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Leukocyte telomere length, mitochondrial DNA copy number, and coronary artery disease risk and severity: A two-stage case-control study of 3064 Chinese subjects

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HIGHLIGHTS

- Lower leukocyte telomere length (TL) and mitochondrial DNA copy number (mtDNA-CN) independently increased coronary artery disease (CAD) risk.
- The aggregated score of TL and mtDNA-CN may better predict CAD risk and severity.
- The aggregated score may inversely correlate with markers of oxidative stress.

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ABSTRACT

Background and aims: Leukocyte telomere length (TL) and mitochondrial DNA copy number (mtDNA-CN), as hallmarks of cellular aging, may be involved in the development of coronary artery disease (CAD) by modulating oxidative stress. This study aimed to investigate the effects of leukocyte TL and mtDNA-CN alone or in combination on CAD risk and severity in the Chinese population.

Methods: In this two-stage case-control study with 1511 CAD patients and 1553 controls, leukocyte TL and mtDNA-CN were determined by a quantitative PCR assay. Three oxidative parameters, including leukocyte 8-hydroxy-2′-deoxyguanosine (8-OHdG), plasma malondialdehyde, and plasma reactive oxygen species (ROS), were quantified by ELISA or colorimetric kits in a subset of 129 cases and 129 controls.

Results: In the combined cohort, each 1-SD decrease in TL and mtDNA-CN was significantly associated with a 1.17-fold and 1.14-fold increased risk of CAD ($p < 0.001$ for all), respectively, after adjusting for confounders. The aggregated score, which reflected the cumulative dosage of the tertiles of TL and mtDNA-CN, showed inverse dose-response correlations with CAD risk ($p_{trend} < 0.001$), and severity, as determined by the severity of clinical presentations ($p_{trend} = 0.037$), the presence of multi-vessel CAD ($p_{trend} = 0.004$), and modified Gensini scores ($p_{trend} = 0.009$). Similar dose-response relations of the aggregated score to leukocyte 8-OHdG and plasma ROS were also identified.

Conclusions: Our data suggested reductions in both TL and mtDNA-CN as independent risk factors for CAD. The combination of TL and mtDNA-CN might jointly contribute to CAD risk, CAD severity, and oxidative stress.

1. Introduction

Coronary artery disease (CAD), the leading cause of morbidity and mortality worldwide [1], is a continuum that extends from the deposition of lipoproteins in coronary arteries to the development of plaque vulnerability and ultimately to myocardial infarction (MI) [2]. A crucial pathway for this continuum is the accelerated process of cellular aging resulting from oxidative stress and progressive inflammation [3,4]. Thus, as two hallmarks of cardiovascular aging [5,6], telomere attrition and mitochondrial dysfunction, may involve the initiation and progression of CAD [4].

Telomeres, the nucleoprotein complexes that cap chromosomal ends and maintain genomic stability, progressively shorten with consecutive cell divisions [7]. Uncontrolled telomeres attrition may trigger

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chromosomal degradation, replicative senescence, or oxidative stress in somatic cells, which have been implicated in age-related diseases, including CAD [8]. Mitochondrial are cellular energy-producing organelles involved in the generation of adenosine triphosphate and regulation of oxidative stress [9]. Mitochondrial dysfunction, caused by mitochondrial DNA (mtDNA) damage with advancing age, may promote atherogenesis through an increase in reactive oxygen species (ROS) production, which in turn induces abnormal mitochondrial abundance and forms a vicious cycle of mitochondrial impairment [10]. MtDNA copy number (mtDNA-CN), a determinant of mtDNA content per cell, serves as a surrogate marker of mitochondrial function [11], with casual effects on mitochondrial respiration and mitochondrial enzyme activity during vascular aging [12,13]. Recently, growing evidence showed that telomere attrition and mitochondrial dysfunction were statistically linked [14] and functionally co-regulated [15,16], suggesting that alterations in telomere length (TL) and mtDNA-CN might reflect the cumulative exposure to oxidative stress and joint involvement in the progression of CAD.

In most epidemiological studies for Caucasians, shortened leukocyte TL was considered as a causal risk factor for atherosclerotic cardiovascular disease [5], especially for MI [17]. While in Chinese, established data yielded mixed results. In Yang et al. study, mean leukocyte TL was first reported to predict incident CAD in 441 Chinese subjects [18]. However, this significant finding was failed to replicate in a Chinese cohort of 858 patients with first stroke, highlighting the necessity of larger studies to address the association between leukocyte TL and CAD risk in the Chinese population [19]. For mtDNA-CN, a recent meta-analysis of three multiethnic cohorts indeed showed that leukocyte mtDNA-CN could be a potential indicator to improve risk prediction and reclassification for primary prevention of CAD [20]. Yet it is still unclear whether leukocyte mtDNA-CN, in combination with TL, may jointly increase their predictive values for CAD risk.

Hence, to gain a comprehensive assessment of how telomere attrition and mitochondrial dysfunction modulated CAD, we investigated the effects of leukocyte TL and mtDNA-CN alone or in combination on CAD risk, CAD severity, cardiometabolic phenotypes, and oxidative parameters (ROS levels, DNA and lipid oxidative damage) in a two-stage case-control study with 3064 Chinese subjects.

