Platelet activation in angina at rest. Evidence by paired measurement of plasma beta-thromboglobulin and platelet factor 4*

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KEY WORDS: Platelets, beta-thromboglobulin, platelet factor 4, angina at rest, coronary atherosclerosis.

Indexes of in vivo platelet activation, beta-thromboglobulin and platelet factor 4 were measured in triplicate in plasma from venous blood of 69 patients with proven ischaemic heart disease (IHD), discarding samples with a ratio of the plasma concentrations of the two proteins < 2.6, in order to rule out sampling artifacts. Compared with 60 control volunteers, differences were not significant [for beta-thromboglobulin controls (ng ml⁻¹, mean ± SD) 27.8 ± 8.6, ischaemic patients 32.3 ± 17.1; for platelet factor 4 controls 4.3 ± 1.4, ischaemic patients 5.9 ± 5.7]. However, when patients were stratified according to disease activity (Group I—patients without spontaneous ischaemic episodes at rest during 4 days of continuous electrocardiographic monitoring; Group II—patients with < 1 ischaemic episode/day; Group III—patients with > 1 episode/day), these indexes were increased in 'active' patients (for beta-thromboglobulin, in Group II—32.4 ± 10.5 ng ml⁻¹, P < 0.05 vs. Group I; in Group III—42.6 ± 14.6 ng ml⁻¹, P < 0.01 vs. Group I, P < 0.05 vs. control. Platelet factor 4 was increased only in Group III—8.9 ± 7.2 ng ml⁻¹, P < 0.05 vs. control). Beta-thromboglobulin and platelet factor 4 were 25.0 ± 6.7 ng ml⁻¹ and 4.9 ± 4.8 ng ml⁻¹, respectively, in Group I (P = NS vs. control). A relationship with the number of spontaneous ischaemic episodes at rest was confirmed by linear regression analysis (in Group III patients for beta-thromboglobulin: τ = 0.76, P < 0.01, and for platelet factor 4 τ = 0.62, P < 0.01). Levels were not elevated in patients with previous myocardial infarction without ischaemia at rest and/or patients with stable angina, and were not influenced by the occurrence of a positive exercise stress test. Coronary angiograms of ischaemic patients were analyzed to assess the extent and severity of atherosclerotic involvement: for both extent and severity, involvement was similar in the three groups. These data support the hypothesis of the occurrence of platelet activation in patients with spontaneous angina at rest, but not in other subsets of IHD patients, and establish the possibility of detecting in vivo platelet activation in IHD by means of such circulating markers.

Introduction

Activation of blood platelets in the coronary circulation as a result of vessel-wall injury is currently considered one of the pathogenetic mechanisms of ischaemic syndromes¹⁻³, but its demonstration by circulating markers has remained elusive. The development of sensitive radioimmunoassays for the platelet-specific proteins beta-thromboglobulin and platelet factor 4, promised to be a powerful tool for the easy detection of platelet activation in the circulation. However, in spite of the widespread use of these assays as a result of their commercial availability, and the multiplication of reports concerning plasma concentrations of these proteins in ischaemic heart disease (IHD) patients, this promise has not yet been fulfilled. Although most reports have shown elevated levels in IHD, large overlaps with control populations and even conflicting data in specific subsets of IHD have cast skepticism on their possible usefulness as diagnostic or predictive tools. Possible reasons for these conflicting reports include methodological problems connected with the spurious release of platelet products occurring during blood sampling, and the heterogeneity of IHD population, implicating differing importance

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of platelet-related events in different subsets of the disease. We have used repeated paired measurements of plasma concentrations of these proteins trying to exploit elements of knowledge recently acquired of their kinetics in an effort to rule out sampling artifacts and have characterized specific subgroups of the IHD population to limit patient selection variables. We searched and obtained evidence for in vivo platelet activation in patients with spontaneous episodes of ischaemia at rest, a subset in which recent independent pathological and angiographic data point to a direct platelet pathogenetic involvement.

Methods

SUBJECTS

The control population consisted of 40 normal healthy volunteers, aged (mean ± SD) 45 ± 9 years (range 20–62 years) 22 females, 18 males, with good accessibility to superficial antecubital veins for clean venipuncture. The patient population consisted of 69 patients admitted to our Coronary Care Unit for the evaluation of chest pain. Clinical characteristics of patients are detailed in Table 1.

