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ORIGINAL ARTICLE

Associations of plasma fibrinogen assays, C-reactive protein and interleukin-6 with previous myocardial infarction

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Summary. *Background*: The association of plasma fibrinogen with myocardial infarction (MI) may (like that of C-reactive protein, CRP) be a marker of subclinical inflammation, mediated by cytokines such as interleukin-6 (IL-6). There are well-recognized discrepancies between commonly performed fibrinogen assays. Increased ratio of clottable fibrinogen to intact fibrinogen (measured by a recently developed immunoassay) has been proposed as a measure of hyperfunctional fibrinogen, and is elevated in acute MI. *Objective*: To compare the associations of intact fibrinogen and four routine fibrinogen assays (two von Clauss assays; one prothrombin-time derived; and one immunonephelometric) in a case–control study of previous MI. *Patients/methods*: Cases ($n = 399$) were recruited 3–9 months after their event; 413 controls were age- and sex-matched from the case–control study local population. Intact fibrinogen was measured in 50% of subjects. *Results*: All routine fibrinogen assays showed high intercorrelations ($r = 0.82$–0.93) and significant ($P < 0.0001$) increased mean levels in cases vs. controls. These four routine assays correlated only moderately with intact fibrinogen ($r = 0.45$–0.62), while intact fibrinogen showed only a small, nonsignificant increase in cases vs. controls. Consequently, the ratio of each of the four routine assays to the intact fibrinogen assay was significantly higher ($P < 0.0003$) in cases vs. controls. Each fibrinogen assay correlated with plasma levels of CRP and IL-6 (which were also elevated in cases vs. controls). Each routine fibrinogen assay remained significantly elevated in cases vs. controls after further adjustment for C-reactive protein and interleukin-6. *Conclusions*: These data provide evidence for acquired, increased hyperfunctional plasma fibrinogen in MI survivors, which is not associated with markers of inflammatory reactions. The causes and significance of these results remain to be established in prospective studies.

Keywords: fibrinogen, inflammation, myocardial infarction.

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Introduction

Epidemiological studies and meta-analyses have shown that plasma fibrinogen levels are associated with both prevalent and incident coronary heart disease (CHD), including myocardial infarction (MI) [1–7]. Although there is evidence for heritability of plasma fibrinogen levels [8], there is little evidence that the association of plasma fibrinogen with CHD is due to increased prevalence of genetic polymorphisms associated with higher plasma fibrinogen levels [9,10]. It is therefore possible that the association of plasma fibrinogen with CHD is instead caused by modifications to intact fibrinogen following its synthesis in the liver and release into circulating plasma [11], as a plasma protein marker of subclinical inflammation (like C-reactive protein, CRP), mediated by cytokines such as interleukin-6 (IL-6) [7].

Plasma fibrinogen heterogeneity is due to heterogeneities in all three constituent chains of fibrinogen, which result from different transcriptional/translational products and post-translational modifications (e.g. partial digestion by thrombin, plasmin and leukocyte elastase) [11]. Based on molecular weights, the fibrinogen molecules can be subdivided in three main groups: high molecular weight (HMW), low molecular weight (LMW) and low molecular weight prime (LMW′). Normally these comprise, respectively, 70%, 25% and 5% of total fibrinogen. HMW fibrinogen clots much faster than LMW, and consequently, relative increases in HMW (at constant total fibrinogen) will lead to apparent increases in fibrinogen as assessed by the routine clotting rate assay of von Clauss [12], in which both initial rate (F1) and final extent (F2) measures can be derived by waveform analysis [13]. Other routine fibrinogen assays include the prothrombin time-derived assay (PTF) [14]; and the immunonephelometric assay [15]. When the ratio of values found with the von Clauss assay and those obtained using a recently

developed ELISA for the total of high molecular weight (HMW) and low molecular weight (LMW) fibrinogen (intact fibrinogen, ITF) was calculated, normal donors had a ratio close to 1, whereas patients with acute MI had a mean ratio of 1.6 before thrombolytic therapy, rising to 2.1 at 24–72 h after therapy [16–19]. It was suggested that this increased ratio represents 'hyperfunctional' fibrinogen (i.e. faster than normal clotting) in acute MI, perhaps because of a shift in the ratio of HMW : LMW fibrinogen [11,20]. It was also suggested that this ratio should be evaluated in epidemiological studies of MI, as a potentially more sophisticated marker of fibrinogen activity than routine assays such as the von Clauss assay [11].

