The associations of interleukin-6 (IL-6) and downstream inflammatory markers with risk of cardiovascular disease: the Caerphilly Study
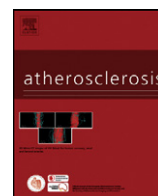
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The associations of interleukin-6 (IL-6) and downstream inflammatory markers with risk of cardiovascular disease: The Caerphilly Study

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A B S T R A C T

Objectives: Interleukin-6 (IL-6) is a key pro-inflammatory cytokine which mediates expression of several 'downstream' inflammatory markers and may play a role in atherothrombosis. However, it is not yet known whether IL-6 plays a role in mediating the associations of each marker with risk of coronary heart disease (CHD) or ischaemic stroke (IS).

Methods and results: We examined the role of IL-6 and several "downstream" markers of inflammation (leucocyte counts, plasma and serum viscosity, fibrinogen, C-reactive protein, α1-antitrypsin and α2-macroglobulin) with risk of subsequent CHD, IS, and a combined endpoint (CHD/IS) in a population of British men. 2208 men aged 45–64 years were followed for a median of 13.4 years and 486 men had experienced a cardiovascular event. In age-adjusted analyses, most inflammatory markers were significantly associated with risk of CHD or CHD/IS, but for IS associations were weaker. On multivariable analyses, including conventional risk factors, associations of serum viscosity, α2-macroglobulin and leucocyte count became non-significant for CHD and CHD/IS, while no inflammatory marker retained a significant association with risk of IS. In contrast, IL-6 retained a significant association with CHD and CHD/IS and, after adjustment for IL-6, hazard ratios for downstream inflammatory markers were attenuated to non-significance.

Conclusions: These findings suggest that IL-6 may play a role in mediating the associations of circulating inflammatory markers with risk of CHD in men. Further studies are required to assess whether this is also the case for risk of IS, and for CHD/IS in women.

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1. Introduction

There is increasing evidence that inflammation plays an important role in atherosclerosis and its clinical complications: cardiovascular disease (CVD) including coronary heart disease (CHD) and ischaemic stroke [1]. Previous reports from the prospective Caerphilly Study have shown that circulating inflammatory markers including fibrinogen, plasma viscosity and leucocyte count [2,3]; C-reactive protein (CRP) [4]; and α1-antitrypsin and α2-macroglobulin [5] are associated with CHD risk. These results are consistent with recent meta-analyses of data from several prospective studies (including Caerphilly) for fibrinogen [6], plasma viscosity [7], leucocyte count [8] and CRP [9,10]. Interleukin-6 (IL-6) is a key pro-inflammatory cytokine which mediates expression of all these "downstream" inflammatory markers [11,12] (Fig. 2). A recent systematic review of 17 prospective studies showed an overall odds ratio of 1.61 (95%CI 1.42,1.83) for subsequent risk of CHD for a two standard deviation increase in circulating levels of IL-6 [12]. Correction for within-person variability, corresponding to the long-term average IL-6 values increased the odds ratio to 3.34 (95%CI 2.45,4.56). Detailed analysis of data from two of these cohorts suggested that IL-6 has comparable associations with subsequent CHD to classical risk factors such as systolic blood pressure and total cholesterol. Hence it has been suggested that IL-6 may play a key role in mediating the associations of "downstream" inflammatory markers (such as CRP) with risk of CHD [12], and possibly also of IS, for which little data are available.

It is, however, not yet established whether increased IL-6 expression might mediate the associations of other "downstream" markers with risk of CHD and IS. The aims of the present study were therefore to examine the associations of IL-6 with CHD and IS events, and to estimate the effect of these on the associations

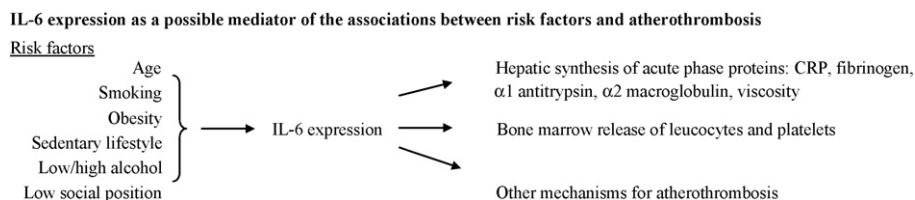**IL-6 expression as a possible mediator of the associations between risk factors and atherothrombosis**

Fig. 1. IL-6 expression as a possible mediator of the associations between risk factors and atherothrombosis.

of several "downstream" inflammatory markers with CHD and IS, which were updated from our previous reports [2–5].