2. Materials and methods

2.1. Study population

This was an ongoing two-stage case-control study, with a total of 1511 CAD patients and 1553 controls. The discovery stage, composed of 506 CAD patients and 509 controls, was assembled from The First Affiliated Hospital of Zhengzhou University at Henan Province, northern China between August 2017 and September 2018. The replication stage included 1005 cases and 1044 controls, recruited from two clinical centers (Zhongnan Hospital of Wuhan University and Wuhan Asia Heart Hospital) at Hubei Province, central China between September 2012 and May 2017. All participants self-reported as Han Chinese.

CAD was defined as angiographically demonstrated stenosis of > 50% in major coronary vessel, with or without clinical symptoms of angina, negative changes of cardiac biomarkers, and typical electrocardiographic patterns. Then, based on the severity of clinical presentations of CAD, each case was classified into three groups: (1) stable angina pectoris (SAP); (2) unstable angina pectoris (UAP); (3) MI [21]. Finally, CAD severity was further quantified by modified Gensini scores and the presence of multi-vessel CAD (Supplementary Materials and Methods) [22,23].

The controls in the discovery stage were the subjects without detectable atherosclerotic stenosis, as validated by angiography. The control group in the replication stage consisted of the general population without cardiovascular disease via physical examination and electrocardiogram. In both stages, the controls were frequency-matched to the cases on age (± 5 years), sex, and residential area.

Subjects were excluded if they 1) underwent percutaneous coronary intervention, coronary bypass surgery or angioplasty; 2) had cardiac diseases like cardiomyopathy, valvular or congenital heart disease, heart failure, coronary spasm, or myocardial bridge; 3) had systemic diseases including malignancy, autoimmune disease, liver or kidney disease. The study protocol followed the declaration of Helsinki, and local ethics committees approved each stage of the study. Participants provided written informed consents accordingly.

2.2. Records of cardiovascular risk factors and cardiometabolic phenotypes

For each participant, a predefined scale was designed to record the following demographics: (1) six conventional cardiovascular risk factors including smoking habit, alcohol drinking habit, overweight/obesity, history of hypertension, history of type 2 diabetes mellitus (T2DM), and history of dyslipidemia; (2) 12 biomarkers (if available) within five categories of cardiometabolic phenotypes, i.e. blood lipids (triglycerides [TG], total cholesterol [TC], low-density lipoprotein cholesterol [LDL-c], high-density lipoprotein cholesterol [HDL-c]), blood glucose (fasting plasma glucose [FPG], hemoglobin A1c [HbA1c]), blood pressure (systolic blood pressure [SBP], diastolic blood pressure [DBP]), inflammatory (C-reactive protein [CRP], homocysteine [HCY], D-dimer), and adiposity measures (body mass index [BMI]). Out of 18 demographics, 14 had complete data of 3064 participants; four (HbA1c, CRP, HCY, D-dimer) had missing values (mostly due to insufficient sample volumes), with the missing rates ranging from 6.5% to 7.5%. Detailed summaries for these demographics were given in Supplementary Materials and Methods.

2.3. Detection of leukocyte TL and mtDNA-CN

Total DNA was isolated from peripheral blood leukocytes using a phenol-chloroform method. Relative TL and mtDNA-CN were measured on a LC480 SYBR Green System (Roche, Indianapolis, IN, USA) using a previously described quantitative PCR (qPCR) assay [24,25], which calculated the copy number ratios of telomere repeats (for TL) or mitochondrial *ND2* gene (for mtDNA-CN) to a single-copy nuclear gene (hemoglobin beta, *HGB*) in experimental samples versus a calibrator sample. The calibrator was a mixed DNA sample pooled from 10 randomly selected healthy controls [26].

Briefly, the seven-point standard curve, produced by a 2-fold serially diluted reference DNA (concentrations: 0.3125–20 ng) [25], was created in each batch to measure the ratio of TL/mtDNA-CN content to *HGB* content. The R^2 for each standard curve should be greater than 0.99, with acceptable standard deviations (SDs) of the Cq values of < 0.25 and sufficient qPCR efficiencies of > 1.95 [25,27]. Then, based on a $2^{-\Delta\Delta Cq}$ formula [28], the ratio of each sample was normalized with the calibrator to quantify leukocyte TL and mtDNA-CN. All samples were assayed in triplicate and four quality controls were inserted into each plate to analyze variability. To control position effects, qPCR reactions for each target were invariably conducted on separate 384-well plates with the same samples in the same wells [25]. qPCR procedures were executed by investigators blinded to clinical data and disease status. Primer sequences and qPCR conditions for TL and mtDNA-CN measurements were summarized in Supplementary Table 1.

2.4. Measurement of oxidative parameters in leukocyte and plasma samples

Three oxidative parameters were measured in a subset of 129 cases and 129 controls randomly selected from the entire population. The amount of leukocyte 8-hydroxy-2′-deoxyguanosine (8-OHdG), reflecting the degree of oxidative DNA damage, was quantified by a competitive ELISA assay, as described by our previous reports

Table 1
Associations of leukocyte TL, mtDNA-CN, and their aggregated score with CAD risk in our two-stage case-control study.

