

The mediating role of inflammation in the association between cigarette smoking and intima-media thickness

The Guangzhou biobank cohort study

Tao Wang, MS^a, Chao Qiang Jiang, MD^b, Lin Xu, PhD^{a,c,*}, Wei Sen Zhang, PhD^b, Feng Zhu, PhD^b, Ya Li Jin, MPhil^b, G. Neil Thomas, PhD^d, Kar Keung Cheng, PhD^d, Tai Hing Lam, MD^{b,c}

Abstract

Cigarette smoking is associated with thicker carotid intima-media thickness (IMT), probably partly through inflammatory pathways. However, to what extent does inflammation mediate the smoking-carotid atherosclerosis association is unclear. We investigated the mediating effect of inflammation on the association between cigarette smoking and carotid IMT, and quantified the respective contributions of inflammatory markers to this association.

A total of 1752 participants from Guangzhou Biobank Cohort Study-Cardiovascular Disease Sub-cohort (GBCS-CVD) were included. Using causal mediation analysis under the counterfactual framework, we decomposed total effects of cigarette smoking on IMT into indirect effects (through inflammatory response) and direct effects (not through inflammatory response).

After adjusting for traditional risk factors, the indirect effects of per 10⁹/L increment in leukocyte and granulocyte, per mg/L increment in high-sensitivity C-reactive protein (hs-CRP), and per mg/dL increment in fibrinogen on carotid IMT was 0.0028 mm (95% confidence interval [CI], 0.0011–0.0047), 0.0019 mm (95% CI, 0.0006–0.0034), 0.0017 mm (95% CI, 0.0006–0.003), and 0.001 mm (95% CI, 0.0001–0.0021), respectively. No evidence for a mediating role of lymphocyte was found. The proportion of the smoking-IMT association mediated by leukocyte, granulocyte, hs-CRP, and fibrinogen was 12.57% (95% CI, 8.50%–22.11%), 8.50% (95% CI, 5.76%–15.09%), 7.64% (95% CI, 5.20%–13.79%), and 4.48% (95% CI, 3.04%–8.03%), respectively. Restricting data analysis to men showed similar results.

The effects of cigarette smoking on IMT were partly mediated by leukocyte, hs-CRP, and fibrinogen. The mediating role of leukocyte was likely mainly driven by higher granulocyte.

Abbreviations: ACME = average causal mediation effect, ADE = average direct effect, ANOVA = analysis of variance, ATE = averaged total effects, BMI = body mass index, CI = confidence interval, CVD = cardiovascular disease, GBCS = The Guangzhou Biobank Cohort Study, GBCS-CVD = The Guangzhou Biobank Cohort Study-Cardiovascular Disease Sub-cohort, hs-CRP = high-sensitivity C-reactive protein, IMT = intima-media thickness, IPAQ = The International Physical Activity Questionnaire, SD = standard deviation, SI = sequential ignorability.

Keywords: atherosclerosis, cigarette smoking, inflammation, mediation analysis

1. Introduction

Cigarette smoking causes the development and progression of atherosclerosis,^[1,2] but the underlying mechanisms have yet to be confirmed.^[3,4] There are several potential mechanisms that could

be relevant, that is, inducing endothelial dysfunction, modifying lipid profile, and increasing inflammatory response and thrombosis.^[5,6] Thickening of carotid intima-media thickness (IMT) is a surrogate marker of early atherosclerosis and predicts the

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^a School of Public Health, Sun Yat-sen University, ^b Guangzhou No.12 Hospital, Guangzhou, China, ^c School of Public Health, the University of Hong Kong, Hong Kong, ^d Institute of Applied Health Research, University of Birmingham, Birmingham, UK.

* Correspondence: Lin Xu, School of Public Health, Sun Yat-sen University, 2nd Zhongshan Road, Guangzhou, Guangdong Province, China (e-mail: xulin27@mail.sysu.edu.cn).

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development of cardiovascular disease (CVD), independent of traditional CVD risk factors.^[7,8] While there was general agreement with the adverse effects of cigarette smoking and high inflammation on carotid IMT,^[1,2,9,10] whether and to what extent inflammation mediates the smoking–atherosclerosis association remains unclear.

Fibrinogen is a marker of inflammation and thrombosis. It has been associated with a higher risk of CVD^[11] and thicker IMT in some,^[12,13] but not all studies.^[14,15] Previous studies showed that smokers had higher levels of fibrinogen than non-smokers,^[16,17] and smoking might modify the association of fibrinogen with carotid IMT.^[9] However, whether fibrinogen mediates the association between smoking and carotid atherosclerosis remains unclear.

As much evidence, including our previous papers from the Guangzhou Biobank Cohort Study (GBCS),^[18,19] has shown the detrimental effects of smoking on carotid IMT,^[1,2] knowledge on the underlying mechanisms will be helpful for intervention strategies against smoking-induced atherosclerosis. We took advantage of the well-established GBCS using causal mediation analysis^[20,21] to investigate the mediating effects of several common inflammatory markers on the association between cigarette smoking and carotid IMT.