2. Methods

The general design and methods of the Caerphilly Study have been described elsewhere [3,4,13]. Briefly, at each examination the men were invited to attend an afternoon or evening clinic where a detailed medical and lifestyle history was obtained, the London School of Hygiene and Tropical Medicine (LSHTM) chest pain questionnaire was administered, a full 12 lead electrocardiogram (ECG) was recorded, and height, weight and blood pressure measured. The men were invited to return, fasting, to an early morning clinic where a blood sample was taken.

2.1. Study population

The original cohort of 2512 men aged 45–59 years was recruited between 1979 and 1983, and since then they have been re-examined at five yearly intervals. The men who are the subject of this report are 2398 men seen at the first re-examination between 1984 and 1988, when they were aged 49–65 years [4].

2.2. Blood collection, storage and analysis

Blood was taken between 7.00 am and 10.00 am for 91% of the men. It was taken before 7.00 am for 7% and between 10.00 am and 11.00 am for the remaining 2%. The blood was collected without venous stasis into evacuated containers using a 19-gauge butterfly needle and Sarstedt monovette adaptors. Centrifugation was carried out within 1 h. Fresh EDTA plasma samples were used for the measurement of nephelometric fibrinogen, total and differential white cell counts (in the Department of Haematology, Southmead Hospital, Bristol), as previously described [3]. Plasma and serum viscosity were measured using a Harkness viscometer as described previously [14], and serum α1-antitrypsin and α2-macroglobulin were measured by protein electrophoresis [15]. Citrated plasma stored at $-70\,°C$ was used for the measurement of high-sensitivity CRP [4] and IL-6. IL-6 was measured by a high-sensitivity ELISA assay (R&D Systems, Oxford, UK). The intra- and inter-assay coefficients of variation were 7% and 8%, respectively. Due to attrition of stored plasma samples [4], CRP and IL-6 measurements were performed in 1511 and 1369 men, respectively. The overwhelming majority of demographic and major risk factors showed non-significant differences between subjects in whom Il-6 or CRP measurements were performed. However, total cholesterol levels averaged 5.58 mmol/L in subjects with IL-6 measured and 5.71 mmol/L in those without this measure ($P < 0.004$).

2.3. Prospective CHD and stroke

Follow-up was from 1984–88 to 31/12/2000, at a median interval of 13.4 years (IQR 10.1, 14.8). All men were flagged with the National Health Service Central Registry and we used death certificates coded to ICD (9th revision) 410–414 and to ICD (9th revision) 430–438 as our definition of fatal CHD and stroke, respectively. Pre-existing CHD was defined either by a past clinical history of myocardial infarction or angina or by using the London School of Hygiene and Tropical Medicine (LSHTM) chest pain questionnaire. We added to the LSHTM questionnaire some questions about admission to hospital with chest pain. Subjects who had ECG evidence of past myocardial infarction were also included as described previously [13]. Past history of stroke was obtained from the clinical history for each subject. These, together with lists from Hospital Activity Analysis of all men admitted to local hospitals with a diagnosis of ICD 410–414 (9th revision), were used as the basis for a search of hospital notes for events meeting standard World Health Organisation (1996) criteria for acute myocardial infarction (MI). Similarly non-fatal ischaemic stroke events were obtained by a search of hospital records with a diagnosis of ICD 430–436 and by questionnaire, and validated from hospital and general practitioner records as described elsewhere [13].

3. Statistical methods

Men with non-fasting blood samples (173 cases), men coded as having haemorrhagic stroke (13 cases) and men with no follow-up history after the baseline (4 cases) were excluded resulting in 2208 subjects for analysis.

All inflammatory markers were found to be positively skewed and required logarithmic transformations to approximate to normality. These variables were summarised using the geometric mean (GM).

The three endpoints used in the analysis were CHD event, IS event and either type of event (CVD). For each endpoint a Cox proportional hazard model with strata defined by the presence or absence of CVD at baseline was used to give the hazard ratios for the inflammatory markers divided into thirds of distribution. The assumption of hazard proportionality was assessed by using cumulative hazard plots and by tests of interaction between categorised variables and time in the Cox model. Effects of inflammatory variables were considered in crude form (adjusted for age only) and also after adjustment for age, smoking (never, past or current), diabetes, systolic blood pressure, body mass index, total cholesterol, HDL cholesterol, total triglycerides and family history of premature CHD before 55 years of age. In each analysis a test for linear trend in hazard ratios across the thirds of the inflammatory variable was used to assess significance. To allow for possible bias arising from the exclusion of cases with missing values on the inflammatory variables, a multiple imputation methodology was used [16]. This approach regresses each inflammatory variable on all other variables to obtain a prediction equation from which missing values are estimated. The process proceeds in an iterative way, with each final imputed value incorporating an amount of error appropriate for that inflammatory variable. This imputation process was repeated to give 10 sets of complete data. The estimates from Cox models for each of these 10 sets of complete data were then combined to give a single hazard ratio for each inflammatory variable [17].