Exclusion criteria were: (1) any evidence of atherosclerotic disease other than coronary artery disease; (2) history of recent (<10 days) intake of non-steroidal anti-inflammatory agents or other platelet-inhibitory drugs; (3) altered renal function (serum creatinine > 1·3 mg dl⁻¹); (4) recent (<1 month) cardiac catheterization; (5) absence of easily accessible antecubital veins. The inclusion criterion was the objective evidence of myocardial ischaemia. This was obtained by any of the following: (1) previous myocardial infarction, documented unequivocally by electrocardiogram and enzymes (N=31); (2) transient spontaneous myocardial ischaemia (ST-segment elevation N=15, ST-segment depression N=25); or (3) transient induced myocardial ischaemia (exercise stress test N=37, ergonovine test N=7, dipyridamole-echo test N=8).

The mean age of the selected population was 53 ± 8 years: 65 patients were male and 4 were female (Table 1).

PROTOCOL

All patients were submitted to four-day continuous one-lead electrocardiographic monitoring in the

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<td>Mean age (years)</td>
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Significant difference between: *Groups I and II, †between Groups II and III, and ‡between Groups I and III, as evaluated with the Fisher exact test on actual frequencies.
Coronary Care Unit in the absence of any therapy other than sublingual nitrates, if needed, to relieve angina. In particular, the administration of long-acting nitrates, beta blockers or calcium antagonists, known possibly to alter platelet function, was halted for the two days before and, subsequently, throughout the sampling period. Spontaneous ischaemia was identified by the occurrence of spontaneous displacement (elevation or depression) of the ST segment by more than 1.5 mV, with or without chest pain. The majority (65%) of recorded episodes were asymptomatic (representing 'silent' ischaemia) in agreement with our previous findings[7].

At the same morning hour (between 8 and 9 a.m.) for three successive days a blood sample was obtained from the patients. All samples were obtained at least 1 h remote from an episode of myocardial ischaemia. Duplicate sampling was also obtained from seven patients before (10 min and 3 min) and after (30 s and 10 min) a multistage exercise stress test performed on a bicycle ergometer.

**BLOOD SAMPLING AND PLASMA DETERMINATIONS**

Blood was drawn into polypropylene syringes, by a double-syringe technique, in order to minimize thrombin formation and platelet activation due to tissue factor release, with minimal venous stasis, through a 19-G needle. Immediately after drawing, blood was gently transferred to a precooled siliconized glass tube containing the anticoagulant —antiplatelet solution, consisting of ethylenediaminotetraacetic acid, procaine, and 2-chloroadenosine ('Thrombotect', Abbott Italia, Rome), 0.3 ml per 4.7 ml of blood, avoiding vacuum aspiration. Blood was subsequently centrifuged at 3000 g for 1 h; the intermediate third of plasma was subsequently aspirated[8] and kept frozen in plastic tubes at —30°C until assayed. Assays were performed by the commercially available radioimmunoassay kits for beta-thromboglobulin (Amersham International, Buckingham, U.K.) and platelet factor 4 (Abbott Italia). Minimum detectable levels were 10 ng ml⁻¹ for beta-thromboglobulin, and 2.5 ng ml⁻¹ for platelet factor 4.

**EVALUATION OF PLASMA MEASUREMENT**

In order to minimize further the artifactual contribution due to incorrect sampling or blood processing, the following two criteria were established *a priori* on the basis of previous experience and used throughout the analysis of results.

1. Discard all values in which the beta-thromboglobulin/platelet factor 4 ratio was < 2.6. General reasons for discarding values when the beta-thromboglobulin/platelet factor 4 ratio is low have been explained by Kaplan and Owen[9]. The specific reasons to set the cut-off point at 2.6 were considerations on the distribution of these ratios in our control population. This distribution was normal, with a mean ± SD equal to 6.9 ± 2.1 (2.6 represents the mean value minus two SD). Therefore, this criterion would cut off values lower than the 95% confidence limits of the ratio distribution in control subjects. This procedure led us to discard 9% of all paired beta-thromboglobulin and platelet factor 4 measurements obtained in any phase of the present study. Discarded values were homogeneously distributed among the various patient categories and subsets of the study.

