value <0.05 was considered statistically significant.

Literature review and meta-analysis

Prospective studies of the association between any of the 5 cytokines and risk of non-fatal MI or CHD death that were published before 31 December 2012 were sought using computer-based databases (MEDLINE, EMBASE, and Science Citation Index), by scanning the reference lists of articles identified for all relevant studies and review articles (including meta-analyses) and hand searching of relevant journals, without language restriction. The computer-based

searches combined free and MeSH search terms and combinations of key words related to the cytokines (e.g., ''interleukin-6'', ''IL-6'', "interleukin-18", "IL-18", "matrix metalloproteinase-9", "MMP-9", "soluble CD40 ligand", "sCD40L", "tumour necrosis factor–alpha", "TNF-alpha") and coronary disease (e.g.,''coronary heart disease'', ''myocardial ischemia'', ''myocardial infarction'', ''CHD'', ''heart attack'', ''MI'') without restricting language or publication date. Studies were eligible for inclusion if they had at least one year of follow-up; had recruited participants from approximately general populations (i.e., did not select participants on the basis of pre-existing disease at baseline); and had assessed non-fatal MI or CHD death as outcome. Study level characteristics and participant characteristics were extracted using a standardised data extraction form, including information on: geographic location, publication date, sample population, sampling methods (i.e., complete, random, etc.), years of baseline survey, number of participants, number of non-fatal MI or CHD death cases, age range or mean age, percentage of males, duration of follow up, and assay/sample characteristics (including source, type, sample storage, sample type and fasting status). Also extracted were the reported measures of association (i.e. hazard ratios, odds ratios, or other measure of relative risk) and their confidence intervals or standard errors, units of comparison, and degree of adjustment for confounders. Information from multivariable models adjusted for conventional risk factors for CHD was prioritised for inclusion in the meta-analysis (as opposed to models further adjusted for inflammatory markers or socio-economic variables, as such information was more limited within studies). The reported relative risks were standardised to correspond to comparison of relative risk (RR) per 1-SD higher values of each cytokine,[9, 29] and were pooled across studies using random-effects meta-analysis. Heterogeneity between studies was quantified using the I^2 statistic.[30] Potential publication and small-study bias was visually assessed from funnel plots, complemented by formal statistical tests of funnel plot asymmetry and use of trim and fill procedure to assess impact.[31] For IL-6, we updated our previous meta-analysis of 17 studies[9] with new information from recently published studies. Two authors (PG and SRKS) abstracted the details sought for each marker and any discrepancies were resolved through discussion with a third author (SK).

Within-person variability

The extent of long-term within person variability in log-transformed cytokine levels was assessed based on regression dilution ratios (RDRs) previously reported in the BRHS study[16, 17, 19, 20] or calculated from serial measurements available from 300 participants in the Reykjavik study an average of 12 years apart.[9] The RDR quantifies the extent to which a single measurement of a biomarker reflects the long-term average (i.e. "usual") level of the biomarker, and correction for long-term within person variability in log-transformed cytokine levels was achieved dividing the regression coefficients (i.e. log RRs) for association with baseline levels by the estimated RDR.[32]

All analyses were conducted using Stata version 11 software (Stata Corp, College Station, Texas).

Results

Results from the RCPH cohorts

Overall, 1514 participants contributed to the analyses comprising 807 participants in the randomly sampled sub-cohort and a further 707 incident CHD cases included as part of the case-sample enrichment in the case-cohort design.[26, 27] Within the random sub-cohort, there were 126 incident CHD cases recorded over 12,570 person-years of follow-up (median 9 years), giving a crude incidence rate of 10 (95% CI: 7 – 12) per 1000 person-years. Hence, in total, the analyses involved 833 CHD cases and 681 non-cases. **Table 1** summarises the participant characteristics by incident CHD status. Compared to non-cases, participants with incident CHD were more likely to be older, male, current smokers, and to have diabetes at baseline. They also had higher BMI, higher systolic and diastolic blood pressure and higher levels of proatherogenic lipids. Log_e levels of cytokines were generally higher amongst individuals with CHD than those without. CRP measurements were available in half of the participants. There were generally weak positive pair-wise correlations between individual cytokines (**Table 2**), with the exception of modest correlations between log_e IL-6 and log_e TNF-α (r = 0.22, 95% CI 0.17 – 0.24), and between log_e MMP-9 and log_e sCD40L (r = 0.31, 95% CI 0.26 – 0.37) and stronger correlations between log_e CRP and each of log_e IL6 (r = 0.48, 95% CI 0.42 – 0.54) and log_e MMP9 (r = 0.31, 95% CI 0.23 – 0.38). Weak correlations were also observed between cytokines and other continuous variables, with the exception of statistically significant correlations between log_e IL-6 and log_e IL-18 with age, BMI, HDL-cholesterol and log_e triglycerides; and between log_e CRP and the preceding factors plus blood pressure (**Table 2**). Concentrations of most cytokines were generally lower in women than men (sCD40L higher), higher among current than never/ex-smokers, lower in current vs. never/ex-drinkers, higher in people with vs. without diabetes, and lower in people of higher socioeconomic position adjusted for cohort, age, and sex (**Table 2**).

There were log-linear relationships between 4 of the cytokines studied and incident CHD risk (**Figure 1**), with the age- and sex-adjusted HRs (95% CI) for all CHD per 1-SD higher log-transformed levels being 1.37 (1.21, 1.54) for IL-6, 1.26 (1.11, 1.44) for IL-18, 1.30 (1.16, 1.46) for MMP-9, and 1.13 (1.01, 1.27) for TNF-α, but no association was seen with sCD40L [1.01 (0.89, 1.15)]. A modest attenuation in HRs was observed upon additional adjustment for several potential confounders (blood pressure, smoking, history of diabetes, BMI, log_e triglycerides, non-HDL cholesterol, HDL cholesterol, alcohol consumption and socioeconomic position), with the adjusted HRs being 1.26 (1.08, 1.46) for IL-6, 1.12 (0.95, 1.31) for IL-18, 1.21 (1.05, 1.39) for MMP-9, 1.14 (1.00, 1.31) for TNF-α and 0.93 (0.78, 1.11) for sCD40L (**Table 3**). The above findings were similar in analyses restricted to non-fatal MI and CHD death outcomes only (**Figure 1** and **Table 3**). In further comparative analyses restricted to the smaller subset of participants with information on CRP, somewhat stronger dose-response associations were observed for baseline log_e CRP than the preceding cytokines (**eFigure 1**)

and, as expected, further adjustment for $\log_e$ CRP levels more markedly attenuated the associations between $\log_e$ IL-6 and CHD (**eTable 3**), due to the strong correlation between the two (r = 0.48). However, because CRP is downstream of IL-6 in the inflammation pathway, such attenuation may be indicative of mediation rather than confounding per se.