Variables	Cases	Controls	Model					
			Model 1[a]		Model 2[b]		Model 3[c]	
			OR (95%CI)	*p*	OR (95%CI)	*p*	OR (95%CI)	*p*
TL z scores with CAD risk								
Discovery stage	506	509						
Each 1-SD decrease			1.29 (1.12–1.48)	< 0.001	1.28 (1.11–1.47)	< 0.001	1.26 (1.08–1.46)	0.003
Replication stage	1005	1044						
Each 1-SD decrease			1.14 (1.04–1.26)	0.007	1.14 (1.04–1.26)	0.008	1.14 (1.03–1.26)	0.011
Combined cohort	1511	1553						
Each 1-SD decrease			1.19 (1.10–1.29)	< 0.001	1.18 (1.09–1.29)	< 0.001	1.17 (1.08–1.28)	< 0.001
By tertiles in controls								
T1	598 (39.6)	520 (33.5)	1.46 (1.22–1.74)	< 0.001	1.44 (1.21–1.72)	< 0.001	1.42 (1.18–1.71)	< 0.001
T2	513 (34.0)	527 (33.9)	1.23 (1.03–1.47)	0.023	1.23 (1.03–1.48)	0.022	1.16 (0.96–1.40)	0.120
T3	400 (26.4)	506 (32.6)	Reference		Reference		Reference	
p trend			< 0.001		< 0.001		< 0.001	
mtDNA-CN z scores with CAD risk								
Discovery stage	506	509						
Each 1-SD decrease			1.20 (1.06–1.36)	0.005	1.18 (1.04–1.35)	< 0.001	1.16 (1.01–1.33)	0.032
Replication stage	1005	1044						
Each 1-SD decrease			1.16 (1.06–1.26)	0.001	1.15 (1.06–1.26)	0.001	1.14 (1.04–1.25)	0.004
Combined cohort	1511	1553						
Each 1-SD decrease			1.17 (1.09–1.26)	< 0.001	1.16 (1.08–1.25)	< 0.001	1.14 (1.06–1.23)	< 0.001
By tertiles in controls								
T1	612 (40.5)	525 (33.8)	1.45 (1.22–1.73)	< 0.001	1.43 (1.20–1.70)	< 0.001	1.35 (1.12–1.62)	0.001
T2	487 (32.2)	515 (33.2)	1.18 (0.98–1.41)	0.074	1.19 (0.99–1.43)	0.054	1.19 (0.99–1.44)	0.066
T3	412 (27.3)	513 (33.0)	Reference		Reference		Reference	
p trend			< 0.001		< 0.001		0.006	
Aggregated scores of TL and mtDNA tertiles with CAD risk								
Combined cohort	1511	1553						
Per dosage decrease			1.20 (1.13–1.28)	< 0.001	1.20 (1.12–1.27)	< 0.001	1.18 (1.10–1.26)	< 0.001
Ordinary variable								
2 dosages	261 (17.3)	175 (11.3)	2.19 (1.61–2.98)	< 0.001	2.14 (1.57–2.93)	< 0.001	1.89 (1.37–2.61)	< 0.001
3 dosages	376 (24.9)	341 (22.0)	1.62 (1.22–2.15)	< 0.001	1.60 (1.21–2.13)	< 0.001	1.44 (1.07–1.94)	0.016
4 dosages	483 (32.0)	532 (34.3)	1.33 (1.01–1.75)	0.039	1.33 (1.01–1.75)	0.043	1.23 (0.92–1.63)	0.157
5 dosages	282 (18.6)	345 (22.2)	1.20 (0.90–1.60)	0.218	1.21 (0.91–1.62)	0.194	1.07 (0.79–1.44)	0.680
6 dosages	109 (7.2)	160 (10.2)	1 (Reference)		1 (Reference)		1 (Reference)	
p trend	612 (40.5)	175 (11.3)		< 0.001		< 0.001		< 0.001

TL: telomere length, mtDNA-CN: mitochondrial DNA copy number; CAD: coronary artery disease.
[a] Crude model without any covariates.
[b] Adjusted for age, WBC count, and plate number.
[c] Adjusted for age, WBC count, plate number, sex, smoking habit, alcohol drinking habit, overweight/obesity, hypertension, T2DM, and dyslipidemia.

(Supplementary Materials and Methods) [21,29]. Plasma malondialdehyde (MDA), a well-established biomarker for lipid peroxidation [30], was quantified by a thiobarbituric acid reactive substances (TBARS) assay, as described by Nie et al. (Supplementary Materials and Methods) [31]. Total ROS levels from freshly isolated plasma samples were determined by a Peroxide Assay Kit (Biomedical Research Service, UB, NY, USA) [31,32], based on the manufacturer's instructions (Supplementary Materials and Methods).

2.5. Statistical analysis

To standardize the distributions of TL and mtDNA-CN data across two stages, we calculated the z scores, which expressed the levels of TL and mtDNA-CN as the units of their respective SDs. The effects of leukocyte TL and mtDNA-CN on CAD risk and severity were analyzed by unconditional logistic regression with three different adjustment models. Model 1 was the crude model without any covariates; model 2 adjusted for variables that potentially influenced the levels of TL and mtDNA-CN, including age [24], white blood cell (WBC) count [33], and plate number; model 3 additionally adjusted for sex and six conventional CAD risk factors including smoking habit, alcohol drinking habit, overweight/obesity, hypertension, T2DM, and dyslipidemia. For each model, the z scores of TL and mtDNA-CN were first analyzed as continuous variables, and then as ordinal variables using tertile values of controls as cutoff points. We also performed sensitivity analyses by excluding outliers of TL and mtDNA-CN ($2 < z\ scores < -2$), as well as by stratifying participants based on conventional CAD risk factors. The differences in odds ratios (ORs) between different subgroups were assessed by the χ^2-based Q test.