We therefore performed a comparison of the association with previous MI (and its major risk factors) of four different routine fibrinogen assays [12–15], as well as the ELISA for intact fibrinogen [15]. (Intact fibrinogen in this assay is defined as the sum of HMW and LMW fibrinogen, and excludes early degradation products such as fragments X and Y. HMW is a family of fibrinogen molecules comprising newly synthesized molecules with fully intact Aα chains (610 amino acids long), and partly proteolyzed molecules in which up to 10% of the Aα chains are missing at their carboxyterminal ends. LMW fibrinogen is a family of molecules in which one Aα chain is proteolyzed as described above, and the other Aα chain for up to 60%). We compared these assays with each other, studied the ratios of each of the four routine assays to the intact fibrinogen assay, and investigated their relationships to age, sex, smoking habit, and the inflammatory markers, CRP and IL-6, and to MI status.

Subjects and methods

The aim of the Glasgow Myocardial Infarction Study (GLAMIS) is to establish associations of plasma hemostatic and inflammatory variables with previous MI and conventional risk factors in a case–control study, and with incident CHD events in a subsequent follow-up study. This study overlaps with the Glasgow extension of the ECTIM study of genetic polymorphisms associated with MI [21]. The aim was to recruit all men and women with MI in the North Glasgow MONICA study [22], diagnosed by MONICA criteria [23] from July 1994, between 3 and 9 months after the event when acute-phase protein reactions in acute MI had settled. Cases were patients with MI in this population survey who were still alive, contactable and gave consent (75% response rate). Controls were selected from a random sample of the same north Glasgow population, obtained from general practice registers, and frequency-matched for sex and age (within 1 year), who had no history or electrocardiograph evidence of MI. Written informed consent was obtained from all participants, and the study was approved by the local research ethics committee.

Participants completed a general health questionnaire including the Rose chest pain questionnaire, a drug and past medical history, a smoking and alcohol history, and a detailed family history [23]. Weight and height were measured for calculation of body mass index (weight/height^2); and an ECG and blood pressure were also recorded [23]. A forearm venous sample was taken after a full overnight fast. Lipid assays were measured as previously described [23]. For assay of fibrinogen and inflammatory variables, blood was anticoagulated with trisodium citrate (0.11 mol L^{-1}, 9 : 1 v/v). Anticoagulated samples were centrifuged at $2000 \times g$ for 10 min at room temperature within 2 h of sampling, and aliquots stored at $-70\,^{\circ}C$ until assay.

Fibrinogen assays were performed in citrated plasma. The von Clauss assay was performed on an MDA-180 analyzer (Organon Teknika, Cambridge, UK) using the manufacturer's reagents and standards, and two endpoints recorded: the initial rate (F1) and final extent (F2) [13]. The prothrombin-derived fibrinogen assay [14] was performed on an ACL 300 Research Coagulometer (Instrumentation Laboratory, Warrington, UK) using the manufacturer's reagents and standards. The immunonephelometric fibrinogen assay [15], and an ultra-sensitive assay for CRP [24], were performed on a nephelometer (Dade-Behring, Marburg, Germany) using the manufacturer's reagents and standards. For logistic reasons, the ELISA for intact fibrinogen [16] was only performed in a random 50% subsample. IL-6 was assayed in citrated plasma using a sensitive ELISA assay (R & D Systems, Abingdon, Oxon, UK) [25].

Statistical analysis

Since all fibrinogen assays gave highly skewed results, cases and controls were compared on the logarithmic scale (back-transforming for presentation). Ratios of assay values were reasonably symmetrical in distribution and so did not require such treatment. Kappa statistics [26] were used to compare the assay results according to rank-order thirds.

Results

The total number of subjects potentially available for analysis was 995 (490 cases and 505 controls). Of these, 399 cases and 413 controls had suitable blood samples for measurement of fibrinogen and inflammatory variables, and thus entry to GLAMIS. As noted, above, intact fibrinogen was assayed in 50% of subjects.