2. Methods

2.1. Study populations

The GBCS is a 3-way collaboration among the Guangzhou Number 12 Hospital in Guangzhou, China, and the Universities of Hong Kong and Birmingham. The study aims to examine the effects of genetic, environmental, lifestyle, and occupational factors on health problems and chronic diseases. The Guangzhou Biobank Cohort Study-Cardiovascular Disease Sub-cohort (GBCS-CVD), nested in the GBCS, included 1996 participants (992 [49.7%] men and 1004 [50.3%] women) examined during September 2006 to December 2007. A detailed description of the CVD sub-cohort has been published elsewhere.^[22,23] The study received ethical approval from the Guangzhou Medical Ethics Committee of the Chinese Medical Association, Guangzhou, China. All participants provided written informed consent before participation. All methods were performed in accordance with the relevant guidelines and regulations.

2.2. Exposure measurement

A computer-assisted standardized questionnaire was used to collect information of demographic characteristics, socioeconomic position, occupational exposure, family and personal disease history, and lifestyle factors including smoking status, alcohol use, and physical activity measured by the International Physical Activity Questionnaire (IPAQ). Reliability of the questionnaire was evaluated by recalling 200 randomly selected participants for re-interview and the results were satisfactory.^[24] Body height, weight, waist and hip circumference, and blood pressure were measured following standardized procedure. Body mass index (BMI) was calculated by weight (kg) divided by the square of body height (m²). Venous blood samples were collected after fasting for at least 8 hours for assay of lipids, fibrinogen, and high-sensitivity C-reactive protein (hs-CRP) levels. Assay of complete blood count including leukocyte count and differential (granulocyte and lymphocyte count) was performed in an automated hematology analyzer (KX-21, SYSMEX, Japan).

2.3. Study outcome

Carotid intima-media thickness (IMT) were measured by carotid B-mode color ultrasonography using ALT HDI 3000 mainframe with a high-resolution, linear array scanner (medium frequency 7.5 MHz) by a specialist physician.^[25] The operators were registered ultrasound doctors who had a professional certificate for color Doppler ultrasound measurement awarded by the Ministry of Health of China. All scans were performed following a predetermined, standardized scanning protocol for the right and left carotid arteries using images of the far wall of the distal 10 mm of the common carotid arteries. Three scanning angles, with the image focused on the posterior wall, were recorded from the angle showing the greatest distance between the lumen-intima interface and the media-adventitia interface. Carotid IMT measurements were performed off-line with the use of automated image analysis software. All scans were analyzed by the same physician, blinded to participants' information. Details of our carotid IMT measurement and research findings have been reported elsewhere.^[26,27]

2.4. Mediation analysis

To estimate the contribution of inflammation to the association between smoking and IMT, we used causal mediation analysis under the counterfactual framework, which can decompose the averaged total effects (ATE) into indirect (average causal mediation effect, ACME) and direct effects (average direct effect, ADE).^[28] Let $M_i(t)$ denotes the potential value of a mediator of interest for unit i under the positive exposure status $T_i=t$, $Y_i(t, m)$ denotes the potential outcome if the positive exposure status and mediating variables equal to t and m . As only one outcome was considered in the current study, the observed mediating and outcome variables were represented as $M_i=M_i(T_i)$ and $Y_i=Y_i(T_i, M_i[T_i])$, respectively. Following the potential outcomes notation, the indirect effects (δ_i) and direct effects (ξ_i) for each unit i and each treatment status $t=(0, 1)$ were defined as $\delta_i(t)=Y_i(t, M_i[1]) - Y_i(t, M_i[0])$ and $\xi_i(t)=Y_i(1, M_i[t]) - Y_i(0, M_i[t])$, respectively, and the ACME was defined as $\bar{\delta}(t)=E(Y_i[t, M_i1] - Y_i[t, M_i0])$. Similarly, the ADE was defined as $\bar{\xi}(t)=E(Y_i1, M_i(t) - Y_i0, M_i(t))$. As it is implausible to observe a counterfactual outcome $Y_i(t, M_i[1-t])$ with one observational unit, the estimation of ACME and ADE requires an additional assumption known as sequential ignorability (SI).^[21] The SI assumes that, firstly, given the observed pretreatment confounders, the treatment assignment is ignorable, and sequentially, given the actual treatment status and pretreatment confounders, the observed mediating status is ignorable. The causal effects could be estimated as function of the sensitivity parameter ρ .^[20,29] The equation between ρ and the coefficients of determination as below, $\rho^2 = R_M^{2*} R_Y^{2*} = \tilde{R}_M^2 \tilde{R}_Y^2 / ([1 - R_M^2][1 - R_Y^2])$, where R_M^{2*} and R_Y^{2*} represent the percentage of residual variance that is explained by the unmeasured confounders in the mediator and in the outcome, \tilde{R}_M^2 and \tilde{R}_Y^2 represent the proportion of variance that is explained by the unobserved confounder in the mediator and in the outcome. Values of ρ different from zero may imply that the SI assumption is violated, indicating that estimation of the mediation effect may be biased. In this study, we used the mediation package in Stata for the mediation analysis.^[30]

2.5. Statistical analysis

Continuous variables were presented as means (standard deviations [SD]), or medians [25th to 75th quartiles] for variables that were not normally distributed. Categorical variables were

presented as frequencies (percentages). Chi-square tests or analysis of variance (ANVOA) were used to compare participants' baseline characteristics by cigarette smoking status (never, former, and current smoking). The average IMT of the left and right common carotid artery was used for data analysis. Cigarette smoking was considered as the exposure variable, inflammation indicated by leukocyte and differential count (granulocyte and lymphocyte count), hs-CRP, and fibrinogen were considered as potential mediators, and IMT was considered as the study outcome. All tests of significance were 2-tailed, with $P < .05$ as statistically significant.

