

## Serum caeruloplasmin as a coronary risk factor in the elderly: the Rotterdam Study

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Serum Cu and caeruloplasmin levels have been suggested to be independent risk factors for CHD operating through oxidative modification of LDL. However, given its function as an acute-phase protein, the question has been raised whether an elevated caeruloplasmin level is not merely an indicator of inflammation. In the current study, we investigated whether serum caeruloplasmin was associated with subsequent myocardial infarction, taking into account indices of inflammation. The study population consisted of 210 cases of first myocardial infarction and controls, frequency-matched on age (5-year categories) and sex, selected from the population-based cohort of the Rotterdam Study. Serum caeruloplasmin levels were significantly elevated in cases of myocardial infarction compared with controls (510 (SD 110) v. 470 (SD 100) mg/l;  $P = 0.007$ ). Risk of myocardial infarction for the highest compared with the lowest quartile of caeruloplasmin was 2.46 (95% CI 1.04, 6.00;  $P_{\text{trend}} = 0.043$ ) after adjustment for age, sex, BMI, pack-years smoked, serum cholesterol, systolic blood pressure, and income. The relative risk was most evident in current smokers. Adjustment for C-reactive protein and leucocyte count reduced the excess risk by 33%. This suggests that a substantial part of the observed association between serum caeruloplasmin and CHD may be attributed to inflammation processes rather than to the pro-oxidant activity of caeruloplasmin.

### Serum caeruloplasmin: Myocardial infarction: Inflammation

Elevated serum caeruloplasmin levels have been found in patients with cardiovascular disorders including arteriosclerosis, abdominal aneurysms, unstable angina, and vasculitis and peripheral artery disease (Fox *et al.* 1995). Several prospective studies have indicated that serum Cu or caeruloplasmin level may be an independent risk factor for cardiovascular disease (Kok *et al.* 1988; Salonen *et al.* 1991a,b; Reunanen *et al.* 1992, 1996; Mäntärrä *et al.* 1994). The increased risk has been attributed to the pro-oxidant activity of caeruloplasmin and recent experimental studies demonstrating the ability of caeruloplasmin oxidatively to modify LDL (Ehrenwald *et al.* 1994; Lamb & Leake, 1994), seem to underline this notion. However, the question has been raised whether elevated caeruloplasmin is not merely an indicator of inflammation, given its acute-phase protein property. So far, most studies investigating the association between serum caeruloplasmin or Cu and cardiovascular

disorders have lacked information on indicators of inflammation. In the current study we obtained information on C-reactive protein and leucocyte count as indicators of inflammation. We examined whether a high serum caeruloplasmin level is associated with an increased risk of myocardial infarction, taking into account possible markers of inflammation.

### Subjects and methods

#### Study population and case ascertainment

The Rotterdam Study is a community-based prospective cohort study of 7983 persons (response rate 78%) aged 55 years and over, living in Ommoord, an urban district in Rotterdam, the Netherlands. The aim of the study is to investigate the incidence of and the risk factors for chronic

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and disabling diseases, as described elsewhere (Hofman *et al.* 1991). The study was approved by the Medical Ethics Committee of the Erasmus University, and written informed consent was obtained from all participants.

Follow-up for CHD started after the baseline survey in 1990, and until April 1996 (mean 4 years) follow-up information was available for 94% of the cohort. With respect to the vital status of participants, information was obtained at regular intervals from the municipal health service in Rotterdam. Information on fatal and non-fatal end-points was obtained from the general practitioners working in the study district of Ommoord. All possible events reported by the general practitioners were verified by research physicians from the Rotterdam Study through records of the participating general practitioners and medical specialists. Cause and circumstances of death were obtained by questionnaire from the general practitioner and by scrutinizing information from hospital discharge records in case of admittance or referral, shortly after reporting of death by the municipal health service or the general practitioner. Classification of fatal and non-fatal events was based on the International Classification of Diseases (World Health Organization, 1992). For the present analysis only cases of first myocardial infarction (ICD-10:I21–I24) were selected. All events were classified independently by two research physicians. If there was disagreement, a consensus was reached in a special session. Finally, all these events were verified by a cardiovascular disease expert. In cases of discrepancy, the judgement by this expert was considered definite.

