

Tissue factor pathway inhibitor, natural coagulation inhibitors and hemostatic activation markers in patients with acute coronary syndromes

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ABSTRACT

Objective: This study aims at characterizing the hemostatic changes, in a large cohort of Saudi Arab patients with acute coronary syndromes.

Methods: We consecutively enrolled 389 patients (unstable angina [UA]: n=181; myocardial infarction [MI]: n=208) in this study at King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia in the period from April 2000 to November 2001. We collected blood samples before coronary angiography. Controls (n=101) were healthy males and females. All hemostatic assays were undertaken using enzyme linked immunosorbent assay based techniques and commercial kits.

Results: The mean plasma levels of both bound and free tissue factor pathway inhibitors (TFPI) were significantly higher and to comparable levels, in patients with MI and UA, than in healthy control levels. Markers of thrombin generation: the mean levels of prothrombin fraction 1+2, thrombin antithrombin complexes, and

D-Dimer were very significantly elevated in the 2 patients groups than in controls. Proteins C and antithrombin III showed statistically significant reduction especially in patients with MI. Plasminogen activator inhibitor levels were significantly elevated in the 2 patient groups, but were higher in MI patients. The mean levels of fibrinogen and D-Dimer as well thrombin antithrombin complex were higher and the levels of free tissue factor pathway inhibitor were lower in patients with 3-vessel coronary artery disease than those with single and double vessel disease.

Conclusion: The results of this study confirm the existence, and to a similar extent, of a hypercoagulable state in Saudi patients with MI than UA and in those with 3-vessel coronary artery disease than those with one or 2-vessel disease.

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It is now well accepted that rupture of lipid-rich plaques with subsequent thrombosis is the most important mechanism underlying the onset of acute coronary syndromes (ACS), and that the clinical

presentation of plaque rupture will depend on the extent of the acute coronary thrombus and the degree of compromise of the blood flow to the myocardium.¹ Therefore, an individual with ACS

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may experience unstable angina (UA), myocardial infarction (MI) or sudden death.^{1,2} The acute thrombotic process involves the activation of platelets and the coagulation system, and both play a critical role in the etiology and clinical progress of ACS.^{3,4} Thrombus formation involves a chain of sequential reactions that transform a number of coagulation factors present as precursors in plasma into the active form that end in thrombin generation, and fibrin clot formation.⁵ Since the hemostatic system is under the control of complex loops of positive and negative feedback of pro- and anticoagulant factors, the formation of a clot could only be the result of a predominance of prothrombotic (hypercoagulable) state. Therefore, a closer understanding of the dynamics of hemostatic factors in any thrombotic disorder can only be ascertained by measuring the plasma levels of as many hemostatic factors as possible. In this respect, many previous studies have been undertaken in an attempt to characterize the contribution of hemostatic abnormalities to the etiology of coronary artery disease (CAD) and more recently whether the measured levels of hemostatic markers will be of value in the clinical progress and outcome after the occurrence of ACS, whether UA or MI. Despite the plethora of available literature, there are wide disagreements in the results of various studies. This could be attributable to many factors, most important among which is variation in hemostatic methodologies, as well as the small size of the test samples. There is also the reported ethnic/geographical variation, not only in the incidence of CAD but also the hemostatic risk factors closely related to the etiology of CAD.^{6,7} The current study is the in-hospital component of a nationwide research project investigating various aspects of CAD in Saudi Arabia; including epidemiological factors and various population characteristics already known to underlie the etiology of CAD. The study aims at characterizing the hemostatic changes (especially endothelial release products: tissue factor pathway inhibitor (TFPI), hemostatic activation markers and natural coagulation inhibitors) in a large cohort of patients admitted to a hospital with suspected UA and MI and before undergoing coronary angiography.

Methods. Three hundred and eighty-nine patients diagnosed with ACS (UA=181 and MI=208) were enrolled consecutively in this study, at King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia in the period from April 2000 to November 2001. Their physical characteristics are shown in **Table 1**. Patients with ACS presented with chest pain resulting from acute myocardial ischemia. Acute coronary syndromes encompass ST segment MI, non-ST-segment MI

and UA. Myocardial infarction and UA were defined according to the consensus document of the Joint European Society of Cardiology/American College of Cardiology Committee for the re-definition of MI, and the American College of Cardiology/American Heart Association Guidelines for Management of Patients with UA and non-ST-segment elevation MI.⁸⁻¹⁰ They were studied after admission to hospital, and just before they undergo coronary angiography. The final diagnosis was only uncovered after the hemostatic assay results were recorded, and therefore, the Coagulation Laboratory staff was unaware of the diagnosis when the assays were performed.