3. Results

Of 1996 participants in the GBCS-CVD sub-cohort, 1752 with complete information of interest were included in the current study, including 869 (49.6%) women and 883 (50.4%) men. Table 1 shows that 1210 (69.06%) were never, 237 (13.53%) were former, and 305 (17.51%) were current smokers. Former smokers were older than never or current smokers ($P < .001$). Most former and current smokers were men (98.31%, 97.38%, respectively). Compared with never smokers, current smokers were less physically active, had more with manual occupation and alcohol use, and had lower levels of BMI and low density lipoprotein (LDL) cholesterol (all $P < .05$). They also had higher levels of leukocyte, lymphocyte, granulocyte, hs-CRP, fibrinogen, and triglycerides, and a thicker IMT (all $P < .001$).

Table 2 shows that, after adjustment for sex, age, occupation, education, physical activity, alcohol use, triglycerides, and LDL cholesterol, the association of cigarette smoking with IMT was

significantly mediated by leukocyte, granulocyte, hs-CRP, and fibrinogen. The indirect effect of leukocyte, granulocyte, hs-CRP, and fibrinogen on carotid IMT was 0.0028 mm (95% confidence interval [CI], 0.0011–0.0047), 0.0019 mm (95% CI, 0.0006–0.0034), 0.0017 mm (95% CI, 0.0006–0.003), and 0.001 mm (95% CI, 0.0001–0.0021), respectively. The proportion of the association between cigarette smoking and IMT mediated by leukocyte, granulocyte, hs-CRP, and fibrinogen was 12.57% (95% CI, 8.50%–22.11%), 8.50% (95% CI, 5.76%–15.09%), 7.64% (95% CI, 5.20%–13.79%), and 4.48% (95% CI 3.04%, 8.03%), respectively. No evidence for a mediating role of lymphocyte was found. As few women (4.31%) smoked in our study population, we conducted sensitivity analyses by excluding women and found similar results (Table 3). In men, the proportion of the cigarette smoking–IMT association mediated by leukocyte, granulocyte, hs-CRP, and fibrinogen was 13.07% (95% CI, 8.96%–22.78%), 9.04% (95% CI, 6.20%–16.01%), 6.77% (4.69%–12.04%), and 7.46% (95% CI, 5.16%–13.26%), respectively (Table 3). To eliminate any influence due to the use of anti-inflammation agents, we also conducted sensitivity analysis excluding 310 participants using blood pressure lowering agents and 165 using nonsteroidal anti-inflammatory drugs from the mediation analysis and found that the results remained (Table 4).

Results of sensitivity analyses support the robustness of the mediation results. Table 5 shows that for the point estimate of the ACME by leukocyte to be zero, ρ value should at least be 0.0768, which means that the direction of ACME by leukocyte would remain positive unless the ρ value > 0.0768 . The products of the

Table 1
Demographic characteristics of the participants by smoking status in 1752 participants in GBCS-CVD.

	Smoking status			P-value
	Never (n = 1210)	Former (n = 237)	Current (n = 305)	
Age, y, mean (SD)	57.99 (6.41)	63.39 (7.00)	60.51 (6.27)	<.001
Men, n (%)	353 (29.17)	233 (98.31)	297 (97.38)	<.001
Occupation, n (%)				.02
Manual	281 (23.22)	70 (29.54)	92 (30.16)	
Non-manual	464 (38.35)	93 (39.24)	115 (37.7)	
Others	465 (38.43)	74 (31.22)	98 (32.13)	
Education, n (%)				.09
≤Primary	302 (24.96)	70 (29.54)	95 (31.15)	
Middle school	744 (61.49)	142 (59.92)	180 (59.02)	
≥College	164 (13.55)	25 (10.55)	30 (9.84)	
Alcohol drinking, n (%)				<.001
Current	658 (54.74)	169 (71.61)	223 (73.36)	
Former	27 (2.25)	13 (5.51)	10 (3.29)	
Never	517 (43.01)	54 (22.88)	71 (23.36)	
IPAQ physical activity, n (%)				<.001
High	759 (62.73)	143 (60.34)	148 (48.52)	
Moderate	337 (27.85)	71 (29.96)	115 (37.7)	
Low	114 (9.42)	23 (9.7)	42 (13.77)	
BMI, kg/m ² , mean (SD)	23.75 (2.98)	24.02 (2.83)	23.16 (3.12)	.002
Leukocyte, ×10 ⁹ /L, mean (SD)	6.12 (1.46)	6.65 (1.69)	7.23 (1.59)	<.001
Lymphocyte, ×10 ⁹ /L, mean (SD)	2.12 (0.58)	2.11 (0.56)	2.35 (0.67)	<.001
Granulocyte, ×10 ⁹ /L, mean (SD)	3.63 (1.15)	4.06 (1.49)	4.33 (1.22)	<.001
hs-CRP, mg/L, geometric mean (95% CI)	1.32 (1.25–1.41)	1.79 (1.55–2.07)	1.58 (1.40–1.79)	<.001
Fibrinogen, mg/dL, mean (SD)	296.99 (77.16)	304.77 (104.35)	314.56 (90.07)	<.001
Triglycerides, mmol/L, geometric mean (95% CI)	1.50 (1.46–1.55)	1.68 (1.57–1.79)	1.56 (1.46–1.66)	<.001
LDL cholesterol, mmol/L, mean (SD)	3.43 (0.69)	3.31 (0.64)	3.21 (0.63)	<.001
IMT, mm, mean (SD)	0.71 (0.12)	0.80 (0.15)	0.80 (0.15)	<.001