To assess the interaction effects of TL and mtDNA-CN on CAD risk, the likelihood ratio test was first conducted to compare the statistical significance of the interaction term of "TL × mtDNA-CN ". Then, an aggregated score, reflecting the cumulative dosage of TL and mtDNA-CN for each subject, was created by adding the tertiles of TL and mtDNA-CN together [34]. This aggregated score, with values ranging from 2 to 6, was analyzed as an ordinary variable in regression models. Finally, we calculated the C statistic to evaluate whether the addition of the aggregated score to the 2013 ACC/AHA Pooled Cohort Equation (PCE) [35] could improve discrimination for CAD events.

The Spearman coefficients were calculated to assess the pairwise correlations among TL, mtDNA-CN, age and WBC count. Multivariable linear regression (model 3) was conducted to analyze the associations of TL and mtDNA-CN with cardiometabolic phenotypes, as well as the effects of oxidative parameters (8-OHdG, MDA, and ROS) on TL, mtDNA-CN, CAD risk, and CAD severity. Considering that TL, mtDNA-CN, and their aggregated score were the three primary targets in this study, the significance threshold was set as $p < 0.017$ (0.05/3) based on the Bonferroni correction for multiple testing. All above tests were conducted by the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Power analyses were performed with the PS 3.0 program (Vanderbilt

University, Nashville, TN, USA).

3. Results

3.1. Study characteristics

The averaged CVs across all 27 plates were 3.1% for the TL assays, and 2.9% for the mtDNA-CN assays. Consistent with the previous reports, we observed inverse correlations of age with both TL ($r = -0.061$, $p < 0.001$) and mtDNA-CN ($r = -0.043$, $p = 0.016$), as well as a negative relationship between WBC count and TL ($r = -0.046$, $p = 0.011$). TL and mtDNA-CN were marginally interrelated ($r = 0.034$, $p = 0.059$). Demographics of two stages are summarized in Supplementary Table 2.

3.2. Independent effect of leukocyte TL on CAD risk

In the discovery stage from Henan Province, northern China, each 1-SD decrease in TL z scores was associated with a 1.29-fold ($p = 4.87 \times 10^{-4}$) increased risk of CAD in the crude model. After progressive adjustment for age, WBC count, plate number (model 2), sex, and six CAD risk factors (model 3), a significant inverse correlation of TL z scores with CAD risk was still identified (Table 1). Then, in a larger cohort from Hubei Province, central China, logistic regression analyses, with and without adjustment for covariates, consistently replicated the inverse association between TL z scores and CAD risk, with modest ORs of ~1.14. To fully increase the statistical power, a meta-analysis of two cohorts was performed, suggesting a 17% ($p = 1.87 \times 10^{-4}$) increase in CAD risk per 1-SD decrease in TL z scores after model 3 adjustment. When participants were stratified by age (> 60 years vs ≤ 60 years), the ORs per 1-SD decrement in TL z scores were significantly different between older and younger participants (OR: 1.31 vs 1.07, $p = 0.013$, Fig. 1A), implying a potential interaction between TL and age in modulating CAD risk. When TL z scores were analyzed as an ordinary variable, the risk of developing CAD was 1.42-fold ($p = 1.90 \times 10^{-4}$) higher in the bottom as compared with the top tertile of TL z scores (model 3, Table 1). Given the OR of 1.42 and a significant threshold of 0.017, the combined cohort could provide a statistical power of 98.9% to address the association. Sensitivity analyses by excluding outliers of TL z scores achieved similar results (Supplementary Table 3).

3.3. Independent effect of leukocyte mtDNA-CN on CAD risk

In both discovery (OR: 1.20 per SD decrease, $p = 0.005$) and replication stages (OR: 1.16, $p = 0.001$), the ORs from the crude model consistently suggested an inverse correlation between the continuous z scores of mtDNA-CN and CAD risk. Additional adjustments in model 2 and model 3 only slightly attenuated the effect sizes (OR: 1.14–1.18, Table 1). In meta-analyses of two cohorts, a decrease of one SD in mtDNA-CN z scores was associated with a 1.14-fold ($p = 5.25 \times 10^{-4}$) increase in CAD risk after model 3 adjustment. In multivariate stratification analyses, with per SD decrement in mtDNA-CN z scores, smokers were at 32% and nonsmokers were only at 8% increased risk of CAD, with a p value of 0.012 for interaction analysis (Fig. 1B). In a comparison of bottom *versus* top tertile of mtDNA-CN z scores, the adjusted OR for CAD risk was 1.35 (model 3), with a p value of 0.001 and a statistical power of 94.8%. Overall results remained after removing outliers of mtDNA-CN (Supplementary Table 4).