Literature-based meta-analysis

Including the current study, we found a total of 29 studies eligible for the meta-analysis (comprising 25 studies for IL-6 (7 new), 7 studies for IL-18, 5 studies for MMP-9, 3 studies for sCD40L, and 7 studies for TNF-α; flow chart in **eFigure 2** and study characteristics in **eTable 4**). As shown in **eTable 4**, the new prospective cohort studies involved around 17,000 participants without known cardiovascular disease at baseline and 3000 incident non-fatal MI or CHD death cases and the previous meta-analysis of IL-6 involved 17 studies and 5730 such cases.[9] The studies were mainly based in general populations in Europe or North America. Approximately half of all studies were case-control studies nested within prospective cohorts. All studies used the same definition for non-fatal MI or CHD death and ascertainment was based on standard criteria, generally involving review of medical records to assess evidence of confirmation by cardiac markers, electrocardiograms, and ICD codes among other standard diagnostic criteria (**eTable 5**). In most studies, ELISA was used as the assay method for analysing the various inflammatory cytokines (**eTable 4**), although further information such as duration of sample storage and assay reproducibility (coefficient of variation) were infrequently reported (**eTable 5**). **Figure 2** shows the forest plot for the updated meta-analysis of association of IL-6 and CHD and **Figure 3** shows similar findings for the other cytokines. The pooled RRs (95% CI) for non-fatal MI or CHD death per 1-SD higher baseline cytokine levels adjusted for conventional CHD risk factors were: 1.25 (1.19, 1.32) for IL-6, 1.13 (1.05, 1.20) for IL-18, 1.07 (0.97, 1.19) for MMP-9, 1.09 (0.97, 1.19) for sCD40L, and 1.17 (1.09, 1.25) for TNF-α (**Figure 3**). There was no statistically significant between-study heterogeneity observed as evidenced by the low I^2 statistic, although the power to detect heterogeneity may have been limited by the small number of studies available for each cytokine (n≤7), other than IL-6 (n=25, including studies in previous meta-analysis,[9] **Figure 2**). Assessment of potential bias from small studies suggested there some slight concern for IL-6 (P = 0.024) and MMP-9 (P = 0.039), although a visual inspection of the degree of asymmetry in the funnel plots (**eFigure 3**) and use of a trim-and-fill procedure[31] to assess potential impact suggested that the asymmetry may not be of material importance, with the pooled RR estimates expected to be 1.19 (1.12, 1.25) for IL-6 and 1.08 (0.97, 1.19) for MMP-9 in the absence of asymmetry.

Correction for long-term within person variability

The extent of long-term within person variability in levels of the cytokines studied appeared to be substantial based on the generally low regression dilution ratios reported by some of the published studies (**eTable 6**). Such variability suggests a potential underestimation of the true magnitude of association with non-fatal MI or CHD death, as indicated by comparison of the

observed associations estimated with baseline levels against those inferred for long-term average ("usual") levels (**eFigure 4**) based on the pooled regression dilution ratios available from the BRHS and Reykjavik studies (**eTable 6**).

Discussion

The current results indicate that circulating levels of several different pro-inflammatory cytokines in initially healthy people are associated with risk of CHD outcomes in an approximately log-linear manner. These associations appear to be largely independent of several conventional and emerging cardiovascular risk factors. Furthermore, updated meta-analyses reinforce the validity and generalisability of these new data, suggesting that a 1 SD higher baseline level for each of IL-6, IL-18 and TNF-α is associated with about 10-25% higher risk of non-fatal MI or CHD death. Analyses that make allowances for the considerable fluctuations that we observed of these analytes within individuals over a few years would increase the estimated size of these associations with CHD (see below). Similar associations have previously been reported for CRP and fibrinogen based on meta-analysis of individual participant data,[3, 4] hence we only analysed the limited data available on CRP in our primary study for making direct internal comparisons. Collectively, the current study establishes robust observational associations of several pro-inflammatory cytokines with the risk of CHD, which lends further support to the inflammation hypothesis in vascular disease. Although this does not establish causality in CHD for any of the analytes, the comprehensive evaluation of their associations with CHD should be important, given the evolving literature on cytokines as potential drug targets[33, 34] and gaps in translating basic scientific findings into clinical practice.[1]

IL-6 is involved in the systemic inflammatory response, but also engages in local tissue inflammation[35] and promotes differentiation of naïve T-helper cells into Th17 cells,[36] a cell type that has been implicated in auto-immune conditions such as rheumatoid arthritis. TNF-α is a pro-inflammatory cytokine, which, similar to IL-6, has been implicated in auto-immune diseases.[37] Both IL-6 and TNF-α signalling pathways are targets of drugs (such as tocilizumab and etanercept) which are used to treat auto-immune diseases.[37, 38] Moreover, there have been suggestions of an increased incidence of CVD in patients with rheumatoid arthritis,[39] and non-randomized data from biologic registries suggest TNF-α blocking therapy could reduce CVD risk in these patients,[37] although such data have well-recognized limitations.[40] It is of interest, therefore, that two of the agents (ie, canakinumab, a monoclonal antibody to IL-1β, and low dose methotrexate) being tested in phase 3 trials of CVD prevention lower circulating IL-6 levels,[12, 41] while low dose methotrexate therapy also lowers TNF-α levels.[12] Furthermore, consistent with previous studies, we found circulating IL-18 levels to be positively associated with CHD incidence. By contrast, we found no significant associations of sCD40L and MMP-9 levels with CHD in the aggregate of available prospective studies, suggesting that positive findings in previous non-prospective data may have been liable to potential biases, eg, "reverse causality".[42-45]

Our study had several strengths. First, whereas most previous studies have concurrently considered only a few cytokines, we were able to measure simultaneously multiple pro-

inflammatory cytokines in a common subset of participants in one central laboratory, facilitating uniform comparison of the observed associations across markers. Furthermore, we excluded all individuals having baseline CVD to minimize any reverse association bias. Additionally, we combined data from previously published prospective studies, yielding qualitatively similar results to those observed in the new data. Moreover, to help identify independent associations, we adjusted for several conventional and emerging risk factors for cardiovascular disease, including all the inflammatory cytokines studied (although we acknowledge the possibility of "over-adjustment").