All analyses were carried out using SPSS version 14 and Stata release 9.

Table 1
Geometric means for inflammatory markers by smoking habit, diabetes and presence of cardiovascular disease at baseline examination.

	n	Smoking status			P trend	Diabetes status		P	Cardiovascular disease at baseline		P
		Never (n_{max} 402)	Ex (n_{max} 843)	Current (n_{max} 957)		No (n_{max} 1523)	Yes (n_{max} 73)		No (n_{max} 2135)	Yes (n_{max} 685)	
Interleukin-6 (pg/mL)	1392	1.42	1.68	2.30	<0.001	1.87	2.07	NS	1.77	2.13	<0.001
CRP (mg/L)	1511	1.09	1.69	2.14	<0.001	1.72	1.91	NS	1.62	1.96	<0.01
Nephelometric fibrinogen (g/L)	2167	3.64	3.79	4.06	<0.001	3.88	3.78	NS	3.84	3.95	<0.01
Plasma viscosity (mPa s)	2142	1.86	1.89	1.90	<0.001	1.89	1.93	<0.05	1.89	1.91	<0.001
Serum viscosity (mPa s)	2118	1.64	1.66	1.65	NS	1.65	1.67	NS	1.65	1.67	<0.001
α2-Macroglobulin (g/100 mL)	2039	1.45	1.52	1.58	<0.001	1.53	1.77	<0.001	1.51	1.59	<0.001
α1-Antitrypsin (g/100 mL)	2041	1.50	1.56	1.72	<0.001	1.62	1.52	<0.05	1.60	1.65	<0.05
Leucocytes (10^9 L^{-1})	2146	6.32	6.80	8.19	<0.001	7.27	7.37	NS	7.20	7.45	<0.01
Neutrophils (10^9 L^{-1})	2146	3.42	3.70	4.60	<0.001	4.01	4.14	NS	3.95	4.17	<0.001
Lymphocytes (10^9 L^{-1})	2146	2.14	2.26	2.60	<0.001	2.38	2.33	NS	2.38	2.38	NS
Monocytes (10^9 L^{-1})	2033	0.37	0.40	0.46	<0.001	0.42	0.41	NS	0.41	0.43	<0.01
Eosinophils (10^9 L^{-1})	2061	0.17	0.19	0.22	<0.001	0.20	0.20	NS	0.20	0.20	NS
Basophils (10^9 L^{-1})	2031	0.05	0.05	0.06	<0.001	0.05	0.05	NS	0.05	0.06	<0.01

NS $P > 0.05$.

Table 2
Associations between IL-6 and other inflammatory marker, between inflammatory markers and cardiovascular risk factors and between inflammatory markers and neutrophil subsets.

	Interleukin-6	C-reactive protein	Fibrinogen	Plasma viscosity	Serum viscosity	α2-Macroglobulin	α1-Antitrypsin	Leucocytes	Neutrophils
(a) Correlation coefficients for associations between inflammatory markers									
C-reactive protein	0.50**								
Fibrinogen	0.36**	0.51**							
Plasma viscosity	0.35**	0.48**	0.51**						
Serum viscosity	0.25**	0.35**	0.32**	0.75**					
α2-Macroglobulin	0.14**	0.17**	0.20**	0.35**	0.33**				
α1-Antitrypsin	0.28**	0.37**	0.27**	0.26**	0.15**	0.11**			
Leucocytes	0.32**	0.34**	0.29**	0.26**	0.15**	0.14**	0.17**		
Neutrophils	0.31**	0.37**	0.29**	0.24**	0.13**	0.12**	0.17**	0.88**	
(b) Correlation coefficients for associations between inflammatory markers and cardiovascular risk factors									
Age	0.16**	0.14**	0.15**	0.12**	0.05*	0.13**	0.12**	0.01	0.03
Body mass index	0.00	0.15**	0.05*	0.06**	0.11**	−0.02	−0.08**	−0.05*	−0.05*
Systolic BP	0.09**	0.13**	0.07**	0.10**	0.12**	0.13**	0.03	0.05*	0.05*
Triglycerides	0.04	0.11**	0.10**	0.25**	0.27**	0.27**	0.00	0.10**	0.07**
Total cholesterol	−0.05	0.07*	0.13**	0.24**	0.27**	0.20**	0.01	0.02	0.01
HDL cholesterol	−0.12**	−0.14**	−0.11**	−0.09**	−0.08**	−0.11**	−0.05*	−0.11**	−0.10**
(c) Correlation coefficients for associations between inflammatory markers and neutrophil subsets									
Lymphocytes	0.09**	0.12**	0.13**	0.14**	0.10**	0.10**	0.07**	0.70**	0.34**
Monocytes	0.19**	0.23**	0.17**	0.14**	0.10**	0.10**	0.13**	0.60**	0.50**
Eosinophils	0.11**	0.11**	0.10**	0.06**	0.03	0.03	0.05*	0.36**	0.23**
Basophils	0.10**	0.11**	0.09**	0.11**	0.06**	0.11**	0.05*	0.37**	0.28**