3.4. Cumulative effect of TL and mtDNA-CN on CAD risk

When the combined cohort was stratified by the tertiles of TL, the adjusted ORs (model 3) of mtDNA-CN reductions for CAD risk were progressively increased in subjects with short, medium, and long TL, implying an interaction between TL and mtDNA in developing CAD risk (Supplementary Table 5). Then, to quantify the effects of the cumulative dosage of TL and mtDNA-CN on CAD risk, an aggregated score of these two aging markers was included into the logistic regression model. Generally, the risk of developing CAD progressively increased with the decreasing dosage of the aggregated score ($p_{trend} < 0.001$, Table 1). Particularly, subjects with the lowest (two dosages) and the second-lowest (three dosages) aggregated score had a 1.89-fold ($p = 1.19 \times 10^{-4}$) and a 1.44-fold ($p = 0.016$) increased risk of CAD, respectively, compared with participants with the highest aggregated score (six dosages). Finally, when adding the aggregated score to the 2013 ACC/AHA PCE risk equation, the C statistic for discrimination of CAD events improved by 3.4% compared to the PCE alone, by 1.9% compared to the model only including PCE and TL, and by 2.5% compared to another model including PCE and mtDNA-CN, suggesting that the combination of TL and mtDNA-CN was more effective to discriminate CAD events than each marker alone (Supplementary Table 6).

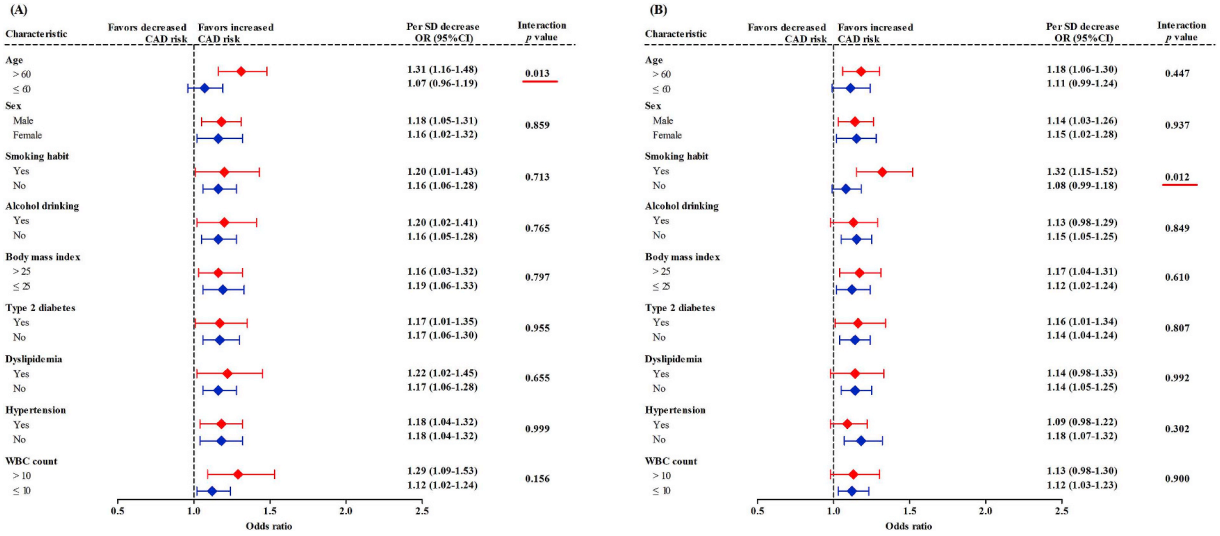

Fig. 1. Association of each 1-SD decrease in leukocyte TL (A) and mtDNA-CN z (B) scores with CAD risk stratified by conventional CAD risk factors.
ORs and their 95%CIs adjusted for age, WBC count, plate number, sex, smoking habit, alcohol drinking habit, overweight/obesity, hypertension, T2DM, and dyslipidemia. $p_{interaction}$ values were obtained from the χ^2-based Q test.

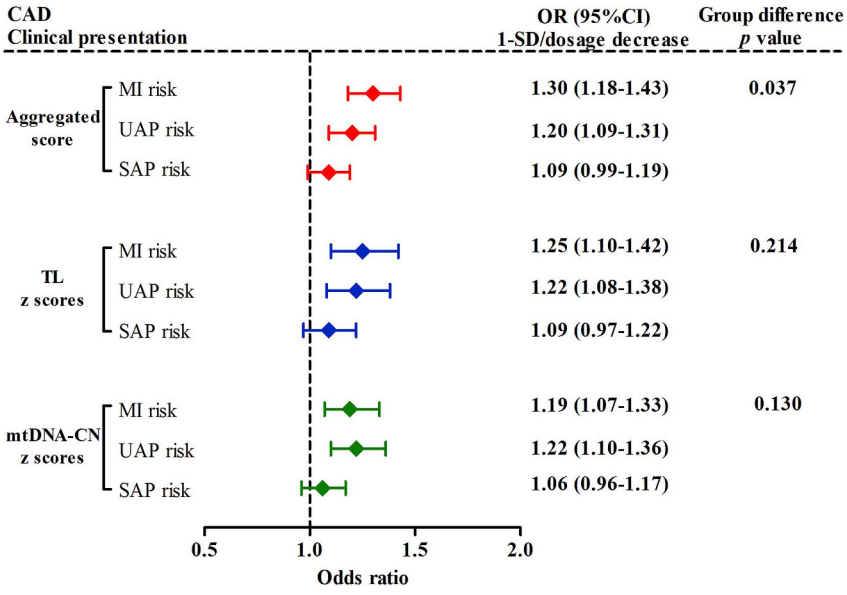

Fig. 2. Associations of leukocyte TL, mtDNA-CN, and their aggregated score with clinical presentations of CAD.
ORs and their 95%CIs adjusted for age, WBC count, plate number, sex, smoking habit, alcohol drinking habit, overweight/obesity, hypertension, T2DM, and dyslipidemia. *p* values for group difference were obtained from the χ^2-based Q test.