Blood collection and plasma preparation. A total of 15ml of blood were collected by venepuncture using a plastic syringe and 21-gauge needle, directly into Vacutainer tubes containing 0.5ml sodium citrate (3.8%, 0.11M) to give a ratio of 9 volumes blood to one volume citrate. Proper mixing of blood with the anticoagulant was attained by gentle inversion. The usual precautions of selecting an easily accessible vein in the antecubital fossa and using the minimum of venous stasis were observed. Blood samples were transported within 2 hours of collection to the Coagulation Laboratory, Physiology Department, College of Medicine and King Khalid University Hospital. The blood sample tubes were centrifuged at 3000 rpm (1000G), for 15 minutes, in a refrigerated (4-6°C) centrifuge (Beckman, model J-68). Platelet poor plasma (PPP) was separated using plastic pipettes and aliquots of plasma was immediately stored at -40°C, until analysis, in patches, later. Before assays plasma specimens were thawed at 37°C for 15 minutes.

Control subjects. The controls for the hemostatic parameters were all healthy blood donors (n=101) aged 17-59 years (mean±SD was 34.14 ± 1.9 years).

Laboratory assays. Fibrinogen Assay: The turbidometric assay of Ellis and Stransky,¹¹ the normal laboratory values are 150-400 mg%.

Antithrombin III (ATIII): The chromogenic quantitative determination of ATIII activity level in plasma (Stachrom ATIII Colorimetric assay kits, Diagnostic Stago, France), was used.

Protein C: The enzyme-linked immunosorbent assay (ELISA) was employed using the Asserachrom Protein C kits, Diagnostic Stago, France).

Protein S (bound and free): The Asserachrom Protein S kits (Diagnostic Stago, France), which is an ELISA, for the quantitative determination of both total and free protein S.

Tissue factor pathway inhibitor (TFPI): (total and free): The Asserachrom kit (Diagnostic Stago, France) which is an ELISA was employed for the measurement of both free and total TFPI.

Thrombomodulin (TM): The Asserachrom kit (Diagnostic Stago, France) is an enzyme immunoassay, which measures plasma soluble thrombomodulin.

Thrombin antithrombin complex (TAT complex) and prothrombin fragment 1+2 (F1+2): were assayed using the Enzygnost TAT micro and the Enzygnost F 1+2 micro (Dade Behring, Germany); both are ELISA techniques.

D-Dimer: The Asserachrom D-Dimer kit (Diagnostic Stago, France) which is an ELISA procedure.

Plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (tPA): were assayed using ELISA Asserachrom PAI-1 kit (Diagnostic Stago, France).

Statistical methods. Data were analyzed using SPSS (version 10.0). Analysis of variance and Tukey post hoc multiple range test were employed to determine the significant differences between the different categories of patients with ACS.

Results. **Table 2** shows the total TFPI levels were significantly elevated in patients with MI and UA, when compared to healthy control levels. Free TFPI levels were elevated to comparable levels MI and UA, when compared to healthy control levels. The levels of TMD were significantly higher in patients with MI and UA, when compared to healthy control levels.

Markers of hemostatic activation. The mean levels of the following 3 markers were very significantly elevated in the 2 patients groups (**Table 2**).

Table 1 - Summary of the physical characteristics of patients with acute coronary syndrome, expressed as mean±SD.

Characteristics	Unstable angina (n=181)	Myocardial infarction (n= 208)	p-value
Age (years)	54.5±14.8	53.4±16.3	0.772
BMI (Kg/m ²)	28.3±3.9	28.4±5.6	0.668
Male/female ratio	79.6:20.4	75:25	0.230
Smoking (%)	27.5	26.1	0.646
Hypertension (%)	40	48.1	0.459
Diabetes (%)	34.8	44.2	0.380
Hypercholesterolemia (> 5.2mmol/l) (%)	43.6	41.5	0.444
BMI - body mass index			

There was statistically significant reduction in the mean levels of protein C (PrC) in MI (87.9%) and UA (90.9%), when compared with the mean in healthy controls (103.5%).

There were no significant alterations in the level of total protein S (PrS) in MI (95.4%) and UA (96.4%), as compared to the healthy controls (94.4%).

