NON-INVASIVE MEASURES OF CARDIOVASCULAR FUNCTION AND HEALTH: SPECIAL CONSIDERATIONS FOR ASSESSING LIFESTYLE BEHAVIOURS

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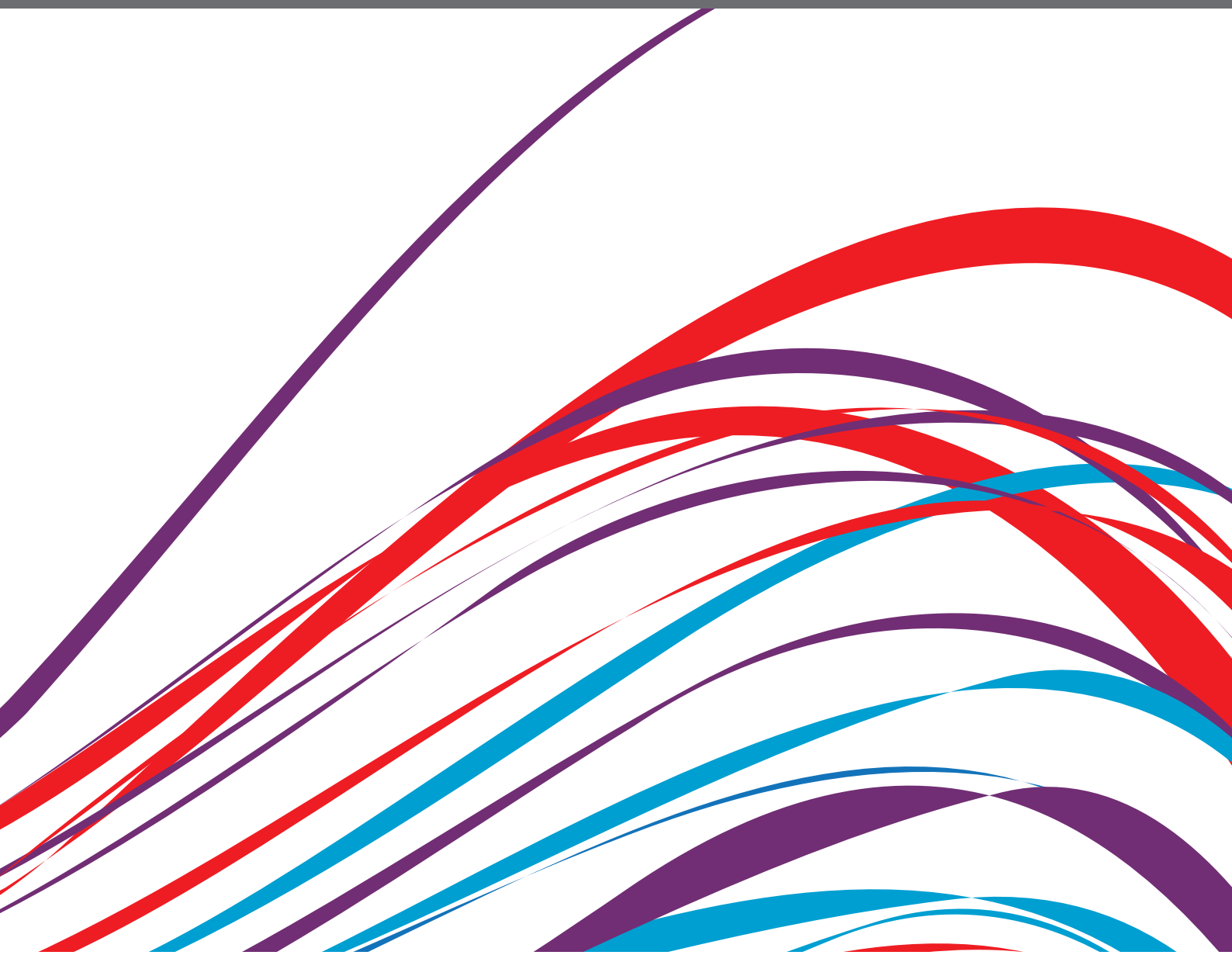

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NON-INVASIVE MEASURES OF CARDIOVASCULAR FUNCTION AND HEALTH: SPECIAL CONSIDERATIONS FOR ASSESSING LIFESTYLE BEHAVIOURS

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EDITORIAL
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Editorial: Non-Invasive Measures of Cardiovascular Function and Health: Special Considerations for Assessing Lifestyle Behaviours

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Keywords: sedentary, vascular–diagnosis, vascular function, arterial stiffness, biomarkers

Editorial on the Research Topic

Non-Invasive Measures of Cardiovascular Function and Health: Special Considerations for Assessing Lifestyle Behaviours

In recent decades it has become clear that lifestyle behaviors such as diet (1), risky alcohol consumption (2), cigarette smoking (Wang et al.), sedentary behavior (3), and physical inactivity (4) are all factors contributing to cardiovascular disease (CVD) incidence and mortality. And, as cardiovascular diseases remain the largest cause of death in the Western world, gaining further knowledge of cardiovascular dysfunction in response to these lifestyle behaviors is of paramount importance. This Research Topic which includes 14 research papers (**Figure 1**), has helped further our knowledge in three important areas of lifestyle behaviors and cardiovascular physiology: First, it has highlighted the importance several modifiable, behavioral risk factors associated with CVD (El-Battrawy et al.; Wang et al.; Zuo et al.), second, it has expanded our understanding of a number of biomarkers and risk scores which are related to CVD (Bo et al.; Cang et al.; Hsu et al.; Tsai et al.; Ying et al.), third it helped us to better understand and interpret markers of arterial stiffness (Elliot et al.; Lane-Cordova and Bouknight; Stone et al.) and lastly, it has allowed us to provide guidance on how to assess cardiovascular function in response to a common and biologically novel lifestyle behavior, prolonged sitting (Stoner et al.). Collectively this new information is an important step forwards in improving our understanding of CVD, and allows us to work toward the development of public health policy aimed at reducing the CVD burden.

In order to inform public health policy surrounding cardiovascular health and modifiable lifestyle behaviors, robust RCT trials need to be conducted (5). As such, simple but reliable non-invasive measures of assessing key markers of vascular health are needed. One key marker of vascular health, which predicts CVD and mortality is carotid-femoral pulse wave velocity (cfPWV) the gold standard measure of arterial stiffness (6). In our topic, Elliot et al. reported that in order to be able to conduct reliable cfPWV measurements, a novice operator needed a familiarization session, guided practice measurements on 5 people followed by measurements with >30 people; demonstrating the simplicity and novice operator reliability of this measure, its highlights the potential use of cfPWV in large RCTs and epidemiological studies. Stone et al. built upon this work by demonstrating that heart-femoral PWV (hfPWV), which is simpler to assess than cfPWV, and is less likely to be affected by subject-level factors such as carotid plaque, is strongly associated with cfPWV. As such, hfPWV may be an even simpler alternative to cfPWV in the identification of CVD risk in both clinical and epidemiological settings (Stone et al.). Assessments of PWV were part of the physiology toolbox developed by Stoner et al. The authors described the need for a set of study

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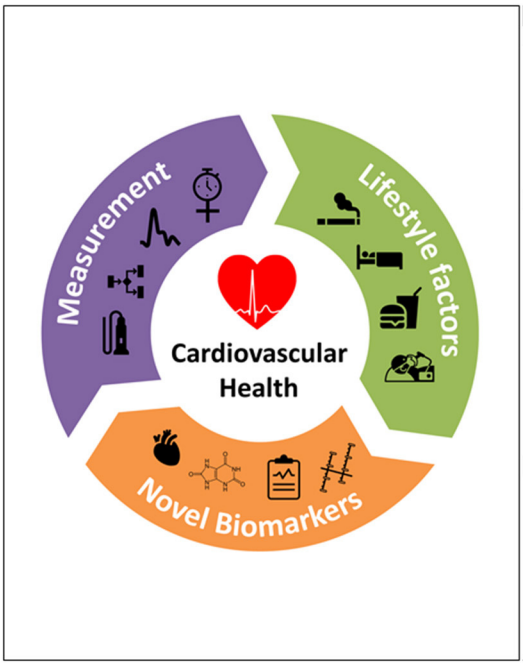

Article	Highlights
Associations between lifestyle factors and cardiovascular health	
Castro et al. (17)	Social jet lag contributes to cardiometabolic disease in children, but is a readily modifiable target.
He et al. (16)	Obstructive sleep apnea-hypopnea syndrome is associated with increased risk of mortality in patients with myocardial infarction, in the absence of obstructive coronary artery disease.
Wang et al. (3)	Smokers with cardiovascular disease have an increased risk of all-cause, cardiovascular disease and cancer mortality, but this risk decreases with smoking cessation.
Zuo et al (7)	Postoperative smoking and hyperglycemia had an increased risk of left internal mammary artery graft stenosis.
Novel biomarkers for cardiovascular health measurement	
Battrawy et al. (6)	Temporal pattern of Takotsubo Syndrome events is an independent predictor of lower-event free survival, particularly those occurring in the fourth quarter of year.
Cang et al. (9)	Abnormal serum uric acid levels are independent risk factors for all-cause and cardiovascular disease mortality.
Hsu et al. (10)	Athletes with the red blood cell hybrid protein GP.Mur were found to have lower flow-mediated dilation than athletes without the GP.Mur protein.
Bo et al. (11)	The nomogram is a simple and effective tool in predicting the occurrence of in-hospital major adverse cardiovascular and cerebrovascular events in acute coronary syndrome patients.
Tsai et al (8)	Left atrial size is not an independent predictor of atrial fibrillation stroke, but CHA_2DS_2-VASc score may be a useful alternative for evaluating risk of stroke in atrial fibrillation patients.
Ying et al. (12)	Increased number of ideal cardiovascular health metrics is associated with lower incidence of ischemic stroke in hypertensive patients, particularly those with hyperhomocysteinemia.
Considerations for cardiovascular health measurement	
Elliott et al. (13)	At least 30 training measurements are recommended for novices to take acceptable augmentation index measurements, after a familiarization.
Cordova et al. (15)	Sensitive assessments of vascular structure and function are needed to evaluate the success of lifestyle interventions in premenopausal women, with consideration for menstrual cycle phase.
Stone et al. (14)	Change in heart-femoral pulse wave velocity is strongly associated with change in carotid-femoral pulse-wave velocity, and may be a simple alternative for the determination of cardiovascular risk.
Stoner et al. (16)	Well-controlled sitting exposure studies, contemplating the criteria used to aid policy development, are needed to accelerate guideline development.

FIGURE 1 | Study highlights for the non-invasive measures of cardiovascular function and health: special considerations for assessing lifestyle behaviors Research Topic.

guidelines/considerations for assessing the physiological responses to acute sitting exposure, a novel and biologically distinct CVD risk factor (Stoner et al.). This work marks an important milestone in the need for the research community to improve cross-study methodological standardization.

In addition to non-invasive assessments of arterial function, this topic also collectively looked at a range of lifestyle and behavioral risk factors associated with CVD. Whilst some data reinforced evidence highlighting that the earlier and less an individual smoke, the lower the chances of premature death from CVD (Wang et al.), other research focused on more novel risk factors such as sleep apnea (He et al.). He et al. report an increased risk of mortality from obstructive sleep apnea-hypopnea syndrome in patients with myocardial infarction in the absence of obstructive coronary artery disease. Another novel predictor of cardiovascular health reported within this Research Topic, is social jetlag in children (Castro et al.). Castro et al. reported that social jetlag, defined as the difference between a person's social rhythms and circadian clock, is linked to both vascular health and cholesterol in preadolescent children. Whilst the authors did not determine biological paucity of this relationship, the findings are of great importance for the early stages of public health policy development.

This Research Topic has answered a number of prominent research questions surrounding non-invasive measures of cardiovascular function and health within the context of assessing lifestyle behaviors. However, it has highlighted several important gaps in the literature which warrant further research. For example, given that hfPWV is less likely than cfPWV to be affected by within subject factors such as the presence of carotid plaques, understanding whether it can be quickly and reliably taught in novices would help with justifying its use in the design of large cohort RCTs or epidemiological studies.

AUTHOR CONTRIBUTIONS

SF: design, writing, and topic editor. DC: proof reading and topic editor. KS: proof reading and figure construction. LS: design, proof reading, and topic editor. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Inter-operator Reliability for Measuring Pulse Wave Velocity and Augmentation Index

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Background: Arterial stiffness is a reversible precursor to hypertension. However, research is needed to determine the minimum amount of training required before acceptable arterial stiffness measurements are collected by novice operators.

Objective: To compare novice vs. experienced operator measurements over a 2-week training period to assess when expert-like measures are achieved by the novice operator.

Method: Forty-one participants (18 males, 23 females, age: 46.6 ± 14.9 years; BMI: 25.2 ± 3.8; systolic blood pressure: 122.8 ± 14.7 mmHg) received alternating novice and experienced operator arterial stiffness assessments. Measurements included: pulse wave velocity (PWV; using the automatic-capture time-periods of 5-, 10-, and 20-s) and augmentation index (AIx75) measurements using the SphygmoCor XCEL System v1 (AtCor Medical Pty Ltd., Sydney, Australia). Data were chronologically arranged into quintiles.