BMI = body mass index, CI = confidence interval, GBCS-CVD = The Guangzhou biobank cohort study-cardiovascular disease sub-cohort, hs-CRP = high sensitivity C-reactive protein, IMT = intima-media thickness, IPAQ = international physical activity questionnaire, LDL = low density lipoprotein, SD = standard deviation.

Table 2**Association between smoking and carotid IMT (mm) with mediation by leukocyte, lymphocyte, granulocyte, hs-CRP, and fibrinogen in 1752 participants in GBCS-CVD.**

Mediators	Indirect effect (ACME) Estimate (95% CI)	Direct effect (ADE) Estimate (95% CI)	Total effect Estimate (95% CI)	Proportion via mediation % (95% CI)
Leukocyte, $\times 10^9/L$	0.0028 (0.0011, 0.0047)	0.0195 (0.0092, 0.0293)	0.0222 (0.0125, 0.0325)	12.57% (8.50%, 22.11%)
Lymphocyte, $\times 10^9/L$	0.0009 (-0.0003, 0.0024)	0.0213 (0.0111, 0.0311)	0.0223 (0.0122, 0.0324)	4.24% (2.89%, 7.68%)
Granulocyte, $\times 10^9/L$	0.0019 (0.0006, 0.0034)	0.0204 (0.0102, 0.0302)	0.0223 (0.0124, 0.0324)	8.50% (5.76%, 15.09%)
hs-CRP, mg/L	0.0017 (0.0006, 0.003)	0.0206 (0.0104, 0.0303)	0.0223 (0.0122, 0.0324)	7.64% (5.20%, 13.79%)
Fibrinogen, mg/dL	0.0010 (0.0001, 0.0021)	0.0213 (0.0111, 0.031)	0.0223 (0.0123, 0.0325)	4.48% (3.04%, 8.03%)

The mediation analysis models were adjusted for age, sex, drinking status, education, occupation, BMI, physical activity, low density lipoprotein cholesterol, and triglycerides.

ACME = average causal mediation effect, ADE = average direct effect, CI = confidence interval, GBCS-CVD = The Guangzhou biobank cohort study-cardiovascular disease sub-cohort, hs-CRP = high sensitivity C-reactive protein, IMT = intima-media thickness.

Table 3**Association between smoking and carotid IMT (mm) with mediation by leukocyte, lymphocyte, granulocyte, hs-CRP, fibrinogen in 883 men in GBCS-CVD.**

Mediators	Indirect effect (ACME) Estimate (95% CI)	Direct effect (ADE) Estimate (95% CI)	Total effect Estimate (95% CI)	Proportion via mediation % (95% CI)
Leukocyte, $\times 10^9/L$	0.0033 (0.0007, 0.0063)	0.0222 (0.0105, 0.0336)	0.0255 (0.0146, 0.0372)	13.07% (8.96%, 22.78%)
Lymphocyte, $\times 10^9/L$	0.0008 (-0.0012, 0.0029)	0.0247 (0.0131, 0.036)	0.0255 (0.0143, 0.0368)	3.25% (2.25%, 5.81%)
Granulocyte, $\times 10^9/L$	0.0023 (0.0003, 0.0046)	0.0232 (0.0116, 0.0345)	0.0255 (0.0144, 0.0372)	9.04% (6.20%, 16.01%)
hs-CRP, mg/L	0.0017 (0.0003, 0.0036)	0.0238 (0.0123, 0.035)	0.0255 (0.0144, 0.037)	6.77% (4.69%, 12.04%)
Fibrinogen, mg/dL	0.0019 (0.0003, 0.0039)	0.0236 (0.0121, 0.0348)	0.0255 (0.0144, 0.037)	7.46% (5.16%, 13.26%)

The mediation analysis models were adjusted for age, drinking status, education, occupation, BMI, physical activity, low density lipoprotein cholesterol and triglyceride.

