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ORIGINAL ARTICLE

Circulating Thrombotic Risk Factors in Young Patients with Coronary Artery Disease Who Are on Statins and Anti-platelet Drugs

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Abstract Thrombotic risk factors may contribute to premature coronary artery disease (CAD), in addition to the conventional risk factors. There is paucity of data on studies evaluating the role of thrombotic factors in premature CAD in Indian patients. Thus a case–control study was performed to evaluate the role of thrombotic and atherogenic factors in young patients with angiographically proven CAD who are on treatment with statins and anti-platelet drugs. 152 patients (≤55 years) with angiographically proven CAD and 102 asymptomatic controls were recruited. Clinical and biochemical data were obtained in both groups. Blood levels of thrombotic factors-fibrinogen, antithrombin-III, tissue-plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1), von-Willebrand factor (v-WF), lipoprotein(a) [Lp(a)] and homocysteine were analyzed. Patients had high levels of conventional CAD risk factors (diabetes mellitus, smoking, hypertension, dyslipidemia and positive family history) compared to controls. Logistic regression analysis revealed that low antithrombin-III (odds ratio/OR 11.2; 95 % confidence interval/CI 2.29–54.01), high fibrinogen (OR 6.04; 95 % CI 1.09–33.21) and high Lp(a) (OR 4.54; 95 % CI 0.92–22.56), as important, independent risk factors in patients. PAI-1(OR 0.15; 95 % CI 0.03–0.69) levels were significantly lower in patients. But other thrombotic risk factors studied (t-PA, v-WF and homocysteine) were comparable among patients and controls. The treatment using statins and anti-platelet drugs might be contributing to the control of some of the thrombotic risk factors. The strategies aiming at lowering the levels of thrombotic risk factors along with conventional risk factors may be useful in primary and secondary prevention of CAD.

Keywords Coronary artery disease (CAD) · Premature CAD · Thrombotic factors · India

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Introduction

Cardiovascular disease (CVD) is a worldwide health epidemic. Over 80 % of CVD deaths take place in low- and middle-income countries and occur almost equally in men and women. The number of people, who die from heart disease and stroke are projected to reach 23.3 million by 2030 [1]. Death due to cardiovascular disease strikes Indians at an earlier age and thus kills or disable many in their productive mid-life [2]. Coronary artery disease (CAD) is the major contributor for CVD.

Indians have the highest risk rates for CAD among all ethnic groups [3, 4], and are also reported to have higher prevalence of the risk factors for CAD at young ages [4, 5]. Factors responsible for premature CAD in Indian subjects could be multiple—ranging from social, economic, psychological (stress), lifestyle (smoking, sedentary lifestyle, improper diet), biological (abnormal lipids, hypertension,

diabetes, obesity [5], and thrombotic risk factors [6, 7]. There are many reports from India which analyzed different thrombotic factors, including fibrinogen [8], tissue-plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1) [7], von-Willebrand factor (v-WF) [9], antithrombin-III [10], lipoprotein (a) [Lp(a)] [10, 11] and homocysteine [8, 12] in premature CAD. Except a few [7, 13, 14], many of these studies are from north-Indian population [3, 6, 8, 10, 11, 15].

Studies on thrombotic and inflammatory markers in CAD are sparse from southern India. Southern states of India, is considered to be having higher prevalence of CAD and higher prevalence of conventional CAD risk factors (tobacco use, hypertension, diabetes mellitus, dyslipidemia and family history of premature CAD) compared to other states of India [16, 17]. Prevalence rate of CAD in the south Indian state of Kerala is reported to be 11 and 7 % in urban and rural population, respectively [17].

Lipid lowering drugs including statins and anti-platelet drugs like aspirin and clopidogrel are essential both for the primary and secondary prevention of CAD [18, 19]. But acute coronary events recur in patients even while they are under treatment [20]. Thrombotic risk factors may also play major role in the advancement of CAD, especially in those with premature CAD and those with positive family history. It may also contribute to recurrence of events on follow-up [21, 22].

Our study is a case–control study, performed to evaluate the role of thrombotic and atherogenic factors in young angiographically proven CAD patients who are on anti-platelet drugs and statins for at least 3 months.

Materials and Methods

Subject Selection

The study was carried out by the Departments of Cardiology, Biochemistry and Thrombosis Research Unit of the Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), a tertiary care hospital in the southern state of Kerala, India, during the period—October 2011 to December 2013.