3.5. Effects of TL and mtDNA-CN alone or in combination on the severity of the clinical presentation of CAD

Overall, when cases in the combined cohort were categorized by the clinical presentation of CAD, TL, mtDNA-CN, and their aggregated score consistently showed inverse correlations with MI and UAP but not with SAP, either as continuous or ordinary variables (Supplementary Table 7). Notably, the adjusted ORs (model 3) for each 1-dosage decrease in the aggregated score increased with the worsening severity of clinical presentations: SAP, OR = 1.09 ($p = 0.057$); UAP, OR = 1.20 ($p = 1.37 \times 10^{-4}$); and MI, OR = 1.30 ($p = 1.26 \times 10^{-7}$), suggesting a potential cumulative effect of TL and mtDNA-CN on CAD severity ($p_{trend} = 0.037$, Fig. 2).

3.6. Effects of TL and mtDNA-CN alone or in combination on CAD severity

Next, CAD severity was further dichotomous based on the median level of modified Gensini scores (> 30 *vs.* ≤ 30) or the presence of multi-vessel CAD (multi-vessel *vs.* single-vessel). In CAD patients from the combined cohort, with each 1-SD decrement in TL z scores, there was a 21% ($p = 0.002$) increased risk of having higher modified Gensini scores and a 25% ($p = 2.66 \times 10^{-4}$) increased risk of developing multi-vessel CAD after model 3 adjustment (Fig. 3). Similar results were obtained when analyzing TL as an ordinary variable. In contrast, there was no significant relationship of mtDNA-CN to either modified Gensini scores or multi-vessel CAD risk. Intriguingly, the aggregated score, reflecting the cumulative dosage of TL and mtDNA-CN, showed an inverse dose-response association with CAD severity (modified Gensini scores: $p_{trend} = 0.009$; multi-vessel CAD: $p_{trend} = 0.004$, Fig. 3). In particular, patients with the lowest dosage (two dosages) of the aggregated score had a 1.78-fold ($p = 0.014$, Fig. 3A) increased risk of higher modified Gensini scores and a 1.94-fold ($p = 0.005$, Fig. 3B) increased risk of multi-vessel CAD, compared with those with the highest dosage (six dosages).

3.7. Effects of TL and mtDNA-CN alone or in combination on cardiometabolic phenotypes

Among 13 cardiometabolic phenotypes investigated, D-dimer ($\beta = -0.063$, $p = 0.016$, Supplementary Table 8) and DBP ($\beta = -0.986$, $p = 0.001$) was inversely related to mtDNA-CN z scores, whereas HDL-c ($\beta = 0.012$, $p = 0.001$) was positively correlated with TL z scores in the whole samples. When analyzing the cumulative effect of TL and mtDNA-CN, the aggregated score showed significant associations with DBP ($\beta = -0.955$, $p < 0.001$) and HDL-c ($\beta = 0.008$, $p = 0.016$), but not with other phenotypes.

3.8. Effects of TL and mtDNA-CN alone or in combination on oxidative parameters

Considering that excessive exposure to oxidative stress is a potential outcome of the cumulative reduction in TL and mtDNA-CN dosages during CAD development, three oxidative parameters, representing the overall state of oxidative stress, were quantified in a subset of 129 cases and 129 controls (Supplementary Table 9). In general, levels of leukocyte 8-OHdG, plasma ROS, and plasma MDA were consistently higher in CAD patients than those in controls ($p < 0.001$ for all, Fig. 4A–C), supporting a direct relation between CAD risk and oxidative stress. Then, a positive correlation between mtDNA-CN z scores and leukocyte 8-OHdG levels was also identified ($\beta = 0.158$, $p = 0.001$, Fig. 4D). More importantly, both leukocyte 8-OHdG ($\beta = 0.171$, $p = 4.40 \times 10^{-5}$, Fig. 4E) and plasma ROS ($\beta = 0.138$, $p = 7.66 \times 10^{-4}$, Fig. 4F) progressively increased across the decreasing dosage of the aggregated score of TL and mtDNA-CN, suggesting a cumulative effect of TL and mtDNA-CN on excessive oxidative stress. Otherwise, plasma MDA levels were not associated with TL, mtDNA-CN, and their aggregated score.

4. Discussion

Given the vital roles of telomeres in regulation of chromosomal integrity, cell division, and oxidative stress [7], emerging studies aimed to investigate the effects of leukocyte TL on CAD development [5,17]. However, due to small sample size of established data, the results in the Chinese population were often inconsistent, with some reports finding an inverse relation between TL and CAD risk [18,36,37], and others reporting the opposite, null association at all [19,38,39]. In the present study, using a two-stage case-control design of 3064 Chinese subjects, we first identified a significantly inverse association not only with CAD risk, but also with CAD severity as determined by modified Gensini scores and presence of multi-vessel CAD. Then, the interaction between TL shortening and older age was found to substantially increase CAD risk. Finally, consistent with previous reports [24,33], we observed a positive correlation between TL and HDL-c levels.