Results: The intraclass correlation coefficient for PWV substantially improved from quintile 1 ($r < 0.8$) to quintile 2 and beyond (typically $r > 0.8$) while AIx75 improved consistently ($r = 0.7$ in quintile 1 and $r = 0.97$ in quintile 5). The coefficient of variation was lowest in quintile 4 (PWV: 4.7–6% across the three measurement time-periods; and 15% for AIx75) but increased in quintile 5 (PWV: 6.2–10.5%; and 25% for AIx75). All measurements demonstrated acceptable to excellent reliability after quintile 2.

Conclusion: To achieve expert-like PWV measurements in this study, the novice operator underwent a familiarization session including guided practice measurements on 5 different people, for 10–15 min per person on two occasions (~2.5 h). The novice operator then required ≥14 practice measurements, with accuracy continuing to improve up to 30 participants. At least 30 training measurements are recommended for novices to take acceptable AIx75 measurements after a familiarization training.

Keywords: pulse wave velocity, augmentation index, pulse wave analysis, training, practice, experience, operator, SphygmoCor XCEL

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INTRODUCTION

Arterial stiffness is one of the earliest predictors of the onset of hypertension (1) and can be gauged by measuring pulse wave velocity (PWV) or pulse wave analysis (PWA). Pulse wave velocity is the gold standard in non-invasive arterial stiffness assessment (2), and is the transit time of a pulse wave measured between two pre-defined anatomical locations [distance traveled (m)/pulse transit

time (s)]. In particular, the carotid-femoral PWV (cf-PWV) provides the most clinically relevant, non-invasive arterial health measurement as the pulse wave passes through the aortic artery (2).

Moreover, PWV is a strong predictor of future cardiovascular events (3), particularly in younger individuals at intermediate risk (4). Increased aortic PWV has been associated with reduced VO_{2max} performance in sedentary, middle aged participants (5), subclinical disease in coronary, lower extremity and cerebral arterial beds (6), and reduced skeletal muscle mass (7, 8). Furthermore, higher PWV has been associated with reduced cognitive ability in hemodialysis patients (9) as well as in older adults (10). The range in subjects receiving arterial stiffness assessments has also been broadened to include neonates (11), children (12, 13), adults (14), the elderly (15), and athletic populations (16). The wide use of these arterial stiffness measurements has enabled the establishment of normal and reference values aiding the assessment and interpretation of arterial health measures (17).

The central pressure waveform and its derived measures such as measurements of central pressure, pulse pressure and the Augmentation index (AIx) collected during the automated PWA provide an indication of peripheral arterial stiffness rather than central arterial stiffness as in PWV. AIx is the ratio of the central augmented pressure to the central pulse pressure expressed as a percentage and provides information on the interaction of the forward moving pressure wave (caused by ventricular contraction), with the backwards-moving reflective wave generated when the forward-moving wave meets a bifurcation in the artery (2). PWA has had broad application to numerous populations and in numerous contexts. For example, higher AIx has been associated with increased cardiovascular risk (18, 19), lower cardiorespiratory fitness (20), and increased clinical severity of coronary artery disease and percutaneous coronary intervention treatment in patients with a high Framingham risk score (18). In addition, higher AIx has also been associated with normotensive kidney disease (21), headaches and migraines in obese participants (22), and lower academic and motor performance in adolescents (23).

The rise in arterial stiffness research over the past few decades results from the availability of reasonably portable, relatively easy to use, time-efficient, and non-invasive devices. Recently, AtCor Medical Pty Ltd. released the SphygmoCor XCEL device which uses cuff-based volumetric displacement to detect the brachial and femoral pulse waves rather than applanation tonometry (24). These cuff-based measurements for PWA have vastly reduced measurement complexity and measurement time while providing validated methods of collecting arterial stiffness (25, 26).

Increased accessibility to more automated arterial stiffness measurement devices has meant that measuring arterial stiffness is no longer limited to a few highly-trained specialists. For example, some studies have reported measurements taken by relatively novice operators (27, 28). While these studies have reported good repeatability between novice operators, no comparisons were made between novice vs. experienced operators. To our knowledge, no evidence-based recommendations exist for the training periods required to achieve reliable expert-level measures of arterial stiffness. Therefore, there is a wide range in what constitutes a "well-trained" operator. For example, reported PWA training periods range from 2-day theoretical/practical workshops and 35 practice measurements (27), to 110 PWA measurements (29), while PWV measurements have been measured after as little as 15 practice measurements in work colleagues (28).

Subtle differences in tonometer technique, software literacy, and confidence of the operator may reduce measurement accuracy amongst novice operators. As Vlachopoulos et al. (30) found that a 1 m/s increase in PWV corresponded to an increase in cardiovascular risk by ∼15% (after adjusting for age, sex, and risk factors). Any technique-related PWV measurement inaccuracy could have implications for clinical decision-making by health practitioners and the outcomes of care for their patients.

Regarding the SphygmoCor XCEL in particular, there are several measurement options in the module's capture settings. For example, if the operator selects the "automatic capture" option for their assessment of PWV, the operator may opt for an automatic capture period of 5, 10, or 20 s (as opposed to the operator selecting when to capture the pulse waves) wherein the software will capture recorded pulse waves over this duration, provided they are of sufficient quality. Aside from a recommendation to use the 20 s automatic capture for patients with unstable heart rates (31), very little advice is provided in the Operator's Manual regarding the best capture setting to use for consistent results. Reporting measurement capture duration settings is unconventional in arterial stiffness studies, thus little is known about which of these time-periods would provide the most reliable results when used by a novice operator.

Therefore, the first aim of this study was to monitor novice vs. experienced operator differences chronologically to determine the minimum training time required by novice SphygmoCor XCEL operators to reach expert-level accuracy. The second aim was to compare the differences between the inter-operator variability between 5, 10, and 20 s measurement capture times to provide a recommendation on which capture period is most reliable during the learning process.

In this study, the cf-PWV was measured using carotid applanation tonometry. Although it is not affected by wave reflection, it requires skill by the operator to obtain accurate applanation (flattening of the arterial wall) which can be difficult since the artery can move freely under the sensor and needs to be stabilized by pressure on nearby neck structures (32). PWA was measured using volumetric cuff displacement of the peripheral pressure waveforms to generate a corresponding central aortic pressure waveform (31, 33). One might assume that there would be more inter-operator variability of cf-PWV which requires accurate tonometer placement and pressure, as opposed to the automated measure of AIx which only requires proper cuff placement.

MATERIALS AND METHODS

Participants

Participants were recruited from posters at a university's recreation center and through word-of-mouth. Inclusion criteria was participants ≥18 years of age who were able to attend a

scheduled appointment in the morning. Exclusion criteria was participants who had a double mastectomy, were hypotensive or had or were treated for any cardiovascular disease (CVD) or peripheral artery disease (PAD). Hypotensives were excluded since they have a harder pulse to transduce, and are one of the more challenging patient groups for novice operators. Including hypotensive participants would give a distinct advantage to the more experienced operator, thus unduly impacting the inter-operator differences. Participants with CVD or PAD were excluded as some may have irregularities in blood pressure which could change for each operator, again, impacting inter-operator differences.

Forty-five participants were initially enrolled in the study, but four participants were later excluded from the dataset due to technical error (novice operator failed to move inflation hose from arm cuff to thigh cuff) ($n = 2$), environmental disruption causing disruption of measurement ($n = 1$) and the inability to capture PWV data by both operators ($n = 1$) due to high adipose tissue in neck (details in recommendations and conclusion).

The final dataset comprised 41 participants (18 males, 23 females, age: 46.6 ± 14.9 years; weight: 74.9 ± 14.8 kg; height: 171.9 ± 7.8 cm; BMI: 25.2 ± 3.8; systolic blood pressure: 122.8 ± 14.7 mmHg; diastolic blood pressure: 76.0 ± 8.9 mmHg; resting heart rate: 57 ± 9.2 bpm). Seventy-eight percent of the participants considered themselves physically active (≥150 min/week), with the remaining 22% physically active for <150 min/week. Ethical approval was obtained from the local university's Human Ethics Committee, and all participants provided written informed consent.

Procedures

To understand the differences between an experienced and novice operator, 2 exercise-scientists collected data by serving as the device operators for this research project. The first operator had ∼1 year experience with regular intervals of collecting PWV and AIx measurements using the SphygmoCor XCEL, testing over 80 people in total. The second operator had no experience collecting arterial health measures prior to this study. The novice operator was asked to read the entire operator's manual for SphygmoCor XCEL System v1 to familiarize herself with the device, measurement protocol and to become familiar with the software. The novice operator received 1-h of measurement training which comprised of technical information as well as PWA and PWV measurement demonstrations provided by the experienced operator and other trained operators from the research team. The novice operator then practiced on five different people, for 10–15 min per person on two occasions (2 just after the technical information and demonstration, and 3 on the morning prior to the research-related data collection). During these two training sessions, the experienced operator as well as two other trained operators from the research team provided feedback to the novice operator during and after testing and they answered her questions as needed. To ensure comparable familiarization prior to the examination of the test participants, the experienced operator also attended the technical information session, and performed similar "practice" measurements on the same preparation participants for 10–15 min each.

The novice and experienced operators alternated in their testing order whereby the operator who was first to collect data from the first participant would then be second to collect from the second participant and so on. Operators were not privy to each other's measurements and results. Each operator was allocated a maximum of 30 min to capture all the recordings which were conducted in the following order: height and body composition analysis, PWA, 5-s PWV, 10-s PWV, and 20-s PWV. Between operator recordings, the participant was asked to stand up and walk around for a few minutes to mimic the starting test conditions for the second operator as the participant walked to the first test from the building's reception.

The second operator then re-measured for height and body composition. All testing sessions followed identical protocols to reduce or eliminate any protocol-related measurement errors between operators. Pulse wave velocity and AIx data from each operator were recorded in an Excel spreadsheet. This grouping allowed for the comparison of measurement agreement between the novice and experienced operator over time to determine the effect of operator experience on measurement accuracy.

Arterial Stiffness Assessment

Pulse wave velocity and PWA are two of the most commonly reported arterial stiffness measurements (34), and therefore comprised the focal measurements of arterial stiffness in the present study. Participant conditions were standardized according to the recommendations listed by the expert consensus document on arterial stiffness (2).

All measurements were conducted between 5:30 and 11:30 a.m. from Monday to Friday for 2 consecutive weeks. Participants were instructed to avoid strenuous physical activity for 12 h, tobacco and caffeine for 4 h, and to have fasted from food and alcohol for 6 h prior to assessment (35). All prescribed medications were continued as usual. Following arrival, the participants were familiarized with the lab space, equipment, operators, and research protocols. Height (SECA220 stadiometer, SECA GMBH & Co., Germany) and body composition (InBody 230, Biospace, Seoul, South Korea) were then measured.

Pulse Wave Analysis (PWA)

Supine PWA was assessed automatically using the SphygmoCor XCEL System v1 (AtCor Medical Pty Ltd., Sydney, Australia) technology and software (SphygmoCor XCEL Software Version: 1.2). Briefly, a pneumatic blood pressure cuff connected to the SphygmoCor® EXCEL was fitted firmly over the participant's right upper arm and aligned over the brachial artery. The participant then rested for 5-min in a supine position. The Sphygmocor XCEL software was used to initiate the cuff inflation for the automatic PWA measurement. In this assessment, the brachial cuff automatically inflates to measure brachial systolic and diastolic pressure, then it deflates and automatically re-inflates after 5 s to capture the PWA waveform (35). The participant's brachial blood pressure, and AIx measurements were then recorded separately onto an Excel spreadsheet. As heart rate has a profound effect on AIx (36), the AIx was normalized to a heart rate of 75 bpm (AIx75).

Pulse Wave Velocity (PWV)

Measurement sites for the PWV analysis were the right carotid artery and right femoral artery (2). The direct method was used to determine the PWV distance as this technique is less complex (less risk of compounding error) than the subtraction method and is more commonly used in arterial stiffness research (17). The location of the strongest carotid pulse was palpated and then marked on the participant's neck. A femoral cuff was then fitted as high up as possible on the participants' right thigh over either thin clothing or bare skin. The distance between the carotid mark and the top edge of the femoral cuff was measured using SECA 207 folding calipers (Hamburg, Germany) designed to increase measurement accuracy in individuals with large breast size or abdominal obesity (2, 37, 38). The distance between the femoral artery and the femoral cuff was then measured by flexing the participant's hip to locate the participant's inguinal crease, and then measuring the distance between the top edge of the femoral cuff and the likely location of the femoral artery in the inguinal crease. The straight line "femoral to cuff" and the "carotid to cuff" distances, were entered into the SphygmoCor software. The "femoral to cuff" measurement is automatically subtracted from the "carotid to cuff" measure to indicate the carotid-femoral distance (typically 500–800 mm) which is the distance used in calculating PWV.

The brachial blood pressure was recorded after the PWA measurement. Then, the capture time (either 5, 10, or 20 s) was selected in the SphygmoCor software. The tonometer was positioned over the marked location with the strongest carotid pulse previously detected by the operator. To standardize recordings and reduce subjectivity, the device was set to automatically capture the pulse wave recording after high quality waveforms had been recorded during the specified capture time (i.e., 5, 10, or 20 s). An "automatic capture" was attempted for a full cuff inflation/deflation cycle, plus the first 20 s of the second cuff inflation. Thereafter, if an automatic capture was unsuccessful but the waveforms appeared to be of sufficient quality to the operator, a manual capture measurement was taken. The operator indicated whether a manual or automatic measurement was taken. Regardless of the automatic or manual capture, the measurement quality was assessed automatically by the SphygmoCor software (based on consistent pulse peaks, troughs and amplitude), and were indicated with either a green "quality controlled" tick, or a red cross indicating a lower quality measure (35). All data for inclusion in this research project were required to have a quality-controlled tick. Any manual capture which did not meet the quality control standards was repeated.