The association between serum caeruloplasmin level and risk of myocardial infarction was examined by use of a nested case–control design. Cases and controls were drawn from the population-based Rotterdam Study. For every subject with a myocardial infarction during follow-up ( $n$  202) a control without a myocardial infarction was selected. Age strata (5-year interval) and sex were used as matching variables. For determination of serum caeruloplasmin and C-reactive protein levels frozen sera were available for 255 subjects, 111 myocardial infarction cases and 144 controls. Blood samples were not available for all cases allocated to this study, particularly for cases in the beginning of the follow-up period. Exclusion of subjects with a history of myocardial infarction at baseline resulted in a study population of 210 subjects (eighty-three cases and 127 controls) for the present analysis.

### Measurements

Baseline information on current health status, medical history, drug use, education, income, and smoking behaviour was obtained with a computerized questionnaire during a home interview. Height and weight were measured, and BMI (weight in kg/height in m<sup>2</sup>) was calculated as a measure of obesity. Sitting blood pressure was measured on the right upper arm with a random zerosphygmomanometer. The average of two measurements was used in the analysis. A venepuncture was performed and haematological variables were obtained by standard clinical laboratory procedures. Serum total and HDL-cholesterol concentrations were determined by an automated enzymic procedure.

Alcohol intake was estimated by use of a semi-quantitative food-frequency questionnaire assessing habitual food intake during the past year (Klipstein-Grobusch *et al.* 1998). Frozen sera, preserved at  $-20^{\circ}$ , and collected from the cases and the controls simultaneously at study baseline, were used to determine serum concentrations of caeruloplasmin and C-reactive protein. Sera from cases and controls were analysed in the same run. Serum caeruloplasmin concentrations and C-reactive protein were determined by kinetic nephelometry by use of a Beckman-Array system (Munich, Germany). For five subjects (cases and controls) C-reactive protein could not be determined due to insufficient serum for analysis. Interassay CV were 4.6% for caeruloplasmin and 3.8% for C-reactive protein.

### Data analysis

Associations between serum caeruloplasmin concentrations and risk factors for ischaemic heart disease were estimated by use of Pearson's correlation coefficient (continuous variables) and ANOVA (categorical variables) adjusted for age and sex. The association between quartiles of serum caeruloplasmin and risk of myocardial infarction was investigated by multiple logistic regression comparing risk of myocardial infarction in the upper quartiles with the lowest quartile of serum caeruloplasmin. Analyses were initially adjusted for age and sex and subsequently for BMI, pack-years smoked, equivalent household income (five categories), serum cholesterol, and systolic blood pressure. Stratification procedures were used to evaluate whether smoking status, hypertension (systolic blood pressure  $>140$  mmHg or diastolic blood pressure  $>90$  mmHg or use of antihypertensive medication), hypercholesterolaemia (serum cholesterol levels  $>6.5$  mmol/l), or HDL-cholesterol levels below the median, modified the association between serum caeruloplasmin and myocardial infarction. We next examined to what extent caeruloplasmin reflects increased inflammation. First we included measures of inflammation in our multivariate logistic regression model, leucocyte count as continuous, and C-reactive protein as a categorized variable using a cut-off point of 6 g/l. Second, we assessed whether exclusion of subjects with C-reactive protein levels greater than 6 g/l modified the serum caeruloplasmin–myocardial infarction association. Associations are expressed as odds ratios with 95% CI. Results were considered statistically different at the two-sided 0.05 alpha level. Statistical analyses was performed using the Statistical Analysis Systems statistical software package version 6.11 (SAS Institute, Cary, NC, USA).

### Results

Baseline characteristics of cases and controls of myocardial infarction are shown in Table 1. Significant differences were seen for leucocyte count, percentage of subjects with C-reactive protein levels greater than 6 g/l, and income of at least 3000 Hfl (about £ 900)/month. Compared with controls, cases of myocardial infarction had non-significantly higher serum levels of cholesterol, had smoked more pack-years, and were more often current smokers.