2. Out of the two or three values available for each patient during any phase of the study, the one with the lowest platelet factor 4 was considered to be the best index of *in vivo* platelet activation. Such selection was made based on the consideration that, while there may be many reasons for spurious elevation of platelet factor 4, there would be none for artefactual lowering of the values. The only known reasons for non-artefactual platelet factor 4 elevation, i.e. elevation as a consequence of very recent *in vivo* platelet activation and administration of heparin[10] were avoided by refraining from platelet sampling in the immediate proximity to an ischaemic episode and from heparin treatment.

**CORONARY ANGIOGRAPHY**

Coronary angiography was performed in all patients using the conventional Judkins technique. Angiography results were evaluated by two independent observers and a quantification of the atherosclerotic involvement was performed deriving two arbitrary scores of 'extent' and 'severity' of coronary atherosclerosis. To this purpose, the main stem of the left coronary artery, three thirds of the left anterior descending, the first diagonal and the first septal branches of the left anterior descending, two halves of the left circumflex coronary artery, the marginal branch, three thirds of the right coronary artery and the posterior descending coronary artery were all considered as independent vessel segments. The atherosclerosis extent score was derived by dividing the number of vessel segments showing any type of profile alteration by the total number of segments visualized. The atherosclerosis severity score was derived by dividing the number of segments showing > 90% stenosis (diameter reduction) by the total number of segments visualized.
The mean number of visualized segments for each angiogram was 11 (range 7-13). The variability of this number is due to the impossibility of analyzing totally occluded, non-perfused vessel segments in some patients. No attempt was made to identify the possible thrombotic nature of atherosclerotic lesions based on angiographic morphology.

PATIENT STRATIFICATION ACCORDING TO DISEASE ACTIVITY

Grouping of patients was performed according to the number of spontaneous ischaemic episodes detected by continuous electrocardiographic monitoring for four days, in the absence of any therapy apart from short-acting nitrates. Groups were defined as follows.

Group I: absence of resting angina (N = 29). These consisted of: (1) previous myocardial infarction without present evidence of ischaemia (N = 7); (2) stable angina without previous myocardial infarction (N = 13); and (3) stable angina with previous myocardial infarction (N = 9).

Group II: moderately frequent resting angina (> 1 spontaneous episode/day) (N = 19).

Group III: severe resting angina (> 1 spontaneous episode/day) (N = 21).

Clinical characteristics of patients were mostly homogeneously distributed among the three groups, as detailed in Table 1.

STATISTICAL ANALYSIS

Characteristics of subgroups were compared by the Fisher exact test. Values of beta-thromboglobulin and platelet factor 4 in ischaemic heart disease patients showed marked skewness of distribution towards high values, so that log transformation of data was used to apply tests implying normal distributions. Comparisons of beta-thromboglobulin and platelet factor 4 values among groups were performed using one-way analysis of variance, and subsequent individual comparisons were performed using the Duncan test; for paired comparisons the non-parametric Wilcoxon rank sum test and for two-sample comparisons the Mann-Whitney test were used. Linear regression was performed to correlate the number of ischaemic episodes with beta-thromboglobulin and platelet factor 4 values. All values were expressed as mean ± SD.

Results

CONTROL POPULATION

Beta-thromboglobulin and platelet factor 4 values in normal controls were 27.8 ± 6.6 ng ml⁻¹ and 4.3 ± 1.4 ng ml⁻¹, respectively (see Fig. 1). There was no trend towards an increase of values as a function of age. Values were comparable in males and in females (for beta-thromboglobulin in males 28.3 ± 9.2 ng ml⁻¹ and in females 26.8 ± 6.7 ng ml⁻¹; for platelet factor 4 in males 4.9 ± 1.6 ng ml⁻¹ and in females 4.0 ± 1.6 ng ml⁻¹).

PATIENT POPULATION

When the IHD population was considered together (Fig. 1) there was a slight non-significant increase of mean values compared to controls. The larger standard deviation of the ischaemic group suggested a greater degree of heterogeneity within this group as compared to controls. The subgroup analysis showed that, relative to controls, values were higher in patients with significant activity and, particularly, in the group with > 1 episode/day of spontaneous ischaemia (Fig. 2).