Table 1 shows demographic data and conventional risk factors in MI cases and controls. Cases were well-matched for age and sex; had similar percentages of current smokers but a significantly higher percentage of ex-smokers; had lower blood pressure and HDL cholesterol; and had higher levels of LDL cholesterol, triglycerides, diabetes, and body mass index.

Table 2 shows age-and sex-adjusted plasma levels of fibrinogen assays, ratios and inflammatory markers in cases and controls. The von Clauss (extent, F2) and nephelometric assays gave lower means than the other assays. Fibrinogen assays were higher in cases than controls by each assay; however, the intact fibrinogen assay showed the least distinction between cases and controls, and, alone, was not statistically significant ($P = 0.10$). In consequence, the ratios of all three assays of clottable fibrinogen to intact fibrinogen were significantly higher in cases

Table 1 Demographic data and conventional risk factors in cases and controls

	Cases	Controls	*P*-value
n	399	413	
Males (%)	74.4	72.6	0.56
Age (years)	54.6 ± 7.5	55.0 ± 7.4	0.45
Smoker (%)			
Current	46.9	46.5	
Ex-	42.6	28.0	
Never	10.6	25.6	< 0.0001
Blood pressure (mmHg)			
Systolic	123.2 ± 21.9	130.6 ± 20.1	< 0.0001
Diastolic	78.4 ± 12.9	83.0 ± 11.4	< 0.0001
Cholesterol (mmol L^{-1})			
Total	5.82 ± 1.18	5.73 ± 1.05	0.23
HDL	1.10 ± 0.29	1.35 ± 0.43	< 0.0001
LDL	3.73 ± 1.07	3.65 ± 0.96	0.03
Triglyceride (mmol L^{-1})	2.24 ± 1.44	2.05 ± 1.29	< 0.0001
Diabetes (%)	11.5	2.2	< 0.0001
Body mass index (kg m^{-2})	27.5 ± 4.85	26.3 ± 4.58	0.0004

Data given as (%) or mean ± SD.

than controls ($P < 0.0003$); as was the ratio of the nephelometric assay to intact fibrinogen ($P = 0.02$). CRP and IL-6 levels were also significantly higher in cases than controls ($P < 0.0001$).

As expected, the three clottable fibrinogen assays showed high intercorrelations (Spearman $r = 0.90$–0.92); while each of these showed lower correlations with the immunonephelometric assay (Spearman $r = 0.82$–0.85). These four assays each showed markedly lower correlations with the intact fibrinogen assay (Spearman $r = 0.45$–0.62). CRP and IL-6 assays showed similar correlations with each other and with the four routine fibrinogen assays ($r = 0.43$–0.58); and lower correlations with intact fibrinogen ($r = 0.31$–0.36) and with fibrinogen ratios ($r = 0.10$–0.28). Age showed weak correlations with all five fibrinogen assays ($r = 0.17$–0.18), but not with fibrinogen ratios. Correlations of fibrinogen assays and ratios with age, blood pressure, lipids and body mass index were generally weak (data not shown).

After age-adjustment, women had consistently higher fibrinogen assays than men, except for the intact fibrinogen assay (data not shown). There were no consistent differences in the sex effect on fibrinogen assays between cases and controls. Current smokers had the highest values for all four routine fibrinogen assays, but not for intact fibrinogen, both in cases and controls (data not shown). Ex- and non-smokers (never smoked) had similar values, with no consistent differences between them, save that PTF was higher for ex-smokers for both cases and controls. Cases had higher values than controls in all subgroups of fibrinogen assay and smoking habit. Age-adjusted Spearman correlations with expired-air carbon monoxide (an objective measure of tobacco inhalation) were significant for all four routine fibrinogen assays (r for F1 0.14, F2 0.20, PTF 0.17, NF 0.13). Overall, there were no significant correlations between fibrinogen ratios and smoking habit.