Statistics

Participants' data were ordered chronologically according to test date and time. Data were then arranged into quintiles to detect any longitudinal differences between the experienced and novice operators.

A simple linear regression was used to determine the validity of the PWA (AIx75) and PWV (m/s) between the experienced (criterion measure) and the novice (practical measure) operators. Measurements were all log transformed prior to analysis, and then back-transformed into a coefficient of variation (CV; Standard deviation expressed as a % of the mean) to reduce non-uniformity of errors (39). Where a dataset had a negative value, a constant (absolute of the maximum negative value +1) was added to all observations in the dataset such that the smallest observation was always 1.

The CV and the validity correlation coefficient (Pearson correlation) were presented to reflect the prediction error, and variable alignment, respectively. The validity correlations were interpreted using the following scale: <0.1, trivial; 0.1–0.3, small; 0.3–0.5, moderate; 0.5–0.7 large; 0.7–0.9, very large; >0.9 extremely large to indicate the level of agreement between the two operators. Correlations >0.9 are considered acceptable for validity studies (39).

Finally, the difference in the experienced and novice operator's measurements was determined, along with the average and SD of these differences for each quintile. The accuracy of the measurement was assessed using both the mean difference and the SD and was interpreted according to the ARTERY Society Guidelines whereby: Excellent: mean difference $\leq$ 0.5 m/s and SD $\leq$ 0.8 m/s; acceptable: mean difference $<$ 1.0 m/s and SD $<$ 1.5 m/s; and poor: mean difference $\geq$ 1.0 m/s or SD $>$ 1.5 m/s (40).

RESULTS

For PWA, the quintile-derived averages for the AIx75 measurements were closer between novice and experience operators than the AIx measurements (see **Table 1**). Statistics in **Table 1** indicate that all quintile-derived PWV averages across all time-periods were similar between novice and experienced operators. Measurement similarity between averages worsened in quintile 5 in all measurements (**Table 1**). In all quintiles, the experienced operator had a distinct measurement advantage given this person was much more trained than the novice operator. As a result, there was a positive bias in the mean of the differences between operators as indicated in **Figure 1** with all mean differences being plotted above 0.

During measurement capture, the novice operator relied on predominantly manual capture measurements in the first quintile. In Quintiles 2–5 both the novice and experienced operators relied predominantly (>75%) on automatically captured measurement (**Figure 1**).

On the whole, measurement reliability (intraclass correlation) improved and measurement variability (coefficient of variation) decreased across the first 4 quintiles, particularly between quintiles 1 and 2 (**Table 2**). Measurement variability between operators in the 5, 10, and 20 s PWV measurement periods all demonstrated "acceptable" agreement from quintile 2 onwards (**Figure 2**), despite worsening in quintile 5 (**Table 2**).

DISCUSSION

The current research is the first to examine the variation in PWA and PWV measurements between a novice and experienced operator. Furthermore, this research is the first to report the differences in measurement accuracy between PWV recording lengths of different durations. Our study found that

TABLE 1 | Means and standard deviations for PWA and PWV measurements recorded by an experienced (criterion) and novice (practical) operator.

	Quintile 1 ($n = 8$)		Quintile 2 ($n = 8$)		Quintile 3 ($n = 9$)*		Quintile 4 ($n = 8$)		Quintile 5 ($n = 8$)	
	Criterion	**Practical**	**Criterion**	**Practical**	**Criterion**	**Practical**	**Criterion**	**Practical**	**Criterion**	**Practical**
PULSE WAVE ANALYSIS										
AIx	18.6 ± 14.9	17.8 ± 11.3	15.4 ± 10.1	19.0 ± 11.0	29.4 ± 11.4	29.1 ± 9.1	20.1 ± 8.4	18.9 ± 10.8	20.9 ± 14.3	14.0 ± 14.1
AIx75	10.9 ± 14.0	9.6 ± 9.9	9.5 ± 9.8	8.4 ± 9.7	21.3 ± 13.0	21.9 ± 10.7	11.3 ± 10.0	11.1 ± 12.9	12.0 ± 17.6	4.8 ± 15.9
PULSE WAVE VELOCITY										
5 s PWV (m/s)	9.8 ± 1.6	8.5 ± 3.0	9.6 ± 1.12	9.2 ± 0.8	10.1 ± 1.7	10.4 ± 1.8	9.6 ± 1.0	9.3 ± 1.1	9.4 ± 1.2	8.8 ± 1.3
10 s PWV (m/s)	10.1 ± 1.3	9.6 ± 1.6	9.6 ± 1.7	9.4 ± 1.6	10.0 ± 2.1	9.9 ± 1.6	9.5 ± 0.9	9.3 ± 1.2	9.6 ± 1.2	8.7 ± 1.2
20 s PWV (m/s)	10.1 ± 1.4	9.0 ± 2.7	9.8 ± 1.5	9.7 ± 1.6	10.1 ± 1.9	10.0 ± 1.6	9.6 ± 0.9	9.4 ± 1.2	9.4 ±1.3	8.8 ± 1.2

Data were arranged chronologically based on the date and time of the participant's assessment, and then divided into quintiles in order to assess the progressive effect of learning on measurement accuracy. Data are presented as mean ± standard deviation.
**10 and 20 s PWV quintile 3 groups had 8 participants.*

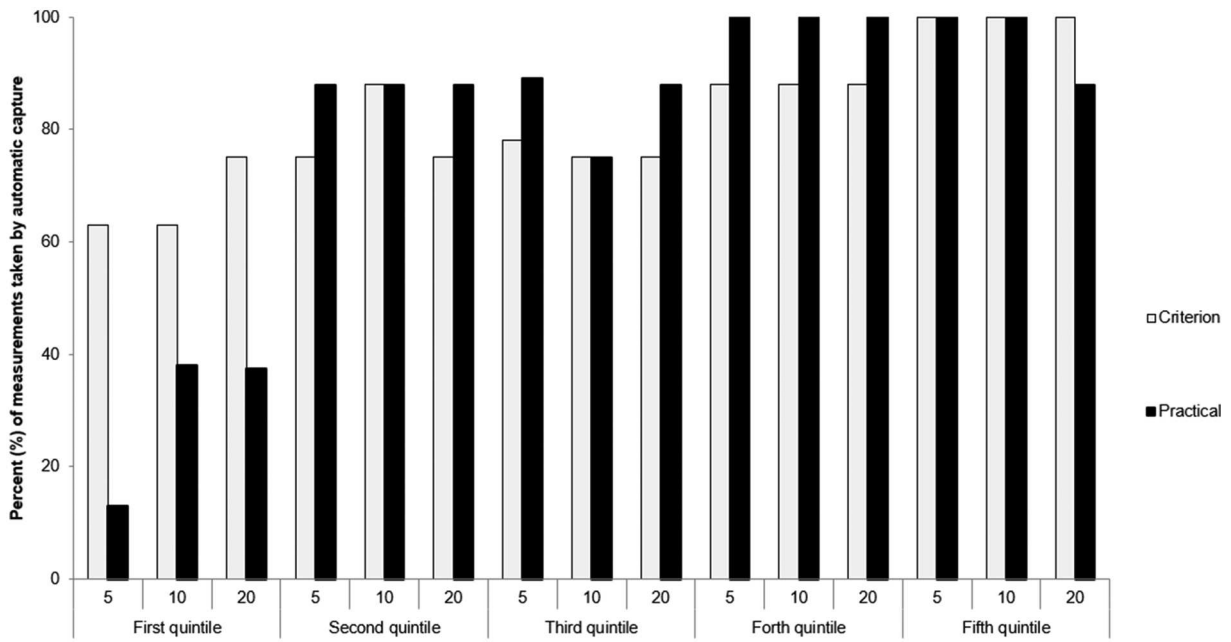

FIGURE 1 | Proportion of "automatic captures" for the experienced (criterion) and novice (practical) operators during the 3 time recording windows (5, 10, 20 s). Quintiles were ordered chronologically according to test date and time (Quintile 1 contains first participants tested).

measurement agreement and accuracy substantially improved between Quintiles 1 and 2 and was typically maintained or improved (with small variability) from quintiles 2–4. These findings indicate a very steep initial learning curve (over quintile 1 + 5 pre-study practice measurements), where after small variations in agreement between experienced and novice operators occurred. The measurement accuracy was similar between 5, 10, and 20 s recording periods in quintiles 2–5.

In the last quintile, the agreement for both AIx75 and PWV worsened. It is possible that over the course of the intensive 2-week data-collection period, which started at 5:30 a.m. and typically persisted for 6 h, that the operators were demonstrating signs of mental fatigue. The data collected over a 2-week period was evenly spread with a minimum of 3 and a maximum of 6 participants measured per morning, accounting for ∼20 participants per week. Monday was a practice testing day where 3 of the 5 training participants were tested as a way to run through the official testing protocol with the novice and expert operators measuring in random order. Tuesday marked the first day of data collection which would be used for analysis and this lasted for two consecutive work weeks with the most popular day to test being Thursday. In order to minimize operator fatigue and measurement errors, the maximum number of participants tested was kept to 6 per morning. Tuesdays and Fridays had 3–4 participants where Monday, Wednesday, and Thursday had 5–6 participants. Both operators found that an even spread of 3–6 participants per morning allowed for learning through training repetition without inducing too much measurement

TABLE 2 | Typical error and validity correlation of the log-transformed data for main measures of PWA and PWV between the experienced (criterion) and novice (practical) operators.

	Quintile 1		Quintile 2		Quintile 3		Quintile 4		Quintile 5	
	Validity correlation (90% CL) qualitative interpretation	**CV (%)**	**Validity correlation (90% CL) qualitative interpretation**	**CV (%)**	**Validity correlation (90% CL) qualitative interpretation**	**CV (%)**	**Validity correlation (90% CL) qualitative interpretation**	**CV (%)**	**Validity correlation (90% CL) qualitative interpretation**	**CV (%)**
5 s PWV	0.5 (−0.2 – 0.9) Moderate	16.1 (10.9 – 33.2)	0.8 (0.4 – 0.96) Very large	7.3 (5 – 14.4)	0.95 (0.8 – 0.99)* Extremely large	6.2 (4.4 – 11.5)	0.9 (0.6 – 0.97) Very large	4.8 (3.3 – 9.5)	0.6 (−0.0 – 0.9) Large	10.5 (7.1 – 21.0)
10 s PWV	0.8 (0.4 – 0.95) Very large	8.2 (5.6 – 16.3)	0.8 (0.4 – 0.96) Very large	10.5 (7.1 – 21.0)	0.9 (0.7 – 0.98)* Extremely large	9.1 (6.2 – 18.1)	0.9 (0.5 – 0.97) Very large	4.7 (3.2 – 9.2)	0.9 (0.6 – 0.97) Very large	6.2 (4.2 – 12.2)
20 s PWV	0.2 (−0.5 – 0.8) Small	14.8 (10 – 30.2)	0.9 (0.7 – 0.98)* Extremely large	6.9 (4.7 – 13.7)	0.9 (0.7 – 0.98)* Extremely large	8.6 (5.9 – 17.2)	0.8 (0.4 – 0.95) Very large	6 (4.1 – 11.9)	0.9 (0.5 – 0.97) Very large	7.4 (5 – 14.6)
AIx75	0.7 (0.1 – 0.9) Large	67.5 (42.8 – 168.5)	0.8 (0.3 – 0.94) Very large	39.3 (25.7 – 88.7)	0.8 (0.4 – 0.9) Very large	25.5 (17.4 – 50.5)	0.97 (0.88 – 0.99)* Extremely large	15 (10.2 – 30.8)	0.97 (0.87 – 0.99)* Extremely large	25.3 (16.9 – 54.1)

$r > 0.9$; Data were arranged chronologically based on the date and time of the participant's assessment, and then divided into quintiles in order to assess the progressive effect of learning on performance. CL, Confidence limits; qualitative interpretation: the magnitude of the agreement between the novice and experienced operator's measurements; CV, coefficient of variation; 5 s PWV, pulse wave velocity measured over a 5-s window; 10 s PWV, pulse wave velocity measured over a 10-s window; 20 s PWV, pulse wave velocity measured over a 20-s window.

fatigue. Some of the common effects of mental fatigue include changes in mood, task motivation, and performance deficit (41). As the effects of mental fatigue may be more pronounced in unfamiliar tasks vs. automatic tasks [as cited by van der Linden et al., Broadbent and Broadbent, and Hockey (41–43)], the accumulating increase in mental fatigue may have had a greater impact on the novice operator compared to the experienced operator. The imbalance in this effect may be responsible for the increase in the measurement variability in quintile 5.