Significant correlation between the number of ischaemic episodes and both beta-thromboglobulin and platelet factor 4 values was demonstrated using linear regression analysis in Group III patients (Fig. 3). Within Groups II and III (patients with episodes...
of angina at rest), patients with ST-segment elevation (Prinzmetal angina) showed the highest values (for beta-thromboglobulin $43.7 \pm 15$ ng ml$^{-1}$, $N=15$; compared to $33.9 \pm 19.1$ ng ml$^{-1}$, $N=25$, in patients with ST-segment depression). However, these patients also showed the largest number of spontaneous episodes, being the majority (13 out of 21) of Group III patients.

The responsiveness of patients to ergonovine testing was positively correlated with beta-thromboglobulin and platelet factor 4 values. Plasma concentrations of beta-thromboglobulin were $38.8 \pm 8.2$ ng ml$^{-1}$ for ergonovine-positive ($N=7$) and $27.8 \pm 6.9$ ng ml$^{-1}$ for ergonovine-negative patients ($N=7$, $P<0.01$). Similarly, platelet factor 4 concentrations were higher in ergonovine-positive ($5.8 \pm 1.4$ ng ml$^{-1}$) than in ergonovine-negative patients ($4.0 \pm 1.2$ ng ml$^{-1}$, $P<0.05$). Positivity to ergonovine testing was only observed in patients in Group II ($N=2$) and III ($N=5$).

Subanalysis of Group I patients showed no difference among patients with stable angina, previous myocardial infarction, previous myocardial infarction plus stable angina (values for beta-thromboglobulin: $26.2 \pm 9.1$, $25.0 \pm 12.4$ and $27.0 \pm 8.7$ ng ml$^{-1}$, respectively).

**EFFORT STRESS TEST AND CORONARY ATHEROSCLEROSIS**

There was no difference in beta-thromboglobulin and platelet factor 4 values according to positivity or negativity of the exercise stress testing (values in patients with positive exercise stress test—for beta-thromboglobulin $33.9 \pm 16.5$ ng ml$^{-1}$, for platelet factor 4 $5.9 \pm 5.2$ ng ml$^{-1}$, $N=34$; in patients with a negative exercise stress test—for beta-thromboglobulin $34.3 \pm 19.2$ ng ml$^{-1}$; for platelet
factor $7.3 \pm 6.4$ ng ml$^{-1}$, $N=18$). Values obtained after the positive exercise testing were not significantly different from pre-test values (for beta-thromboglobulin $36.4 \pm 24.9$ ng ml$^{-1}$ before, and $37.6 \pm 15.8$ ng ml$^{-1}$ after, $N=9$). Results pertaining to the extent and severity of coronary atherosclerosis in the patient population studied are shown in Fig. 4. There was no difference in the extent or severity of coronary artery lesions according to patient groupings.

**EFFECT OF PLASMA MEASUREMENT SELECTION ON RESULTS**

Although selection criteria for ruling-in/ruling-out plasma measurements on the basis of beta-thromboglobulin/platelet factor 4 ratio and of lowest platelet factor 4 values were established before the beginning of the study based upon considerations previously outlined (see Methods), an evaluation of the influences of these criteria was also performed (i) including the 9% of subjects discarded because of beta-thromboglobulin/platelet factor 4 ratio <2.6, and (ii) averaging multiple determinations of beta-thromboglobulin or platelet factor 4 instead of taking the couple of values with lowest platelet factor 4. As expected, both conditions caused some decrease of the discriminating
power of the measurements. Inclusion of patients with beta-thromboglobulin/platelet factor 4 ratio <2.6 did not allow differentiation between the control and Group II and between IHD I and IHD II. Differences between the control and IHD III, and between IHD I and IHD III were still, however, significant (P<0.05) for both comparisons, Duncan test after analysis of variance). Correlation coefficients between the number of ischaemic episodes in Group III and beta-thromboglobulin or platelet factor 4 decreased to 0.60 (P<0.01) and 0.36 (P=NS), respectively. Conversely, averaging the multiple determinations of beta-thromboglobulin and platelet factor 4 had minor effects, because between-group discrimination was still always possible and correlation coefficients between the number of ischaemic episodes in Group III and beta-thromboglobulin or platelet factor 4 decreased to 0.57 (P<0.01) for beta-thromboglobulin, but actually increased to 0.70 (P<0.01) for platelet factor 4, therefore still remaining significant. Between-day variability of the two determinations in the subjects studied was (mean coefficient of variation) 22.6% for beta-thromboglobulin and 32.6% for platelet factor 4, as compared to <12% inter- and intra-assay variability of the radioimmunoassay. None of the selection criteria for measurements altered significantly the results in relation to ergonovine testing positivity or negativity.

