Indian Heart Journal 71 (2019) 459–463

Contents lists available at ScienceDirect

Indian Heart Journal

journal homepage: www.elsevier.com/locate/ihj

Original Article

Correlation of urinary cotinine with cardiovascular risk factors in pan masala tobacco users

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ARTICLE INFO

Article history:
Received 3 March 2019
Accepted 12 October 2019
Available online 22 October 2019

Keywords:
Blood pressure
Cardiovascular
Lipid-profile
Pan masala tobacco
Urinary cotinine

ABSTRACT

Background: Tobacco consumption is considered as one of the major risk factors for cardiovascular (CV) morbidity. However, the effect of paan masala tobacco (PMT) (a type of smokeless tobacco) consumption has not been well studied in our context. Our study is aimed to find an association of CV risk factors between PMT users and nonusers and to correlate those parameters with urinary cotinine level, a degradation product of nicotine occurring in tobacco.

Methods: This comparative cross-sectional study was carried out among 200 participants. The effect of PMT use on CV risk factors such as blood pressure (BP), lipid profile, and body mass index was measured against urine cotinine level. Statistical tests used were χ^2 test for categorical variable, independent *t*-test, Mann–Whitney U test, and Spearman's correlation applied for numerical variable, and multivariate regression analysis was performed as required. The level of significance was set at $p < 0.05$.

Result: Mean BP, total cholesterol, triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and median cotinine level were found to be significantly higher in PMT users than in controls ($p < 0.001$). Urinary cotinine level was positively correlated with mean BP, TC, TG, and LDL-C in PMT users ($p < 0.001$). Similarly, the odds of having hypercholesterolemia and increased diastolic BP was also significantly higher in PMT users ($p < 0.001$).

Conclusion: PMT use has an adverse effect on CV risk parameters and there is a rational of cotinine measurement for screening CV risk among PMT users.

1. Introduction

Cardiovascular disease (CVD) has become epidemic in developing countries. This is attributed to excessive consumption of tobacco which contributed to 10% of all CVDs, globally.[1] The direct relation between smoking and cardiovascular (CV) death is well documented. Although incidence of smoking has fallen in developed countries, by contrast, middle- and low-income countries in South and Southeast Asia are experiencing increased smoking rate and excessive consumption of smokeless tobacco (ST).[2–4] ST, such as gutkha, betel quid, etc., is consumed without burning the tobacco. They are taken in different ways such as chewing, spitting, dipping, applying to teeth and gums, and snuff[2,5] which has become more prevalent in developing countries of South Asian regions such as India, Nepal, etc.[2,6] Situated in Southeast Asian region, Nepal is a low-income country bordering India where the prevalence of ST use is high. As reported by various studies, there is rising trend, with 7.2% in 2001 to 19.1% in 2011.[2,7] ST such as paan masala tobacco (PMT) provides an excellent opportunity to study the impact of nicotine exposure on health, especially CVD, without

Abbreviations: CV, Cardiovascular; CVD, Cardiovascular Disease; PMT, Pan Masala Tobacco; BP, Blood Pressure; TC, Total Cholesterol; G, Triglyceride; BMI, Body Mass Index; DBP, Diastolic Blood Pressure; SBP, Systolic Blood Pressure; ST, Smokeless Tobacco; LDL/LDL-C, Low-Density Lipoprotein Cholesterol; HDL/HDL-C, High-Density Lipoprotein Cholesterol.

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https://doi.org/10.1016/j.ihj.2019.10.001

concomitant exposure to carbon monoxide and combustion products of tobacco smoke.