* Correlation is significant ($P < 0.05$; two-tailed).
** Correlation is significant ($P < 0.01$; two-tailed).

Table 3
Hazard ratios for first events after baseline by thirds of inflammatory variables in 2208 fasting subjects obtained from Cox models stratified by cardiovascular disease status at baseline.

	Prospective cardiovascular disease			Prospective ischaemic stroke			Prospective coronary heart disease		
		Hazard ratio (95%CI)			Hazard ratio (95%CI)			Hazard ratio (95%CI)	
	Events	Crude[a]	Adjusted[b]	Events	Crude[a]	Adjusted[b]	Events	Crude[a]	Adjusted[b]
Interleukin-6									
Low <1.38 pg/mL	69	1.00‡	1.00†	21	1.00	1.00	48	1.00‡	1.00†
Mid 1.38–2.21 pg/mL	99	1.32(0.97,1.81)	1.20(0.86,1.65)	24	1.03(0.57,1.86)	0.91(0.49,1.67)	75	1.45(1.01,2.09)	1.34(0.91,1.97)
High >2.21 pg/mL	127	1.83(1.36,2.47)	1.50(1.08,2.07)	33	1.50(0.86,2.62)	1.30(0.71,2.37)	94	1.98(1.39,2.82)	1.59(1.08,2.34)
C-reactive protein									
Low <1.08 mg/L	76	1.00‡	1.00‡	22	1.00	1.00	54	1.00‡	1.00†
Mid 1.08–2.60 mg/L	121	1.63(1.22,2.17)	1.44(1.07,1.94)	32	1.47(0.85,2.53)	1.42(0.80,2.51)	89	1.69(1.21,2.38)	1.44(1.01,2.05)
High >2.60 mg/L	139	1.94(1.46,2.57)	1.56(1.15,2.11)	32	1.48(0.85,2.56)	1.46(0.81,2.61)	107	2.13(1.53,2.97)	1.58(1.11,2.25)
Fibrinogen									
Low <3.58 g/L	123	1.00‡	1.00†	30	1.00	1.00	93	1.00‡	1.00
Mid 3.58–4.20 g/L	154	1.27(1.00,1.61)	1.17(0.92,1.49)	50	1.66(1.05,2.61)	1.66(1.04,2.63)	104	1.14(0.86,1.51)	1.02(0.77,1.36)
High >4.20 g/L	199	1.70(1.36,2.14)	1.32(1.04,1.67)	48	1.59(1.00,2.52)	1.51(0.93,2.45)	151	1.74(1.34,2.27)	1.26(0.96,1.66)
Plasma viscosity									
Low <1.85 mPa s	127	1.00‡	1.00†	40	1.00	1.00	87	1.00‡	1.00†
Mid 1.85–1.93 mPa s	145	1.20(0.95,1.53)	1.08(0.85,1.38)	39	1.03(0.67,1.61)	0.98(0.62,1.54)	106	1.28(0.96,1.70)	1.13(0.85,1.52)
High >1.93 mPa s	197	1.72(1.37,2.15)	1.33(1.05,1.68)	50	1.34(0.88,2.04)	1.23(0.79,1.90)	147	1.89(1.45,2.47)	1.36(1.03,1.81)
Serum viscosity									
Low <1.62 mPa s	119	1.00‡	1.00	34	1.00	1.00	85	1.00‡	1.00
Mid 1.62–1.68 mPa s	164	1.13(0.89,1.43)	0.99(0.78,1.27)	43	1.03(0.66,1.62)	0.96(0.61,1.53)	121	1.17(0.89,1.55)	1.00(0.75,1.34)
High >1.68 mPa s	179	1.38(1.09,1.74)	1.12(0.87,1.43)	49	1.27(0.82,1.98)	1.21(0.76,1.92)	130	1.42(1.08,1.87)	1.07(0.80,1.44)
α2-Macroglobulin									
Low <1.38 g/100 mL	119	1.00‡	1.00	37	1.00	1.00	82	1.00‡	1.00
Mid 1.38–1.66 g/100 mL	147	1.88(0.93,1.51)	1.02(0.79,1.31)	44	1.15(0.74,1.78)	1.08(0.68,1.70)	103	1.21(0.90,1.61)	1.01(0.75,1.36)