Discussion

In the past many studies have addressed the issue of platelet activation in IHD[12]. However, difficulties in methodological interpretations and significant variations among different tests have hampered the possibility of a clear definition of the topic. Measurement of plasma concentrations of beta-thromboglobulin and platelet factor 4 made possible by sensitive and specific radioimmunoassays[13-19], has been proposed as a novel approach to the problem. Beta-thromboglobulin and platelet factor 4 are proteins specific for platelets (and their precursors megakaryocytes), and secreted upon platelet stimulation. Measurement of their plasma levels has been proposed to reflect the actual occurrence of platelet secretion in vivo[9]. Verification of this assumption has been obtained in the past years in situations where a clear enhancement of platelet consumption is established, such as thrombotic thrombocytopenic purpura and idiopathic thrombocytopenic purpura[22], disseminated intravascular coagulation[18-19], and extracorporeal circulation[20]. However, findings in IHD, whereby important pathophysiological implications were expected to be drawn, have been equivocal. In chronic IHD, platelet release products have been reported as elevated in some studies[21-23], but not in others[24,25]. Elevations[26,27] as well as normal values[28-30] have been reported in exercise-induced myocardial ischemia. Confusion also exists for acute myocardial infarction and acute phases of IHD, where negative reports have appeared[31-33], although most studies showed elevation in platelet-specific proteins[33,34].

The present study helps to clarify previously conflicting data documenting an increase of platelet release products in a specific subset of ischaemic patients, i.e. patients with frequent spontaneous ischaemia at rest. Since levels are normal in asymptomatic patients with previous myocardial infarction and patients with stable effort angina (group I), a pooling of the entire IHD population would not have revealed changes in beta-thromboglobulin and platelet factor 4 values, due to dilution of the 'active' patients into the overall population of IHD patients. On the other hand, when the population of patients with angina at rest is isolated, the increase in plasma levels of platelet-specific proteins becomes evident. For the population with more than one ischaemic episode per day a linear relationship between plasma levels of the proteins and the number of ischaemic episodes is found. This is particularly striking taking into account the specificity problems connected with peripheral sampling in patients with presumable organ-specific types of lesion. The novelty of this finding is at least partially attributable to our selection criteria in which we excluded subjects with other clinical evidence of atherosclerotic involvement besides coronary artery disease. Although suggestions of increase in platelet-specific proteins in active IHD syndromes have been given previously, the present study is, to our knowledge, the first demonstration of such a straightforward direct relationship with frequency of ischemia.

METHODOLOGICAL PROBLEMS OF BETA-THROMBoglobULIN AND PLATELET FACTOR 4 DETERMINATIONS

Some of the discrepancies in results obtained in other studies may be attributed to methodological problems. Choice of a proper anti-activation-anticoagulant solution[42], speed and time of centrifugation[33] and the use of platelet factor 4 as a
marker of artifactual release are all factors likely to contribute to the decrease of scattering by elimination of spuriously high values. The low values reported by us in normal controls are the result of this combined approach. The choice of discarding measurements when the beta-thromboglobulin/platelet factor 4 ratio was <2.6 is based logically, on the statistical considerations outlined. The use of this conservative approach, although possibly decreasing sensitivity of the method, would be expected to result in increased specificity, i.e. the ability to detect only ‘true’ in vivo activations. Operatively, on the basis of our findings, we suggest the use of multiple paired measurements of beta-thromboglobulin and platelet factor 4 and to use our criteria, or similar ones, in the evaluation of results. The approach illustrated here is the most conservative one used to date in the evaluation of platelet specific proteins in IHD.