The mean concentrations of ATIII also displayed significant reduction in patients with MI (93.5%) and UA (93.8%), as compared to the mean levels in healthy controls (102.4%).

The levels of both PAI and tPA were significantly elevated in patients with UA as well those with MI, when compared to healthy control levels. However, PAI levels were higher in MI than UA.

There was statistically significant elevation of fibrinogen level in patients with MI and UA, as compared to the healthy reference fibrinogen levels.

Further analysis hemostatic parameters according to the results of coronary angiography showed that only the free form of TFPI was lowest (18.6%) in the 3 coronary vessel disease when compared to those with single and double vessel disease (27.5%; $p<0.01$). The fluctuations in the other hemostatic parameters were not of significance.

Table 2 - Summary of the hemostatic variables in patients with acute coronary syndrome, expressed as mean±SD.

Hemostatic variables	Unstable angina	Myocardial infarction	Control
Thrombomodulin (ng/ml)	43.3±19.9 ^a	45.8±20.8 ^a	33±9.1 ^b
Fibrinogen (mg%)	424.8±161.8 ^a	389.3±178.6 ^a	298.6±76.6 ^b
PAI (IU/ml)	27.9±18.3 ^a	32.4±19.8 ^a	20.4±11.1 ^b
tPA (IU/ml)	10.9±6.4 ^a	10.8± 5.9 ^a	7.1±2.8 ^b
Total TFPI (ng/ml)	59.8±22.4 ^a	57.7±21.4 ^a	48.2±13.9 ^b
Free TFPI (ng/ml)	16.7±9.1 ^a	15.4±8.3 ^a	12.4±4.7 ^b
F1+2 (nmol/l)	3.6±3.8 ^a	3.1±3.6 ^a	0.8±0.3 ^b
TAT (ug/ml)	15.2±15.1 ^a	11.7±12.9 ^a	1.8±1.6 ^b
D-Dimer (ug/ml)	551.3±290.7 ^a	532.1±317.9 ^a	52.9±45.0 ^b
PrC (%)	90.9±25.1 ^a	87.9±23.6 ^a	103.5±21.5 ^b
PrS (%)	96.4±19.4 ^a	95.4±18.1 ^a	94.5±20.1 ^b
ATIII (%)	98.3±15.1 ^a	93.5±13.1 ^a	102.6±13.8 ^b
Different alphabetical letters (a and b) indicate statistically significant difference between patients and controls. PAI - plasminogen activator inhibitor, tPA - tissue plasminogen inhibitor, TAT - thrombin antithrombin complex, F1+2 - prothrombin fragment 1+2, PrC - protein C, PrS - protein S, ATIII - antithrombin III			

DISCUSSION. Since thrombus formation, which underlies the onset of ACS, involves the activation of the hemostatic system and generation of thrombin, measurement of the plasma levels of markers of thrombin generation (F1+2, TAT and D-Dimer), have proved to be valuable monitors of the progress of the thrombotic process *in vivo*.^{4,12} In the current study the blood levels of these markers were very significantly elevated above normal levels in patients with ACS (MI and UA), before they underwent coronary angiography. This agrees with many earlier studies.¹³⁻¹⁵ Many follow-up studies found the levels of these hemostatic activation markers remain elevated up to 2 years after MI.^{16,17} Also measurements of these markers were shown to be useful in predicting the clinical outcome of ACS, whether new AMI and or death.^{16,18} The results of the current study in the Saudi Arab population, is in line with the weight of evidence in the literature that thrombin generation is instrumental in the pathogenesis of ACS, whether MI or UA. However, our results in such a large cohort found the elevated levels of these hemostatic activation markers of comparable magnitude in UA and MI; this disagrees with other earlier and smaller studies that found the levels of these markers higher in MI than UA.

The extrinsic tissue factor (TF) pathway of the coagulation mechanism has also been a focus of interest in research on the hyper-coagulability associated with ACS. The TF is considered to be a major initiator/regulator of coagulation *in vivo* and rupture of the atherosclerotic plaques, which contain TF-synthesizing cells, results in the exposure of TF activity, activation of the extrinsic coagulation pathway, thrombin generation and fibrin deposition.^{19,20} The activity of TF is controlled by the TFPI that inhibits the initial reaction in the extrinsic tissue factor coagulation pathway. In the current study the levels of TFPI, both total and free displayed significant elevation in patients with MI and UA, when compared to the levels in healthy controls. This is in line with the results of many recent studies in patients with UA and MI.^{14,21-24} Most of these earlier smaller studies found the levels of TFPI to be higher in MI than in UA, which contrasts our finding of similar levels of both forms of TFPI in patients with UA and MI. Racial differences could account to the disagreement in the results. The content and procoagulant activity of TF in human coronary atherosclerotic plaques varies widely, but is higher in the plaques extracted from patients with UA, MI or histologic/angiographic evidence of coronary thrombosis, than in those taken from patients with stable angina or uncomplicated coronary lesions.²⁰ Therefore, variations in TF content and activity may be responsible for not only the different thrombotic complications of coronary atherosclerotic plaque