Functional studies have shown that telomere attrition, as a mediator between mitochondrial dysfunction and apoptosis [40], might result

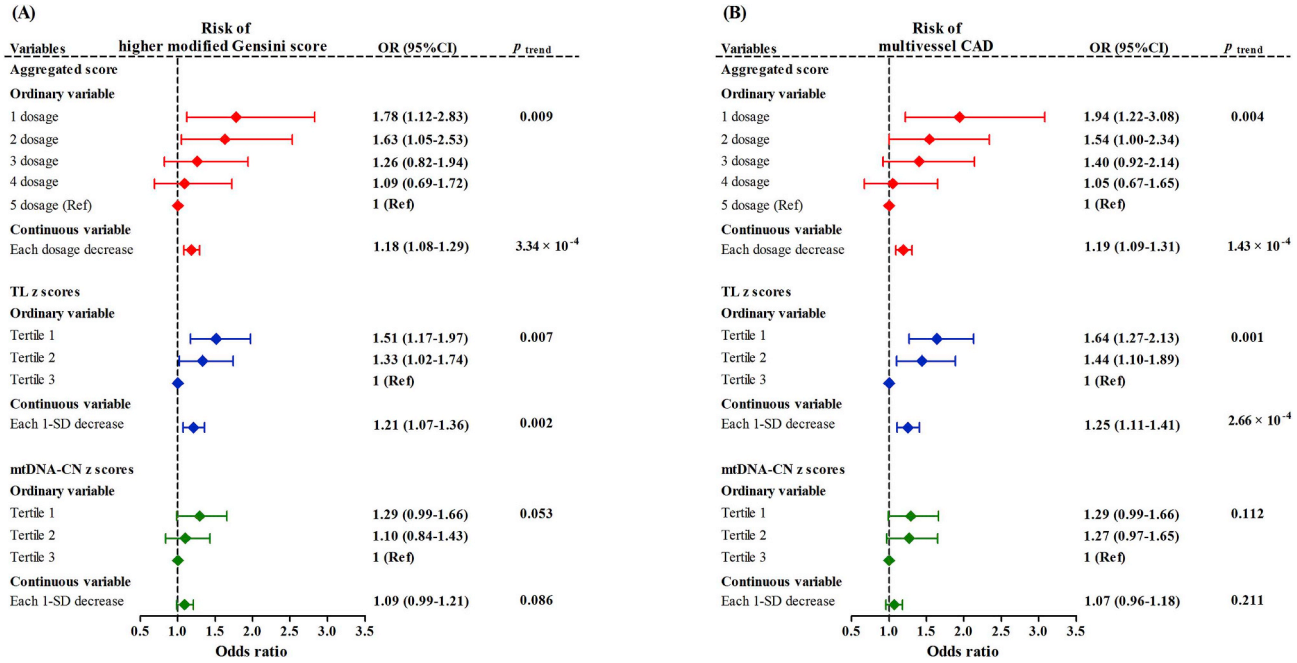

Fig. 3. Associations of leukocyte TL, mtDNA-CN, and their aggregated score with higher modified Gensini score (A) and presence of multivessel CAD (B) in CAD patients.
ORs and their 95%CIs adjusted for age, WBC count, plate number, sex, smoking habit, alcohol drinking habit, overweight/obesity, hypertension, T2DM, and dyslipidemia.

from the excessive production of ROS by comprised mitochondria, which further oxidized proteins necessary for telomere maintenance, broke polyguanosine sequences in telomere repeats, and consequently induced telomere damage [40,41]. Correspondingly, leukocyte mtDNA-CN, as a surrogate of mitochondria function, has been reported to change with mitochondria DNA damage *ex vivo* [42], and inversely correlate with CAD risk in population studies [20,43]. In the current study, we first found leukocyte mtDNA-CN was marginally related to leukocyte TL, supporting the notion that TL and mtDNA-CN might be co-regulated [15,16]. Then, we observed an inverse association between mtDNA-CN and CAD risk in the Chinese population, especially in smokers. Considering that the common feature between exposure to cigarette smoke and mtDNA-CN reduction was uncontrolled oxidative stress [44], we then quantified three oxidative parameters in leucocytes and plasma, and successfully identified an inverse correlation between mtDNA-CN and leukocyte 8-OHdG levels. This finding, combined with *in vivo* evidence that the interaction of a decline in mitochondrial biogenesis with ROS production induced cardiac phenotypes like