The study was approved by the Institutional Ethics Committee and written informed consent was obtained from all the participants before blood sample collection. The major objective was to evaluate thrombotic risk factors in young patients (age less than or equal to 55 years), who were admitted and treated in this hospital. In this study 152 patients with angiographically proven disease were consecutively enrolled from a database of 5500 young CAD patients and the data was compared to 102 asymptomatic healthy volunteers of the same age group. Controls were healthy volunteers, who were free living subjects recruited from the hospital at the same time as that of patients. CAD patients were on treatment with cholesterol lowering drugs, statins (mainly atorvastatin) and other standard anti-platelet agents (aspirin and/or clopidogrel) and anti-anginal drugs (nitrates, beta blockers and/or calcium channel blockers), wherever indicated.

Data Collection

Data regarding conventional risk factors such as positive family history (determined as those whose parents and/siblings had any incidence of CVD before the age of 60) [23], tobacco smoking (current and ex-smokers), diabetes mellitus (those with fasting blood glucose level above 127 mg/dl or those taking anti-diabetic medication) [24], dyslipidemia [25] and hypertension (those whose systolic/diastolic pressure above 140/90 mm of Hg and or taking hypertensive drugs) [26] were collected. Data of patients were obtained from hospital records, while data regarding controls were obtained using a structured questionnaire.

Blood Sample Collection

Blood samples were collected from patients and controls after over-night fasting of 12 h. Patients were admitted to the hospital for elective procedures after 3 months of onset of symptoms and after at least 3 months of treatment with statins (mainly atorvastatin) and anti-platelets (aspirin and clopidogrel). Ten milliliter of blood was collected from each subject and was transferred to appropriate vacutainer tubes. Fresh citrated plasma and serum were used for the assays of fibrinogen and lipid profile respectively. For rest of the analyses, plasma and serum samples were aliquoted and stored at −20 or −80 °C and the thrombotic parameters were analyzed within 1–6 months of sample collection. All the biochemical and thrombotic factors were analyzed using accredited procedure in accredited laboratories, following basic guidelines.

Laboratory Investigations

Biochemical Assays

Fasting blood glucose was determined by hexokinase and glucose-6-phosphate dehydrogenase method (GLU method-Flex Reagent Cartridge-SIEMENS, Dimension Clinical Chemistry System). Total cholesterol was estimated by cholesterol esterase, cholesterol oxidase and horse radish peroxidase method (CHOL method-Flex Reagent Cartridge-SIEMENS, Dimension Clinical Chemistry System). Triglycerides were assayed using

Lipoprotein lipase, Glycerol Kinase and glycerol-3- phosphate oxidase method (TGL method-Flex Reagent Cartridge-SIEMENS, Dimension Clinical Chemistry System). HDL-C was analyzed by enzymatic end point method (Direct method using ASPEN HDL-C KIT). LDL-C was derived using Friedewald equation [27].

Assays of Thrombotic Markers

Citrated plasma was used for following assays except for, Lp(a) and homocysteine. Fibrinogen was measured by Clauss method [28] using FIBRI-PREST-2 reagent kit in semi-automated machine of Diagnostica Stago, France—*Starline*. PAI-1, v-WF and t-PA were determined using ELISA kits: ASSERACHROM®PAI-1, ASSERACHROM®VWF:Ag and ASSERACHROM®tPA, respectively of Diagnostica Stago, France.

Antithrombin-III (AT-III) assay [STACHROM® ATIII reagent kit] was measured using *STA- compact* auto analyzer of Diagnostica Stago, France. The facility was provided by Advanced Clinical Research Laboratory, Medical College Thiruvananthapuram, Kerala, India.

Serum homocysteine was quantified by enzymatic assay using reagent kit from Accurex Biomedicals, in semi-automated analyser, [Nexgen of Span Diagnostics]. Serum Lp(a) was estimated using Latex turbidimetry method [Lp(a)- turbilatex from SPINREACT, Spain] in UV-spectrometer [Shimadzu].

Statistical Analysis

Continuous variables were expressed as mean along with standard deviation (SD). Unpaired Student's *t* test was used to compare the difference between mean values. Binary logistic regression analysis was used to estimate odds ratios and 95 % confidence interval (CI) for group comparisons. $p < 0.05$ was defined significant. Pearson's correlation analysis was also carried to study the relation between different parameters. Microsoft-office Excel and GraphPad prism demo 5 was used for statistical analysis. Logistic regression analysis was performed using STATA/IC 11.2 version software.