Major CV risk factors include cigarette smoking, hypertension (HTN) (BP > 140/90 mmHg), low high-density lipoprotein cholesterol (HDL-C) [<1.0 mmol/L (<40 mg/dl)], diabetes mellitus, family history of premature coronary heart disease, etc. Other factors are lifestyle-related such as obesity (body mass index [BMI]>30 kg/m^2), physical inactivity, and atherogenic diet.[8] These CVD risk factors are more prevalent in tobacco chewers[6,9] because nicotine and other different chemicals produced by the use of tobacco are speculated to be associated with CVD and its risk factors as well as other conditions such as cancers.[5,10] Nicotine is readily absorbed through the mucosal lining of mouth and is atherogenic with potential to disturb hemodynamic aspects of CV system; however, the exact mechanism is unknown.[11,12] The action of chewing and sucking for long time results in continuous absorption of nicotine which leads to its maximum level in blood similar to or even higher than that after cigarette smoking producing prolonged and excessive exposure to nicotine in PMT users and more risk of CVD.[9,13]

Studies carried out to find the relation between tobacco consumption and CV accidents or its risk factors mostly focused on western population and the results are still inconclusive.[2] Several studies performed in different countries have shown the incidence of metabolic syndrome, HTN, dyslipidemia, and diabetes in chronic tobacco users.[14,15] However, many of these research studies still suggest the need of rigorous studies to establish strong association and pathophysiological process of tobacco on CV system. In the meantime, studies from South Asian region contribute less to this problem. In addition, the case–control studies evaluating the effect of tobacco on CVD or its risk factors are very limited.[2] The most studied constituents of smoked or smokeless tobacco is nicotine, 80% of which breaks down to cotinine inside the human body with a half-life of about 17 h, much longer than nicotine which is about 30 min to 2 h. Therefore, estimation of cotinine level in urine can be a sensitive estimate of tobacco exposure.[3,11,16]

Most of the studies conducted focused on smokers and few on STs. Thus, the effect of ST on health is not well established. Furthermore, there are limited studies cited in the literature regarding PMT as a risk factor for CV morbidity in our context. Even though taxation is increased in PMT, prevalence of users is increasing. To create awareness regarding the health hazards about its use, we have conducted a cross-sectional study in which we measured urinary cotinine levels among PMT users and established its correlation with CV risk factors.

2. Materials and methods

2.1. Study design and population

This is a community-based comparative cross-sectional study, which was carried out during a period of one year from July 2013 to June 2014. A total of 200 samples were taken, of which 130 were PMT users and 70 were nonusers. Informed and written consent were obtained from all the participants. Ethical clearance was acquired from the Institutional Ethical Review Board at the start of the study.

Those who were more than 18 years of age and using only PMT (gutkha, khaini, paan, etc.) for at least 6 months were included; however, the smokers were excluded. Control subjects (n = 70) who did not use any forms of tobacco were included for comparison. Those who were not willing to take part in the study and with any self-reported acute illness, diagnosed cardiac, diabetes, renal, or hepatic disease, current treatment for cardiac or BP-related morbidities were excluded. Individuals with recent history of heavy alcohol and heavy diet were followed up after a week of normal diet to control the effect on lipid parameters. All the study subjects were instructed in advance to have normal diet and 8 h of fasting before blood sample collection.

2.2. Anthropometric and biochemical measurement

General clinical examination was performed including the measurement of BP, height, and weight. BMI was calculated as the ratio of weight in kilograms per square meter. BMI was further classified as normal <25 kg/m^2, overweight 25–29.99 kg/m^2, and obese ≥30 kg/m^2.[17] Five millimeters (5 ml) of blood sample after at least 8 h of fasting was collected from all the subjects in plain vials with all the aseptic precautions. Blood samples were allowed to clot and were centrifuged for 10 min at 3000 rpm and stored at −20 °C until assayed.

2.3. Estimation of biochemical parameters

Serum total cholesterol was assayed by enzymatic (CHOD-PAP) method using a kit from AGAPPE Diagnostics **[Warnick G].** In this method, free cholesterol is oxidized by cholesterol oxidase. The liberated hydrogen peroxide (H_2O_2) then couples with 4-aminoantipyrine and phenol in the presence of peroxidase to form a colored compound which is measured at 500 nm in UV–visible spectrophotometer.

