ORIGINAL ARTICLE

Conventional Risk Factors, Telomere Length, and Ischemic Heart disease: Insights into the Mediation Analysis

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ABSTRACT

Telomere length is regarded as a potential biomarker of biological ageing and is associated with various age-related diseases, such as ischemic heart disease (IHD), myocardial infarction, peripheral vascular disease, and cancer. As there is a paucity of study that deals with this influence, this study aimed to assess how the cardiovascular risk factors influence the risk of IHD by performing mediation analysis. A total of 407 males were included in the study. IHD was diagnosed through echocardiography and coronary angiography by determining the number of coronary vessels involved. Demographic data, clinical history, and laboratory investigations such as random blood sugar (RBS), fasting lipid profile, serum creatinine, and serum urea levels of all the subjects were measured and recorded. Serum uric acid and blood urea nitrogen (BUN) levels were significantly higher in IHD subjects compared to non-IHD subjects ($P < 0.05$). Body mass index (BMI), glycosylated hemoglobin (HbA1c), RBS, serum uric acid, serum creatinine, BUN, total cholesterol, triglycerides, and telomere length significantly differed between subjects with and without IHD ($P < 0.05$). Further, telomere length ($P < 0.001$), BMI ($P < 0.001$), and total cholesterol level ($P < 0.001$) were risk factors that significantly affected the incidence of IHD, as proved by logistic regression. It indicates that shorter telomeres contribute to increased risk of IHD, influenced by BMI, HbA1c, BUN, total cholesterol levels, and RBS ($P < 0.001$). The study established a link between telomere shortening, conventional risk factors, and IHD; moreover, the study takes care in the role of mediation analysis which is a novel idea as little is done in this area of biostatistics with telomere length. Overall, this further establishes that telomeres length might serve as the promising biomarkers in predicting the risk of IHD.

Key words: Blood urea nitrogen, ischemic heart disease, real-time polymerase chain reaction, serum uric acid, telomere length

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Background

Ischemic heart disease (IHD) accounts for about 8.1 million deaths, globally, every year.[1] In this modernizing world, where people have got accustomed to unhealthy diet, lack of physical inactivity, increased alcohol consumption and smoking, lifestyle disorders such as type II diabetes mellitus and hypertension, and raised levels of cholesterol, triglycerides, serum low-density lipoprotein, homocysteine, and C-reactive protein are one the rise and are identified as the potential risk factors of IHD.[2] These risk factors, in turn, lead to the shortening of telomeres and play a crucial role in the pathogenesis of age-related cardiovascular diseases.[3,4] However, the underlying mechanism is still hypothetical.

Several observational studies have reported that shortening of telomere length increases the risk of IHD, myocardial infarction, and early death.[5] A prospective study conducted among white

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individuals in Copenhagen stated that both the genetic and nongenetic factors affect the telomere length in IHD subjects.[6] However, there is a paucity of data in determining the relationship between telomere length and IHD along with other established risk factors of IHD that affect telomere length. Hence, this impelled us to explore the relationship of telomere length and conventional risk factors with IHD through mediation analysis.

Methods

This present case–control study was conducted at the Department of Cardiology, Jawaharlal Nehru Medical College, KAHER, Belagavi, Karnataka, India. Before commencement of the study, ethical approval was obtained from the Institutional Medical, Ethical Committee. Written informed consent was obtained from the subjects before their involvement in the study. A total of 407 males (IHD subjects: 205; non-IHD subjects: 202) were included; females and subjects with a history of chronic alcoholism and systemic illnesses were excluded from the study. Females were excluded as they possess many changes concerning menopause and metabolism compared to male. IHD was diagnosed through echocardiography and coronary angiography.

Demographic data including age and gender of the subjects were recorded. The clinical data collected from the subjects included body height and weight, systolic and diastolic blood pressure, history of smoking, and history of medications used. Body height and weight were measured for the calculation of body mass index (BMI) using a standard protocol.[7] Blood pressure was recorded using a sphygmomanometer.