Our study also had potential limitations. First, because we used stored samples, we cannot rule out protein degradation between sample collection and assay, especially for self-activable enzymes like MMP-9.[46] However, randomisation of samples from CHD cases and non-cases within assay plates and blinding of laboratory technicians to case/control status of the samples should have ensured that samples were treated alike during sample handling and assay. Moreover independent studies employing accelerated stability testing protocols have concluded that serum samples for determination of cytokines such as IL-6 and sIL-6R can be stored at -20°C or less for several years without affecting recovery rates,[47] although such evidence was lacking for serum levels of the other cytokines we studied. Furthermore, despite known diurnal variation in the concentrations of some of the cytokines studied,[48] we believe that this would also have similarly affected both CHD cases and non-cases. Second, our principal analysis used a single baseline measurement of each cytokine to study its association with incident CHD. However, our reproducibility sub-studies of cytokine measurements made in the same individuals an average of 12 years apart in the Reykjavik[9] cohort and 4 years apart in the British Regional Heart Study[16, 17, 19, 20] suggest that we could have substantially underestimated any underlying aetiological associations in CHD. Conversely, the poor reproducibility of some cytokines may limit their utility for use in clinical practice as reliable risk indicators based on just a single measurement. Third, information on some extensively-studied downstream measures of inflammation (such as CRP) was only available in half of the participants in our primary study (due to exhaustion of serum samples), which limited the power for direct comparisons with other cytokines, besides inability to adequately adjust for the impact of differential measurement errors in different cytokines when making such aetiological comparisons. Fourth, atherosclerosis and plaque rupture are complex processes involving the interplay between several inflammatory substances at different stages of their evolution, hence the associations may somewhat differ according to the nature of the endpoints studied. To enhance power, we used a composite coronary endpoint as our primary outcome, but sub-analyses focusing solely on non-fatal MI or CHD death yielded very similar findings to those overall. Due to limited information, we could not assess the extent to which the duration of sample storage and differences in assay reproducibility may have influenced our meta-analysis findings; nevertheless, there was little variation seen in the few reported estimates of assay reproducibility. Finally, future observational analyses will wish to evaluate these cytokines in

much larger prospective studies with extensive concomitant genetic data to enable causal evaluation (eg, Mendelian randomization[49]).

In conclusion, several different pro-inflammatory cytokines are each associated with CHD risk independent of conventional risk factors and in an approximately log-linear manner. Although the current findings lend further support to the inflammation hypothesis in vascular disease, causality remains to be established for these cytokines in CHD.

Acknowledgements

Dr Mathew Walker and Mr Carsten Agger provided assistance with data management. We thank Dr Paul Welsh and Ms Estelle Poorhang for technical assistance, and Dr Barbara Jefferis for providing clarifications on information sought about the BRHS and BWHHS studies.

Funding

This work was supported by a grant from the British Heart Foundation (RG/08/014), the U.K. Medical Research Council, and the U.K. National Institute of Health Research Cambridge Biomedical Research Centre. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of Interest

None declared.

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Tables and Figures

Table 1. Summary of baseline characteristics of participants by incident CHD status.

	Cases		Non-cases		Difference[†]
Variable	n	Mean (SD) or %	n	Mean (SD) or %	p
Demographics and lifestyle					
Age at survey (years)	833	57.4 (7.2)	681	48.2 (9.8)	<0.0001
Sex					<0.0001
Male	556	67%	334	49%	
Female	277	33%	347	51%	
Smoking status					<0.0001
Never/former	369	44%	373	55%	
Current	462	56%	306	45%	
Alcohol consumption					0.002
Never/former	207	25%	132	19%	
Current	624	75%	547	81%	
History of diabetes					<0.0001
No	699	92%	600	98%	
Yes	58	8%	13	2%	
Socioeconomic position					0.032
Low	167	26%	232	29%	
Low/Medium	189	30%	235	30%	
Medium	150	23%	189	24%	
Medium/High	92	14%	102	13%	
High	42	7%	36	5%	
Physical measurements					
Systolic blood pressure (mmHg)	832	138 (20)	680	128 (18)	<0.001
Diastolic blood pressure (mmHg)	832	84 (11)	681	81 (10)	0.022
Pulse rate (count/min)	833	69 (11)	680	67 (10)	0.002
Body mass index (kg/m^2)	827	26 (4.2)	678	25 (4.4)	<0.0001
Lipids					
Total cholesterol (mmol/l)	829	6.63 (1.21)	680	6.14 (1.14)	<0.0001
LDL cholesterol (mmol/l)	809	4.52 (1.09)	670	4.00 (1.02)	<0.0001
Non HDL cholesterol (mmol/l)	827	5.33 (1.23)	677	4.63 (1.16)	<0.0001
HDL cholesterol (mmol/l)	827	1.30 (0.37)	677	1.49 (0.42)	<0.0001
Log_e triglycerides (log_e mmol/l)	829	0.44 (0.49)	680	0.19 (0.48)	<0.0001
Inflammatory cytokines					
Log_e IL-6 (log_e ng/l)	732	0.86 (0.67)	594	0.58 (0.68)	<0.0001
Log_e IL-18 (log_e ng/l)	740	5.71 (0.42)	596	5.55 (0.40)	<0.0001
Log_e MMP-9 (log_e µg/l)	748	5.97 (0.57)	612	5.87 (0.56)	<0.0001
Log_e sCD40L (log_e ng/l)	652	8.79 (0.75)	529	8.80 (0.75)	0.938
Log_e TNF-α (log_e ng/l)	738	0.43 (0.39)	595	0.40 (0.45)	0.244
Log_e CRP (log_e mg/l)	389	0.94 (1.02)	369	0.22 (1.15)	<0.0001

[†] p-value for difference in risk marker distribution between cases and non-cases adjusted for cohort, age, and sex.