Serum triglyceride (TG) was measured by enzymatic (GPO-PAP) method using a kit from AGAPPE Diagnostics **[Jacob NJ]**. In this method, glycerol is liberated from TG by lipoprotein lipase. In the presence of glycerol kinase and glycerol oxidase, glycerol is converted to dihydroxyacetone phosphate with the liberation of H_2O_2. The liberated H_2O_2 then couples with 4-aminoantipyrine and p-chlorophenol in the presence of peroxidase to form a colored compound which is measured at 500 nm in UV–visible spectrophotometer.

Serum low-density lipoprotein cholesterol (LDL-C) was measured by homogenous, direct method using a kit from Gesan Production S.R.L, Italy **[Jacob NJ]**. In this method, chylomicrons, VLDL, and HDL fractions are eliminated from the solution. After various enzymatic reactions and in the presence of specific surfactants, remaining LDL-C was measured specifically by a color formation, at 600 nm in UV–visible spectrophotometer.

Serum HDL-C was measured by homogenous, direct method using a kit from Gesan Production S.R.L., Italy **[Jacob NJ]**. In this method, chylomicrons, VLDL, and LDL fractions are eliminated from the solution. After various enzymatic reactions and in the presence of specific surfactants, remaining HDL-C was measured specifically by a color formation, at 600 nm in UV–visible spectrophotometer.

The optimal values of lipid parameters were as per the manual given in their respective kits. According to them, TC (mg/dl) < 200, TG (mg/dl) < 150, HDL-C (mg/dl) ≥ 60, and LDL-C (mg/dl) < 100 were considered optimal or low risk of CVD. Dyslipidemia was defined as increase level of TG, TC, and LDL-C with a decrease in HDL-C.

2.4. Urinary cotinine level measurement

Early morning urine samples ten milliliters (10 ml) each were collected in clean dry plastic containers. Fresh urine did not require any special handling or pretreatment. The specimens were frozen at −20 °C until assayed. Specimens previously frozen were thawed, equilibrated to room temperature, and mixed thoroughly before testing. Cotinine kit was purchased from the Calbiotech, Spring Valley, Canada, a solid-phase competitive ELISA. In this method, the sample and cotinine enzyme conjugate are added to the wells

coated with anticotinine antibody. Cotinine in the samples competes with a cotinine enzyme (HRP) conjugate for binding sites. Unbound cotinine and cotinine enzyme conjugate was washed off. On the addition of the substrate, the intensity of color was inversely proportional to the concentration of cotinine in the sample. The cutoff level of urinary cotinine was considered to be 31.5 ng/mL.[18]

Participant's data were collected on the basis of standard questionnaire ensuring all the ethical consideration. A structured questionnaire including name, age, sex, height, weight, PMT use, its duration, and quantity was filled up after taking informed consent from the subjects.

2.5. Statistical analysis

Data were analyzed in Statistical Package of Social Science (SPSS) 11.5. Independent T-test and Mann–Whitney U-test were applied to find out the significant difference between CV risk factors and other related variables. Spearman's correlation was applied for correlation analysis between urine cotinine level and various CV risk factor parameters. In all these tests, p value < 0.05 was deemed significant. Multivariate analysis was done for those variables which were significant in bivariate analysis, at 80% CI ($p < 0.2$) to find out the significant groups of variable.

3. Results

3.1. Baseline characteristics of participants

Table 1 contains the demographic variable of study subjects. Most of the study subjects were in their thirties with more males than females. Although most of the (about 90%) PMT users have different level of awareness regarding the effects of using PMT, however, they were chronic user of PMT and consume around 10 packets of tobacco in a day.