A 5-ml blood sample drawn from the antecubital vein of each subject and collected in a vacutainer containing a small amount of ethylenediaminetetraacetic acid was used for all the tests. Laboratory investigations, such as random blood sugar (RBS); glycosylated hemoglobin (HbA1c); serum creatinine, uric acid, and blood urea nitrogen (BUN) levels; and high-density lipoprotein (HDL), total cholesterol, and triglyceride levels of the subjects were measured.

Ejection fraction was assessed using echocardiography. Radial artery size for all the subjects was measured using an ultrasound machine. Other related data pertaining to IHD subjects including coronary artery anomalies, history of anxiety, the coronary vessels involved, and type of angiographic catheters used were also recorded.

DNA extraction, amplification, and measurement

DNA was extracted from the peripheral blood leukocytes using Qiagen DNA Mini Kit (QIAGEN Inc., California, United States).[8] Quantitative real-time (RT)-polymerase chain reaction (PCR) (Applied Biosystems, California, United States) was used to measure the telomere length of the extracted DNA as a T/S ratio.[9] Cycling conditions for both telomere and single-copy gene (36B4) amplicons were as follows: 10 min at 95°C, 40 cycles at 95°C for 15 seconds, and 1 min at 60°C; followed by a melting curve with little modifications as described previously.[9] 36B4 serves as a reference gene in the conventional quantitative PCR technique in the measurement of telomere length. All samples were analyzed in the ABI StepOnePlus RT-PCR system with SDS version StepOnePlus software (Applied Biosystems, California, and United States).

Descriptive analysis (categorical variables counts and percentages and continuous variables: mean ± standard deviation) was done for the study characteristics and risk factor assessments of the subjects using Microsoft excel spreadsheet and R version 3.5.1. Comparison of the data was done by Chi-square (in case of categorical variables) and independent *t*-test (for continuous variables). Median differences between the groups were analyzed by Wilcoxon signed-rank test.

With respect to telomere length data, boxplots were used to compare the telomere length among the groups and Pearson's correlation was performed to assess the correlation of the conventional risk factors with telomere length of both the groups and their effect on the telomere length was evaluated by performing multivariate linear regression. With respect to IHD patients, telomere length was dichotomized into two categories based on the median value to evaluate the significant factors affecting its length.

Logistic regression analysis was used to assess the relationship of risk factors with IHD status. Logistic regression model was built by taking the status of IHD (present/absent) as response and others as regressors using age as covariates for all the models. Model selection was done using sequential addition of variables. For mediation analysis, the status of IHD taken as a response, elomere length is taken as a predictor. Three versions of the same patient were considered (i.e. except for the difference described hereafter, these three versions of the patient are exactly the same). In the first version, the patient has IHD and the risk factor levels (mediators) have the natural levels of a patient with IHD. In the second version, the patient has IHD, but the risk factor levels are set to the value of a patient without IHD. In the third version, the patient does not have IHD and the risk factor levels have the natural levels of a patient without IHD. The total effect will be the difference in outcome between the patient in versions 1 and 3. The natural direct effect will be the difference in outcome between the patient in versions 2 and 3. The indirect effect will be the difference in outcome between the patient in versions 1 and 2. The interpretation of these effects is as follows: the indirect effect is the (relative) difference in telomere length that can be attributed to mediation through the risk factor, whereas the natural direct effect is the (relative) difference in telomere length that can be attributed to a direct path from IHD to telomere length. This path will include mediation through other risk factors that are not included in the analysis. Finally, the total effect is the (relative) difference in telomere length between those who have IHD compared with those without IHD, which equals the sum of the natural direct effect and indirect effect. The risk factors used as mediators in this model are BMI, HbA1c, RBS, uric acid, BUN, HDL, and total cholesterol. The total, direct and indirect effects are reported as estimate with 95% confidence intervals. To obtain 95% CIs for the total effect and (combined) indirect effect, bootstrapping was used with 1000 bootstrap samples. The bounds of the 95% CI were based on the 2.5 and 97.5 percentiles of the different distributions of effects.