Table 2. Associations between inflammatory cytokines and CHD risk markers.

			Partial correlation (95% CI) with row variable or percent difference (95% CI) compared to reference category [§]					
Variable	n	Mean (SD)	IL-6 (ng/l)	IL-18 (ng/l)	MMP-9 (µg/l)	sCD40L (ng/l)	TNF-alpha (ng/l)	Log CRP (mg/l)
Demographics and lifestyle								
Age at survey (years)	1514	52.9 (9.3)	0.28 (0.22, 0.34)	0.15 (0.08, 0.21)	0.01 (-0.05, 0.08)	0.00 (-0.07, 0.08)	-0.08 (-0.14, -0.01)	0.24 (0.17, 0.30)
Sex								
Male	1514	890 (59%)	Ref	Ref	Ref	Ref	Ref	Ref
Female	1514	624 (41%)	-10% (-16%, -3%)	-16% (-19%, -12%)	-5% (-11%, 1%)	30% (20%, 42%)	-3% (-7%, 2%)	-8% (-22%, 8%)
Smoking status								
Never/former	1510	742 (49%)	Ref	Ref	Ref	Ref	Ref	Ref
Current	1510	768 (51%)	18% (9%, 26%)	9% (5%, 14%)	32% (25%, 40%)	13% (4%, 23%)	0% (-4%, 5%)	58% (36%, 85%)
Alcohol consumption								
Never/former	1510	339 (22%)	Ref	Ref	Ref	Ref	Ref	Ref
Current	1510	1171 (78%)	-11% (-19%, -2%)	-2% (-8%, 3%)	4% (-4%, 12%)	7% (-4%, 19%)	-1% (-7%, 5%)	-12% (-28%, 8%)
History of diabetes								
No	1370	1299 (95%)	Ref	Ref	Ref	Ref	Ref	Ref
Yes	1370	71 (5%)	23% (3%, 46%)	3% (-7%, 14%)	-9% (-21%, 5%)	-8% (-23%, 11%)	-3% (-13%, 8%)	57% (6%, 133%)
Socioeconomic position								
Low	1434	399 (28%)	Ref	Ref	Ref	Ref	Ref	Ref
Low/Medium	1434	424 (30%)	-12% (-20%, -3%)	-7% (-12%, -1%)	-3% (-11%, 5%)	-5% (-16%, 7%)	-4% (-10%, 2%)	-2% (-21%, 20%)
Medium	1434	339 (24%)	-17% (-25%, -8%)	-8% (-14%, -2%)	-4% (-12%, 5%)	2% (-10%, 15%)	2% (-5%, 9%)	2% (-20%, 29%)
Medium/High	1434	194 (14%)	-12% (-22%, 0%)	-14% (-20%, -7%)	-11% (-20%, -1%)	-4% (-17%, 12%)	-7% (-14%, 0%)	3% (-22%, 36%)
High	1434	78 (5%)	-27% (-38%, -13%)	-14% (-22%, -5%)	-11% (-23%, 3%)	4% (-16%, 29%)	-6% (-16%, 5%)	-14% (-42%, 28%)
Physical measurements								
Systolic blood pressure (mmHg)	1512	134 (20)	0.01 (-0.04, 0.06)	0.04 (-0.01, 0.09)	-0.04 (-0.09, 0.02)	-0.03 (-0.09, 0.02)	0.00 (-0.05, 0.06)	0.15 (0.08, 0.22)
Diastolic blood pressure (mmHg)	1513	83 (11)	0.01 (-0.05, 0.06)	0.07 (0.02, 0.12)	-0.05 (-0.11, -0.00)	-0.04 (-0.10, 0.02)	0.03 (-0.03, 0.08)	0.12 (0.05, 0.19)
Pulse rate (count/min)	1513	68 (11)	0.12 (0.07, 0.17)	0.06 (0.01, 0.12)	0.04 (-0.01, 0.10)	0.01 (-0.05, 0.07)	0.04 (-0.01, 0.10)	0.20 (0.13, 0.27)
Body mass index (kg/m^2)	1505	25.8 (4.3)	0.12 (0.07, 0.18)	0.04 (-0.01, 0.10)	-0.02 (-0.08, 0.03)	-0.00 (-0.06, 0.06)	0.08 (0.02, 0.13)	0.30 (0.24, 0.37)
Lipids								
Total cholesterol (mmol/l)	1509	6.41 (1.20)	-0.02 (-0.08, 0.03)	0.03 (-0.03, 0.08)	0.02 (-0.04, 0.07)	-0.01 (-0.07, 0.04)	0.00 (-0.05, 0.06)	0.05 (-0.02, 0.12)
LDL cholesterol (mmol/l)	1479	4.29 (1.09)	-0.02 (-0.08, 0.03)	0.02 (-0.03, 0.08)	0.03 (-0.02, 0.09)	-0.01 (-0.07, 0.04)	0.01 (-0.04, 0.07)	0.05 (-0.02, 0.12)
Non HDL cholesterol (mmol/l)	1504	5.02 (1.25)	0.02 (-0.03, 0.08)	0.09 (0.03, 0.14)	0.03 (-0.03, 0.08)	-0.00 (-0.06, 0.05)	0.04 (-0.02, 0.09)	0.13 (0.06, 0.20)
HDL cholesterol (mmol/l)	1504	1.39 (0.41)	-0.14 (-0.19, -0.09)	-0.21 (-0.26, -0.16)	-0.03 (-0.09, 0.02)	-0.03 (-0.09, 0.02)	-0.10 (-0.16, -0.05)	-0.23 (-0.30, -0.16)
Log_e triglycerides (log_e mmol/l)	1509	0.33 (0.50)	0.11 (0.05, 0.16)	0.20 (0.14, 0.25)	0.03 (-0.02, 0.08)	0.02 (-0.03, 0.08)	0.08 (0.02, 0.13)	0.28 (0.21, 0.34)
Inflammatory cytokines								
Log_e IL-6 (log_e ng/l)	1326	0.73 (0.69)	-	0.11 (0.06, 0.17)	0.16 (0.11, 0.21)	0.04 (-0.02, 0.09)	0.22 (0.17, 0.27)	0.48 (0.42, 0.54)
Log_e IL-18 (log_e ng/l)	1336	5.64 (0.42)	0.11 (0.06, 0.17)	-	0.07 (0.02, 0.13)	0.03 (-0.03, 0.09)	0.17 (0.12, 0.23)	0.18 (0.10, 0.26)
Log_e MMP-9 (log_e µg/l)	1360	5.93 (0.57)	0.16 (0.11, 0.21)	0.07 (0.02, 0.13)	-	0.31 (0.26, 0.37)	0.09 (0.03, 0.14)	0.31 (0.23, 0.38)
Log_e sCD40L (log_e ng/l)	1181	8.79 (0.75)	0.04 (-0.02, 0.09)	0.03 (-0.03, 0.09)	0.31 (0.26, 0.37)	-	0.10 (0.04, 0.16)	0.10 (0.01, 0.19)
Log_e TNF-α (log_e ng/l)	1333	0.42 (0.42)	0.22 (0.17, 0.27)	0.17 (0.12, 0.23)	0.09 (0.03, 0.14)	0.10 (0.04, 0.16)	-	0.10 (0.02, 0.17)
Log_e CRP (log_e mg/l)	758	0.59 (1.14)	0.48 (0.42, 0.54)	0.18 (0.10, 0.26)	0.31 (0.23, 0.38)	0.10 (0.01, 0.19)	0.10 (0.02, 0.17)	-