Serum caeruloplasmin levels in the case–control population ranged from 220 to 790 mg/l with averages of 370, 460, 520,

**Table 1.** Baseline characteristics of cases of myocardial infarction and controls (Mean values and standard deviations, or percentage distribution)

Variables	Cases of myocardial infarction (n 83)		Controls (n 127)		P =*
	Mean	SD	Mean	SD	
Men (%)	45.8		44.1		NS
Age (years)	76.4	8.7	76.8	9.0	NS
BMI (kg/m <sup>2</sup> )	26.2	2.8	25.9	3.5	NS
Waist : hip ratio	0.94	0.09	0.92	0.08	NS
Serum caeruloplasmin (mg/l)	510	110	470	100	0.007
Leucocyte count (10 <sup>9</sup> /l)	7.34	2.20	6.70	1.73	0.020
Serum cholesterol (mmol/l)	6.67	1.37	6.46	1.37	NS
Serum HDL-cholesterol (mmol/l)	1.22	0.26	1.28	0.32	NS
Systolic blood pressure (mmHg)	148	22	143	23	NS
Diastolic blood pressure (mmHg)	73	14	74	12.2	NS
Pack-years smoked (%)	17.9	26.6	12.9	27.0	NS
Current smokers (%)	24.1		17.3		NS
C-reactive protein > 6 g/l (%)	21.3		9.9		0.019
High income (%)†	1.2		9.5		0.013

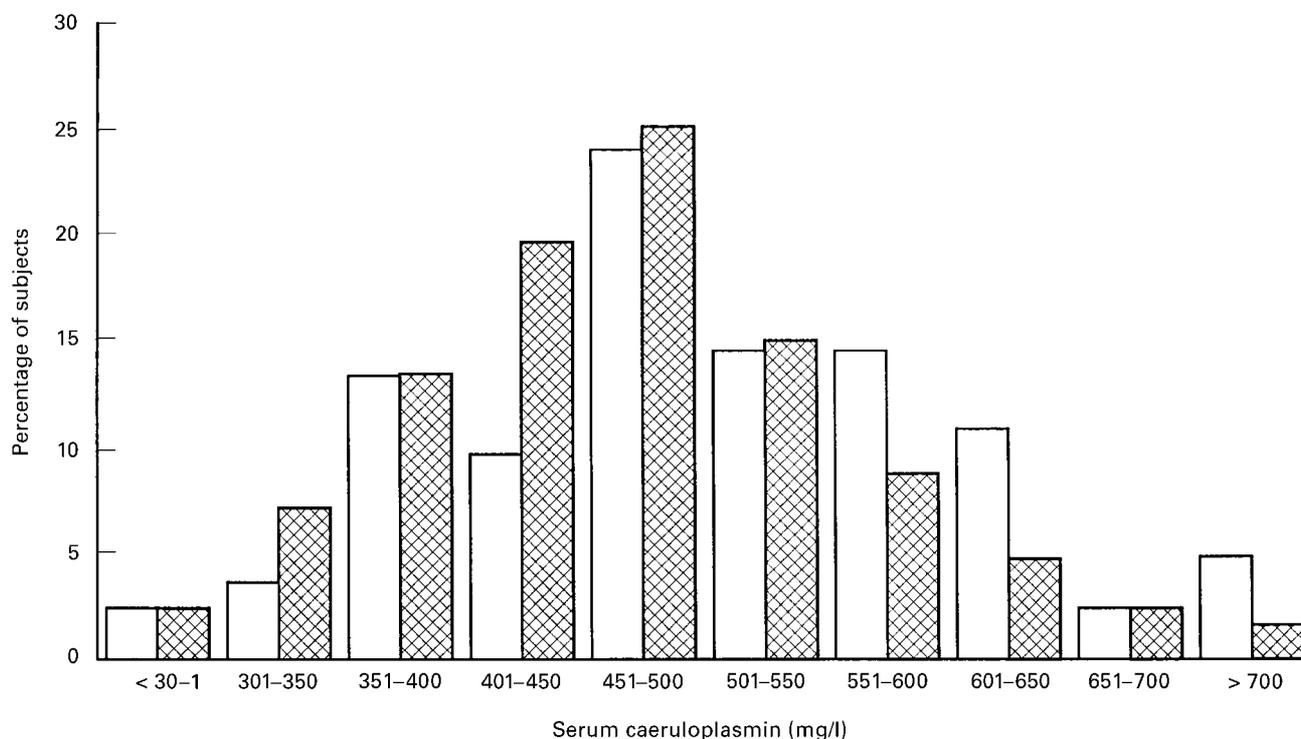
\*For continuous variables: ANOVA adjusted for age and sex; for categorical variables: Mantel-Haenszel Chi-square test adjusted for age and sex.