Since mediation analysis essentially highlights the direct and indirect effects of the exposure and outcome, in the present study, the

effect of telomere length on the risk of IHD occurrence was the direct effect and effect of conventional risk factors assessed in the study on the risk of IHD when influenced by telomere length was considered the indirect effect. Blood pressure and age were considered to be confounding factors for IHD and telomere length, IHD and mediators, and telomere length and mediators' associations, as per the report of Sakboonyarat and Rangsin. Calculation of 95% confidence intervals (CIs) for single effects was done using robust standard errors; for composite effects, 95% CI were derived from robust variance-covariance estimates with the delta method. The mediation analysis was done using NCSS 2020 software version 20.0.2. The level of statistical significance considered for the two-sided *P* value was 0.05.

Results

A total of 407 subjects with (205) and without (202) IHD were assessed in the study. Baseline demographic and clinical characteristics of subjects in IHD and non-IHD subsets are shown in Table 1. The telomere length in different age groups is represented in Figure 1.

A statistically significant difference was observed between the IHD and non-IHD subjects in terms of telomere length ($P < 0.001$) and levels of HbA1c ($P < 0.001$), RBS ($P < 0.001$), serum uric acid ($P < 0.001$), creatinine ($P < 0.013$), BUN ($P < 0.04$), total cholesterol ($P < 0.001$), and total triglycerides ($P < 0.001$). Logistic regression demonstrated that telomere length, BMI, and total cholesterol were the risk factors significantly affecting the IHD subjects. For every unit increase in the telomere length and BMI, the odds of possessing IHD were decreased by 12.5 and 1.13 units, respectively, whereas for every unit increase in total cholesterol, the odds of occurring IHD were increased by1.015 units [Table 1].

The telomere length in IHD and normal subjects is shown in Figure 2. The telomere length in IHD subjects was less as compared to non-IHD subjects. In nearly half of the IHD

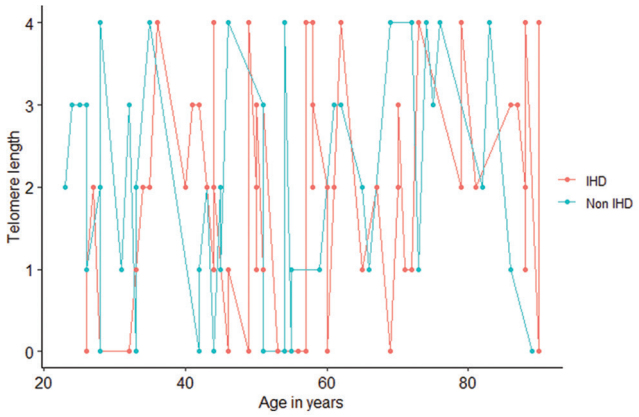

Figure 1: Telomere length concerning age groups

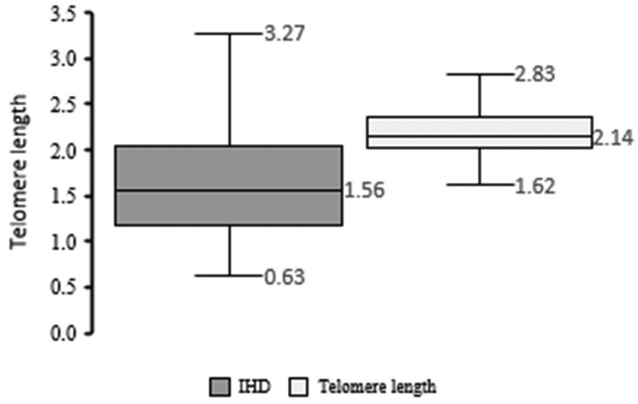

Figure 2: Comparison of telomere length in ischemic and nonischemic subjects

Table 1: Baseline differences between ischemic and nonischemic subjects and assessment of risk factors affecting subjects with ischemic heart disease