Results

Demography, Conventional Risk Factors and Biochemical Analysis

Majority of the patients (84 %) were males. Patients had significantly higher rate of dyslipidemia (as low HDL-C), history of tobacco smoking, diabetes mellitus, hypertension and positive family history of premature CAD compared to controls (Table 1). Among conventional risk factors, smoking was an important risk factor among male patients (68 % of the male patients gave a history of either current or past smoking).

Table 1 Basic characteristics of patients and controls

	Patients (N: 152)	Controls (N: 102)	*p* value
Age: Mean ± SD	45.8 ± 6.6	43.4 ± 9.0	0.027
	Frequency (%)	Frequency (%)	
Sex			
Male	127 (84)	77 (76)	0.113
Female	25 (16)	25 (24)	
Conventional risk factors			
Positive family history to CAD	54 (36)	17 (17)	<0.05
Smoking	85 (56)	13 (13)	<0.001
Diabetes mellitus	89 (59)	17 (17)	<0.001
Hypertension	79 (52)	5 (5)	<0.001
Low HDL	142 (93)	52 (52)	<0.001

N number of subjects, % percentage of subjects from the total population of patients or controls, *SD* standard deviation

All patients were on statins and dietary restrictions and hence had lower levels of total cholesterol, triglycerides and LDL-C and the values were even lower than the controls who were free living subjects with no dietary restriction and/or any drugs. But mean fasting blood sugar and low HDL-C were significantly higher in patients compared to controls. (Table 2).

Thrombotic Factors

Thrombotic risk factor analysis showed that mean levels of fibrinogen and Lp(a) were significantly higher in patients compared to controls. Another major finding was that mean value of antithrombin-III, an antithrombotic factor was significantly lower in patients.

PAI-1, (an inhibitor of t-PA and u-PA/urokinase- plasminogen activator), and hence an inhibitor of fibrinolysis, was found significantly lower in the patient group. There were no significant differences between the levels of t-PA, v-WF, and homocysteine, between patients and controls (Table 3).

Logistic regression analysis to find out the most significant risk factors associated with CAD revealed that low antithrombin-III, high fibrinogen and high Lp(a) levels are the important risk factors (Table 4).

55 % of our patients had at least three of the five thrombotic risk factors (levels of high fibrinogen, lipoprotein (a), homocysteine, v-WF and low levels of antithrombin-III) and 91 % had at least two thrombotic risk factors.

Table 2 Fasting blood sugar and lipid profile of patients and controls

Parameters (units)	Patients: Mean ± SD	Controls: Mean ± SD	*p* value
Fasting blood sugar (mg/dl)	159 ± 85	100 ± 33	<0.001
Total cholesterol (mg/dl)	145 ± 52	219 ± 45	<0.001
Triglycerides (mg/dl)	127 ± 54	147 ± 69	<0.05
HDL-C (mg/dl)	34 ± 8	41 ± 7	<0.001
LDL-C (mg/dl)	83 ± 33	151 ± 54	<0.001

Table 3 Thrombotic risk factor analysis (unpaired *t* test)

Parameters (normal range)	Patients		Controls		*p* value
	Frequency (n)	Mean ± SD	Frequency (n)	Mean ± SD	
Fibrinogen (2–4 g/l)	149	3.94 ± 1.71	90	3.14 ± 0.88	<0.001
Lipoprotein (a) (<30 mg/dl)	149	38.59 ± 33.52	99	21.43 ± 16.78	<0.001
Antithrombin-III (80–120 %)	103	60.51 ± 38.24	78	93.22 ± 31.58	<0.001
Von-Willebrand factor (55–200 %)	106	146.02 ± 108.66	53	130.05 ± 87.87	0.36
Plasminogen activator inhibitor-1 (4–43 ng/ml)	107	27.42 ± 15.27	53	38.97 ± 22.19	<0.001
Tissue-plasminogen activator (2–12 ng/ml)	45	14.27 ± 8.67	30	15.71 ± 9.71	0.51
Homocysteine (<30 μM/ml)	45	25.06 ± 13.35	33	22.94 ± 13.86	0.50

SD standard deviation, *n* total number of subjects in which test were done within the total population of patients and controls. Unpaired *t* test of thrombotic risk factors with mean and Standard deviation represented as mean ± SD. Normal range is detailed in square brackets