Alternatively, personal factors such as overconfidence, or, more specifically, overestimation of one's actual performance (44) may have reduced measurement agreement in the last quintile. Overestimation is often associated with a second form of overconfidence known as overplacement, wherein a person believes their performance to be better in relation to others (44). Interestingly, some research has indicated that an increase in personal familiarity without an increase in observing others in a similar context increases overplacement (44). However, overestimation is more likely to arise from imbalanced information about one's own performance compared to others (specifically that one has worse knowledge of others than of oneself) (44) than of self-seeking or wishful thinking (45). Unfortunately, overconfidence can result in poorer decision-making and poorer outcomes (45) than if better self-regulation and monitoring strategies are employed (46). In the present study, the novice operator gained familiarity with the equipment over the course of the study, but, to avoid research bias, was not privy to the experienced researcher's measurement sessions. This research design, while scientifically thorough, possibly contributed to overconfidence and, ironically, to poorer learning outcomes on the part of the novice operator.

PWV Comparison With Other Studies

Our findings support those of Grillo and colleagues who suggested that acceptable—excellent measurement recordings require a relatively short learning period (47). Their CV using the SphygmoCor Vx in patients with high cardiovascular risk was higher (CV = 9.5%) than the CV in the present study after a similar amount of training (2 weeks). The SphygmoCor XCEL intra-test reliability reported by Hwang et al. (26) was considerably smaller than our study (stronger validity correlation: $r > 0.99$ vs. $r \sim 0.9$ in our study) however, no CV of the intra-test measurement differences were presented. The closer measurement repeatability in Hwang et al.'s (26) study is possibly due to a single, well-experienced researcher taking all measurements.

The inter-operator PWV agreement in quintiles 2–4 in our study support previous research. For example, our levels of agreement were slightly higher than those reported by experienced operators measuring PWV in patients with kidney failure (48). That is, Frimodt-Møller et al. (48) reported a mean inter-operator difference in aortic PWV of 0.3 m/s compared to a range of 1–4 m/s in quintiles 2–4 our study. However, our study reported better levels of agreement when compared to Grillo et al. (47) who assessed PWV in patients with high cardiovascular risk. Even when Grillo et al. (47) analyzed inter-operator variability in

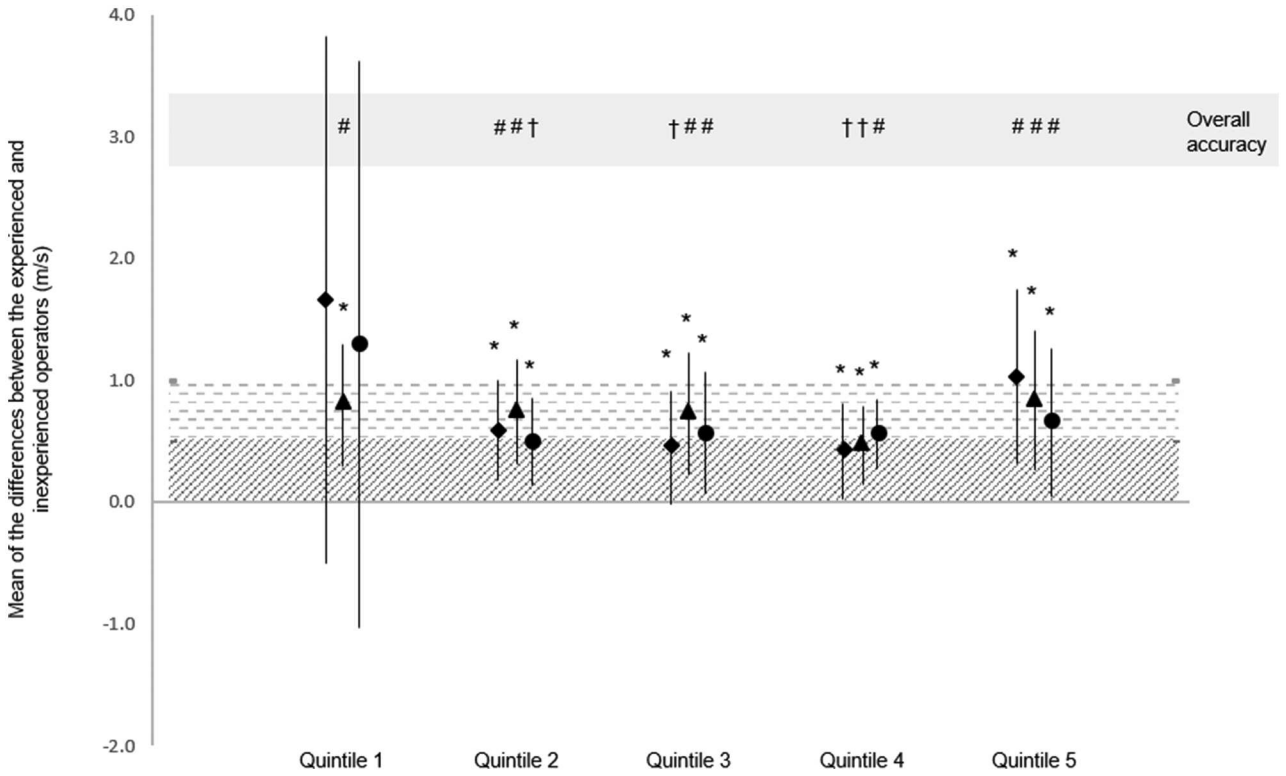

FIGURE 2 | Mean difference and SD of the mean differences between experienced and novice operator, and the qualitative outcome. Data represent the mean of the differences in PWV measured by experienced and novice operators, and the SD of the mean differences. Accuracy of the mean differences is represented using diagonal stripes (excellent accuracy) and horizontal stripes (acceptable accuracy). *Excellent SD accuracy. Overall accuracy is the worse of either the mean difference or SD qualitative interpretation: †Excellent overall accuracy; #Acceptable overall accuracy. ##PWV from 5 s recording; ††PWV from 10 s recording; ###PWV from 20 s recording. Quintiles were ordered chronologically according to test date and time (Quintile 1 contains first participants tested).

a sub-set of patients with reasonable arterial compliance (i.e., a PWV of <10 m/s), our CV typically remained lower than their data (PWV < 10 m/s = CV of 8.5% vs. PWV > 10m/s = CV of 10.4%; compared to a CV range of 4.7–10.5 in quintiles 2–5 in our study).

The participants in our study were middle-aged, mostly physically active, and boarderline normal/overweight, and with generally healthy blood pressure. Therefore, we would have anticipated that the mean PWV values would lie closer to the reference value of 7.2 m/s reported by Mattace-Raso et al. (17). Instead the PWV in our population was only slightly lower than the mean PWV of older patients with kidney disease [PWV ∼ 10.5 m/s (47)], and similar to an older population with chronic kidney disease [PWV = 9.9 m/s (48)]. While both Grillo et al. (47) and Frimodt-Møller et al. (48) used Sphygmocor devices to monitor the aortic (carotid-femoral) PWV, their devices were less automated earlier models than the device used in the present study. As different technologies are known to produce different measurements and variances (47), some of the differences seen between studies may reflect equipment discrepancies.

PWV and Recording Window

The SphygmoCor XCEL device offers three different measurement durations for the capture of PWV waveforms (5, 10, or 20 s). The operator manual indicates that while 5 s is the default setting, longer recording times may be required for participants with slower respiratory cycles and/or with more variable heart rates (31). The "capture time" is not always reported in journal articles and little is known about the differences in measurement agreement or variability between these time selections. Our study found that inter-operator agreement improved considerably in all measurement periods between quintile 1 and 2, suggesting that there is a strong initial learning effect regardless of automatic capture time. The 10-s time-period was the only measurement that yielded acceptable agreement between the novice and experienced researchers in quintile 1 and so may be preferable for training operators. By quintile 4, both the 5- and 10- s recordings had "excellent" acceptibility, very large correlation coefficients and a CV < 5%. However, the 5-s measurement was most succeptible to inaccuracy in quintiles 1 and 5 which may indicate that this recording window may be inappropriate for novice operators, or during times where mental fatigue (41) or overestimation (44) may be exhibited.

PWA Comparison to Other Studies

Our measures of AIx75 in a reasonably healthy population were similar to those reported in other healthy poplations,

including the "healthy participants" [AIx = 20.2 (26)], and pregnant females [AIx75 = 11.7 (29)]. Conversely, our data were considerably lower than those taken in ambulatory hospital patients [AIx75 ∼ 19 (27)], or patients with high cardiovascular risk [AIx75= 26.6 (49)].

The AIx or AIx75 measurement appears to be a measurement with considerably higher variation than any of the PWV measures. This has also been reported in other studies with Magda et al. (49) reporting excellent PWV inter-operator variability of 2.5% but only satisfactory inter-operator variability of 8.4% for AIx. Indeed, Magda et al. reported an even higher intra-operator difference of 17.8% for AIx which further demonstrates the varaibility of this measure. The CVs reported by Magda et al. (49) were about half the size of the present study. These could have been attributed to the longer experience (∼35 practice measurments, and a 2-day training workshop) of the data collectors in their study, or to the differences in the Complior and SphygmoCor devices.

The health of the participants my also influence the level of inter-operator AIx75 agreement. Our study (quintiles 2–4) reported slightly better mean inter-operator differences in AIx75 than studies using kidney disease patients (48) or ambulatory hospital patients (27). On the other hand, our study reports ICCs for AIx that are similar to those of Hwang et al. (26) in their population of healthy adults of a similar age to our participants. In particular, Hwang and colleagues reported similar ICCs (r = 0.98) to ours ($r = 0.97$ in quintiles 4 and 5), as well as reporting similar mean differences over consecutive measurements (AIx range: ∼1.1 in Hwang et al.'s study which was similar to the mean difference range in quintiles 3 and 4 in our study).

Despite excellent coefficients of variation and small differences in mean, the CV associated with AIx are reasonably large (15–25% in the last 3 quintiles in our study). However, when the average AIx75 measurements taken in triplicate was used, the trained nurses (2-day workshop and ∼35 practice measurements) demonstrated an inter-technican AIx difference of 0.1 (29). Therefore, an average of 2–3 AIx measurements may be more reliable than taking only one measurement.

There is little research available regarding potential reasons for the considerably higher variability in AIx. The AIx is a complex measurement that requires accurate capture of the pulse waveform including accurate measurement of the first and second systolic peaks as well as the pulse pressure (2). As our arteries are dynamic organs which are continually responding to alterations in shear stress (50, 51) through both flow-mediated dilation and constriction (50), the participant's internal and external environments may have transient effects on the augmentation index. This measurement complexity combined with the automatic nature of the measurement could be responsible for higher variability. That is, while using the tonometer to assess PWV, the operator may make slight adjustments to the tonometer placement or pressure in order to maximize the quality of the waveform capture. However, once the cuff has been fastened to the participant's upper arm in the assessment of AIx, the operator has very little control over the quality of the measurement as it is no longer possible to adjust or correct cuff pressure or placement in response to subtle changes in physiological landmarks or for any external stimuli that may interefere with the measurement. However, with greater practice, cuff placement and fit may become more consistent, and instructions to the particpant may become clearer, all resulting in a more accurate measurement. Unfortunately there is no research available to support or refute this suggestion and further work in this area is required.

Automatic Capture Findings

Recordings using the equipment's "automatic capture" function was prioritized over manual measurements to standardize outcomes. To this end, the novice operator depended primarily on the manual capture technique in the first quintile ($n = 8$), but by the second quintile demonstrated similar automatic capture proportions to the experienced operator (**Figure 1**). There was also a gradual increase in the proportion of the experienced operators' "automatic capture" measurements from the first to the fifth quintile. These data suggest that experienced operators may also benefit from practice measurements prior to a large research study.

Limitations

The limitations associated with reliability and validity studies is the manner in which the levels of agreement have been interpreted. While we have elected to examine variability between researchers using the ICC and CV, others have preferred to use Bland-Altman plots. Generally the studies using Bland-Altman plots have reported high PWA reproducibility (26, 27, 29, 48). However, our interpretation of reliability using the CV to represent the typical error of the estimate or prediction error, and the ICC to link the assessments (52), appears to be more conservative particularly regarding the AIx75 outcomes. These differences in methodological approaches makes comparisons between studies more challenging. In the end, we attempted to compare our reliability with others using primarily the inter-operator difference in means, which does not account for any variation in the measurements and therefore can only provide limited substance to our comparison.

Another limitation is that participant numbers in each of the quintiles ($n = 8$) was small, and therefore may reduce the statistical power of the analyses. A power calculation was made a priori. The sample size needed in this inter-operator reliability study with two operators was designed to achieve a kappa value of K = 0.80, with an assumed probability of positive ratings of 0.30, and a desired width of the CI at $w = 0.20$, with a 0.95 level of confidence as suggested by Shoukri et al. (53) equates to a total sample size of 117 instead of the 41 recruited. The sample was limited as the study took place at a university which was nearing a major holiday so fewer participants were available than anticipated. However, having smaller groups does provide better insight into the individual variation for each participant, which is something that is important in a practical or clinical context. Furthermore, some data was necessarily excluded from the dataset due to equipment failure or major errors in technique related to the learning process but rendering the data inaccurate.

Moreover, the participant cohort studied was atypical for the majority of research studies and patient work conducted in

clinical settings, as they were healthy (free from cardiovascular disease) and generally physically fit (78% physically active). Therefore, training recommendations in this study are merely the minimum recommendations and might need to be higher for novice operators aiming to accurately record PWV from unfit and unhealthy populations, i.e., with cardiovascular diseases.