High >1.66 g/100 mL	183	1.46(1.15,1.84)	1.07(0.83,1.38)	42	1.05(0.67,1.65)	0.95(0.59,1.54)	141	1.64(1.25,2.16)	1.12(0.83,1.52)
α1-Antitrypsin									
Low <1.55 g/100 mL	105	1.00‡	1.00‡	30	1.00	1.00	75	1.00‡	1.00‡
Mid 1.55–1.78 g/100 mL	162	1.52(1.19,1.94)	1.48(1.15,1.91)	45	1.47(0.93,2.33)	1.44(0.90,2.31)	117	1.53(1.15,2.05)	1.52(1.13,2.04)
High >1.78 g/100 mL	182	1.77(1.39,2.25)	1.49(1.15,1.92)	48	1.57(0.99,2.49)	1.39(0.86,2.26)	134	1.85(1.39,2.46)	1.54(1.14,2.08)
Leucocytes									
Low <3.50 × 10⁹ L⁻¹	117	1.00‡	1.00‡	39	1.00	1.00†	78	1.00‡	1.00‡
Mid 3.50–9.70 × 10⁹ L⁻¹	158	1.37(1.08,1.75)	1.29(1.01,1.66)	37	1.00(0.64,1.57)	0.94(0.59,1.50)	121	1.56(1.17,2.07)	1.47(1.09,1.99)
High >9.70 × 10⁹ L⁻¹	192	1.76(1.40,2.22)	1.49(1.15,1.92)	53	1.50(0.99,2.27)	1.27(0.80,2.01)	139	1.89(.143,2.50)	1.58(1.16,2.16)
Neutrophils									
Low <3.50 × 10⁹ L⁻¹	103	1.00‡	1.00‡	36	1.00	1.00†	67	1.00‡	1.00‡
Mid 3.50–4.51 × 10⁹ L⁻¹	175	1.73(1.35,2.20)	1.56(1.21,2.01)	44	1.30(0.83,2.02)	1.24(0.78,1.95)	131	1.95(1.45,2.62)	1.72(1.27,2.34)
High >4.51 × 10⁹ L⁻¹	189	1.95(1.53,2.48)	1.61(1.24,2.10)	49	1.47(0.95,2.26)	1.23(0.77,1.97)	140	2.20(1.64,2.95)	1.80(1.31,2.48)
Lymphocytes									
Low <2.09 × 10⁹ L⁻¹	152	1.00†	1.00	47	1.00	1.00	105	1.00‡	1.00
Mid 2.09–2.68 × 10⁹ L⁻¹	139	0.93(0.74,1.17)	0.83(0.66,1.06)	36	0.81(0.52,1.25)	0.74(0.47,1.16)	103	0.98(0.75,1.29)	0.87(0.66,1.16)
High >2.68 × 10⁹ L⁻¹	176	1.26(1.02,1.57)	1.06(0.84,1.33)	46	1.10(0.73,1.65)	0.94(0.61,1.45)	130	1.34(1.03,1.73)	1.09(0.83,1.44)
Monocytes									
Low <0.37 × 10⁹ L⁻¹	130	1.00†	1.00	42	1.00	1.00	88	1.00‡	1.00
Mid 0.37–0.48 × 10⁹ L⁻¹	141	1.08(0.85,1.37)	0.96(0.75,1.23)	40	0.96(0.62,1.48)	0.87(0.56,1.36)	101	1.13(0.85,1.51)	1.01(0.75,1.36)
High >0.48 × 10⁹ L⁻¹	166	1.30(1.03,1.64)	1.12(0.88,1.42)	41	0.99(0.64,1.53)	0.81(0.51,1.27)	125	1.45(1.10,1.91)	1.27(0.95,1.69)
Eosinophils									
Low <0.16 × 10⁹ L⁻¹	147	1.00	1.00	43	1.00	1.00	104	1.00	1.00
Mid 0.16–0.25 × 10⁹ L⁻¹	151	1.03(0.82,1.30)	0.95(0.75,1.21)	48	1.10(0.73,1.67)	0.96(0.63,1.48)	103	1.01(0.77,1.32)	0.94(0.71,1.26)
High >0.25 × 10⁹ L⁻¹	146	0.99(0.79,1.25)	0.95(0.75,1.21)	33	0.75(0.48,1.19)	0.66(0.41,1.05)	113	1.09(0.84,1.43)	1.09(0.82,1.44)
Basophils									
Low <0.05 × 10⁹ L⁻¹	134	1.00	1.00	39	1.00	1.00	95	1.00	1.00
Mid 0.05–0.07 × 10⁹ L⁻¹	140	1.08(0.85,1.37)	1.08(0.85,1.38)	34	0.90(0.57,1.43)	0.86(0.54,1.38)	106	1.16(0.88,1.53)	1.18(0.88,1.56)
High >0.07 × 10⁹ L⁻¹	162	1.24(0.98,1.56)	1.14(0.89,1.45)	50	1.36(0.90,2.08)	1.28(0.83,1.98)	112	1.19(0.91,1.57)	1.07(0.80,1.43)