Factors	Non-IHD	IHD	P^a	OR	P^d
Smoking[b]	48 (23.76%)	44 (21.46%)	0.66	0.750	0.348
Age (years) [c]	60.3±8.05	61.1±8.23	0.32	1.011	0.430
Telomere length[c]	2.18±0.26	1.66±0.56	<0.001*	0.080	<0.001*
BMI (kg/m2) [c]	23.46±3.81	22.12±2.62	0.002*	0.882	0.001*
HbA1c levels (%) [c]	4.96±0.33	7.65±2.64	<0.001*	-	-
RBS (mg/dL) [c]	100.22±3.67	177.21±73.97	<0.001*	-	-
Serum uric acid (3.0-8.2 mg/dL) [c]	6.31±1.59	8.97±4.08	<0.001*	-	-
Serum creatinine (0.6-1.2 mg/dL) [c]	0.99±0.15	1.19±0.4	<0.001*	1.003	0.844
BUN (7-20 mg/dL) [c]	13.69±2.98	22.11±7.04	<0.001*	-	-
HDL (mg/dL) [c]	39.72±6.76	38.75±6.82	0.41	1.006	0.762
Total cholesterol (<200 mg/dL) [c]	166.17±21.49	180.89±34.62	<0.001*	1.015	<0.001*
Triglycerides (<150 mg/dL) [c]	120.99±24.42	131.18±35.2	<0.001*	0.997	0.514
Radial artery size (mm) [c]	1.61±0.28	1.62±0.28	0.75	1.300	0.547
Ejection fraction (%) [c]	53.48±10.4	52.9±10.06	0.566	0.991	0.414

*Significant, Data are expressed as mean±SD for continuous variables and percentages for categorical variable, [a]*P* calculated was the baseline difference between ischemic and nonischemic subjects, [b]Chi-square test, [c]Unpaired *t*-test, [d]Logistic regression was used to assess the risk factors affecting the ischemic status. IHD: Ischemic heart disease, SD: Standard deviation, BMI: Body mass index, HDL: High-density lipoprotein, RBS: Random blood sugar, BUN: Blood urea nitrogen, OR: Odds ratio, HbA1c: Glycosylated hemoglobin

Table 2: Factors affecting telomere length among ischemic and nonischemic subjects

Variables	Telomere length			
	IHD subjects		Non-IHD subjects	
	Estimate	*P*	Estimate	*P*
Age (years)	0.0007	0.6106	−0.0005	0.2082
BMI (kg/m²)	0.0003	0.9393	−0.0010	0.2143
Smoking	−0.0108	0.0003*	−0.0132	0.0559
HbA1c levels (%)	0.0063	0.3211	0.0268	0.0621
RBS (mg/dL)	−0.0001	0.7400	−0.0008	0.5017
Serum uric acid (mg/dL)	−0.1241	<0.001*	−0.0330	<0.001*
Serum creatinine (mg/dL)	−0.0255	0.4402	−0.1837	<0.001*
BUN (mg/dL)	−0.0077	0.0013*	−0.0462	<0.001*
HDL (mg/dL)	0.0005	0.7903	−0.0008	0.0761
Total cholesterol (mg/dL)	0.0009	0.0025*	−0.0000	0.8523
Triglycerides (mg/dL)	−0.0009	0.0022*	−0.0001	0.5906
Radial artery size (mm)	−0.0036	0.9308	−0.0021	0.8429
Ejection fraction (%)	0.0002	0.8801	0.0004	0.1332
Anomalies				
Intima tissue	−0.0372	0.8011	-	-
Parallel	−0.0320	0.4920	-	-
Loop anomaly	−0.0107	0.9410	-	-
Occlusion	0.3647	0.4512	-	-
Parallel left radial	−0.0743	0.2774	-	-
Coronary vessel involvement				
1	0.0062	0.8084	-	-
3	0.0222	0.6042	-	-
Drug history				
Aspirin+clopidogrel+pantoprazole	0.0309	0.2565	-	-
Aspirin+atorvastatin+metoprolol+pantoprazole	−0.0257	0.3593	-	-
Catheter used (French 6f)	−0.0472	0.0347*	-	-
Anxiety				
Moderate	0.0360	0.1942	-	-
Severe	0.0157	0.6052	-	-

*Significant. BMI: Body mass index, RBS: Random blood sugar, BUN: Blood urea nitrogen, HDL: High-density lipoprotein, IHD: Ischemic heart disease, HbA1c: Glycosylated hemoglobin

subjects, the telomere length was distributed between 1.18 and 2.04 with a median telomere length of 1.56.