† Equivalent household income > 3000 Hfl (€900).

and 640 mg/l in subsequent quartiles. Caeruloplasmin concentration was significantly higher in cases of myocardial infarction than controls (510 (SD 110) v. 470 (SD 100) mg/l;  $P=0.007$ ). The distribution of serum caeruloplasmin for cases and controls is shown in Fig. 1, indicating a shift towards higher serum caeruloplasmin levels for incident cases of myocardial infarction compared with controls.

Serum caeruloplasmin adjusted for age and sex correlated significantly with serum cholesterol ( $r\ 0.25$ ,  $P=0.0002$ ),

leucocyte count ( $r\ 0.24$ ,  $P=0.0004$ ), and pack-years smoked ( $r\ 0.24$ ,  $P=0.0004$ ), and was significantly inversely associated with serum HDL-cholesterol ( $r\ 0.17$ ,  $P=0.0170$ ). No correlations were observed between caeruloplasmin and age, BMI, waist : hip ratio, diastolic and systolic blood pressures, and alcohol intake. Mean caeruloplasmin was observed to be significantly higher (572 (SD 85) v. 470 (SD 94) mg/l;  $P<0.0001$ ) for subjects with a serum C-reactive protein level of at least 6 g/l. These



**Fig. 1.** Serum caeruloplasmin levels among myocardial infarction cases (□) and controls (▨) in the Rotterdam Study.

**Table 2.** Risk of myocardial infarction (MI) according to quartiles of serum caeruloplasmin concentration in the Rotterdam Study (Values are odds ratios (OR) and 95% CI)

	Quartiles							
	1*	2		3		4		<i>P</i> value for trend
No. of MI . . .	20	16	20	27				
Serum caeruloplasmin (mg/l) . . .	< 0.420	0.420–0.489		0.490–0.560		> 0.560		
	OR	OR	(95% CI)	OR	(95% CI)	OR	(95% CI)	
Age and sex adjusted	1	1.07	(0.47, 2.42)	1.45	(0.65, 3.25)	2.96	(1.29, 6.84)	0.010
Multivariate adjusted†	1	0.97	(0.42, 2.22)	1.31	(0.58, 2.98)	2.59	(1.10, 6.24)	0.029
Multivariate adjusted‡	1	0.99	(0.43, 2.27)	1.28	(0.57, 2.93)	2.46	(1.04, 6.00)	0.043
Adjusted for C-reactive protein‡	1	0.96	(0.41, 2.22)	1.15	(0.49, 2.68)	1.97	(0.76, 5.18)	0.189
Adjusted for leucocyte count‡	1	0.91	(0.39, 2.11)	1.15	(0.50, 2.66)	2.02	(0.82, 5.06)	0.127

\*Reference (lowest) category.

†Adjusted for age, sex, BMI, pack-years smoked, and equivalent household income (five categories).