3.2. Association of cardiovascular risk factors and cotinine level between cases and controls

Table 2 shows the comparison of mean level CV risk factors such as BMI, SBP, DBP, TC, TG, HDL, LDL, and median level of cotinine between PMT user cases and nonuser controls. The median level of cotinine, a marker of tobacco exposure was substantially increased in PMT users but low level occurred in control groups [2695 (1600–4880) ng/ml vs 6 (4–10) ng/ml respectively, $p < 0.001$].

Table 1
Baseline characteristics of cases and controls.

Variables	Cases (n = 130)	Controls (n = 70)	Total (n = 200)
Age	38.07 ± 6.58	34.24 ± 7.64	36.73 ± 7.18
Gender			
Male	121 (93.1%)	53 (75.7%)	174 (87%)
Female	9 (6.9%)	17 (24.3%)	26 (13%)
Diet			
Vegetarian	12 (9.2%)	11 (15.8%)	23 (11.5%)
Non-Vegetarian	118 (90.7%)	59 (84.2%)	177 (88.5%)
PMT consumption years			
≤ 10 years of usage	66 (50.8%)	0	66 (50.8%)
> 10 years of usage	64 (49.2%)	0	64 (49.2%)
PMT use per day			
≤ 10 packets per day	92 (70.8%)	0	92 (70.8%)
> 10 packets per day	38 (29.2%)	0	38 (29.2%)
Awareness of PMT use			
Full awareness	51 (39.2%)	49 (70%)	100 (50.0%)
Limited awareness	67 (51.5%)	17 (24.3%)	84 (42.0%)
Unaware	12 (9.2%)	04 (5.7%)	16 (8.0%)

PMT, paan masala tobacco.

Table 2
Association of cardiovascular risk factors between cases and controls.

Variable	Cases (n = 130)	Controls (n = 70)	p value
BMI (kg/m^2)	24.99 ± 3.73	24.09 ± 3.12	0.085[a]
SBP (mm Hg)	129.23 ± 12.14	116.64 ± 8.29	<0.001[a]
DBP (mm Hg)	90.12 ± 8.99	78.29 ± 7.42	<0.001[a]
TC (mg/dl)	213.91 ± 44.71	163.57 ± 31.58	<0.001[a]
TG (mg/dl)	157.12 ± 75.93	121.59 ± 45.90	<0.001[a]
LDL-C (mg/dl)	136.72 ± 41.03	94.67 ± 26.22	<0.001[a]
HDL-C (mg/dl)	45.09 ± 9.08	47.87 ± 9.13	0.041[a]
Cotinine (ng/ml)	2695 (1600–4880)	6 (4–10)	<0.001[b]

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triacylglycerol; LDL-C, low-density lipoprotein cholesterol; HDL-C, low-density lipoprotein cholesterol.
Date expressed as mean ± SD, except cotinine which is expressed as median (IQR).
p value < 0.05—significant; p value < 0.001—highly significant.
[a] Independent T-test.
[b] Mann–Whitney U test.

Table 3
Correlation of different parameters with cotinine in PMT user cases.

Variable	Correlation-coefficients (r)	P value
Duration in years	0.284	0.479
Quantity in pkt/day	0.637	<0.001
BMI (kg/m^2)	0.050	<0.001
SBP (mm Hg)	0.458	<0.001
DBP (mm Hg)	0.480	<0.001
TC (mg/dl)	0.523	<0.001
TG (mg/dl)	0.297	<0.001
LDL-C (mg/dl)	0.500	<0.001
HDL-C (mg/dl)	−0.620	0.385

Spearman's rho correlation. p value < 0.001 highly significant.
BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triacylglycerol; LDL-C, low-density lipoprotein cholesterol; HDL-C, low-density lipoprotein cholesterol.

Besides, PMT users were significantly hypertensive than controls. Both, mean SBP and DBP were significantly higher in cases than in controls ($p < 0.001$). By contrast, BMI was nearly identical in both groups and no significant different was seen ($p = 0.085$). Similarly, PMT users showed significant dyslipidemia as compared with controls. The parameters of lipid profile such as TC, TG, and LDL were considerably high in PMT users compared with controls ($p < 0.001$), with slightly low level of HDL (called good cholesterol) in PMT users ($p = 0.041$).