The factors influencing the telomere length among IHD and non-IHD subjects are shown in Table 2.

Of all the the IHD risk factors assessed, levels of serum uric acid and BUN were significantly correlated with IHD and non-IHD subjects ($r = \pm 0.6$). Telomere length significantly shortened with increasing levels of BUN (IHD: $R = -0.93944$; $P < 0.001$; non-IHD: $R = -0.96251$; $P < 0.001$) and serum uric acid (IHD: $R = -0.79104$; $P < 0.001$; Non-IHD: $R = -0.97774$; $P < 0.001$) in IHD subjects. Correlation of telomere length with levels of BUN and serum uric acid among IHD and non-IHD subjects is shown in Figure 3.

Among the 205 IHD subjects, 103 had telomere length <1.56, whereas 102 had telomere length >1.56. Hence, the analysis was performed in two parts, i.e. upper median (>1.56) and lower median (<1.56). On multivariate linear regression analysis, smoking and levels of serum uric acid and triglycerides were independent predictors that significantly affected the telomere length among IHD subjects in the lower median. For every unit increase in serum uric acid and triglyceride levels, the telomere length decreased by 0.1257 and 0.0013 units, respectively, whereas in IHD subjects in the upper median, the serum uric acid level was the only independent predictor that significantly affected the telomere length; for every unit increase in uric acid level, the telomere length decreased by 0.0927 units [Table 3].

The total effect of IHD on telomere length was a decreased risk with an estimate of −9.87 (95% CI −18.17, −6.06). The direct effect and indirect effect contributions were −4.86 (95% CI −10.30, −0.34) and −5.01 (95% CI − 17.65, 0.40), respectively [Table 4]. The largest mediated effect contributing to the indirect effect was uric acid (0.75, 95% CI 0.34, −1.07). The second largest mediated effect was through total cholesterol (−0.03, 95% CI −0.05, −0.01), followed closely by the presence of RBS (−0.27, 95% CI −0.33, −0.21), HbA1c (−0.31, 95% CI − 0.38, 0.23), and BUN (−0.60, 95%

CI −0.76, −0.42). The excess risk was not mediated through BMI and HDL (−0.02, 95% CI − 0.03, 0.002, and 0.001, 95% CI −0.01, 0.02) [Table 4].

Discussion

The current research is focused on the role of telomere length in the development of chronic diseases. The biological mechanism underlying the shortening of telomere and the subsequent risk of cardiovascular diseases is still under debate.[10] The relationship between telomere length and type 2 diabetes mellitus among patients with IHD is reported in the previous study.[5] However, in our study, we investigated the effect of shortening of telomeres on the risk of IHD. In addition, influence of conventional risk factors that were affected by the change in telomere length in IHD subjects was also investigated by performing mediation analysis.

Due to high prevalence of degenerative diseases, men have shorter telomere length as compared to women.[11] In addition, menopausal and early metabolic changes in females are themselves are independent factors for telomerie length changes. Hence, only males were included in the study. Further, the risk of IHD is also higher in cases of males, which impelled us to explore the influence of risk factors of cardiovascular diseases on IHD and telomere length in men. Goglin *et al.*[12] reported that stable coronary artery disease along with telomere shortening was significantly observed in the older population and the male gender ($P < 0.001$). Baseline differences observed between IHD and non-IHD patients were similar to a prospective study conducted by Tian *et al.*,[13] wherein dyslipidemia, smoking, and levels of BMI, triglycerides, total cholesterol, HDL, high-sensitivity C-reactive protein, and blood pressure significantly varied in IHD subjects than in non-IHD subjects with type II diabetes mellitus.[14] Similarly, our study also reported significantly shorter telomeres in IHD subjects as compared to non-IHD subjects. In a prospective case–control study of ischemic heart failure consisting of 27 ischemic heart failure subjects and 24 controls, Wong *et al.*[15] observed significantly shorter telomeres in ischemic heart failure patients than in healthy controls ($P = 0.002$).