Table 4 Logistic regression analysis of thrombotic risk factors

Parameters	Patients	Controls	Binary logistic regression analysis			
	n (%)	n (%)	Un adjusted odds ratio	95 % CI	Adjusted odds ratio[a]	95 % CI
Fibrinogen levels						
Fibrinogen <4 g/l	99 (67)	77 (86)	1			
Fibrinogen >4 g/dl	50 (34)	13 (14)	2.99	1.52–5.90	6.04	1.09–33.21
AT-III levels						
AT III >80 %	40 (39)	61 (78)	1			
AT III <80 %	63 (61)	17 (22)	5.65	2.90–11.02	11.2	2.29–54.01
Lp(a) levels						
Lp(a) <30 mg/dl	83 (56)	77 (78)	1			
Lp(a) >30 mg/dl	66 (44)	22 (22)	2.78	1.57–4.94	4.54	0.92–22.56
PAI-1 levels						
PAI-1 <43 ng/ml	96 (90)	34 (64)	1			
PAI-1 >43 ng/ml	11 (10)	19 (36)	0.21	0.09–0.48	0.15	0.03–0.69

Logistic regression analysis of important thrombotic risk factors with odds ratio (OR) and confidence interval (CI) (*n* number of subjects in which thrombotic risk factors analysis was done representing number of subjects with low and high values. [a]All parameters were adjusted for each other in the multiple logistic regression)

Relationship Between Thrombotic Risk Factors and Conventional Risk Factors

We observed that patients with at least three conventional risk factors had higher prevalence of thrombotic risk factors when compared to controls. The controls had fewer conventional risk factors, except dyslipidemia (Table 1). Among patients who had three or more conventional risk factors, 60 % had low antithrombin-III ($p < 0.05$). Similarly 33 % had high fibrinogen and 43 % of patients had high Lp(a). Controls who were smokers had higher prevalence of the thrombotic risk factor, fibrinogen. 40 %

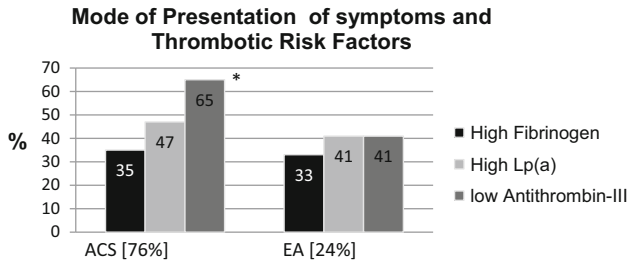

Fig. 1 Thrombotic risk factors in ACS versus EA patients. *ACS* acute coronary syndrome, *EA* effort angina. $*p < 0.05$/significance: Chi square analysis. Acute coronary syndrome patients have increased risk factors [within 74 % of ACS patients, 35, 47 and 65 % had high fibrinogen level, high Lp(a) and low antithrombin-III levels, respectively] compared to effort angina patients (lowered trend of thrombotic risk factors)

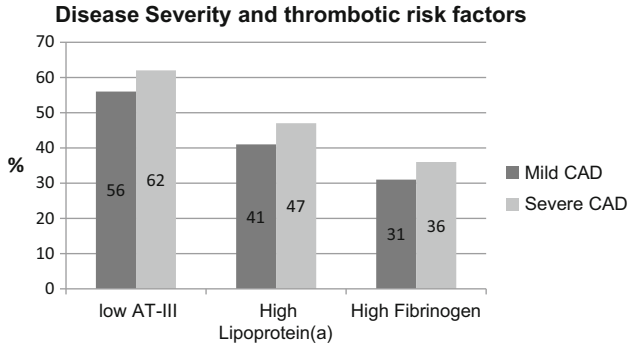

Fig. 2 Thrombotic risk factors were elevated in severe CAD compared to mild but these elevations were not statistically significant

of the smokers in the control group also had low antithrombin-III.

Relationship Between Mode of Presentation of CAD and Thrombotic Risk Factors

Majority of the patients (74 %), were presented with acute coronary syndromes (ACS) and the rest (26 %) with effort angina (EA). Thrombotic risk factors were more prevalent in the ACS group compared to the EA group (Fig. 1), but it reached statistical significance ($p < 0.05$) only in the case antithrombin-III.