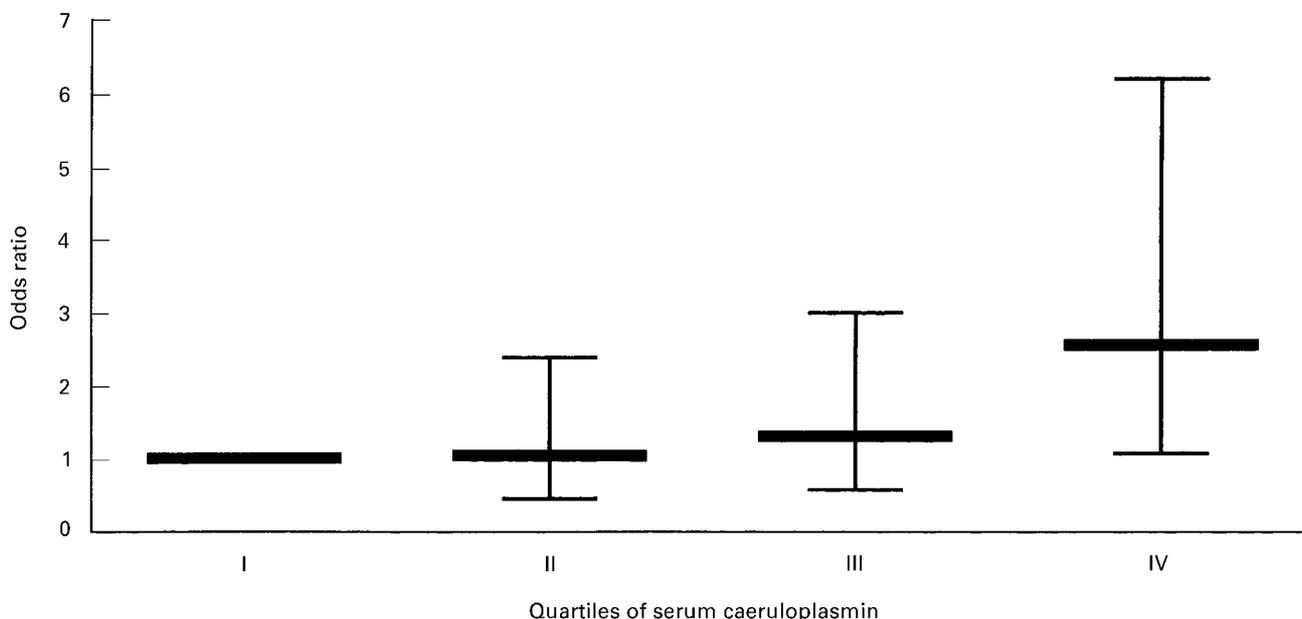
‡Adjusted for age, sex, BMI, pack-years smoked, equivalent household income (five categories), serum cholesterol, and systolic blood pressure.

subjects also reported that they had smoked more pack-years and they were more likely to be current smokers. No correlation between leucocyte count and pack-years smoked was seen.

When adjusted for age and sex, serum caeruloplasmin levels were significantly associated with increased risk of myocardial infarction (Table 2). The odds ratio for the highest compared with the lowest quartile of caeruloplasmin was 2.96 (95% CI 1.29, 6.84;  $P_{\text{trend}} = 0.010$ ). The association remained statistically significant after adjustment for BMI, pack-years smoked, income (five categories), serum cholesterol, and systolic blood pressure (Table 2). Fig. 2 depicts a trend of increasing myocardial infarction risk across the quartiles of serum caeruloplasmin.

We next stratified by smoking status, hypertension, hypercholesterolaemia, and low HDL-cholesterol levels. Results showed a significantly increased risk of myocardial infarction with elevated caeruloplasmin concentration in current smokers compared with former and non-smokers. Risk estimates for quartiles of serum caeruloplasmin in smokers were 1, 2.08 (95% CI 0.29, 19.81), 3.54 (95% CI 0.43, 38.81), and 9.12 (95% CI 1.41, 88.09) respectively ( $P$  value for trend 0.021). For hypertension, hypercholesterolaemia, and low HDL-cholesterol levels no risk modifications were observed (results not shown).

The excess risk of myocardial infarction for the highest compared with the lowest quartile of serum caeruloplasmin was reduced by 33% when leucocyte count was taken into



**Fig. 2.** Odds ratios (with 95% CI indicated by vertical bars) for myocardial infarction according to quartiles of serum caeruloplasmin concentration (adjusted for age, sex, BMI, pack-years smoked, equivalent household income (five categories), alcohol intake (four categories), serum cholesterol and hypertension) in the Rotterdam Study.

account or when C-reactive protein was included in the model (Table 2). For both leucocyte count and C-reactive protein, a non-significantly increased risk of myocardial infarction was observed in the multivariate models. Exclusion of subjects with C-reactive protein levels greater than 6 g/l (twenty-eight subjects), resulted in an age- and sex-adjusted odds ratio of 2.18 (95% CI 0.88, 5.49;  $P_{\text{trend}} = 0.107$ ) for the highest compared with the lowest quartile of serum caeruloplasmin. In the multivariate adjusted model, an odds ratio of 1.73 (95% CI 0.65, 4.73,  $P_{\text{trend}} = 0.300$ ) was observed.