[§] Partial correlation between levels of cytokine and continuous variables or the percentage difference in mean levels of cytokine compared to reference category for categorical variables adjusted for cohort, age, and sex.

Table 3. Hazard ratios for coronary heart disease per 1-SD higher baseline levels of inflammatory cytokines with progressive adjustment for baseline levels of potential confounders.

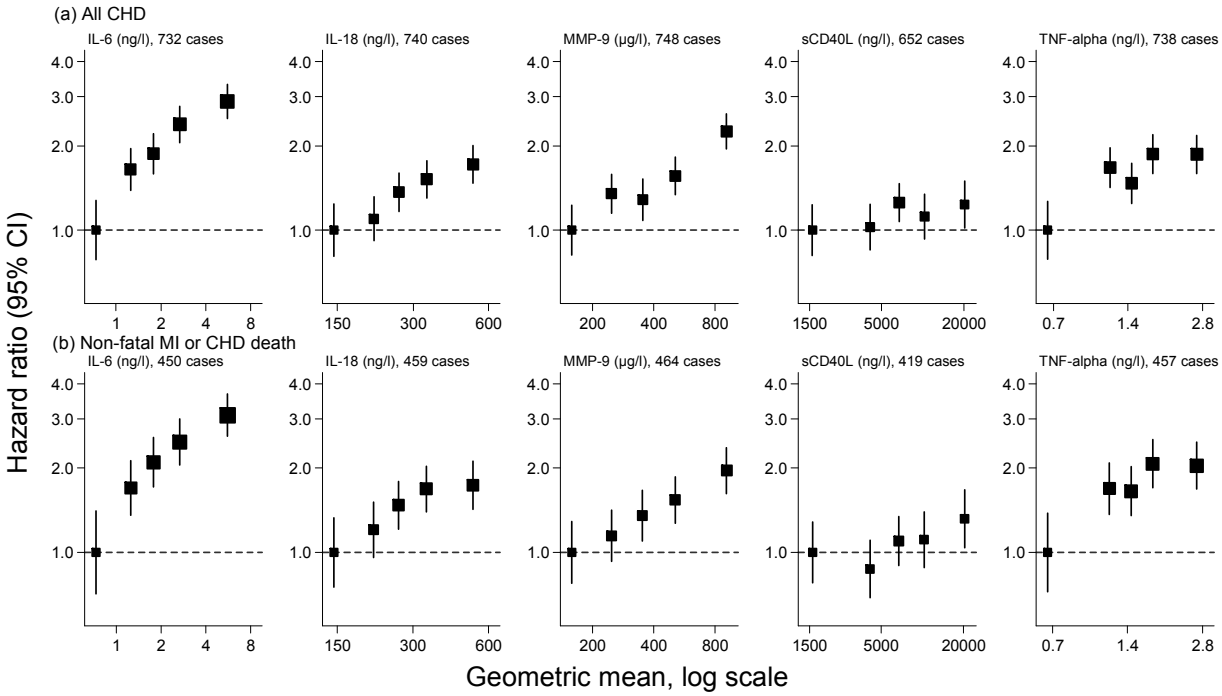
Outcome \ adjustment[¶]	Log_e IL-6 (ng/l)		Log_e IL-18 (ng/l)		Log_e MMP-9 (µg/l)		Log_e sCD40L (ng/l)		Log_e TNF-α (ng/l)	
	HR (95% CI)	Wald χ^2_1	HR (95% CI)	Wald χ^2_1	HR (95% CI)	Wald χ^2_1	HR (95% CI)	Wald χ^2_1	HR (95% CI)	Wald χ^2_1
All CHD (including angina)	**1180 participants, 654 cases**		**1190 participants, 662 cases**		**1214 participants, 670 cases**		**1036 participants, 575 cases**		**1187 participants, 660 cases**	
Adjusted for cohort, sex	1.46 (1.30, 1.64)	41	1.37 (1.20, 1.55)	23	1.27 (1.14, 1.42)	18	1.01 (0.88, 1.15)	0	1.06 (0.95, 1.18)	1
plus age	1.35 (1.18, 1.53)	21	1.29 (1.12, 1.49)	13	1.27 (1.12, 1.44)	14	0.99 (0.85, 1.14)	0	1.13 (1.00, 1.27)	4
plus systolic blood pressure	1.35 (1.18, 1.53)	20	1.29 (1.11, 1.49)	12	1.26 (1.11, 1.44)	12	1.00 (0.86, 1.16)	0	1.14 (1.01, 1.29)	4
plus smoking status	1.30 (1.14, 1.49)	15	1.27 (1.10, 1.47)	10	1.20 (1.05, 1.37)	8	0.98 (0.84, 1.14)	0	1.16 (1.02, 1.31)	5
plus history of diabetes	1.32 (1.15, 1.51)	16	1.25 (1.08, 1.46)	9	1.23 (1.07, 1.41)	9	0.97 (0.83, 1.14)	0	1.17 (1.03, 1.32)	6
plus BMI	1.30 (1.14, 1.50)	14	1.24 (1.07, 1.44)	8	1.23 (1.07, 1.41)	9	0.97 (0.83, 1.14)	0	1.15 (1.01, 1.31)	5
plus log_e triglycerides	1.27 (1.11, 1.46)	11	1.16 (0.99, 1.36)	4	1.22 (1.06, 1.40)	8	0.96 (0.81, 1.12)	0	1.15 (1.01, 1.31)	4
plus total cholesterol	1.28 (1.11, 1.47)	12	1.17 (1.00, 1.36)	4	1.22 (1.06, 1.41)	8	0.97 (0.82, 1.14)	0	1.18 (1.03, 1.35)	6
plus non-HDL cholesterol[§]	1.28 (1.11, 1.48)	12	1.15 (0.99, 1.35)	3	1.22 (1.06, 1.40)	8	0.97 (0.82, 1.14)	0	1.17 (1.02, 1.34)	5
plus HDL cholesterol[§]	1.27 (1.11, 1.47)	11	1.12 (0.96, 1.32)	2	1.22 (1.06, 1.40)	8	0.95 (0.80, 1.13)	0	1.14 (1.00, 1.31)	4
plus alcohol[§]	1.27 (1.10, 1.46)	10	1.12 (0.96, 1.32)	2	1.21 (1.06, 1.40)	7	0.95 (0.80, 1.13)	0	1.14 (1.00, 1.31)	4
plus socioeconomic position[§]	1.26 (1.08, 1.46)	9	1.12 (0.95, 1.31)	2	1.21 (1.05, 1.39)	7	0.93 (0.78, 1.11)	1	1.14 (1.00, 1.31)	4