Thrombotic Risk Factors and Coronary Angiogram Findings

Significant coronary artery disease was defined as narrowing of more than 50 % of the lumen on coronary angiography. The association of the severity of CAD and thrombotic factor levels were also analyzed. Based on Coronary angiography (CAG) findings CAD was graded as minor CAD (50–70 % stenosis); single vessel disease; double and triple-vessel disease, defined as CAD with >70 % lesion in one, two and three coronary arteries respectively. In the study, the patients were segregated into two groups—(a) *Mild CAD* with minor CAD and or single vessel disease (40 %) and (b) *Severe CAD* with double or triple vessel disease (60 %). Though there was a trend, associating the severity of CAD and thrombotic risk factor levels, it did not reach statistical significance (Fig. 2).

Discussion

The patho-biology of coronary artery disease (CAD) is linked with inflammation and thrombosis which may contribute to its premature occurrence in young patients. There are only few studies which evaluated thrombotic risk factors associated with CAD in young Indians [6, 10]. We have found only one study from South India, which evaluated more than two thrombotic risk factors [13]. Present study is unique in that, it evaluated combined thrombotic risk factors [antithrombin-III, fibrinogen, Lp(a), t-PA, PAI-1, v-WF and homocysteine] and assessed its association with conventional risk factors, mode of presentation of symptoms of CAD and disease severity.

Lipid levels of patients were low or normal compared to controls as they were on treatment with statins [18]. HDL-C was low in patients compared to controls as statins can increase HDL-C moderately [29]. The control population involved free living subjects without any exposure to any form of anti-atherogenic drugs, is a probable reason for the phenomenon. In a study on Lp(a) among CAD patients and controls by Rajashekhar et al. [14] total cholesterol and LDL-C levels were elevated in patients while low HDL-C was lower among patients.

Patients with conventional risk factors and controls who were smokers had higher prevalence of thrombotic risk factors. This indicates that conventional risk factors might be contributing to some of the thrombotic risk factors.

Thrombosis plays a critical role in the pathogenesis of acute coronary syndrome (ACS) [30]. In our study, significantly low *antithrombin-III* levels were found in patients compared to controls. Again patients presenting with ACS had significantly low levels of antithrombin-III compared to patients presented with EA. Antithrombin is a serine protease inhibitor which inhibits thrombin and activated factor X. Severe inflammation lead to decrease in antithrombin levels due to its impaired synthesis and degradation by elastase produced by activated neutrophils [31]. This is probably a reason for reduced antithrombin-III in smokers (both patients and controls) in our study population.

In multivariate analysis, low antithrombin-III emerged as an important thrombotic risk factor along with high fibrinogen and high Lp(a). The finding of low

antithrombin-III levels in patients is an important observation from our study. So far there are only sporadic reports of its deficiency in cardiovascular disease [10, 32]. In an earlier study on antithrombin-III and fibrinogen, the risk of cardio-vascular events was positively related to fibrinogen levels and negatively related to antithrombin-III levels in patients who had angina pectoris [33].

Fibrinogen has a predictive role in future coronary events [34]. In our study 33 % of the patients had high fibrinogen levels and all were on treatment with statins. Leibovitz et al. [35] has reported a 16 % reduction in fibrinogen levels in hypercholesterolemia subjects on treatment with statins. In a study from North India [15] fibrinogen was elevated in 58 % of CAD patients who were not on treatment with statins. This may indicate that, statins might be reducing fibrinogen in some of our patients, although we need more evidence. Fibrinogen was high especially among smokers irrespective of whether they were patients or controls indicating that smoking is an important contributing factor for fibrinogen elevation. Smoking alters haemostatic process by multiple mechanisms, which also involves alteration of fibrinogen and creating an imbalance in thrombotic, antithrombotic and anti-fibrinolytic pathways, which can increase oxidative stress [36].

Lp(a) is an apo B containing lipoprotein, with an additional apo(a) apolipoprotein and is also a pro-thrombotic agent involved in fibrinolytic pathway. It is a potent inhibitor of fibrinolysis, due to its structural homology to plasminogen [37]. It was found to be an important risk factor in our patients. Prospective studies indicated an association between elevated Lp(a) and angiographically proven CAD [14], and with disease severity [10]. In our study, mean Lp(a) was significantly elevated, 44 % of the CAD patients showed high Lp(a) levels (30 mg/dl or more). Logistic regression revealed a higher odds ratio (Table 4) for Lp(a) indicating it as an important risk factor in the population. There are reports that aspirin administration can reduce Lp(a) [38], as our patients were on aspirin, this might be the probable reason for reduced Lp(a) in at least 60 % of the patients.