ACME = average causal mediation effect, ADE = average direct effect, CI = confidence interval, GBCS-CVD = The Guangzhou biobank cohort study-cardiovascular disease sub-cohort, hs-CRP = High sensitivity C-reactive protein, IMT = intima-media thickness

Table 4**Association between smoking and carotid IMT (mm) with mediation by leukocyte, lymphocyte, granulocyte, hs-CRP, and fibrinogen in GBCS-CVD, excluding participants using blood pressure lowering agents and nonsteroidal anti-inflammatory drugs.**

Mediators	Indirect effect (ACME) Estimate (95% CI)	Direct effect (ADE) Estimate (95% CI)	Total effect Estimate (95% CI)	Proportion via mediation % (95% CI)
Leukocyte, $\times 10^9/L$	0.0029 (0.0009, 0.0050)	0.0167 (0.0061, 0.0270)	0.0196 (0.0094, 0.0301)	14.83% (9.50%, 30.28%)
Lymphocyte, $\times 10^9/L$	0.0007 (-0.0006, 0.0022)	0.0189 (0.0083, 0.0289)	0.0196 (0.0093, 0.0301)	3.74% (2.41%, 7.85%)
Granulocyte, $\times 10^9/L$	0.0020 (0.0006, 0.0037)	0.0176 (0.0070, 0.0277)	0.0196 (0.0094, 0.0300)	10.58% (6.81%, 21.68%)
hs-CRP, mg/L	0.0018 (0.0006, 0.0032)	0.0188 (0.0074, 0.0278)	0.0196 (0.0093, 0.0301)	9.09% (5.87%, 19.01%)
Fibrinogen, mg/dL	0.0011 (0.0001, 0.0023)	0.0185 (0.0080, 0.0285)	0.0196 (0.0094, 0.0301)	5.73% (3.68%, 11.87%)

The mediation analysis models were adjusted for age, sex, drinking status, education, occupation, BMI, physical activity, low density lipoprotein cholesterol and triglycerides.

ACME = average causal mediation effect, ADE = average direct effect, CI = confidence interval, GBCS-CVD = The Guangzhou biobank cohort study-cardiovascular disease sub-cohort, hs-CRP = High sensitivity C-reactive protein, IMT = intima-media thickness.

Table 5**Sensitive analysis examining the violation of sequential ignorability assumption.**

	ρ at which ACME = 0	$R_M^{2*} R_Y^{2*}$	$\tilde{R}_M^2 \tilde{R}_Y^2$
Leukocyte, $\times 10^9/L$	0.0768	0.0059	0.0042
Lymphocyte, $\times 10^9/L$	0.0343	0.0012	0.0009
Granulocyte, $\times 10^9/L$	0.0712	0.0051	0.0038
hs-CRP, mg/L	0.1015	0.0103	0.0081
Fibrinogen, mg/dL	0.0501	0.0025	0.002

ACME = average causal mediation effect, R_M^{2*} = proportion of the unexplained variance explained by the unobserved confounder in the mediator, R_Y^{2*} = proportion of the unexplained variance explained by the unobserved confounder in the outcome, \tilde{R}_M^2 = proportion of variance that is explained by the unobserved confounder in the mediator, \tilde{R}_Y^2 = proportion of variance that is explained by the unobserved confounder in the outcome.

determination coefficients at which ACME by leukocyte being zero were $R_M^{2*} R_Y^{2*} = 0.0059$ and $\tilde{R}_M^2 \tilde{R}_Y^2 = 0.0042$. This means ACME would be positive unless the confounders explained at least 7.7% of residual variance (square root of 0.0059) in the mediator and in the outcome, though other combinations are possible. Similarly, ACME would be positive unless the omitted confounders explained >6.5% of the original variance (square root of 0.0042) in the mediator and in the outcome.

4. Discussion

Our study provided for the first time a quantitative assessment of respective contributions of several common inflammatory factors to the association between cigarette smoking and IMT based on a well-conducted population-based study. We found that of the

selected inflammatory markers including leukocyte and subtypes, hs-CRP and fibrinogen, leukocyte counts mediated the effects of smoking on IMT by a greatest proportion (12.57%), followed subsequently by hs-CRP (7.64%) and fibrinogen (4.48%). Furthermore, we found that the mediating effect of leukocyte might be mainly due to granulocyte rather than lymphocyte.