plus other cytokines[§$]	1.24 (1.04, 1.47)	6	1.03 (0.86, 1.23)	0	1.25 (1.05, 1.50)	6	0.80 (0.66, 0.98)	5	1.09 (0.92, 1.30)	1
Non-fatal MI or CHD death	**1180 participants, 405 cases**		**1190 participants, 414 cases**		**1214 participants, 419 cases**		**1036 participants, 375 cases**		**1187 participants, 412 cases**	
Adjusted for cohort, sex	1.47 (1.29, 1.67)	34	1.37 (1.18, 1.58)	18	1.27 (1.12, 1.43)	14	1.04 (0.89, 1.22)	0	1.09 (0.96, 1.23)	2
plus age	1.36 (1.18, 1.56)	19	1.29 (1.10, 1.52)	10	1.26 (1.10, 1.45)	11	1.02 (0.86, 1.21)	0	1.17 (1.02, 1.34)	5
plus systolic blood pressure	1.36 (1.18, 1.56)	18	1.29 (1.09, 1.52)	9	1.26 (1.09, 1.45)	10	1.03 (0.87, 1.22)	0	1.18 (1.03, 1.36)	6
plus smoking status	1.31 (1.13, 1.51)	14	1.27 (1.08, 1.50)	8	1.19 (1.03, 1.37)	6	1.02 (0.85, 1.21)	0	1.20 (1.04, 1.38)	7
plus history of diabetes	1.32 (1.14, 1.52)	14	1.26 (1.06, 1.49)	7	1.22 (1.05, 1.41)	7	1.01 (0.84, 1.21)	0	1.22 (1.06, 1.40)	8
plus BMI	1.31 (1.13, 1.52)	13	1.25 (1.05, 1.48)	7	1.22 (1.05, 1.41)	7	1.01 (0.84, 1.21)	0	1.21 (1.04, 1.39)	7
plus log_e triglycerides	1.29 (1.11, 1.50)	11	1.17 (0.98, 1.40)	3	1.22 (1.05, 1.41)	7	1.00 (0.84, 1.20)	0	1.19 (1.03, 1.38)	6
plus total cholesterol	1.30 (1.12, 1.51)	11	1.18 (0.99, 1.40)	3	1.22 (1.05, 1.42)	7	1.02 (0.85, 1.22)	0	1.22 (1.05, 1.42)	7
plus non-HDL cholesterol[§]	1.30 (1.11, 1.51)	11	1.16 (0.97, 1.39)	3	1.21 (1.04, 1.41)	6	1.02 (0.85, 1.23)	0	1.21 (1.04, 1.41)	6
plus HDL cholesterol[§]	1.29 (1.11, 1.50)	11	1.13 (0.95, 1.35)	2	1.21 (1.04, 1.41)	6	1.01 (0.83, 1.22)	0	1.18 (1.02, 1.37)	5
plus alcohol[§]	1.27 (1.09, 1.48)	10	1.13 (0.95, 1.35)	2	1.21 (1.04, 1.40)	6	1.00 (0.83, 1.21)	0	1.18 (1.02, 1.37)	5
plus socioeconomic position[§]	1.29 (1.10, 1.52)	10	1.13 (0.95, 1.36)	3	1.20 (1.03, 1.40)	6	0.98 (0.80, 1.19)	0	1.19 (1.02, 1.39)	5
plus other cytokines[§$]	1.27 (1.06, 1.53)	7	1.06 (0.87, 1.29)	0	1.19 (0.99, 1.43)	3	0.87 (0.70, 1.09)	1	1.12 (0.93, 1.35)	2

[¶] Hazard ratios are presented per 1-SD higher observed levels at baseline for each inflammatory cytokine, progressively adjusted as shown and stratified by cohort. The sequence of adjustment generally corresponded to adjustment for established non-lipid risk factors first, then lipids (omitting one of highly correlated lipid variables) and finally other lifestyle risk factors and cytokines. Analyses were restricted to participants with complete information on the respective inflammatory cytokines and all confounding variables.