RELATIONSHIP BETWEEN PLATELET ACTIVATION, MYOCARDIAL ISCHAEMIA AND CORONARY ATHEROSCLEROSIS

Our study documents the lack of relationship between platelet activation and effort-induced ischaemia, in contrast to some previous findings, but in keeping with more recent reports. Effort-induced ischaemia is predictable in the single patient and, therefore, allows easy pre- and post-ischaemic blood sampling. Because effort-induced ischaemia can be easily provoked and timed, contrary to ischaemia secondary to spasm or thrombosis, it is the ideal model for evaluating the contribution of ischaemia per se to platelet activation. From our results we can infer a minimal or absent involvement of platelets in this type of ischaemia. Accordingly, there is no evidence of any clinical benefit in forms of stable angina pectoris by intervention trials with antiplatelet drugs. The similarity between concentration of platelet proteins obtained before and soon after exercise-induced ischaemia can, therefore, be taken as an important proof that ischaemia per se does not cause platelet activation.

Our coronary angiographic data allow some speculation on the relationship between coronary artery disease and myocardial ischaemia. Atherosclerosis extent and ‘angiographic’ severity were similar in the three groups of IHD patients studied, and this is in line with previous literature. Characters of the atherosclerotic lesion possibly related to clinical activity and instability are not readily apparent on angiographic films, unless specifically sought in some cases. It is evident, however, that angiography has only a limited ability to detect other types of atherosclerosis severity different from lumen obstruction. The presence of qualitative features (erosion, ulceration, intimal haemorrhage, cellular infiltration) can be revealed by angiography only in a limited number of cases and to a limited extent. However, those features, due to the exposure of non-physiological surfaces to flowing blood, determine a situation of platelet activation which might transiently impede flow by various mechanisms and cause ischaemia. Detection of a consequence of atherosclerotic activity, such as platelet activation in IHD patients could, therefore, be considered as useful information, additional and complementary to usual angiographic parameters. The existence of a situation of platelet activation would imply the ‘active’, ‘unstable’ character of the coronary artery lesions underlying the disease.

Our findings concerning platelet release proteins in IHD are now much more in agreement with findings obtained by others using a marker of thrombin formation, fibrinopeptide A, for which a parallel between clinical activity and plasma levels has been found. A recent paper comparing the performance of platelet products and fibrinopeptide A in stable versus unstable angina pectoris patients found a significant increase in only the last parameter in unstable patients. It is noteworthy, however, that platelet products also were somewhat higher in this group. Methodological differences already alluded to and the selection of entry criteria may account for the apparent discrepancy, in this, as well as in other previous negative reports. In none of these studies were multiple paired measurements of beta thromboglobulin and platelet factor 4 performed to discard artifactual elevations. Therefore, taken together these data suggest that both types of indices are parallel and reflect a multifaceted response of blood, in its most reactive cellular and humoral components, to the same alteration of the inner vessel surface.

Our study does not address directly the issue of whether platelet activation in angina at rest is primary or secondary, because the relative unpredictability of the ischaemic episodes in this condition makes adequate time-course studies difficult. Such difficulty is indeed common to all the available literature concerning platelets and IHD, including evidence obtained from recently developed measurements of enzymatic thromboxane metabolites. Our findings are consistent both
with a pathogenetic role for platelets and with the view that platelet activation may be the result of a common pathogenetic step. Caution in interpreting the findings as a cause–effect relationship could come from the appreciation of the difficulty in the interruption of the pathogenetic sequence in angina at rest by ‘pure’ antiplatelet interventions. Other experimental designs are required to resolve the issue.

IMPLICATIONS OF THE FINDINGS

Despite extensive ongoing investigation in this area, it is important to realize that no other methods to evaluate platelet activation in vivo have as yet been introduced, evaluated and made extensively available as has been done with beta-thromboglobulin and platelet factor 4 measurements. Therefore, the best possible use of the potential of these tests should be considered highly desirable. This study proposes a practical way, based on rational considerations, to improve the specificity of these measurements. Important theoretical limitations in sensitivity (peripheral sampling) and specificity (localization of ‘active’ atherosclerosis), may make individual beta-thromboglobulin or platelet factor 4 measurement by peripheral sampling of doubtful value in the individual patient. However, multiple paired measurements of these proteins may be more useful, especially in population studies, for identifying subgroups within the IHD population, in which evidence of platelet involvement can be obtained. In contrast to previous myocardial infarction per se and stable angina, unstable angina is a subgroup of IHD in which platelet activation is detectable by such circulating markers.

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