Previous studies showed that cigarette smoking increased multiple inflammatory markers including leukocyte counts and differential (neutrophil, lymphocyte, monocyte counts), hs-CRP, fibrinogen, and interleukin-6.^[31–33] In addition, cigarette smoking was associated with development and progression of carotid atherosclerosis, probably partly through the inflammation pathway.^[34] Accumulating evidence has shown a positive association between inflammation and carotid atherosclerosis. A meta-analysis based on 20 large prospective cohort studies within the PROG-IMT (individual progression of carotid intima media thickness as a surrogate of vascular risk) collaboration involving 49,097 participants from the general population showed positive associations between 3 markers of inflammation (leukocyte count, hs-CRP, fibrinogen) and carotid common artery (CCA) IMT at baseline.^[10] However, no evidence for an association between baseline inflammatory markers with IMT progression after adjusting for traditional risk factors was found, probably because of imprecise measurements of IMT progression during only a few years (an average follow-up of 3.9 years). A large longitudinal study showed that baseline leukocyte count was significantly associated with thickening of IMT over a 9-year follow up period.^[35] Similarly, some cross-sectional studies also reported a positive association between leukocyte count and IMT in middle-aged free-living men^[36] and in patients with diabetes.^[37,38] Thus, the evidence above supports the pathological pathways from smoking to atherosclerosis through inflammation. However, we found only one study investigating the mediating effect of inflammation on the association between smoking and atherosclerosis. This study showed that the detrimental effects of smoking on progression of carotid IMT did not change after adjusting for hs-CRP and WBC, suggesting hs-CRP and WBC did not mediate the association.^[39] However, in this longitudinal study with 16 years of follow-up, smoking status and inflammatory markers assessed at baseline might change during the follow-up. Misclassification of both exposure and mediating variables was likely to explain the null mediation results. Our findings based on accurate measurement may minimize such misclassification bias and extend our understanding of the mechanisms for the smoking-induced atherosclerosis.

Results of the association between fibrinogen and IMT have been less consistent. The Coronary Artery Risk Development in Young Adults (CARDIA) study^[13] showed that elevated fibrinogen concentrations were independently associated with thicker IMT. Another study of participants free of clinical cardiovascular disease also reported a positive association between plasma fibrinogen concentrations and carotid IMT.^[40] However, in a study of patients with atherosclerosis, no association between fibrinogen and IMT was found.^[14] Our mediation analysis showed that fibrinogen was significantly associated with carotid IMT, and might explain in part the association between cigarette smoking and carotid IMT. The results were similar after excluding women. Some possible mechanisms have been suggested. Cytokines such as interleukin-6 significantly increase fibrinogen transcription, a possible source of these cytokines is the recruitment of macrophages to the lungs due to damage caused by smoking.^[41] Other possible explan-

ations include the effects on plaque composition, blood viscosity, endothelial and smooth muscle cell activation, platelet aggregation and activation, and immune cell recruitment,^[42,43] although the exact mechanisms warrant further investigation.

The strengths of this study include the comprehensive measurement and adjustment of potential confounding factors, and the application of a mediation analysis in quantifying mediation effect due to inflammation. There are some limitations to consider in interpreting our results. First, the causal inference for the associations between smoking and inflammation, as well as inflammation and carotid IMT might not be confirmed in the current cross-sectional analysis. However, much evidence for the causal associations between smoking and inflammation, and between inflammation and carotid IMT have been consistently reported in previous studies.^[34,44,45] Thus, the causal pathways from smoking to atherosclerosis through inflammation is biologically plausible. Secondly, inflammatory factors were measured only at a single time point, which might not reflect a long-term inflammatory response. Third, some inflammatory factors associated with cardiovascular disease in previous studies, such as interleukin 6 or interleukin-1 β ,^[46,47] were not measured in our study. Fourth, the calculation of ACME and ADE effects requires a SI assumption, which is a strong assumption in a non-trial setting. Although adjusting for potential confounders might to some extent mitigate this concern, this assumption cannot be perfectly tested (i.e., it is nonrefutable) by using observational data.^[48] We conducted a sensitivity analysis to examine the violation of the SI assumption and found that the probability for the violation (indicating by the value of ρ and $\tilde{R}_M 2\tilde{R}_Y 2$ for the ACME to be 0) was low, supporting the robustness of the mediation results. Further studies in other settings to replicate our results are warranted. Finally, due to financial constraints, not all GBCS participants had IMT measured, and the sample size in the current analysis was relatively small.

In conclusion, our study showed that the effects of cigarette smoking on IMT were partly mediated by leukocyte, high sensitivity C-reactive protein, and fibrinogen. Of the inflammatory factors, leukocyte appeared to mediate much of the effect of smoking-induced atherosclerosis, and the mediating role was mainly driven by granulocyte rather than lymphocyte.

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The Guangzhou Biobank Cohort Study investigators include: Guangzhou No. 12 Hospital: WSZ, MC, TZ, BL, CQJ (Co-PI); The University of Hong Kong: CMS, SMM, GML, RF, THL (Co-PI); The University of Birmingham: PA, GNT, KKC (Co-PI).

Author contributions

Conceptualization: Lin Xu.

Data curation: Wei Sen Zhang, Feng Zhu, Ya Li Jin.

Formal analysis: Tao Wang, Chao Qiang Jiang, Lin Xu, Tai Hing Lam.

Supervision: Wei Sen Zhang.

Writing – original draft: Tao Wang, Chao Qiang Jiang, Lin Xu, G. Neil Thomas, Kar Keung Cheng, Tai Hing Lam.

Writing – review & editing: Chao Qiang Jiang, Lin Xu, Kar Keung Cheng, Tai Hing Lam.

Tao Wang orcid: 0000-0002-9231-9371

Lin Xu orcid: 0000-0002-0537-922X.