Logistic regression revealed that among all factors, BMI, total cholesterol, and telomere length were the significant factors affecting IHD status. Of these factors, progressive telomere length shortening was the prominent factor increasing the risk of IHD. A prospective study conducted by Masi *et al.*[16] reported that telomere length can predict the risk of IHD disease. Moreover, a recent study conducted by Piplani *et al.*[5] among Caucasian individuals also proved that short telomeres are significantly associated with an increased risk of IHD, both through observation as well as genetically. In contrast, a study conducted by Spyridopoulos *et al.*[17] among the subjects with myocardial infarction revealed that none of the risk factors (history of hypertension and diabetes; smoking; increased levels of cholesterol, white cell count, fibrinogen, C-reactive protein, and homocysteine; and positive history of coronary heart disease and myocardial infarction) affected the relationship between the status of myocardial infarction and telomere length. However, the risk factors investigated in our study were different from those assessed in the study by Nilsson *et al.*[18]

Studies have shown that elevated BMI levels were associated with an increased risk of both fatal and nonfatal IHD.[19,20] However, the effect of fine incremental changes of BMI in the increased risk of IHD is uncertain. A study conducted by Helby *et al.*[6] reported that 1 unit (4 kg/m^2) increase in BMI showed an odds ratio of 1.23 (95% CI, 1.19–1.28) in IHD subjects. Contradictorily, in our study, we reported that a gradual increase in BMI decreases the risk of IHD. Hence, further prospective studies are required to determine the underlying mechanism.

Table 3: Factors affecting telomere length among ischemic subjects

Telomere length	Estimate	*P*
Lower median (<1.56)		
Smoking	−0.1292	0.0064
Serum uric acid	−0.1257	<0.001
Triglycerides	−0.0013	0.0019
Upper median (>1.56)		
Serum uric acid	−0.0927	<0.001

Table 4: Mediation analysis of conventional risk factors as mediators to assess influence of telomere length on ischemic heart disease

Effects	Estimate (CI)	*P*
Total effect	−9.870 (−18.170-−6.064)	-
Direct effect	−4.863 (−10.302-−0.335)	<0.0001*
Indirect effect	−5.007 (−17.645-0.396)	-
Indirect effect through BMI	−0.0153 (−0.0286-0.0023)	0.0557
Indirect effect through HbA1c	−0.3128 (−0.3834-−0.2312)	<0.0001*
Indirect effect through RBS	−0.2749 (−0.3260-−0.2118)	<0.0001*
Indirect effect through uric acid	0.74911 (0.3355-−1.0669)	0.0001*
Indirect effect through BUN	−0.6040 (−0.7585-−0.4203)	<0.0001*
Indirect effect through HDL	0.00128 (−0.0115-0.0152)	0.8414
Indirect effect through total cholesterol	−0.0302 (−0.0494-−0.0095)	0.0033*

CI: Confidence interval, BMI: Body mass index, RBS: Random blood sugar, BUN: Blood urea nitrogen, HDL: High-density lipoprotein, HbA1c: Glycosylated hemoglobin