rupture, but also to the measured plasma levels of TF in various forms of CHD.²⁰ The blood monocytes and endothelial cells are possible additional sources of TF and TFPI.^{18,25}

However, we noted with great interest that the plasma levels of the free physiologically-active form of TFPI is reduced significantly in patients with 3-vessel coronary disease as compared to those with single or double vessel disease. It is reasonable to explain this difference by excessive consumption of this inhibitor in presumably more extensive clotting process, in patients with 3-vessel disease. Small amounts of circulating soluble plasma thrombomodulin are taken to reflect endothelial damage, and on this basis it is considered to be a marker of coronary atherosclerosis.²⁶ Our results of markedly elevated levels of thrombomodulin in with ACS (both UA and MI) are in line with many published studies.²⁷⁻²⁹ In contrast, one study³⁰ found no difference in soluble thrombomodulin levels between patients with and without CAD; however, the same study reported elevated levels of von Willebrand factor (and not thrombomodulin) in patients with various stages of CHD and concluded that vWF is a better marker of coronary atherosclerotic disease than thrombomodulin. The simultaneous elevation of soluble thrombomodulin and TFPI levels indicates significant endothelial activation in patients of ACS.

In the current study, there was significant elevation of PAI-1 and tPA, and to a comparable extent, in patients with UA and MI when compared to controls. This is in line with many previous studies that repeatedly confirm the close association between CHD and inhibited fibrinolysis as shown by elevated levels of PAI-1, which is a known risk factor for CHD.^{30,31} Also, elevated levels of PAI-1 predicted the occurrence of the first acute MI,^{32,33,34} re-stenosis after angioplasty, even in asymptomatic patients^{35,36} subsequent MI in patients with stable angina pectoris,³³ as well as being an outstanding risk factor for recurrent re-infarction.³⁵ In the current study the level of plasma fibrinogen is elevated in patient with ACS, when compared to controls. This is in line with the well-known and widely reported hyperfibrinogenemia as a risk factor for CAD, in different ethnic communities whether Western countries³⁷⁻⁴¹ or Oriental, Chinese⁴² and Indians.⁴³ The levels of the natural coagulation inhibitors, ATIII, Prc and not PrS showed slight but statistically significant reduction below healthy control values. This finding that is also in line with earlier reports,⁴⁴⁻⁴⁶ and supports further the existence of a hypercoagulable state in ACS.

Lastly remains the question of the relationship between race and CHD. This topic has been reviewed in detail recently,⁴⁷ where emphasis was put on the paucity of epidemiological, laboratory and clinical data to clarify the interaction between

race and CHD or ACS, or both. The overwhelming available data was derived from comparative studies between white and black Americans, and very few studies are available in other races. For example, Blacks have twice as high lipoprotein (a) as Whites, but do develop atherosclerosis with the same propensity as Whites.⁴⁷ Thus, there is a need for large scale prospective comparative studies into all aspects of CHD, in various ethnic groups, especially Arabs where the extensive change towards westernized lifestyle is bringing CHD to the forefront of the health problems.

In conclusion, in this large scale study, we measured multiple hemostatic factors in Saudi Arab patients with ACS (UA and AMI) before they undergo coronary angiography. In both patient groups the levels of TFPI, both total and free, markers of thrombin generation (F1+2, TAT and D-Dimer) as well as the fibrinolytic inhibitor, PAI, were significantly elevated above healthy control values. This confirms the existence of an ongoing in vivo activated coagulation process and inhibited fibrinolysis in both AMI and UA. We also noted with interest that the level of TFPI (the prime inhibitor of the extrinsic tissue factor pathway of blood coagulation) is significantly reduced in patients with 3-coronary artery vessel disease as compared to those with one or two vessel diseases. This indicates the excessive consumption of this physiological inhibitor at sites with more extensive thrombotic process.

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