References

- [1] Kunutsor SK, Spee JM, Kieneker LM, et al. Self-reported smoking, urine cotinine, and risk of cardiovascular disease: findings from the PREVENT (Prevention of Renal and Vascular End-Stage Disease) Prospective Cohort Study. *J Am Heart Assoc* 2018;7:e008726.
- [2] McEvoy JW, Blaha MJ, DeFilippis AP, et al. Cigarette smoking and cardiovascular events: role of inflammation and subclinical atherosclerosis from the MultiEthnic Study of Atherosclerosis. *Arterioscler Thromb Vasc Biol* 2015;35:700–9.
- [3] Siasos G, Tsigkou V, Kokkou E, et al. Smoking and atherosclerosis: mechanisms of disease and new therapeutic approaches. *Curr Med Chem* 2014;21:3936–48.
- [4] Unverdorben M, von Holt K, Winkelmann BR. Smoking and atherosclerotic cardiovascular disease: part II: role of cigarette smoking in cardiovascular disease development. *Biomark Med* 2009;3:617–53.
- [5] Ambrose JA, Barua RS. The pathophysiology of cigarette smoking and cardiovascular disease: an update. *J Am Coll Cardiol* 2004;43:1731–7.
- [6] Messner B, Bernhard D. Smoking and cardiovascular disease: mechanisms of endothelial dysfunction and early atherogenesis. *Arterioscler Thromb Vasc Biol* 2014;34:509–15.
- [7] Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature* 2011;473:317–25.
- [8] Lorenz MW, Markus HS, Bots ML, et al. Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis - response. *Circulation* 2007;115:459–67.
- [9] Cho HM, Kang DR, Kim HC, et al. Association between fibrinogen and carotid atherosclerosis according to smoking status in a Korean Male population. *Yonsei Med J* 2015;56:921–7.
- [10] Willeit P, Thompson SG, Agewall S, et al. Inflammatory markers and extent and progression of early atherosclerosis: Meta-analysis of individual-participant-data from 20 prospective studies of the PROG-IMT collaboration. *Eur J Prev Cardiol* 2016;23:194–205.
- [11] Danesh J, Lewington S, et al. Fibrinogen Studies Collaboration Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. *JAMA* 2005;294:1799–809.
- [12] Green D, Chan C, Kang J, et al. Longitudinal assessment of fibrinogen in relation to subclinical cardiovascular disease: the CARDIA study. *J Thromb Haemost* 2010;8:489–95.
- [13] Green D, Foiles N, Chan C, et al. Elevated fibrinogen levels and subsequent subclinical atherosclerosis: the CARDIA Study. *Atherosclerosis* 2009;202:623–31.
- [14] Hayashi S. Significance of plasma D-dimer in relation to the severity of atherosclerosis among patients evaluated by non-invasive indices of cardio-ankle vascular index and carotid intima-media thickness. *Int J Hematol* 2010;92:76–82.
- [15] Sekikawa A, Ueshima H, Kadowaki T, et al. Less subclinical atherosclerosis in Japanese men in Japan than in White men in the United States in the post-World War II birth cohort. *Am J Epidemiol* 2007;165:617–24.
- [16] Bazzano LA, He J, Muntner P, et al. Relationship between cigarette smoking and novel risk factors for cardiovascular disease in the United States. *Ann Intern Med* 2003;138:891–7.
- [17] Kannel WB, D'Agostino RB, Belanger AJ. Fibrinogen, cigarette smoking, and risk of cardiovascular disease: insights from the Framingham Study. *Am Heart J* 1987;113:1006–10.
- [18] Jiang CQ, Xu L, Lam TH, et al. Smoking cessation and carotid atherosclerosis: the Guangzhou Biobank Cohort Study-CVD. *J Epidemiol Community Health* 2010;64:1004–9.
- [19] Jiang CQ, Xu L, Lin JM, et al. [Dose-response relationship between smoking status and carotid atherosclerosis]. *Zhonghua Liu Xing Bing Xue Za Zhi* 2010;31:983–7.
- [20] Imai K, Keele L, Tingley D. A general approach to causal mediation analysis. *Psychol Methods* 2010;15:309–34.
- [21] Imai K, Keele L, Yamamoto T. Identification, inference and sensitivity analysis for causal mediation effects. *Stat Sci* 2010;25:51–71.
- [22] Jiang C, Thomas GN, Lam TH, et al. Cohort profile: The Guangzhou Biobank Cohort Study, a Guangzhou-Hong Kong-Birmingham collaboration. *Int J Epidemiol* 2006;35:844–52.
- [23] Jiang CQ, Lam TH, Lin JM, et al. An overview of the Guangzhou biobank cohort study-cardiovascular disease subcohort (GBCS-CVD): a platform for multidisciplinary collaboration. *J Hum Hypertens* 2010;24:139–50.
- [24] Jiang CQ, Lao XQ, Yin P, et al. Smoking, smoking cessation and aortic arch calcification in older Chinese: the Guangzhou Biobank Cohort Study. *Atherosclerosis* 2009;202:529–34.