As there was only one novice researcher being compared against one experienced researcher, considerable variation might exist between the findings of this study and other studies involving different experience gaps between operators or between other novice operators. For example, the swiftness of the learning response will vary depending on the ability of the operator to learn and understand a new skill (using a tonometer) as well as the ability to keep a steady hand, locate the anatomical landmarks, detect regions of the strongest pulse, measure distances accurately, and react quickly sensitively to physiological changes to capture good data. Future research should consider comparing an experienced operator with a larger number of novice operators.

Finally, as there is only one study which used the SphygmoCor XCEL device (26), we have compared our findings with other studies using different techniques and devices which may add further differences between outcomes. Moreover, this study examines a participant cohort that may vary for what is considered typical in research or clinical work.

Recommendations and Conclusion

Our study reported acceptable—excellent PWV measurement accuracy by a novice operator following as little as 14 practice participants (5 practice participants + 8 participants in quintile 1 and one extra for tonometer measurement difficulty). Counter-intuitively, the automatic AIx75 measurement required more practice before measurement agreement reached acceptable levels of $r > 0.9$ (39). For both AIx75 and PWV, measurement accuracy typically continued to improve over the first 4 quintiles (increased validity correlation and reduced CV). Therefore, for operators who have no experience measuring PWV with the SphygmoCor XCEL, we recommend a practice period of at least 14 participants prior to practical data collection (**Figure 2**), and ideally 30 participants (5 practice participants + first 3 quintiles which yielded excellent overall accuracy for 5 and 10 s time-periods, and borderline-excellent overall accuracy for the 20 s time-period, **Figure 2**) for either research or clinical use. The 10 s measurement capture interval appeared to provide the most accurate measurements for beginner users.

Despite AIx75 being an automated measurement using a pneumatic brachial cuff, the measurement itself is more complex (involving numerous variables detected from a pressure waveform). As such the AIx is associated with a considerably higher inter-operator CV. One critical finding that should be strongly considered for future implications is that both operators experienced a general inability to capture PWV data from one participant who had a seemingly high proportion of neck adipose tissue, making the carotid pulse difficult to detect with the tonometer. Since it is impossible to predict the likelihood of measuring someone with high neck adipose tissue, we advise testing one extra person ($n = 14$) during the PWV training due to potential measurement difficulties but the AIx was unaffected by such a case so it remains unchanged at a recommended minimum of 30 training participants (5 practice measurements + first 3 quintiles for $r > 0.9$, and lowest CV of 15%) prior to clinical data collection.

Mental fatigue, and/or over-confidence may play a role in measurement accuracy, particularly in prolonged measurement intervals. Therefore, we recommend that operators are well-rested at the time of data capture, that the intensity of the measurement periods is moderated by taking regular breaks. Collaboration with experts/support throughout the learning process should be available to encourage a meticulous and reflective attitude to data collection.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Lincoln University Human Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CE and MH designed and planned the study. MH wrote and submitted the ethical application. CE and CL contributed to participant recruitment, data collection, and data entry. MH and CL conducted data analysis. MH, CE, and CL contributed to drafting different sections of the manuscript. All authors finalized the draft and CE submitted the final manuscript.

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Importance of Sensitive Vascular Measurements for Evaluating Effects of Lifestyle in Premenopausal Women

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Premenopausal women generally have a favorable cardiovascular risk profile, owing to young age and the protective effects of estrogen. Rates of hypertension and more advanced cardiovascular disease (CVD) are low in premenopausal women. A large body of epidemiological evidence has shown that lifestyle behaviors in midlife, i.e., cardiorespiratory fitness, physical activity, and healthy diet, are associated with lower risk of overt CVD and adverse cardiovascular outcomes in the future for men and women. Despite differences in future cardiovascular risk, brachial blood pressures might be similar between premenopausal women with favorable vs. unfavorable levels of lifestyle behaviors in early-to-mid-life. Here we make the case for deeper phenotyping by means of vascular function measurements, such as arterial stiffness, augmentation index, and endothelial function, to identify potential mechanistic pathways linking lifestyle behaviors in early-to-mid-adulthood with lifelong CVD risk in women. We describe considerations for vascular function measurement in premenopausal women and opportunities for investigators to fill in knowledge gaps to further our understanding of CVD risk assessment and CVD progression in premenopausal women.

Keywords: hormones, premenopausal women, arterial stiffness, subclinical atherosclerosis parameter, lifestyle and behavior

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INTRODUCTION

Lifestyle interventions sometimes do not elicit significant improvements in brachial blood pressure (BP) in premenopausal women (1, 2). Reasons for the lack of improvement in BP or other traditional cardiovascular disease (CVD) risk factors after behavioral interventions are typically attributed to the general good cardiovascular health of premenopausal women due to younger age and protective effects of ovarian hormones (3). Despite the occasionally null or small effect on some traditional CVD risk factors, i.e., BP, lipids, and body mass index (BMI), following some interventions, lifestyle behaviors in young and middle adulthood have been consistently associated with CVD and traditional CVD risk factors later in life (4–6). In fact, improving any lifestyle behavior or CVD risk factor during adulthood has been linked to lower likelihood of subclinical atherosclerosis in middle age, underscoring the importance of lifestyle for longer-term CVD risk (7).

We propose that more sensitive measures of vascular structure and function are needed to understand how lifestyle influences CVD risk and better evaluate the effects of lifestyle behaviors and lifestyle interventions in premenopausal women. Some studies that employed more sensitive vascular measurements pre- and post- exercise intervention, such as central BP, arterial stiffness and distensibility, carotid intima-media thickness, or ventricular-vascular coupling, reported improvements in some of these measures while brachial BP stayed the same (1, 2, 8–11). Interestingly, changes in different vascular measurements were detected after different types of exercise training (indoor cycling, endurance, or circuit style resistance training), suggesting that subclinical vascular adaptations to exercise might be modality specific. These data illustrate the need for more thorough vascular characterization in larger samples of premenopausal women after different types of exercise interventions (1, 2, 8–11). Effects of endogenous and exogenous ovarian hormones should also be evaluated or accounted for, as some hormones have been linked to vascular structure and function and CVD risk (**Table 1**) and can be measured in clinic and research settings.

RATIONALE FOR COLLECTION OF SENSITIVE MEASUREMENTS OF VASCULAR STRUCTURE AND FUNCTION IN LIFESTYLE INTERVENTIONS

Ascertainment of hard outcomes, i.e., cardiovascular events, morbidity, and mortality, can guide clinical decision making and are used for evaluation of intervention success (24). However, trials designed with such hard outcomes as endpoints require significant time and resources. Other major clinical trials have used traditional CVD risk factors as endpoints (25, 26). Utilizing more proximal outcomes that are on the pathway toward hard outcomes and traditional CVD risk factors, such as less favorable vascular structure and function, as endpoints is attractive (24, 27). Trials designed with these proximal outcomes as endpoints may reduce the numbers of participants required, length of follow-up, and costs of the trial (24). Furthermore, more proximal outcomes, like some measures of vascular structure, and function, might be more malleable to change in response to lifestyle interventions than traditional CVD risk factors and used to gauge the success or potential for such interventions to interrupt the progression toward CVD risk factors or overt CVD (24, 27). The tracking of vascular functional measurements might be particularly important for younger women because there may be abnormalities in these measurements even when CVD risk factors are at normal levels.

With the exception of coronary artery calcium, the value of novel or subclinical CVD risk factors, such as poor endothelial function (measured using flow-mediated dilation), systemic inflammation (c-reactive protein), and ankle-brachial index, beyond traditional CVD risk factors for identifying individuals at high-risk for atherosclerotic CVD endpoints has not been consistently supported (28, 29). However, unlike the Framingham Risk Score used in earlier studies, more recently developed risk prediction algorithms for atherosclerotic CVD include race and/or inflammation to estimate CVD risk in women (30, 31). Guidance from the Pooled Cohort working group suggested that, although the utility of c-reactive protein for assessing CVD risk requires further study and was not included in the primary algorithm, clinicians may consider c-reactive protein levels to guide preventive strategies if a risk-score based treatment decision is unclear (30).

Importantly, the atherosclerotic CVD risk prediction models were calibrated to identify patients at risk for atherosclerotic, but not myocardial, CVD (30). A new risk prediction model calibrated to detect heart failure with preserved ejection fraction (HEFpEF; a myocardial disease), which occurs more frequently in women, is distinct from the atherosclerotic models in that it includes BMI and QRS interval in its algorithm (32). High BMI is a strong risk factor for HEFpEF in women (5). Further, BMI is consistently linked to vascular structure and function throughout the lifespan (33, 34), and the increased rate of aortic stiffening seen in obese adults in a community-based sample was partially attributed to systemic inflammation (33). Thus, the use of sensitive vascular-related measurements that have been related to vascular structure and physiological function, such as c-reactive protein, endothelial function, or arterial stiffness, as trial endpoints that might help reclassify women at intermediate risk for HEFpEF holds promise.

TABLE 1 | Effects of ovarian hormones on vascular function measurements.

Ovarian hormone	Association with vascular measurements	Reference
Estrogen	↓brachial and central BP, ↑vascular reactivity	(12–16)
Progesterone	↓augmentation index, ↓vasoconstriction in response to agonists	(3, 17)
OCP	↑brachial and central BP during non-placebo phases vs. women not using OCP	(15, 18, 19)
AMH	↓in POI, ↑in PCOS, ↓carotid intima-media thickness in POI	(20–23)

BP, blood pressure; OCP, oral contraceptive pill; AMH, anti-Müllerian hormone, a biomarker of ovarian reserve; POI, primary ovarian insufficiency; PCOS, polycystic ovarian syndrome. Female hormones have been linked to vascular structure and function and CVD risk and can be easily measured in clinic. Higher levels of estrogen and progesterone tend to have beneficial effects on the vasculature. Associations of AMH with vascular structure are contextual and may vary between women with POI or PCOS vs. controls.

CONSIDERATIONS FOR DATA COLLECTION IN PREMENOPAUSAL WOMEN

There are challenges associated with collection of vascular function data in premenopausal women. Premenopausal women experience cycling of reproductive hormones, which affect some vascular function measurements, summarized in **Table 1** (35). Estrogen and progesterone have dilating and anti-constricting vascular effects, respectively (3). One study reported lower brachial and central BP and higher vascular reactivity, measured using brachial artery flow-mediated dilation and calf and forearm reactive hyperemia, during the late follicular phase of menstrual cycle, i.e., days 7–14, that immediately precede ovulation and are characterized by a rapid rise in estrogen (12). Augmented brachial flow-mediated dilation and endothelium-independent dilation, assessed by determining dilation in response to sodium nitroprusside, were also observed during the late follicular phase of menstrual cycle in a prior study (13). In contrast, three more recent studies found there was no change in brachial flow-mediated dilation across phases of the menstrual cycle at rest (14, 15) or during handgrip exercise (16). More studies are needed in larger samples and diverse women to clarify the issue; earlier studies either did not report race or reported mostly Caucasian participants with mean BMI under 30 kg/m^2. Augmentation index has been shown to be lower in the late luteal phase when progesterone is at its highest and estrogen is above the levels found in the early follicular phase (17). Beta-stiffness of the carotid artery and central pulse wave velocity were similar throughout the natural menstrual cycle in a recent study that included 33 women (18).

Use of oral contraceptive pills (OCP) might further complicate vascular assessment in premenopausal women and requires thoughtful consideration. While arterial stiffness was similar in women who do and do not use OCP, brachial and central BPs were higher during the non-placebo phases of the pill cycle in women who use OCP (15, 18, 19). Accordingly, the current standard is to account for phase of menstrual cycle or conduct all vascular assessment in the early follicular phase, i.e., days 1–7, when all reproductive hormones are at their lowest points (35, 36). This practice aims to standardize hormone levels, even in women who use cyclic OCP. However, there are multiple hormone preparations on the market, and little is known about how type and duration of OCP use influences measures of vascular function. Thus, further studies and thoughtfully collected data related to these topics are needed.

Premenopausal women may also become pregnant. Pregnancy is associated with profound cardiovascular changes in women, and pregnancy-related pathologies, such as hypertensive disorders of pregnancy, intrauterine growth restriction, and preterm birth, are associated with excess lifelong CVD risk (37). Given that BP tends to go back to the normotensive range, even after hypertensive disorders of pregnancy, and that preterm birth or intrauterine growth restriction can occur even when the mother is normotensive throughout pregnancy, BP alone might not identify women who are progressing toward CVD after an adverse pregnancy outcome (37). More sensitive measures of vascular function are warranted during and after pregnancy (38).

Best practices for collecting vascular structure and function measurements during reproduction have not been established. The current recommendations for assessment of vascular function in non-pregnant adults require an overnight fast preceding testing and specify that vascular testing should be performed with the participant in the supine position (36, 39). A lengthy fast might be an unreasonable request for a pregnant or breastfeeding woman, as pregnancy and breastfeeding are associated with heightened energy needs for the mother or baby. Further, maintaining blood sugar is important for promoting fetal growth, and morning sickness can be exacerbated by low blood sugar.

Pregnant women are advised to avoid lying supine to prevent the weight of the baby and uterus from compressing the vena cava. Lying supine for a 30–45 min testing session past the first trimester would not be acceptable for fetal health and might cause a drop in maternal BP. We propose the use of a reclining, padded table set at a standard 45° angle and conducting testing 1–2 h after a light meal that is low in vasoactive saturated fat and refined carbohydrates (40). The reproducibility of vascular function measurements in pregnant and breastfeeding women using these proposed methods has not yet been rigorously evaluated.