[§] Non-HDL cholesterol has been substituted for total cholesterol in these adjusted models

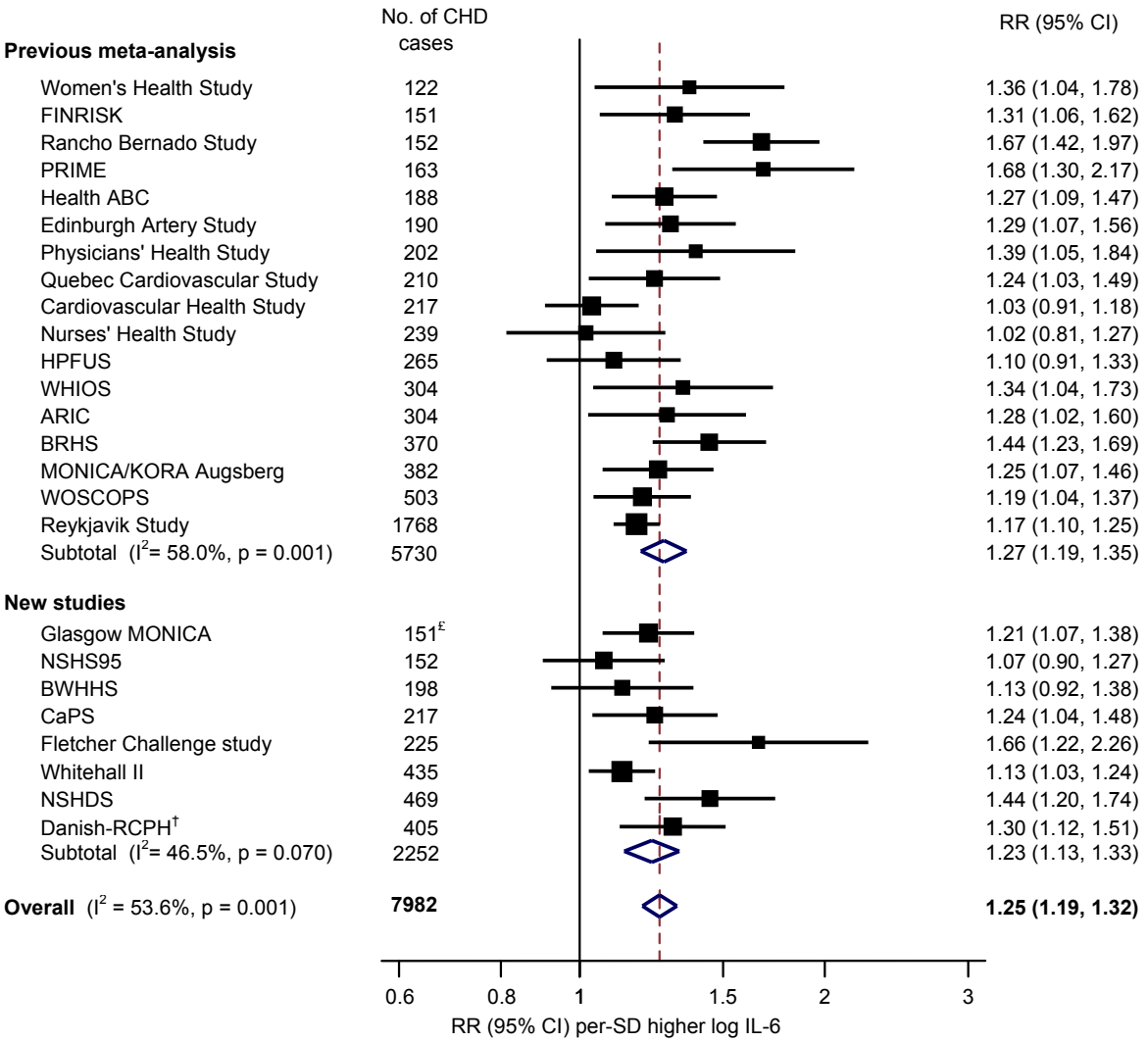
[$] Additional mutual adjustment of the 4 other cytokines based on data from 977 participants and 550 cases for analyses of all CHD outcome, and 355 cases for analysis of non-fatal MI or CHD death outcome.

Figure 1. Hazard ratios for all CHD (top panel)[†] and for non-fatal MI or CHD death (bottom panel)[‡] by fifths of inflammatory cytokine levels adjusted for age and sex and stratified by cohort.