The effects of CHD risk factors and inflammation markers on the relationships between fibrinogen assays, fibrinogen ratios and previous MI were examined by multiple regression analysis, adjusting for a range of cardiovascular risk factors (systolic and diastolic blood pressures, total and high-density lipoprotein cholesterol, triglycerides, body mass index, diabetes, smoking status, CRP and IL-6; Tables 1 and 2). The results differed little from those adjusted only for age and sex (Table 2). All four routine fibrinogen assays remained significantly associated with MI ($P < 0.0001$), as did the three ratios of clottable to intact fibrinogen ($P < 0.002$). The ratio of nephelometric to intact fibrinogen became nonsignificant after this further adjustment. After such adjustment, CRP was no longer significantly associated with MI, whereas IL-6 retained a significant association.

Table 2 Fibrinogen assays, ratios and inflammation markers (C-reactive protein and interleukin-6) in cases and controls

	Cases		Controls			Mean increase	*P*-value for extra adjustment
	n	Mean (95% CI)	*n*	Mean (95% CI)	*P*-value	(%)	
Fibrinogen assays (g L^{-1})							
Clauss rate (F1)	387	4.60 (4.48–4.71)	378	4.13 (4.02–4.23)	< 0.0001	11.4	< 0.0005
Clauss extent (F2)	387	3.43 (3.36–3.51)	378	3.11 (3.04–3.18)	< 0.0001	10.3	< 0.0001
Prothrombin time (PTF)	365	4.58 (4.44–4.72)	363	4.03 (3.92–4.16)	< 0.0001	11.4	< 0.0001
Nephelometric (NF)	302	3.57 (3.48–3.65)	277	3.25 (3.17–3.33)	< 0.0001	9.8	< 0.006
Intact (ITF)	200	4.52 (4.37–4.67)	184	4.35 (4.20–4.50)	0.10	3.9	0.60
Fibrinogen ratios							
F1/ITF	194	107.4 (104.4–110.4)	164	98.6 (95.5–101.8)	< 0.0001	8.9	0.005
F2/ITF	195	77.3 (75.1–79.6)	163	71.6 (69.2–74.0)	0.0003	8.0	0.001
PTF/ITF	178	110.8 (106.5–115.1)	150	96.1 (91.5–100.7)	< 0.0001	15.3	0.0003
NF/ITF	155	81.9 (79.2–84.5)	114	77.2 (74.2–80.1)	0.02	6.1	0.07
Inflammation markers							
C-reactive protein (g L^{-1})	391	3.20 (2.84–3.62)	370	1.98 (1.75–2.24)	< 0.0001	61.6	0.11
Interleukin-6 (ng mL^{-1})	379	2.14 (2.00–2.29)	360	1.56 (1.45–1.67)	< 0.0001	37.2	< 0.0001

Data adjusted for age and sex; and *additionally for systolic and diastolic blood pressure, total and high density lipoprotein cholesterol, triglyceride, body mass indes, diabetes status, smoking status, C-reactive protein, and interleukin-6 (Table 1).

Discussion

In this case–control study of previous MI, we report for the first time that, while four routine assays of plasma fibrinogen (three of clottable fibrinogen [12–14], and an immunonephelometric assay for total fibrinogen [15]) showed significant increases in MI cases compared with controls (as in previous studies [6]) a recently developed immunoassay for intact fibrinogen [16] did not. The latter assay [16] is specific for the total of HMW and LMW fibrinogen released into the circulation by the liver and is insensitive to shifts in the relative amounts in HMW and LMW, whereas functional assays are sensitive to such changes. Our finding that intact fibrinogen is not associated with previous MI is consistent with the lack of an association of genetic polymorphisms for fibrinogen with MI in case–control studies [9,10]. We therefore suggest that the consistent association between routine assays of plasma fibrinogen and prevalent or incident CHD [1–7] may not have a genetic basis; but instead results from an acquired, apparent hyperfibrinogenemia in which intact fibrinogen released into the circulation by the liver undergoes modification to a different extent in persons with CHD than in persons without CHD.