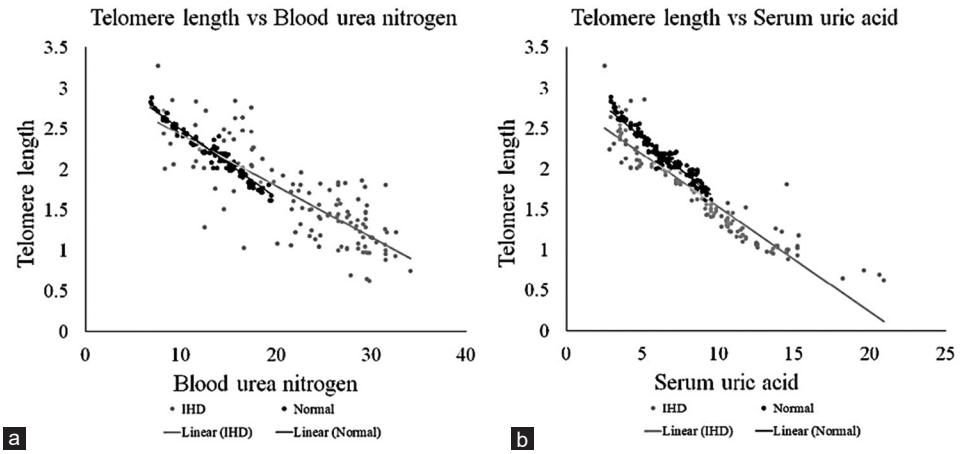

Figure 3: Correlation of telomere length with (a) blood urea nitrogen and (b) serum uric acid]

Further, the effect of conventional risk factors (age, smoking, BMI, HbA1c, RBS, serum uric acid, serum creatinine, BUN, HDL, total cholesterol, and triglycerides levels) on the telomere length among IHD patients was also studied. We observed that smoking, serum uric acid, BUN, total cholesterol, triglycerides levels, and catheter used were the significant factors. Nevertheless, there is a paucity of study that deals with the effect of conventional risk factors on the telomere length among IHD patients. A similar case–control study conducted by Zee *et al*.[14] also investigated the effect of risk factors of coronary heart disease on telomere length. However, neither the risk factors nor the history of smoking explained the effect on telomere length.

The correlational analysis confirmed that telomere length was negatively correlated with levels of serum uric acid and BUN in both IHD and non-IHD subjects. A similar retrospective study conducted by Buxton *et al*.[20] reported that smoking (β–0.752; $P = 0.0002$), history of myocardial infarction (β–0.048; $P < 0.001$), and atrial fibrillation (β–0.029; $P = 0.019$) were the significant factors correlated with the telomere length. Fascinatingly, a study conducted by Tian *et al*.[13] indicated that none of the conventional risk factors correlated significantly with telomere length in patients with coronary artery disease as well as controls. In our study, multivariate analysis performed in quartiles showed that smoking, serum uric acid, and triglyceride levels significantly affected the telomere length in IHD subjects in the lower quartile, whereas only serum uric acid significantly affected the telomere length in IHD subjects in the upper quartile. This suggests that telomere biology is involved in the development of IHD. However, there is a lack of similar kinds of data to support these findings.

Our study adds to the existing evidence that telomere shortening increases the risk of IHD. Further, the mediation analysis performed for the first time in case of IHD related studies, indicated that these risk factors significantly play a role in the development of IHD and are also influenced by the change in telomere length. The selection of the mediators and the confounding factors, i.e. age and blood pressure, was done on the basis of established reports indicating them as the conventional risk factors for IHD.

This is the first study of its kind to report a relationship between the telomere length and risk of IHD among the Indian population. However, the study is primarily limited being single centric. However, multicentric study with large study population may provide better insights into the exact relationship between shortening of telomere length and the risk of IHD. Second, as this was a case–control study, the selection bias of IHD and non-IHD subjects cannot be excluded. Finally, due to case–control design of the present study, we cannot draw definite inferences whether the telomere shortening observed in the subjects was the cause/consequence of IHD. Hence, further clinical studies need to be performed to explicate the current findings considering the onset age of IHD to indicate biological and age-related telomere length.

Conclusion

The study sheds light on a possible link between telomere shortening and IHD. It indicates that shorter telomeres contribute to an increased risk of IHD. Moreover, the study suggests that smoking, BMI, HbA1c, RBS, BUN, total cholesterol levels, and levels of uric acid further influence the telomere length in IHD subjects. Therefore, telomere length might contribute to predicting the risk of IHD.

Ethics approval and consent to participate

The study was submitted to Institutional Ethics Committee with MDC/DOME/180 and after that, samples were recruited on patients who signed the consent form. The trial was registered under Clinical Trial Registry of India: CTRI/2018/02/011663.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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