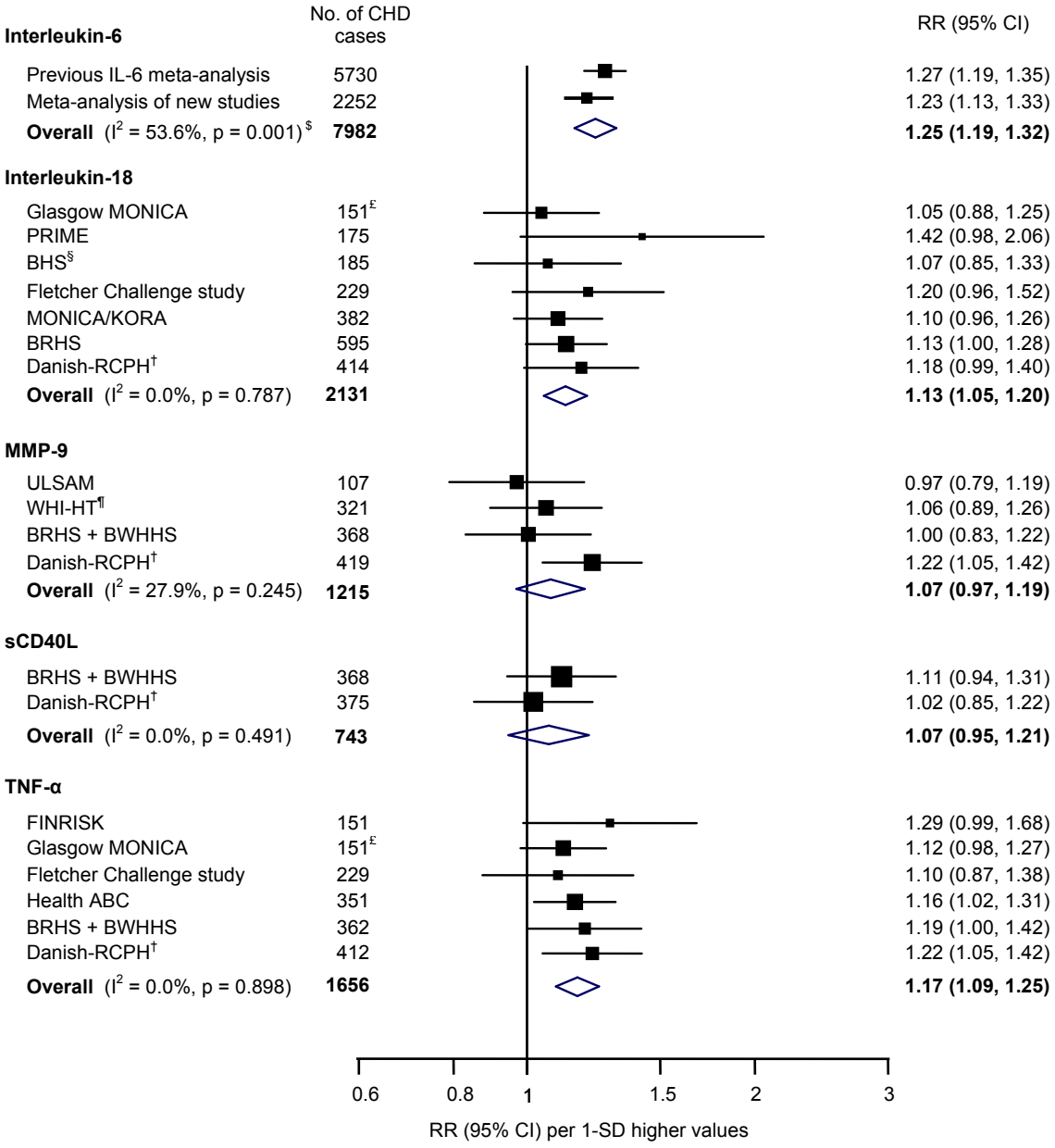
[†] The cut-points defining the fifths of each cytokine were based on the distribution of values observed in the random subcohort, so as to be representative of the expected distribution in the population (i.e. avoiding potential bias due to purposeful over-sampling of cases in the case-cohort design). Assuming a log-linear relationship the age and sex adjusted HR (95% CI) for any CHD per 1-SD higher values were: 1.37 (1.21, 1.54) for IL-6, 1.26 (1.11, 1.44) for IL-18, 1.30 (1.16, 1.46) for MMP-9, 1.01 (0.89, 1.15) for sCD40L and 1.13 (1.01, 1.27) for TNF-α.
[‡]Assuming a log-linear relationship the age and sex adjusted HR (95% CI) for non-fatal MI or CHD death per 1-SD higher values were: 1.38 (1.21, 1.57) for IL-6, 1.27 (1.09, 1.47) for IL-18, 1.27 (1.12, 1.45) for MMP-9, 1.03 (0.88, 1.19) for sCD40L and 1.17 (1.03, 1.33) for TNF-α.

Figure 2. Forest plot for the updated meta-analysis of association of IL-6 and risk of non-fatal MI or CHD death in prospective studies adjusted for conventional risk factors*.



*In most studies, these included age, sex, smoking status, adiposity markers, blood pressure, and/or lipid markers.
†Current study.
£The study reported total number of CVD cases and gave RRs for CHD without stating the number of CHD cases.
ARIC Atherosclerosis Risk in Communities; **BRHS** British Regional Heart Study; **BWHHS** British Women's Heart and Health Study; **CaPS** Caerphilly Prospective Study; **FINRISK** = Finnish National Risk Factor Survey; **HPFUS** Health Professionals' Follow-up Study; **MONICA** MONItoring of trends and determinants in Cardiovascular disease; **NSHDS** Northern Sweden Health and Disease Study; **NSHS95** Canadian Nova Scotia Health Survey; **PRIME** Prospective Epidemiological Study of Myocardial Infarction; **RCPH** Research Centre for Prevention and Health; **WHIOS** Women's Health Initiative Observational Study; **WOSCOPS** West of Scotland Coronary Prevention Study;

Figure 3. Meta-analysis of the associations of inflammatory cytokines and risk of non-fatal MI or CHD death in prospective studies adjusted for conventional risk factors*.



*In most studies, these included age, sex, smoking status, adiposity markers, blood pressure, and/or lipid markers.
†Current study.
$The pooled estimate and heterogeneity statistics consider each of the 17 studies in previous IL-6 meta-analysis separately (**Figure 2**). §Relative risk estimates were further adjusted for log C-reactive protein concentrations in this study in addition to conventional risk factors.
¶A proportion of the study participants had prevalent cardiovascular disease at baseline.
£The study reported total number of CVD cases and gave RRs for CHD without stating the number of CHD cases.
Abbreviations: **BHS**, Busselton Health Study; **BRHS** British Regional Heart Study; **BWHHS** British Women's Heart and Health Study; **FINRISK** Finnish National Risk Factor Survey; **Health ABC** Health, Aging, and Body Composition Study; **PRIME** Prospective Epidemiological Study of Myocardial Infarction; **RCPH** Research Centre for Prevention and Health; **ULSAM** Uppsala Longitudinal Study of Adult Men; **WHI-HT** Women's Health Initiative Hormone Trials;.