### Discussion

In the prospective cohort of the Rotterdam Study we observed a significant association between high baseline levels of serum caeruloplasmin and the subsequent risk of a first myocardial infarction. The association was most evident in current smokers. Serum caeruloplasmin concentration was observed to be highly correlated with indices of inflammation. Exclusion of subjects with elevated C-reactive protein levels reduced the excess risk of myocardial infarction associated with high serum caeruloplasmin levels by 33%.

For each 100 mg/l increase in serum caeruloplasmin we observed an increment of 41% in myocardial infarction risk. Comparable findings of an elevated risk of myocardial infarction (Reunanen *et al.* 1992) and incidence of CHD (Mänttari *et al.* 1994) among persons with high levels of serum caeruloplasmin have been previously reported. Several other studies reported associations of high levels of serum Cu to elevated risk of increased carotid intima-media thickness (Salonen *et al.* 1991b), myocardial infarction (Reunanen *et al.* 1996), and mortality from CHD or cardiovascular disease (Kok *et al.* 1988; Salonen *et al.* 1991a).

Caeruloplasmin, in which 90–95% of serum Cu is bound, is considered to be a physiological inhibitor of lipid peroxidation. However, besides its antioxidant properties such as its ability to scavenge superoxide and other reactive species, and the inhibition of the Fenton reaction by conversion of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  through ferroxidase (EC 1.16.3.1) activity, pro-oxidant activities of caeruloplasmin have been proposed (Ehrenwald *et al.* 1994). To explain the pro-oxidant activity of caeruloplasmin a pathway involving lipid and lipoprotein oxidation has been suggested. Recent findings indicate that caeruloplasmin by itself can oxidise LDL *in vitro* and possibly *in vivo* (Ehrenwald *et al.* 1994; Craig *et al.* 1995). However, accessory factors derived from vascular cells may be modulatory or requisite during lipoprotein oxidation within the vessel wall (Fox *et al.* 1995).

An alternative explanation for the association between elevated serum caeruloplasmin levels and increased incidence of CHD is its property as an acute-phase protein. Since an increase of caeruloplasmin can be mediated by many unspecific factors causing tissue injury, a high caeruloplasmin level may reflect response to injury or inflammation. Endothelial injury and inflammatory processes are thought to be involved in the pathogenesis of atherosclerosis, and markers of inflammation and infection, such as leucocyte count, fibrinogen, and C-reactive protein have been shown to be independent risk factors for CHD (Yarnell *et al.* 1991; Ernst & Resch, 1993; Kuller *et al.*

1996; Rosengren & Wilhelmsen 1996; Haverkate *et al.* 1997). Most previous studies have not taken into account markers of inflammation. We investigated whether the observed associations in our study may have been due to the presence of inflammation. Including leucocyte count or C-reactive protein in the multivariate logistic regression model substantially reduced the risk estimate. We next excluded subjects with clinically elevated C-reactive protein from the analysis. Risk of myocardial infarction with raised serum caeruloplasmin levels was considerably decreased but still present. This suggests that a substantial part of the increased risk associated with high levels of serum caeruloplasmin may be attributed to inflammation processes. The remaining elevated risk may be due to other properties of caeruloplasmin, like its pro-oxidant activity, or it may reflect low-level inflammation being involved in the cardiovascular disease process.

In summary, we observed an elevated risk of myocardial infarction with high serum caeruloplasmin levels in the elderly population of the Rotterdam Study. Adjustment for markers of inflammation substantially reduced the association between serum caeruloplasmin and myocardial infarction. These results suggest that at least part of the observed risk association with high levels of caeruloplasmin can be attributed to its property as an acute-phase protein.