SUBFERTILITY IN REPRODUCTIVE AGED WOMEN AND VASCULAR STRUCTURE AND FUNCTION

Early menopause and conditions related to subfertility have also been associated with higher risk of overt CVD in women (41, 42). Anti-Müllerian hormone (AMH), a biomarker of ovarian reserve, is an established indicator of menopausal onset, and longitudinal data demonstrated that lower baseline and rapidly declining levels of AMH were individually associated with higher risks of CVD and coronary heart disease in women (41). Primary ovarian insufficiency (POI) is a form of early menopause that develops before the age of 40, is characterized by low AMH levels, high follicle stimulating hormone levels, amenorrhea, and hypoestrogenism, and is causally related to higher CVD risk in affected women (42). Women diagnosed with POI achieved fewer pregnancies and exhibited unfavorable lipid profiles and hypertension, and yet they exhibited similar coronary artery calcium scores compared to unaffected controls (42, 43). However, an inverse relationship between AMH levels and femoral and carotid intima-media thickness has been described in women with POI (20). There is little information regarding lifestyle behavior and subclinical atherosclerosis in women with POI.

Women diagnosed with polycystic ovarian syndrome (PCOS), an endocrine disorder characterized by high AMH levels, hyperinsulinemia, amenorrhea or oligomenorrhea, and hyperandrogenism, also present with unfavorable lipid profiles, hypertension, subfertility, higher fasting insulin levels

and arterial stiffness, and have a higher risk for developing CVD (21, 44). Studies have shown that carotid intima-media thickness is higher in women with PCOS compared to age and BMI-matched controls, thus it may be a useful measurement for defining subclinical atherosclerotic progression and may serve as an outcome for lifestyle interventions in this population (22). A clinical trial investigated the effect of metformin on vascular function in women with PCOS and found that arterial stiffness, augmentation index, and endothelial function were all improved after a 12-week course of metformin (23). As lifestyle modification has been shown to be more effective than metformin for preventing cardiometabolic disease in middle aged adults (26), future lifestyle interventions in younger women with PCOS should also test for changes in vascular function.

DISCUSSION AND SUMMARY OF RESEARCH NEEDS

Given their generally healthy traditional CVD risk factor profile, sensitive assessments of vascular structure and function are needed to evaluate the success of lifestyle interventions in premenopausal women. Our understanding of CVD development and progression is lacking in women. Tracking changes in vascular function in response to lifestyle interventions, as well as evaluating associations of vascular structure and function with easily measured sex-specific biomarkers, will critically inform preventive and treatment strategies and risk stratification in women. Cycling hormone levels, OCP use, pregnancy and breastfeeding, and conditions linked to subfertility present challenges to achieving reproducible vascular measurements in premenopausal women and/or caveats for the application of findings of smaller studies. Rather than ignoring potentially important factors related to ovarian hormone levels or excluding women from studies, we suggest that investigators carefully consider these variables when planning research and systematically fill in the knowledge gap. Our specific recommendations for research are as follows:

- Sensitive measures of vascular structure and function should be included in clinical trials involving different types of lifestyle behaviors, i.e., resistance interval and endurance exercise training, weight loss, in premenopausal women.
- Studies should account for the menstrual cycle phase and perform vascular data collection during the early follicular phase when possible.
- Investigators should gather information regarding type and length of oral contraceptive use to use in main analyses or sensitivity analyses.
- Vascular measurement protocols for data collection during pregnancy and post-partum, including meal timing and body posture, must be developed and standardized.
- More lifestyle-based clinical trials should be conducted in women with POI and PCOS to better understand the role of lifestyle in mitigating the excess CVD risk observed with these conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AL-C and SB drafted the manuscript, prepared the table, and critically edited the manuscript. All authors contributed to the article and approved the submitted version.

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Association Between Obstructive Sleep Apnea-Hypopnea Syndrome and Outcomes in Patients With Myocardial Infarction in the Absence of Obstructive Coronary Artery Disease

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Background and Aims: Myocardial infarction in the absence of obstructive coronary artery disease (MINOCA) occurs in 5–10% of all patients with acute myocardial infarction. Obstructive sleep apnea-hypopnea syndrome (OSAHS) is linked to increased cardiovascular morbidity and mortality, but the relationship of OSAHS and outcomes in patients with MINOCA remains unknown. We aimed to evaluate the association between OSAHS and clinical outcomes in patients with MINOCA.

Methods: Between January 2015 and December 2016, we carried out a consecutive cohort study of 583 patients with MINOCA and followed them up for 3 years. An apnea-hypopnea index of $\geq$ 15 events per hour recorded by polysomnography was defined as the diagnostic criterion for OSAHS. The primary end point was all-cause mortality, and the second end point was major adverse cardiovascular or cerebrovascular events (MACCE), a composite of cardiac death, non-fatal myocardial infarction, heart failure, cardiovascular-related rehospitalization, and stroke.

Results: All-cause mortality happened in 69 patients and MACCE occurred in 113 patients during the 3-year follow-up. Kaplan–Meier survival curves indicated the significant relationship of OSAHS with all-cause mortality (log-rank $P = 0.012$) and MACCE (log-rank $P = 0.002$). Multivariate Cox regression analysis indicated OSAHS as an independent predictor of all-cause mortality and MACCE [adjusted hazard ratio: 1.706; 95% confidence interval (CI): 1.286–2.423; $P = 0.008$; and adjusted hazard ratio: 1.733; 95% CI: 1.201–2.389; $P < 0.001$; respectively], independent of age, sex, cardiovascular risk factors and discharge medications.

Conclusions: OSAHS is independently associated with increased risk of all-cause mortality and MACCE in patients with MINOCA. Intervention and treatment should be considered to alleviate OSAHS-associated risk.

Keywords: obstructive sleep apnea-hypopnea syndrome, coronary artery disease, outcome, mortality, major adverse cardiac and cerebral event (MACCE)

INTRODUCTION

Myocardial infarction in the absence of obstructive coronary artery disease (MINOCA) is a distinct entity with multiple causes defined by acute presentation of myocardial infarction with no remarkable stenosis of coronary artery on coronary angiography (stenosis < 50%) (1–3). In June 2019, the American Heart Association (AHA) has systematically described recommendations for contemporary diagnosis and management of MINOCA, and they have broadened our horizon to understand this heterogeneous disease (4). More than 660,000 percutaneous coronary intervention (PCI) procedures were performed in China in 2017, and ∼10% of emergency coronary angiography results showed the absence of evident obstructive coronary stenosis (5). Although the number of initial diagnosis of MINOCA is considerable owing to the huge population and demand for PCI, the clinical outcomes, and follow-up of MINOCA have received minimal attention with the result indicating a portion of individuals may not receive appropriate treatment (6, 7).

Sleep apnea-hypopnea syndrome (SAHS) is a syndrome of nocturnal respiratory interruptions resulting in snoring, sleep fragmentation, and daytime drowsiness, with a prevalence ranging from 5.7 to 17% in adults (8–10). Obstructive SAHS is the most common manifestation of sleep apnea and it is caused by nocturnal upper airway collapse with paradoxical thoracoabdominal movement (8). In several multi-center registries, obstructive sleep apnea was observed in 45.3–65.7% of patients with myocardial infarction or who underwent PCI (11, 12). Accumulating evidence has demonstrated that OSAHS is an emerging risk factor for arteriosclerosis, coronary heart disease, hypertension, stroke, cardiovascular rhythm, and heart failure (HF) (13–17). Numerous studies have shown that continuous positive airway pressure (CPAP) therapy could retard the progression of coronary artery disease (CAD) and decrease the rate of occurrence of cardiovascular mortality, stroke, and non-fatal myocardial infarction (18–21).

To our best knowledge, no published data are currently available concerning the prevalence of OSAHS in patients with MINOCA. In addition, the association between OSAHS and clinical outcomes has not been determined in this population. This study aimed to investigate the prevalence of OSAHS in patients with MINOCA and to evaluate the relationship between OSAHS and clinical outcomes in patients with MINOCA.

METHODS

Study Design and Population

Our study complied with the Declaration of Helsinki and was approved by the Ethics Committee (The First Affiliated Hospital of Jiaxing University, Jiaxing, China). All participants provided their informed written consent before being enrolled in the present study.

A total of 583 patients who had been referred to First Affiliated Hospital of Jiaxing University for emergency coronary angiography and diagnosed with MINOCA were included consecutively in the present study between January 2015 and December 2016. Jiaxing University Hospital has a Chest Pain Center that covers a 4,000 sq. km area and serves about six million residents. MINOCA was diagnosed with the following criteria in line with a position paper of European Society on cardiology: (1) the diagnostic criteria involving the Fourth Universal Definition of Myocardial Infarction criteria; (2) coronary angiography with no artery lesions ≥ 50% in any infarct-related artery; (3) no other clinically overt specific cause that can explain acute presentation (22). The exclusion criteria were extremely strict in the present study: (1) mimic myocardial infarction, such as sepsis, pulmonary embolism, cardiac contusion, overlooked obstructive CAD, coronary emboli/thrombus, Takotsubo syndrome, and myocarditis (4); (2) cognitive disorder or mental confusion; (3) known OSAHS on CPAP therapy; (4) patients' refusal to give a written informed consent. The patients were divided in two groups, namely, the OSAHS, and non-OSAHS groups, in accordance with the evaluation of OSAHS.

Data Collection

We used inpatient record system to collect the data on demography, laboratory, electrocardiogram, echocardiography, coronary angiography, and discharge medication. Data collectors were blinded to the study group assignment. After discharge, all patients were followed-up for 3 years by physicians in First Affiliated Hospital of Jiaxing University. Follow-up was performed by outpatient clinic visit, patient record system, telephone contact or instant messaging (WeChat). In case of failure to contact the patients, we used their family members' contact information and asked patients to visit the outpatient clinic annually. All individuals were advised to contact us immediately if they experience angina or uncomfortable chest stuffiness.

Diagnosis of OSAHS

All enrolled patients were required to complete the Berlin Questionnaire (23). The Chinese version of Berlin Questionnaire

is a convenient and inexpensive self-assessment method, which consists of 10 questions in three categories. The questionnaire assesses the presence of sleep apnea by persistent symptoms of snoring, the severity of daytime sleepiness, and a history of hypertension or a body mass index (BMI) > 30 kg/m^2. A positive score in at least two out of three categories indicates a high risk of OSAHS (23).

All individuals were asked to monitor their overnight sleep by using a polysomnography device (SOMNOmedics GmbH & Co. KG, Germany) with the assistance of pneumology department regardless of their results on the Berlin Questionnaire. Polysomnography is the golden standard for obstructive sleep apnea diagnosis. The apnea-hypopnea index (AHI) was quantified as the average number of apnea and/or hypopnea per hour of the total recorded time. Apnea was defined by a 90% or greater reduction in airflow lasting at least 10 s. Hypopnea was defined as a 30% or greater drop in respiratory airflow lasting $\geq$ 10 s accompanied with an oxygen desaturation $\geq$ 4% (24). Obstructive sleep apnea was defined as the absence of airflow in the presence of paradoxical thoracoabdominal movement. An AHI of > or = 15 events/h was considered prospectively as the criterion for OSAHS (AHI values of 5–15, 15–30 and > 30 indicate mild, moderate and severe symptoms, respectively) (24).

Primary and Secondary End Points

The primary end point in the present study was all-cause mortality. The secondary end point was a major adverse cardiovascular or cerebrovascular events (MACCE), defined as a composite of cardiac death, non-fatal myocardial infarction, HF. cardiovascular-related rehospitalization, and stroke. Cardiac death refers to death with evidence of acute coronary syndrome, cardiac arrhythmia or congestive HF. The diagnosis of stroke was performed in line with the AHA guidelines for the scientific statement of stroke (25). The definition of HF was determined in accordance with the current guidelines published by European Society of Cardiology (26). Cardiovascular-related rehospitalization means any cardiovascular causes for readmission. Mortality and MACCE were first verified by searching the patient record system by means of HaiTai software. Death certificates were obtained for any patients who died during the follow-up period if available. All individuals were then contacted by telephone or instant messaging (WeChat) for assessment of end points. End point confirmations were conducted by two physicians without any knowledge of diagnosis of OSAHS.