The association between antithrombin-III [33], fibrinogen [34] and Lp(a) [10, 14] levels and coronary artery disease severity has been established in many studies. Though our patients showed a positive trend between the thrombotic factor levels and CAD severity, it did not reach statistical significance.

t-PA and *PAI-1* levels are established thrombotic risk factors. A Chennai based study [26], has shown an elevation of t-PA and PAI-1 were associated with CAD along with fibrinogen. Present study could not observe significant difference in mean t-PA levels among patients and controls. But a study from Western India (Mumbai) [7] showed that there was no significant increase in t-PA values in older (>40 years) Acute Myocardial infarction (AMI) patients compared to controls. The study also showed significant lowering of PAI-1 in patients under treatment and with stable angina and explains PAI-1 is correlated with acute events as AMI patients had significantly elevated PAI-1. The study was comparable to our observations, as there was significant decrease in the level of PAI-1 in patients compared to controls. Statin treatment in our patients could be contributing to this. Cell culture studies in human vascular smooth muscle and endothelial cells [39], show that statins reduces PAI-1, and increases t-PA that inhibits release of PAI-1.

In the present study there was no significant difference in the levels of v-WF and homocysteine. Comparable *v-WF* levels in patients and controls could be due to statin therapy in patients. A study on the effects of statins on peripheral atherosclerosis [40] and another study on the effect of statins in hypercholesterolemic subjects has shown that there was 10 % reduction of v-WF levels [41].

Homocysteine is a thiol containing amino acid synthesized as a byproduct of methionine metabolism. Hyperhomocystinemia can damage the endothelium, resulting in a pro-thrombotic state. There are several conflicting reports about the association of the thrombotic risk factor, homocysteine and CAD [12, 42, 43] For homocysteine assay, we used indirect enzymatic assay for the estimation of homocysteine, in which NAD/NADH coupled enzyme reaction was employed. Recently our group [44], have standardized a direct, rapid and sensitive method for homocysteine assay in urine sample (liquid chromatography–tandem mass spectrometry coupled with electro spray ionization).Using this technique, urine samples from CAD patients showed elevated homocysteine levels compared to that of controls.

There were differences in the levels of different thrombotic risk factors among patients and controls. Some were significantly elevated while others were normal and levels of PAI-1 were significantly lower among patients compared to controls. Our patients were taking the anti-atherosclerotic drugs, like statins and anti-platelets for a period of at least 3 months; hence the effect of these drugs in lowering some of thrombotic risk might have contributed to our results.

Future Directions

There could be extensive cross-talk between conventional risk factors and thrombotic risk factors, as our study indicate. The presence of thrombotic risk factors may predispose to the development of recurrent coronary events. Thus treatment targets should focus on reduction of thrombotic risk factors also. Studies leading to the inflammatory role

of these thrombotic risk factors are gaining momentum and efforts should be made to interpret the influence of these factors on leukocyte (monocyte) recruitment, endothelial damage, smooth muscle cell proliferation, tissue factor production, platelet activation and matrix degradation on CAD progression. As evidenced from our study many of the thrombotic risk factor levels were not elevated probably due to treatment with statins and antiplatelet drugs. Therefore, more emphasis should be there on improving the efficacy of current treatment regimes.

Conclusions

Patients with conventional risk factors had more thrombotic risk factors. Low antithrombin-III, high fibrinogen and high lipoprotein (a) levels were found in young Indian patients with angiographically proven CAD who were on treatment with statins and antiplatelet drugs. PAI-1 levels were significantly lower in patients. But t-PA, v-WF and homocysteine levels were comparable among patients and controls. Smoking was found to raise the levels of thrombotic risk factors in both patients and controls. Treatment with statins and antiplatelets may be reason for lower levels of many of the thrombotic risk factors. The strategies aiming at lowering the levels of these thrombotic risk factors may be useful in primary and secondary prevention of CAD, along with strict control of conventional risk factors.

Strengths and Limitations of the Study

We had collected blood from patients who were on treatment with statins and anti-platelet drugs, and we could not make a comparison of patients without the influence of these drugs, as it is unethical to withhold these drugs from patients with CAD. Another major limitation of the study is that the sample size was not uniform for all of the factors. Major reason for this was the financial constrains as most of the reagent kits were expensive. Since this is a case–control study any unknown bias could also have resulted in differences in values. But our study was unique in the fact that it is only study from our region which analyzed combined thrombotic risk factors in the patients and controls along with conventional risk factors, presenting of symptoms and angiographic profile of CAD.

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Compliance with Ethical Standards

Conflict of interest Authors declare no conflict of interest.

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