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### References

- Craig WY, Poulin SE, Palomaki GE, Neveux LM, Ritchie RF & Ledue TB (1995) Oxidation-related analytes and lipid and lipoprotein concentrations in healthy subjects. *Arteriosclerosis Thrombosis and Vascular Biology* **15**, 733–739.
- Ehrenwald E, Chisolm GM & Fox PL (1994) Intact human caeruloplasmin oxidatively modifies low density lipoprotein. *Journal of Clinical Investigation* **93**, 1493–1501.
- Ernst E & Resch KL (1993) Fibrinogen as a cardiovascular risk factor: a meta-analysis and review of the literature. *Annals of Internal Medicine* **118**, 956–963.
- Fox PL, Mukhopadhyay C & Ehrenwald E (1995) Structure, oxidant activity, and cardiovascular mechanisms of human caeruloplasmin. *Life Sciences* **56**, 1749–1758.
- Haverkate F, Thompson SG, Pyke SDM, Gallimore JR & Pepys MB, for the European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group (1997) Production of C-reactive protein and risk of coronary events in stable and unstable angina. *Lancet* **349**, 462–466.
- Hofman A, Grobbee DE, De Jong PTVM & Vandenouwendland FA (1991) Determinants of disease and disability in the elderly: The Rotterdam Elderly Study. *European Journal of Epidemiology* **7**, 403–422.
- Klipstein-Grobusch K, den Breeijen JH, Goldbohm RA, Geleijnse JM, Hofman A, Grobbee DE & Witteman JCM (1998) Dietary assessment in the elderly: validation of a semiquantitative food frequency questionnaire. *European Journal of Clinical Nutrition* **52**, 588–596.
- Kok FJ, van Duijn CM, Hofman A, van der Voet GB, de Wolff FA,

- Paays CHCH & Valkenburg HA (1988) Serum copper and zinc and the risk of death from cancer and cardiovascular disease. *American Journal of Epidemiology* **128**, 352–359.
- Kuller LH, Tracy RP, Shaten J & Meilahn EN, for the MRFIT Research Group (1996) Relation of C-reactive protein and coronary heart disease in the MRFIT nested case–control study. *American Journal of Epidemiology* **144**, 537–547.
- Lamb DJ & Leake DS (1994) Acidic pH enables ceruloplasmin to catalyse the modification of low-density lipoprotein. *FEBS Letters* **338**, 122–126.
- Mänttari M, Manninen V, Huttunen JK, Palosuo T, Ehnholm C, Heinonen OP & Frick MH (1994) Serum ferritin and ceruloplasmin as coronary risk factors. *European Heart Journal* **15**, 1599–1603.
- Reunanen A, Knekt P & Aaran R-K (1992) Serum ceruloplasmin level and the risk of myocardial infarction and stroke. *American Journal of Epidemiology* **136**, 1082–1090.
- Reunanen A, Knekt P, Marniemi J, Mäki J, Maatela J & Aromaa A (1996) Serum calcium, magnesium, copper and zinc and risk of cardiovascular death. *European Journal of Clinical Nutrition* **50**, 431–437.
- Rosengren A & Wilhelmsen L (1996) Fibrinogen, coronary heart disease and mortality from all causes in smokers and nonsmokers. The study of men born in 1933. *Journal of Internal Medicine* **239**, 499–507.
- Salonen JT, Salonen R, Korpela H, Suntioiu S & Tuomilehto J (1991a) Serum copper and the risk of acute myocardial infarction: a prospective study in men in Eastern Finland. *American Journal of Epidemiology* **134**, 268–276.
- Salonen JT, Salonen R, Seppänen K, Kantola M, Suntioinen S & Korpela H (1991b) Interactions of serum copper, selenium, and low density lipoprotein cholesterol in atherogenesis. *British Medical Journal* **302**, 756–760.
- World Health Organization (1992) *International Statistical Classification of Diseases and Related Health Problems*, vol. 1, 10th revision. Geneva: WHO.
- Yarnell JW, Baker IA, Sweetnam PM, Bainton D, O'Brien JR, Whitehead PJ & Elwood PC (1991) Fibrinogen, viscosity, and white blood cell count are major risk factors for ischemic heart disease. The Caerphilly and Speedwell collaborative heart disease studies. *Circulation* **83**, 836–844.