3.3. Correlation of urinary cotinine with different cardiovascular risk parameter

Table 3 illustrates the correlation of cotinine with various risk factors of CVD. It can be seen that mean BMI, SBP, DBP, TC, TG, LDL, and quantity of PMT showed moderate positive correlation with urine cotinine level. Likewise, packet per day and cotinine were strongly and positively correlated. The p value was <0.001 in all these correlations. However, duration of PMT use and HDL did not show any significant correlation.

3.4. Prediction cardiovascular risk factors

Table 4 shows the multivariate logistic regression analysis of different parameters of CV risk factors with cotinine, in PMT users. It indicates significantly high correlation of cotinine with gender, DBP, and TC. PMT users who are male and those having high cotinine level are in higher odds of having high DBP and elevated TC ($p = 0.045$, $p < 0.001$, $p < 0.001$, respectively) which are potential predictors of CVD. Those with high level of cotinine are about 20

Table 4
Univariate and multivariate analysis for predicting higher CV risk factors.

Category		Univariate			Multivariate		
		OR	95% CI	*p* value	OR	95% CI	*p* value
Gender	Female	Ref					
	Male	3.715	1.596–8.647	0.002	3.142	1.027–9.613	0.045
DBP	<90	Ref					
	≥90	15.589	7.205–33.727	<0.001	20.054	6.836–58.830	<0.001
TC	<200	Ref					
	≥200	9.723	4.652–20.324	<0.001	4.315	1.389–13.323	<0.001

p value < 0.05 significant, *p* value < 0.001 highly significant. Variables were entered into the multivariate logistic regression model with forward stepwise method.
DBP, diastolic blood pressure; TC, total cholesterol; CI, confidence interval; OR, odds ratio.

times likely to develop diastolic HTN (OR 20.05; $p < 0.001$) followed by 4 times likely to have hypercholesterolemia (OR: 4.32; $p < 0.001$). Odds ratio were adjusted for age and BMI. Although SBP, TG, and LDL were higher in PMT users and were positively correlated with cotinine level, however, they were found to be associated with cotinine level in univariate analysis only.

4. Discussion

The aim of this study was to determine the association of CV risk factors between PMT users (consuming different ST), and nonusers group and to find correlation of urinary cotinine value with CV risk parameters among PMT users.

According to our study, median cotinine level was significantly higher in PMT users 2695 (1600–4880) ng/ml than in nonusers 64–10 ng/ml. This is in accordance with several studies carried out in individuals taking different forms of ST.[13,19–22] Similarly, studies have reported that the use of ST causes higher nicotine level in serum and urine than smoking cigarettes. Prolonged retention in mouth, continuous absorption, frequent dose, swallowing of tobacco, etc., are considered to be responsible for higher level of nicotine and thus elevated urinary cotinine in ST users.[16,23]

Another important finding of our study is that the PMT users have elevated BP (diastolic BP [DBP] and systolic BP [SBP]) as compared with nonusers which is consistent with various studies carried out at different places.[9,24–27] It is speculated that the role of sustained vasoconstriction due to prolonged nicotine absorption, high salt concentration, and licorice occurring in ST can be a cause of HTN in PMT users.[15,24] A 15-year follow-up study conducted in Sweden found the development of HTN in ST users and were at relative risk of HTN of 1.43; in another population-based study, the risk of having DBP was 1.8 times and SBP was 1.7 times in ST users.[2,28] Similar to these findings, our study also showed the OR of having DBP was 20.1 in PMT users.