[a] Adjusted for age only.
[b] Adjusted for age, smoking, diabetes, systolic blood pressure, body mass index, total cholesterol, HDL cholesterol, total triglycerides and family history of premature CHD.
† Significant test for trend in hazard ratios ($P < 0.05$).
‡ Significant test for trend in hazard ratios ($P < 0.01$).

4. Results

The mean age for the 2208 men was 57 years (standard deviation 4.5 years). By the end of the follow-up 486 men had at least one CVD event (either fatal or non-fatal). Of these, 353 men had experienced a CHD event first, and 133 had an IS event first.

The distributions of the inflammatory markers according to smoking habit, diabetes status and presence of cardiovascular disease at baseline are shown in Table 1. All markers were associated
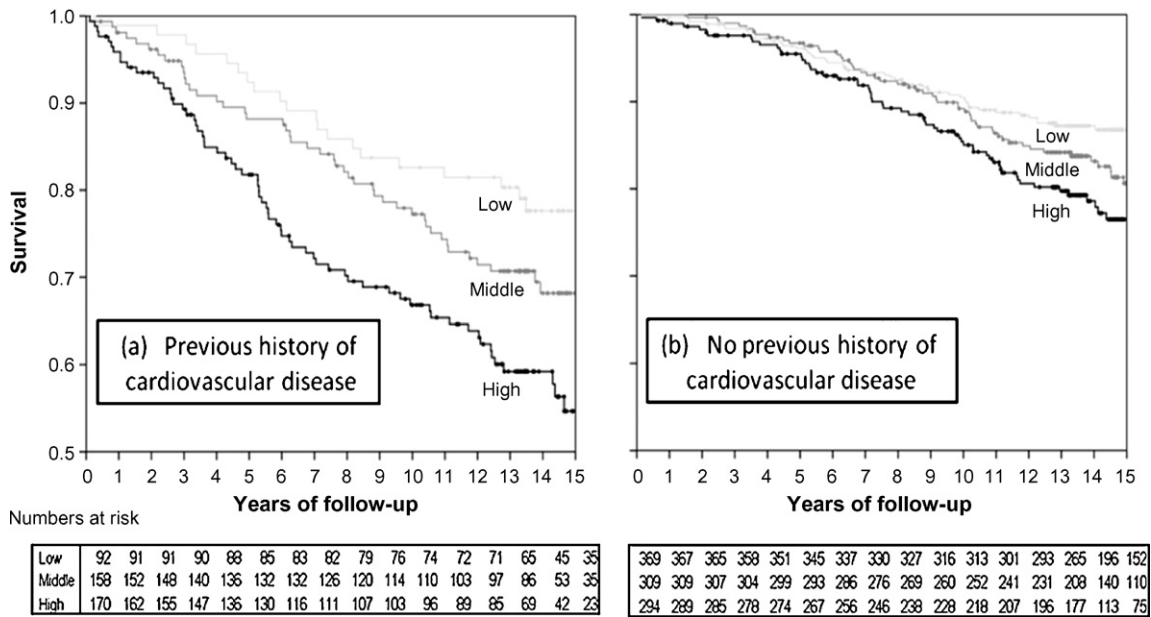
Fig. 2. Survival without a cardiovascular event by third of interleukin-6 distribution for men with and without a history of cardiovascular disease at entry to the study.

with smoking habit and all except lymphocyte and eosinophil counts with baseline CVD. Plasma viscosity, α2-macroglobulin and α1-antitrypsin were associated with diabetes.

Table 2 shows correlations between IL-6, other inflammatory markers, and cardiovascular risk factors. The significant correlations are flagged. Only body mass index (BMI), total triglycerides and total cholesterol were not significantly associated with IL-6. All other inflammatory markers, systolic blood pressure, HDL cholesterol and age were found to be significantly associated with IL-6. The majority of inflammatory markers showed moderate or strong correlations, in agreement with recognised biological associations [11,12].