Statistical Analysis

Data were analyzed with the SPSS version 19.0 software (IBM, SPSS Inc., Chicago, IL). Continuous variables were presented as the mean and standard deviation and categorical variables were expressed as frequencies and percentages. Differences between means were analyzed with Student *t*-test. Differences between proportions were assessed with Chi-square test or Fisher's exact test as appropriate. Kaplan–Meier curves of the survival function were calculated for patients with OSAHS and non-OSAHS, and the two groups were compared with the log-rank test. Multiple Cox proportional hazards model was used to adjust for potential confounders using stepwise regression (modification of the forward selection method) with $P < 0.10$ in the univariate analysis. The proportional hazards assumption was evaluated for Cox regression. Results of Cox regression are presented as hazard ratio (HR) with 95% confidence interval (CI). A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristics of Patients

The study objects included 730 patients with acute myocardial infarction and non-obstructive coronary arteries. A total of 147 patients were excluded from the present trial: 2 with cognitive disorder, 3 with sepsis, 4 with pulmonary embolism, 2 with overlooked obstructive CAD, 4 with coronary emboli/thrombus, 33 with Takotsubo syndrome, 67 with myocarditis, 15 patients on CPAP therapy, and 17 patients who refused to participate in the study. This prospective cohort study recruited 583 patients with MINOCA (**Figure 1**), of which 8 individuals terminated study participation or were lost to follow-up. The Berlin Questionnaires of 237 patients have indicated a high risk of sleep apnea and/or hypopnea, and 158 patients were diagnosed with OSAHS by polysomnography, with a prevalence of 27.1%. Whereas, only 70.9% of the patients in the OSAHS group were classified as high risk by the Berlin Questionnaire. Patient baseline characteristics are listed in **Table 1**. Compared with the control group, OSAHS patients had a higher proportion of males and a history of smoking but without further significant differences regarding age, BMI, cardiovascular risk factors, medications at discharge, laboratory, electrocardiogram, echocardiography, and coronary angiography data. **Table 2** shows the sleep results from OSAHS and non-OSAHS groups. Of 158 OSAHS individuals, 84 presented moderate symptoms, and 74 exhibited severe ones. The mean AHI values were 29.0 $\pm$ 12.2 and 5.3 $\pm$ 4.2 for OSAHS and non-OSAHS groups, respectively. The mean lowest SpO2 values during apnea were 82.7 $\pm$ 7.9 and 91.3 $\pm$ 4.1, respectively. Fifteen patients with moderate or severe OSAHS agreed to receive CPAP treatment. The present study was to investigate the association between untreated OSHAS and clinical outcomes in patients with MINOCA, patients who had received CPAP therapy for more than 1 month during the study were excluded from analysis.

Medications

Medications prescribed at hospital discharge are presented in **Table 1**. No significant differences were observed between the OSAHS group and non-OSAHS group in the use of medications at discharge. On the basis of prescription refill at the latest follow-up, 89.4% of the patients in the OSHAS group and 91.2% of the patients in the non-OSAHS group have insisted on antiplatelet therapy. Besides, a total of 89.6 and 87.4% of patients in the OSHAS group were adherent to statin and beta-blocker therapy, compared with 87.9 and 86.8% in the non-OSAHS group, respectively.

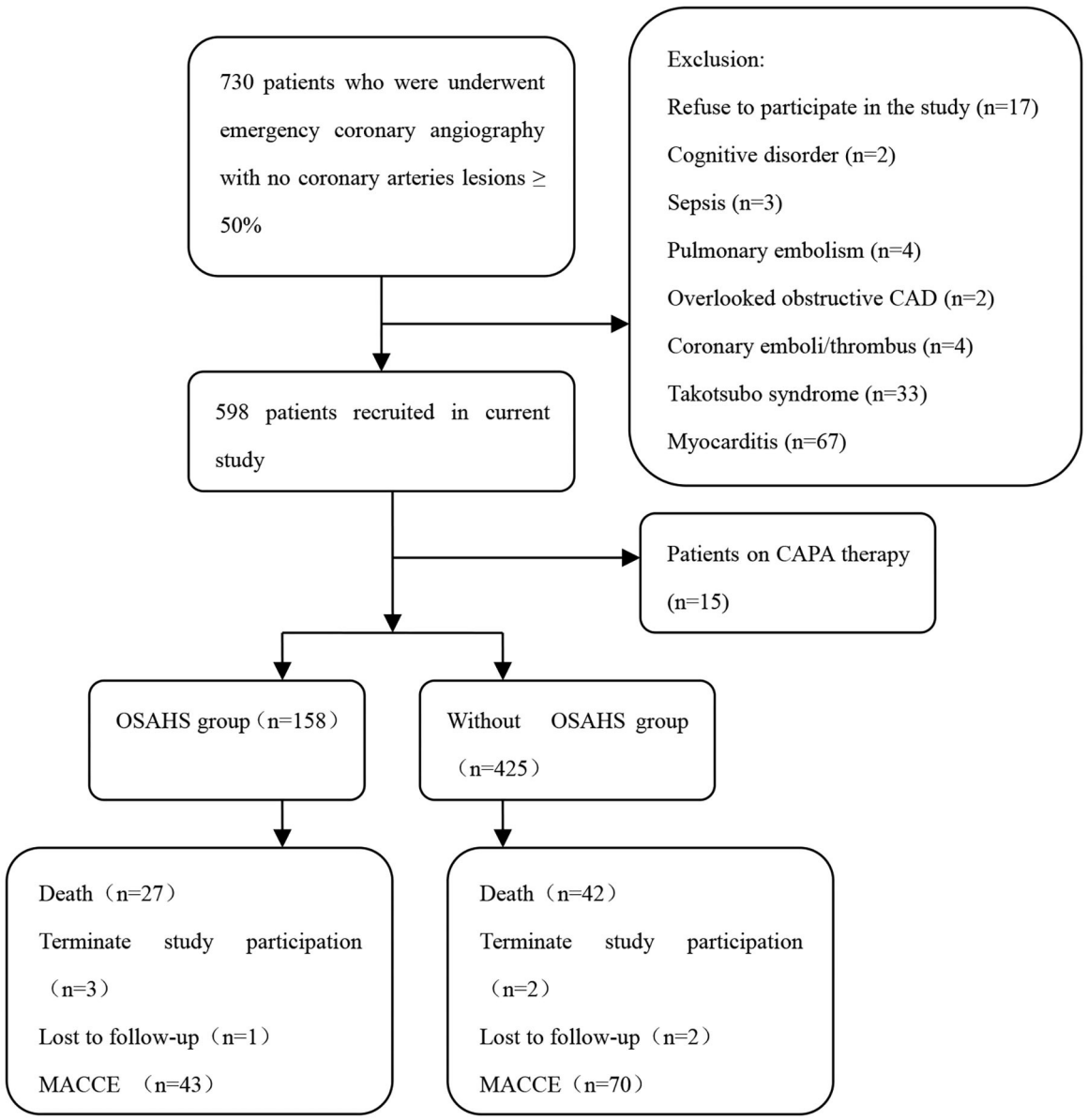

FIGURE 1 | Flowchart of the selection process and dropouts of the current study. CAD, coronary artery disease; CPAP, continuous positive airway pressure; OSAHS, obstructive sleep apnea-hypopnea syndrome; MACCE, major adverse cardiac or cerebrovascular events.

Follow-Up and Clinical Outcomes Medications

A total of 69 all-cause mortality and 113 MACCE events occurred during the 3-year observation period in both groups. MACCE included cardiac death in 51 patients (45.1%), non-fatal myocardial infarction in 11 (9.7%), HF in 4 (3.5%), cardiovascular-related rehospitalization in 24 (21.2%), and stroke in 23 (20.6%). The Kaplan–Meier cumulative survival curves indicated a significant association of OSAHS with all-cause mortality (log-rank $P = 0.012$) and MACCE (log-rank $P = 0.002$) (**Figures 2**, **3**, respectively). In univariate Cox regression analysis, OSAHS predicted the incidence of all-cause mortality and MACCE (unadjusted HR = 1.923; 95% CI: 1.411–2.724; $P < 0.001$; and unadjusted HR = 1.977; 95% CI: 1.365–2.856; $P < 0.001$; respectively). After adjustment for age, sex, cardiovascular risk factors, and medications in multivariate Cox regression analysis, OSAHS remained an independent predictor of all-cause mortality and MACCE (adjusted HR = 1.706; 95% CI: 1.286–2.423; $P = 0.007$; and adjusted HR = 1.733; 95% CI: 1.201–2.389; $P < 0.001$; respectively). The results are summarized in **Tables 3**, **4**. Notably, statins, rennin-angiotensin-aldosterone system (RAAS) inhibitors, and ST-segment elevation myocardial infarction (STEMI) revealed a significant correlation with clinical outcomes, agreeing with the findings of previous studies (27).

TABLE 1 | Baseline characteristics of the study population with or without OSAHS.

Characteristics	With OSAHS (n = 158)	Without OSAHS (n = 425)	P-value
Demographics			
Age, mean ± SD, y	64.5 ± 12.9	63.4 ± 12.7	0.158
Female, n (%)	62 (39.2)	268 (63.1)	<0.001
BMI, mean ± SD, kg/m^2	24.9 ± 3.6	23.7 ± 3.8	0.107
Risk factors, n (%)			
Smoking	52 (32.9)	97 (22.8)	0.013
Hypertension	88 (55.7)	201 (47.3)	0.071
Diabetes	36 (22.8)	84 (19.7)	0.423
COPD	19 (12.0)	38 (8.9)	0.265
Stroke history	9 (5.7)	15 (3.5)	0.242
Heart failure	8 (5.1)	19 (4.5)	0.762
Medications at discharge, n (%)			
Anti-platelets	135 (85.4)	365 (85.9)	0.647
β Blockers	96 (60.8)	246 (57.9)	0.531
RAAS inhibitors	79 (50.0)	203 (47.8)	0.631
Statins	146 (92.4)	379 (89.2)	0.247
Electrocardiographic changes on admission, n (%)			0.708
STEMI	20 (12.7)	49 (11.5)	
NSTEMI	138 (87.3)	376 (88.5)	
Echocardiography, mean ± SD			
LVEF (%)	54.3 ± 13.4	54.9 ± 13.5	0.715
Laboratory parameters on admission, mean ± SD			
Pro-BNP (pg/mL)	986.4 ± 1423.3	919 ± 1356.3	0.778
cTnT (ng/mL)	2.1 ± 1.0	2.0 ± 0.9	0.765
CRP (mg/L)	15.6 ± 4.7	15.7 ± 5.1	0.756
Angiographic data, n (%)			0.760
Normal vessels	18 (11.4)	40 (9.4)	
Stenosis ≤ 30%	72 (45.6)	202 (47.5)	
30% < Stenosis < 50%	68 (43.0)	183 (43.1)	

OSAHS, obstructive sleep apnea-hypopnea syndrome; SD, standard deviation; BMI, body mass index; COPD, chronic obstructive pulmonary disease; RAAS, rennin-angiotensin-aldosterone system; STEMI, ST-segment elevation myocardial infarction; NSTEMI, non-ST-segment elevation myocardial infarction; LVEF, left ventricular ejection fraction; Pro-BNP, pro-brain natriuretic peptide; cTnT, cardiac troponin T; CRP, C reactive protein.

TABLE 2 | The Berlin Questionnaire results and sleep respiratory events recorded by polysomnography.

Characteristics	With OSAHS (n = 158)	Without OSAHS (n = 425)	P-value
AHI, mean ± SD, /h	29.0 ± 12.2	5.3 ± 4.2	<0.001
Baseline SpO2, mean ± SD, %	94.3 ± 4.1	95.0 ± 3.9	0.349
Lowest SpO2, mean ± SD, %	82.7 ± 7.9	91.3 ± 4.1	<0.001
High-risk BQ, n (%)	112 (70.9)	125 (29.4)	<0.001

OSAHS, obstructive sleep apnea-hypopnea syndrome; AHI, apnea hypopnea index; SD, standard deviation; BQ, Berlin Questionnaire.

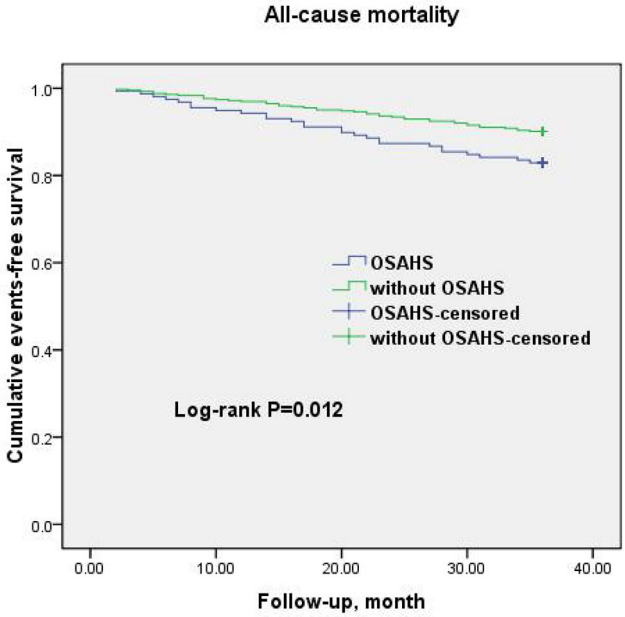

FIGURE 2 | Cumulative event-free survival (Kaplan-Meier curves) for all-cause mortality in OSAHS patients compared to patients without OSAHS.

DISCUSSION

To our best knowledge, this research is the first study to explore the prevalence of OSAHS in patients with MINOCA and demonstrate the possible relationship between OSAHS and clinical outcomes in this population. In this cohort of 583 patients with MINOCA, the prevalence of OSAHS was 27.1%, similar to other CAD population. We observed that OSAHS was associated with an increased risk of all-cause mortality and MACCE comprising cardiac death, non-fatal myocardial infarction, HF, cardiovascular-related rehospitalization, and stroke. After adjusting for demographics, cardiovascular risk factors, and discharge medications, patients with OSAHS exhibited 1.706 times risk of all-cause mortality and 1.733 times risk of MACCE at 3-year follow-up. Compared to our earlier study on depression on all-cause mortality and cardiovascular events in patients with MINOCA, the association between OSAHS and clinical outcomes was not that strong (28).