However, some studies[29–31] have failed to show an association between ST use and HTN. The discrepancies could be due to variation in subject characteristics and their origin, difference in susceptibility of subjects to various factors of HTN, differences in the study designs (cross-sectional vs. prospective) and adjustment for various confounding factors. Further studies are warranted to gain insight and understanding of this matter.

There was significant dyslipidemia in PMT users as compared with controls. Parameters of lipid profile except HDL were correlated with urinary cotinine level (Table 3). Findings of abnormal lipid parameters in ST users and their positive correlation with cotinine were in accordance with existing data.[15,29,30,32–35] In one study,[34] ST users had 2.5 times the adjusted risk of hypercholesterolemia and likewise our study also showed an OR of 4.3 in having hypercholesterolemia in PMT users. Lipolysis induced by nicotine increases free fatty acid concentrations. Increased fatty acid turnover is associated with overproduction of VLDL, TC, TGs, and LDL-C, but lower HDL-C may be responsible for dyslipidemia in PMT users.[36] In contrast to our study, several studies have shown no effect of ST consumption on lipid profile.[2]

It is speculated that tobacco consumers weigh less than nonuser and this can be recovered after cessation of tobacco consumption.[37] However, both cases and controls had normal BMI and the difference was not significant. This could be due to the fact that consuming PMT did not interfere with eating and subjects might have different eating habits. Our result was coherent with other studies,[25,38] but some showed conflicting results where BMI in tobacco user was significantly lower.[15,17,39] It is supposed that educational status, gender, ethnicity, tobacco consumption frequency, etc., affect the relationship between tobacco consumption and BMI. In addition, such relationship can change with time.[39]

We also found a significant positive correlation between urinary cotinine level and TC, TG, LDL, SBP, DBP, and packets per day, while HDL was negatively correlated [Table 3]. Decrease in HDL level with increase in cotinine was found in a study of subjects who are passively exposed to cigarette smoke.[40] By contrast, a study by Jensen et al did not find any positive correlation of urinary cotinine level with plasma lipid concentration in smokers and suggested contributory factors other than tobacco responsible for dyslipidemia.[37] The positive correlation of cotinine with number of packets per day in our study could be explained on the basis of findings of high nicotine level with increased tobacco consumption. Limited number of studies were carried out about the correlation of cotinine with CV risk factors. Our study and several others have shown the presence of dyslipidemia and HTN in tobacco user as compared with nonusers. Some studies, including ours, indicate the correlation of nicotine/cotinine with CVD, whereas some suggest other mechanisms, creating a controversy.

The findings of this study indicate that ST is harmful and should not be used as an alternative to safeguard the adverse effect of smoking. Cotinine level could be used as good prognosis of improving health, but routine use of cotinine assay for screening, diagnostic, and prognostic purpose would be too early to recommend. In a larger prospective and interventional study, dose-dependent effect of ST/cotinine correlating with CVD outcomes could be more informative.

5. Conclusion

Our study suggests that PMT use is closely associated with CV risk factors, which in turn is positively correlated with urinary cotinine level. Thus, cotinine measurement for screening CV risk among PMT users and the positive outcome of reducing cotinine level to decrease CVD mortality can be an important future scope of this study.

6. Limitations

Small sample size due to constrain in time and resource is the main limitation that could limit the significant findings of our

study. Findings of our study are expected in PMT users, but due to small sample size, the findings could be diluted in large population. Furthermore, we were unable to conduct the interventional or prospective study.

Funding

All the required resources was provided by Department of Biochemistry, BPKIHS, Dharan, Nepal.

Availability of data and materials

The datasets analyzed during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate and publication

Ethical approval of this study was granted by Institutional Ethical Review Board (IERB). Written consent was taken from all the participants before sampling and for publication of this study.

Authors' contributions

All the authors have significant contribution for the completion of this study and for its publication with their respective expertise area.

Conflict of interest

All authors have none to declare.

Acknowledgment

The authors are very thankful to the institute, faculties, juniors, seniors, staff, and participants for making this study complete.

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