Fig. 2(a) and (b) shows Kaplan–Meier plots for thirds of the IL-6 distribution in men with and without evidence of CVD at baseline, respectively. Both plots show clear separation of the highest and lowest thirds of the distribution with increasing periods of follow-up. All subsequent analyses were stratified by CVD status at baseline as this variable did not satisfy the proportional hazards assumption.

A stratified Cox proportional hazards analysis, as described in Section 3, was conducted for the three outcome events and the results are presented in Table 3. Tertile cut-points and numbers of events in each category are given. Testing for significant linear trends across the three categories was performed both before and after adjustment for conventional risk factors, and the significant results are flagged.

For the prospective CVD endpoint, the crude analysis showed significant linear trends over the thirds of the distributions of IL-6, CRP, fibrinogen, plasma viscosity, serum viscosity, α2-macroglobulin, α1-antitrypsin, leucocytes, neutrophils, lymphocytes and monocytes. In the adjusted analysis, significant linear trends are shown for IL-6, CRP, fibrinogen, plasma viscosity, α1-antitrypsin, leucocytes and neutrophils.

For the prospective stroke endpoint, only the adjusted analysis showed significant linear trends for leucocyte count and neutrophils. All other inflammatory variables were non-significant.

The patterns between CVD and CHD were essentially similar, reflecting the greater number of CHD events than stroke events in the combined endpoint. For CHD events the pattern of significance in the linear trends was the same as that for CVD events, except that fibrinogen was only significant in the crude analysis.

As there was little evidence of deviations from linearity in the tests of hazard ratios presented in Table 3, further modelling was conducted using the logarithmically transformed inflammatory variables to maximise the power of the analysis. Table 4 presents further analysis of the combined CVD endpoint. Once IL-6 had been forced into the model along with the established risk factors, no other inflammatory variable made a significant contribution to the model. For categorical variables the hazard ratios in Table 4 represent comparisons of subgroups, while for continuous variables they represent the change in hazard associated with a standard deviation change in the variable, logarithmically transformed where indicated.

The final model presented in Table 4 was based on 281 events in 1339 men. To investigate any possible distorting effects of missing values a sensitivity analysis was performed using multiple imputation to estimate values for men with missing inflammatory variables. The results obtained by multiple imputation were similar to those shown in Table 4, a one standard deviation increase in

Table 4

Hazard ratios for a prospective cardiovascular disease event from a Cox regression stratified by cardiovascular disease status at baseline, with forced inclusion of established risk factors and IL-6. Analysis is based on 281 events in 1339 men with complete data on these variables.

Established risk factors	Hazard ratio (95%CI)	P value
Age (per SD)	1.21 (1.07,1.37)	0.002
Smoking		
(Past vs never)	1.06 (0.72,1.56)	0.78
(Current vs never)	1.58 (1.09,2.31)	0.02
Systolic blood pressure (per SD)	1.16 (1.03,1.31)	0.01
Diabetes (Yes vs No)	1.45 (0.87,2.44)	0.16
Total cholesterol (per SD)	1.17 (1.03,1.33)	0.02
HDL cholesterol (per SD)	0.85 (0.74,0.97)	0.02
Triglycerides[a] (per SD)	1.06 (0.92,1.22)	0.42
Body mass index (per SD)	1.00 (0.89,1.14)	0.93
Family history of coronary heart disease under 55 years (Yes vs No)	1.06 (0.74,1.51)	0.76
Inflammatory variables		
Interleukin-6[a] (per SD)	1.12 (1.01,1.25)	0.04

[a] Logarithmically transformed.

IL-6 being associated with a hazard ratio of 1.11 (95%CI 1.00,1.24) $P = 0.05$.

This indicated that, among inflammatory markers, only IL-6 was found to be an independent significant risk factor for a CVD event. Age, current smoking, systolic blood pressure, total cholesterol and HDL cholesterol were also significant risk factors. In the model containing only the conventional risk factors the CVD hazard ratio in the top third of the distribution of predicted risk relative to the bottom third was 2.9. In the model which included additionally IL-6, this ratio increased to 3.0.