- [25] Poredos P. Intima-media thickness: indicator of cardiovascular risk and measure of the extent of atherosclerosis. *Vasc Med* 2004;9:46–54.
- [26] Xu L, Jiang CQ, Lam TH, et al. Impact of impaired fasting glucose and impaired glucose tolerance on arterial stiffness in an older Chinese population: the Guangzhou Biobank Cohort Study-CVD. *Metabolism* 2010;59:367–72.
- [27] Xu L, Jiang CQ, Lam TH, et al. The metabolic syndrome is associated with subclinical atherosclerosis independent of insulin resistance: the Guangzhou Biobank Cohort Study-CVD. *Clin Endocrinol (Oxf)* 2010;73:181–8.
- [28] Imai K, Keele L, Tingley D, et al. Unpacking the Black Box of Causality: learning about causal mechanisms from experimental and observational studies. *Am Polit Sci Rev* 2011;105:765–89.
- [29] Imai K, Tingley D, Yamamoto T. Experimental designs for identifying causal mechanisms. *J R Stat Soc A* 2013;176:5–1.
- [30] Hicks R, Tingley D. Causal mediation analysis. *Stata J* 2011;11:605–19.
- [31] McEvoy JW, Nasir K, DeFilippis AP, et al. Relationship of cigarette smoking with inflammation and subclinical vascular disease: the Multi-Ethnic Study of Atherosclerosis. *Arterioscler Thromb Vasc Biol* 2015;35:1002–10.
- [32] Smith MR, Kinmonth AL, Luben RN, et al. Smoking status and differential white cell count in men and women in the EPIC-Norfolk population. *Atherosclerosis* 2003;169:331–7.
- [33] Tibuakuu M, Kamimura D, Kianoush S, et al. The association between cigarette smoking and inflammation: The Genetic Epidemiology Network of Arteriopathy (GENOA) study. *PLoS One* 2017;12:e0184914.
- [34] Liang LR, Wong ND, Shi P, et al. Cross-sectional and longitudinal association of cigarette smoking with carotid atherosclerosis in Chinese adults. *Prev Med* 2009;49:62–7.
- [35] Chambless LE, Folsom AR, Davis V, et al. Risk factors for progression of common carotid atherosclerosis: the Atherosclerosis Risk in Communities Study, 1987-1998. *Am J Epidemiol* 2002;155:38–47.
- [36] Loimaala A, Rontu R, Vuori I, et al. Blood leukocyte count is a risk factor for intima-media thickening and subclinical carotid atherosclerosis in middle-aged men. *Atherosclerosis* 2006;188:363–9.
- [37] Cardellini M, Marini MA, Frontoni S, et al. Carotid artery intima-media thickness is associated with insulin-mediated glucose disposal in nondiabetic normotensive offspring of type 2 diabetic patients. *Am J Physiol Endocrinol Metab* 2007;292:E347–52.
- [38] Temelkova-Kurktschiev T, Koehler C, Henkel E, et al. Leukocyte count and fibrinogen are associated with carotid and femoral intima-media thickness in a risk population for diabetes. *Cardiovasc Res* 2002;56:277–83.
- [39] Hansen K, Ostling G, Persson M, et al. The effect of smoking on carotid intima-media thickness progression rate and rate of lumen diameter reduction. *Eur J Intern Med* 2016;28:74–9.
- [40] Paramo JA, Beloqui O, Roncal C, et al. Validation of plasma fibrinogen as a marker of carotid atherosclerosis in subjects free of clinical cardiovascular disease. *Haematologica* 2004;89:1226–31.
- [41] Humphries SE, Ye S, Talmud P, et al. European Atherosclerosis Research Study: genotype at the fibrinogen locus (G-455-A beta-gene) is associated with differences in plasma fibrinogen levels in young men and women from different regions in Europe. Evidence for gender-genotype-environment interaction. *Arterioscler Thromb Vasc Biol* 1995;15:96–104.
- [42] Davalos D, Akassoglou K. Fibrinogen as a key regulator of inflammation in disease. *Semin Immunopathol* 2012;34:43–62.
- [43] Tousoulis D, Papageorgiou N, Androulakis E, et al. Fibrinogen and cardiovascular disease: genetics and biomarkers. *Blood Rev* 2011;25:239–45.
- [44] Kume A, Kume T, Masuda K, et al. Dose-dependent effects of cigarette smoke on blood biomarkers in healthy Japanese Volunteers: observations from smoking and non-smoking. *J Health Sci* 2009;55:259–64.
- [45] Sunyer J, Munoz A, Peng Y, et al. Longitudinal relation between smoking and white blood cells. *Am J Epidemiol* 1996;144:734–41.
- [46] Sarwar N, Butterworth AS, et al. Collaboration IRGCFERInterleukin-6 receptor pathways in coronary heart disease: a collaborative meta-analysis of 82 studies. *Lancet* 2012;379:1205–13.
- [47] McCarty S, Frishman W. Interleukin 1beta: a proinflammatory target for preventing atherosclerotic heart disease. *Cardiol Rev* 2014;22:176–81.
- [48] Lancaster T. Identification for prediction and decision. *Econ J* 2009;119:F404–9.