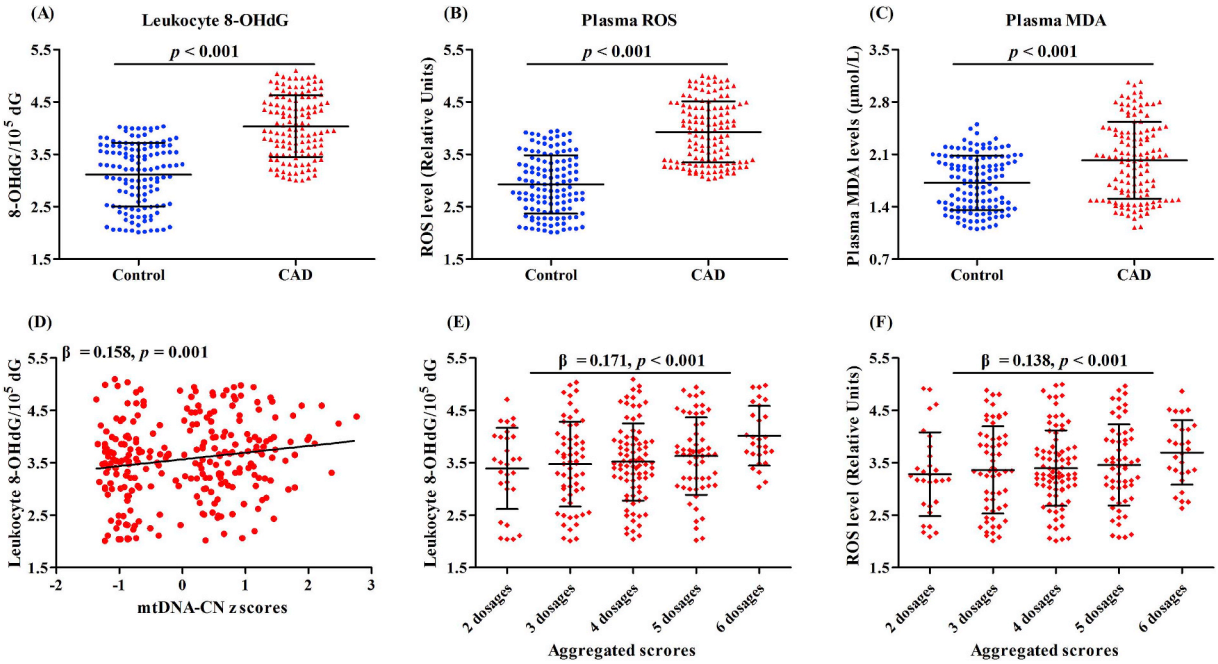

Fig. 4. Correlations of oxidative parameters with CAD risk (A–C), mtDNA-CN (D), and the aggregated scores (E and F).
β and *p* values adjusted for age, WBC count, plate number, sex, smoking habit, alcohol drinking habit, overweight/obesity, hypertension, T2DM, and dyslipidemia.

impairment of systolic and diastolic function [45], suggested a causal link between mtDNA-CN, oxidative stress, and CAD risk. Notably, a recent cross-sectional study of 400 individuals undergoing coronary angiography observed a progressive decrease in mtDNA-CN across the quartiles of Gensini scores [46], which was not fully replicated in our case group with 1511 CAD patients. Considering that our study only calculated modified Gensini scores in patients with coronary stenosis of > 50%, but not in any subjects undergoing angiography, subsequent studies, based on consistent study design and larger sample size, may help to resolve this controversy.

TL and mtDNA-CN have been separately studied as independent predictors in age-related diseases [47], yet, there is growing evidence that these two markers are functionally linked or at least that combining them together may better predict disease development. For instance, in epidemiologic studies, the combination of TL and mtDNA-CN has been reported to monitor cognitive dysfunction in Korean elders [48]. In experimental studies, knockout of either telomerase RNA component (*Terc*) or telomerase reverse transcriptase (*Tert*) genes induced a P53-dependent inhibition of the major coactivators (i.e. *PPARγ*, *PGC-1α*, and *PGC-1β*) of mitochondrial biogenesis, which impaired mitochondrial function in mouse heart [16,47]. In turn, mitochondrial dysfunction could increase ROS production, trigger the retrograde response, and consequently cause telomere attrition *in vivo* [15,40]. In light of this, the present study, for the first time, aimed to investigate the cumulative effect of TL and mtDNA-CN on CAD risk. As a result, we first found that the aggregated score of TL and mtDNA-CN inversely correlated with CAD risk, and more effectively improved discrimination for CAD events than either TL or mtDNA-CN alone. Secondly, the decreasing dosage of the aggregated score progressively increased CAD severity, as determined by the worsening severity of clinical presentations (from SAP to UAP, and to MI), the presence of multi-vessel CAD, and modified Gensini scores. Finally, there was an inverse dose-response relationship of the aggregated score to the levels of leukocyte 8-OHdG and plasma ROS. Taken together, our data suggest that the combination of TL and mtDNA-CN may reflect the cumulative exposure to oxidative stress, and help better predict CAD risk and severity. Future prospective studies are urgent to ensure that this finding was not inflated by reverse causation.

This study had some limitations. First, we could not entirely eliminate the possibility of reverse causation due to the retrospective design, although our study had a relatively large sample size of 3064 Chinese subjects from two districts, and adjusted for most known confounders. Second, we detected TL and mtDNA-CN only in leukocytes, given that the acquired data were easily compared with most established evidence, and that the highly positive correlations have been observed between leukocytes and atherosclerotic plaque tissue for mtDNA-CN [43], and between leukocytes and multiple somatic tissues for TL [49]. Third, cardiac diagnostic imaging, such as optical CT or intravascular ultrasound, was not conducted for controls, so we could not fully exclude subjects with angiographically invisible atheromas from the control group. Lastly, although we tried our best to record each participant's cardiometabolic phenotypes, interpreting these data should be cautious due to the existence of missing variables and lack of repeated measurements at multiple time points.

Overall, this two-stage case-control first showed that the reductions in both TL and mtDNA-CN were two independent risk factors for CAD. Then, for the first time, our data provided evidence that the combination of TL and mtDNA-CN might jointly contribute to CAD risk, CAD severity, and parameters of oxidative stress. These findings warrant replication in future prospective studies.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

XBW and LM designed the experiments, wrote the paper, and draft the manuscript. XBW, NHC, and SZ and performed the experiments. XBW, JFM, and ZJL interpreted the data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.03.010.

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