5. Discussion

This updated report on a major aim of the prospective Caerphilly Study cohort – the association of circulating inflammatory markers with prospective major cardiovascular events (CHD and IS) – has three important new findings. First, we report that circulating levels of the pro-inflammatory cytokine, IL-6, measured in residual plasma samples are significantly associated with risk of CHD (multivariable-adjusted HR for top third versus bottom third), 1.59 (95%CI 1.08,2.34). This association, based on prospective CHD events, is consistent with a recent meta-analysis of several other prospective studies [12] which reported a multivariable-adjusted HR for top third versus bottom third of 1.66 (95%CI 1.51–1.83). Second, we confirm previous reports [12,18–21] that plasma IL-6 levels in the general population are significantly associated with circulating levels of several "downstream" inflammatory markers mediated by IL-6, including CRP, fibrinogen, plasma viscosity and total and neutrophil leucocyte counts; and further report its associations with serum viscosity, α1-antitrypsin and α2-macroglobulin; and with lymphocyte, monocyte, eosinophil and basophil counts (Table 2 and Fig. 1). Third, we show that in multivariable analyses, IL-6 retained a significant association with CHD and total CVD; while after adjustment for IL-6 their associations with "downstream" markers were attenuated to non-significance. We therefore conclude that IL-6 may partly mediate the associations of these downstream inflammatory markers with CVD risk in middle-aged men (Fig. 1).

The associations of IL-6 with prospective CHD are underestimated in this and other studies, because its within-person correlation over a few years was relatively modest, suggesting that long-term "usual" values of IL-6 (i.e. those corrected for regression dilution bias) would be at least twice as strongly associated with CHD risk as the single baseline IL-6 measurement used in our study [12]. This high within-person variability limits the potential value of IL-6 levels as a clinical predictor of CHD risk, as suggested in the current study, in which the addition of IL-6 to predicted risk in the top third of the distribution relative to the bottom third increased the ratio from 2.9 to only 3.0. However, IL-6 may have a potential causal role in atherothrombosis, given its position in the cytokine cascade as a key mediator of downstream inflammatory processes including activation of coagulation, hepatic release of acute phase reactant proteins, and release from bone marrow of leucocytes and platelets (Fig. 2). In support of a pathogenic role in atherosclerosis, we have recently shown that baseline IL-6 levels were more strongly associated than other inflammatory or haemostatic variables with progression of lower limb atherosclerosis over 12 years of follow-up [22]; and have also recently reported that IL-6 and other pro-inflammatory cytokines are associated with several CVD risk factors including social deprivation [21]. In a recent report in Type 2 diabetics IL-6 levels were associated with the degree of coronary artery calcium whereas the inflammatory markers (C-reactive protein and lipoprotein-associated phospholipase A2) were not [23].

Further studies are required to assess the potential causality of IL-6, including Mendelian randomization studies of functional genotypes [12] and IL-6 antagonists [25]. Our finding that the neutrophil count is the major contributor to the association of total leucocyte count with prospective CHD is consistent with most reported studies [8]. While fibrinogen is an important determinant of plasma viscosity and its association with incident CHD, serum reactant proteins such as α2-macroglobulin and α1-antitrypsin also contribute to serum and plasma viscosity (Fig. 2). However, on multivariable analyses serum viscosity and α2-macroglobulin did not remain as significant associates of CHD risk.

Only total leucocyte and neutrophil counts were significantly associated ($P < 0.05$) with risk of ischaemic stroke following multivariable-adjusted analyses (Table 3). All the other inflammatory markers in this study (including IL-6) were not significantly associated with risk of ischaemic stroke. These results are consistent with previous findings for fibrinogen, viscosity and leucocyte count from the Caerphilly and Speedwell cohorts [3,24]. However, at present the limited number of stroke events in this study (and other studies) limits their power to detect associations with circulating inflammatory markers, hence further studies are required. Meanwhile, circulating markers (including IL-6) were associated with the sum of CHD and ischaemic stroke events in the present study.

Strengths of the present study include its prospective nature, the degree of standardisation of time and fasting status of blood sample collection, and the assay of a wider range of other circulating inflammatory markers than those in many previously reported studies of IL-6. Limitations include non-availability of stored plasma samples available for IL-6 assay [4], and their storage for up to 20 years at −70 °C prior to assay. Also IL-6 data were not found to be "missing at random", but multiple imputation was used to investigate the likely effect on the IL-6 hazard ratio and this was found to be minimal. Furthermore, the distributions of IL-6 assays, and their associations with risk factors and with prospective CHD, were similar to those reported in other studies [12]. Finally, as the Caerphilly Study included only males, further studies are required to investigate the relationships between IL-6, downstream inflammatory markers, risk factors and CHD in females as well as in males. At present, the associations of IL-6 with risk factors and with risk of CHD appear similar in males and females [12].

6. Conclusions

We conclude that IL-6 shows comparable associations with risk of CHD as several "downstream" inflammatory markers and may potentially mediate their associations with CHD risk. Further studies on inflammatory markers and risk of ischaemic stroke are required.

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