The three routine assays of clottable fibrinogen used in the present study were the initial rate (F1) and final extent (F2) of clotting in the von Clauss assay [12,13]; and the prothrombin time derived assay [14]. As in previous reports [13,27,28], these three assays were highly correlated, and each showed significant and similar mean increases (10.3–11.4%) in MI cases compared with controls (Table 2). The ratio of each of these assays of clottable fibrinogen to intact fibrinogen was also increased, being higher for the prothrombin-time assay (mean increase 15.3%) than for the von Clauss assay (rate 8.9%, extent 8.0%) (Table 2). While smaller than the increase in von Clauss : intact fibrinogen ratio in acute MI (baseline median ratio 1.6 [16]) these increased ratios of clottable to intact fibrinogen suggest that hyperfunctional fibrinogen (faster than normal clotting) is also present in chronic MI. As in acute MI, this change may reflect an increased percentage of HMW fibrinogen (without significant changes in HMW and LMW fibrinogen) [20] in patients with chronic MI: this hypothesis requires further study. The cause of such a shift is unknown. In inflammatory conditions, fibrinogen synthesis rate increases, whereas the conversion rate of newly synthesized fibrinogen molecules to LMW stays at the same level; consequently, the relative amount of HMW increases. However the increased fibrinogen ratios in patients with chronic MI in the present study were not explained by CRP or IL-6 levels.

The immunonephelometric assay of fibrinogen [15] employs a non-specific antibody against fibrinogen, resulting in an assay of total rather than intact fibrinogen. This assay correlated less strongly with the three assays of clottable fibrinogen than with the intercorrelations of the clottable fibrinogen assays, while all four of these routine fibrinogen assays correlated much less strongly with the intact fibrinogen assay.

Age showed weak correlation with all five fibrinogen assays, consistent with previous reports [3–7], but not with fibrinogen ratios. Women had higher fibrinogen levels than men for all four routine assays, consistent with previous reports [3–7], but not higher intact fibrinogen. There were no significant sex differences in fibrinogen ratios among controls. We confirmed the reports of previous studies [3–7] that current smokers had higher levels of fibrinogen (by all four routine assays) than ex-smokers, or those who had never smoked. Cases had higher values than controls in all subgroups of fibrinogen assays and smoking habit. These correlations with self-reported smoking habit were confirmed by significant correlation with carbon monoxide as an objective measure of smoke exposure. Overall, we observed no significant correlation between smoking and fibrinogen ratios.

We observed that MI cases had higher levels of CRP and IL-6 than matched controls. These results are unlikely to be consequences of recent MI, because cases were studied 3–9 months after the event. They are more likely to reflect chronic low-grade inflammation in persons with chronic vascular disease [5,7,25,29]. We confirmed significant correlations of CRP and IL-6 with age, smoking and other cardiovascular risk factors [29–33], and fibrinogen [5,7,25]. CRP, and IL-6 showed similar correlations with each other, and with all four routine fibrinogen assays, most likely reflecting the role of IL-6 as a key mediator of inflammatory reactions including hepatic synthesis and release of CRP and fibrinogen. The correlations of CRP and IL-6 were weaker for intact fibrinogen and for fibrinogen ratios, suggesting that these are less strong acute-phase reactants, and that the associations of previous MI with increased fibrinogen ratios were not due to inflammatory reactions. Indeed, after adjusting for major CHD risk factors, CRP and IL-6, the associations with previous MI of all four routine fibrinogen assays, and all three ratios of clottable fibrinogen to intact fibrinogen, remained statistically significant (Table 2). After multiple adjustment, the association of CRP with MI was substantially reduced, as in prospective studies [24,29,33]. In contrast, IL-6 retained a significant association with MI. Together with prospective studies that indicate that IL-6 is an independent predictor of CHD [34], these data suggest that IL-6 has a central role in the association of inflammatory markers (including fibrinogen and CRP) with CHD [24,25].

We conclude that patients with previous MI do not have significant increases in plasma levels of intact fibrinogen. Rather, they have increased levels of hyperfunctional thrombin-clottable fibrinogen (assayed by routine methods). These associations are not explained by markers of inflammatory reactions or major cardiovascular risk factors. They may reflect increases in HMW fibrinogen, which clots faster than LMW fibrinogen, as in acute MI. The causes and significance of the acquired, hyperfunctional increased plasma fibrinogen in MI survivors remain to be established in future studies.

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