The previous large multi-center registry, The Sleep and Stent Study (11) has shown that obstructive sleep apnea is independently associated with cardiovascular adverse events in patients with CAD, with the prevalence reaching 45.3%. A similar study conducted by Lee also has reported that sleep apnea prevalence is as high as 65.7% in patients admitted with AMI (12). However, the prevalence of OSAHS in patients with MINOCA is unknown. Although the identical diagnostic criterion of OSAHS was an AHI ≥ 15 in the present study, OSAHS was found in 27.1% of patients with MINOCA, which is relatively lower compared with that of CAD patients described by previous studies. In addition, we observed that only 70.9% of the individuals with OSAHS were classified as high risk by the Berlin Questionnaire. The results suggested that the sensitivity and specificity of Berlin Questionnaire remained controversial.

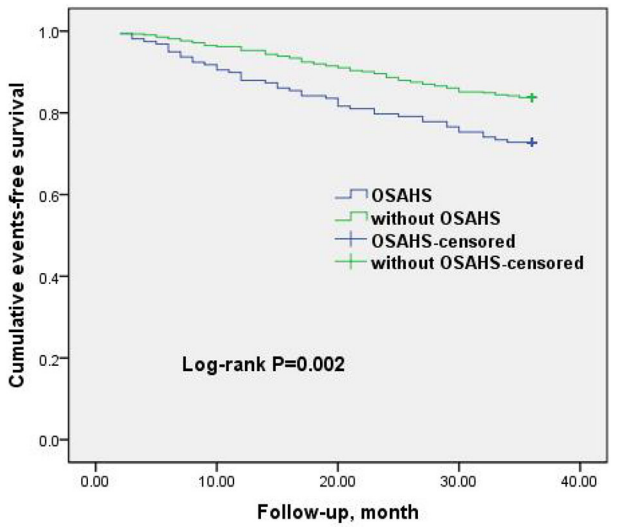

FIGURE 3 | Cumulative event-free survival (Kaplan-Meier curves) for MACCE in OSAHS patients compared to patients without OSAHS.

TABLE 3 | Univariate and multivariate Cox regression of variables influencing All-cause mortality.

Variables	Unadjusted HR (95% CI)	Adjusted HR (95% CI)	*P-value
Heart failure	1.425 (0.926–1.963)	1.236 (0.884–1.855)	0.112
RAAS inhibitors	0.569 (0.364–0.851)	0.664 (0.390–0.927)	<0.001
Statins	0.601 (0.354–0.670)	0.640 (0.339–0.870)	<0.001
STEMI	3.916 (1.422–9.453)	3.766 (1.416–8.952)	<0.001
OSAHS	1.923 (1.411–2.724)	1.706 (1.286–2.423)	0.008

HR, hazard ratio; RAAS, rennin-angiotensin-aldosterone system; STEMI, ST-segment elevation myocardial infarction; OSAHS, obstructive sleep apnea-hypopnea syndrome.
**The P-value is from multivariate model.*

OSAHS is characterized by intermittent upper airway collapse with paradoxical thoracoabdominal movement during sleep, resulting in biochemical and haemodynamic disorders, including sympathetic activation, increased platelet agreeability, hypercoagulability, ultra-inflammation responses, endothelial dysfunction, increased pulmonary vascular resistance, and increased left ventricular load caused by decrease in intrathoracic pressure (27, 29–32). These potential mechanisms together contribute to the progression of atherosclerosis, myocardial infarction, hypertension, arrhythmia, HF, and stroke (8). In the study of Mooe et al. (33) the co-existence of CAD and sleep apnea resulted in 70% relative increase and a 10.7% absolute increase in the composite of death, cerebrovascular events, and myocardial infarction after a median period of 5.1-year follow-up. In one study, obstructive sleep apnea was demonstrated as an independent predictor for clinical outcomes in patients with acute coronary syndrome after PCI (34). The main finding of our observational study is that OSAHS is independently associated with all-cause mortality and MACCE in patients with MINOCA. The total-cause mortality of MINOCA in the 3-year

TABLE 4 | Univariate and multivariate Cox regression of variables influencing MACCE.

Variables	Unadjusted HR (95% CI)	Adjusted HR (95% CI)	*P-value
Heart failure	1.512 (0.968–2.161)	1.202 (0.807–1.575)	0.077
β-Blockers	0.678 (0.589–0.998)	0.712 (0.675–1.100)	0.042
RAAS inhibitors	0.601 (0.396–0.902)	0.624 (0.412–0.942)	<0.001
Statins	0.466 (0.277–0.746)	0.502(0.297–0.802)	<0.001
STEMI	4.903 (2.142–10.877)	4.633 (1.774–9.680)	<0.001
OSAHS	1.977 (1.365–2.856)	1.733 (1.201–2.389)	<0.001

MACCE, major adverse cardiovascular or cerebrovascular events; HR, hazard ratio; RAAS, rennin-angiotensin-aldosterone system; STEMI, ST-segment elevation myocardial infarction; OSAHS, obstructive sleep apnea-hypopnea syndrome.
**The P-value is from multivariate model.*

follow-up was 11.8% in the present study, agreeing with the SWEDEHEART registry, with a mortality of 13.4% after mean follow-up of 4.1 years (35). OSAHS patients had an adjusted OR of 1.733 for the MACCE when compared with patients without sleep apnea. Interestingly, we observed that stroke accounted for one-fifth of MACCE in the current study, 16 in patients with OSAHS and 7 in the control group, which is consistent with previous findings indicating OSAHS as an independent risk factor for stroke (13, 36, 37).

The design of present study has certain strengths. First, all patients with OSAHS were diagnosed with gold standard by supervised polysomnography during hospitalization. Second, we rigorously excluded clinically overt causes for a myocardial injury, such as myocarditis, sepsis, pulmonary embolism, Takotsubo syndrome, an potentially overlooked obstructive CAD from the current study, consistent with the clinical algorithm for the recently introduced diagnosis of MINOCA by AHA (4). Therefore, the definition of MINOCA utilized in the current study was consistent with the statement of contemporary diagnosis and management of patients with MINOCA, so the result is more reliable. Third, the well-known confounding risk and preventive factors that affect clinical outcomes in patients with MINOCA, such as traditional cardiovascular risk factors, STEMI, RAAS inhibitors, and statins, were all used for adjustment. Fourthly, the rate of loss to follow-up was low in the present study owing to the convenient instant messaging app (WeChat).

Several limitations need to be considered in the present study. First, the present study is an observational single Chest Pain Center study, and the results may not be generalizable. Second, the sample size of the current study is relatively small due to the critical inclusion and exclusion criteria. Third, the exclusion of patients who refused to participate in the trial and loss of follow-up could contribute to a bias of the results in present study. Finally, one major limitation is that we did not collect longitudinal data on some study covariates prone to change during follow-up period, such as smoking and use of medications after discharge.

CONCLUSION

OSAHS is common among patients with MINOCA and is independently associated with increased risk of all-cause

mortality and MACCE in patients with MINOCA. The results highlight the importance of management in this population with OSAHS. Intervention and treatment should be considered to alleviate OSAHS-associated risks. Further studies are warranted to determine the effectiveness of CPAP treatment in MINOCA population.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee (The First Affiliated Hospital of Jiaxing University). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

C-JH: conceptualization, methodology, software, investigation, and writing-original draft. L-FC: data curation, formal analysis, and writing- original draft preparation. C-YZ: resources, visualization, and investigation. X-CD: software. Y-JZ: formal analysis, validation, and resources. Y-YY: software and validation. C-LZ: project administration, writing- reviewing, and editing. GQ: validation. H-LH: conceptualization, methodology, writing-reviewing and editing, and supervision. All authors contributed to the article and approved the submitted version.

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Ideal Cardiovascular Health Metrics and Incidence of Ischemic Stroke Among Hypertensive Patients: A Prospective Cohort Study

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Background: This study aimed to assess the relationship between ideal cardiovascular health (CVH) metrics and incident ischemic stroke (IS) in hypertensive patients, especially those with hyperhomocysteinemia (HHcy).

Methods: A prospective cohort study enrolled 5,488 hypertensive patients in Nanshan District of Shenzhen City in southern China from September 2011 to December 2017. CVH metrics were defined according to the American Heart Association. Cox proportional hazards models were used to examine the associations between the number of ideal CVH metrics and the incidence of IS by calculating multivariable-adjusted hazard ratios (HRs) and 95% CI.

Results: During an average follow-up of 5.7 years, 340 IS patients were identified. Compared with those having 0 ideal CVH metrics, the HRs (95% CIs) for IS among those with 1, 2, 3, 4, and 5–6 ideal CVH metrics were 0.62 (0.31–1.25), 0.37 (0.19–0.74), 0.37 (0.18–0.74), 0.34 (0.16–0.71), and 0.28 (0.12–0.63), respectively ($P < 0.001$). An ideal healthy diet score and ideal fasting blood glucose level were independently associated with IS among participants, with HRs (95% CIs) of 0.53 (0.33–0.86) and 0.32 (0.17–0.66), respectively. Additionally, compared with those with normal total homocysteine (tHcy) levels (<15 μmol/L), the HR (95% CI) for IS among participants with HHcy and who had 5–6 ideal CVH metrics was 0.50 (0.27–0.92).

Conclusion: An increased number of ideal CVH metrics was inversely associated with the incidence of IS in hypertensive patients. The participants with HHcy who had 5–6 ideal CVH metrics exhibited a lower IS risk than those with normal tHcy levels.

Keywords: ideal cardiovascular health metrics, ischemic stroke, hypertensive patients, prospective study, hyperhomocysteinemia

INTRODUCTION

Stroke is the leading cause of mortality and disability among adults in China (1) and 87% of stroke-related deaths are caused by ischemic stroke (IS) (2). Evidence suggests that hypertension and hyperhomocysteinemia (HHcy) have a synergistic effect on the risk of IS (3). We previously found that the hazard ratio (HR) (95% CI) of IS caused by HHcy was 2.18 (1.65–2.89) in a hypertensive Chinese population (3). Importantly, ∼80.3% of the hypertensive patients in this Chinese sample had HHcy (4), and the risk of stroke faced by patients with HHcy was 12.7 times higher than that of adults without hypertension and HHcy (5).

The American Heart Association (AHA) has defined ideal cardiovascular health (CVH) metrics. These include four health behaviors [not smoking, a body mass index [BMI] < 24 kg/m^2, physical activity at goal levels, and a healthy diet composition], and three health factors (untreated total cholesterol <11.1 mmol/L, untreated blood pressure <130/80 mmHg, and untreated fasting blood glucose <5.5 mmol/L). Mounting evidence shows an inverse association between the number of ideal CVH metrics and the incidence of stroke across diverse populations and types of patients (6, 7). However, there are limited data to inform ideal CVH metrics that align with the AHA's definition of IS incidence in hypertensive patients, and especially in patients with both hypertension and HHcy. We hypothesized that an increased number of ideal CVH metrics would be associated with a decreased incidence of IS in hypertensive patients, and especially those with HHcy. Therefore, we performed a prospective cohort study to examine whether ideal CVH metrics were associated with a lower risk of IS among Chinese hypertensive patients.

METHODS

Study Population

We performed a community-based, observational, long-term follow-up study to investigate the epidemiology of IS in Chinese hypertensive adults (3). This type of study design has been described previously (3). This prospective study was based on data from the hypertension management information systems of 60 community health service centers (CHSCs) in Nanshan District of Shenzhen City in southern China. The inclusion criteria were as follows: (1) patients who had essential hypertension and were ≥20 years of age; (2) local residents who had lived in Shenzhen for ≥6 months; (3) Chinese citizens who could receive follow-up without difficulty for at least 3 years; and (4) patients whose health records had been established in a CHSC hypertension management information system. The exclusion criteria were as follows: (1) patients with secondary hypertension, cancer, severe liver or kidney disease, or pregnancy; and (2) hypertensive patients with IS.

From April 2010 to September 2011, 5,935 hypertensive patients aged ≥20 years and who had lived in Shenzhen for ≥6 months completed the baseline survey (Supplementary Figure 1). All of the patients were Chinese citizens who could be followed up without difficulty for at least 3 years, and had established health records at the CHSCs. The study was performed according to the guidelines of the Helsinki Declaration and was approved by the Ethics Committee of the Shenzhen Nanshan Center for Chronic Disease Control. Signed informed consent was obtained from all of the participants.

Data Collection

During the baseline survey, we used a validated questionnaire to collect demographic characteristics of the participants. For more details about characteristics, please see Supplementary Table 1.

In addition to the survey items, physical examination data including height and weight were measured at baseline. Height without shoes was measured to the nearest 1 cm with a standard right-angle device. Weight was measured to the nearest 0.1 kg with a spring balance. BMI was calculated as weight/height^2 (kg/m^2). Blood pressure was measured three times at 30-s intervals, using a standardized automatic electronic sphygmomanometer after 5–10 min of rest with the participants in a seated posture, and the average of the three measurements was used in all of the analyses. Essential hypertension was diagnosed based on systolic blood pressure (SBP) ≥ 140 mmHg or diastolic blood pressure (DBP) ≥ 90 mmHg, or self-reported use of antihypertensive medication (8).

Laboratory Methods

Blood samples were collected from the antecubital vein in the morning after an overnight fast. Serum uric acid levels were measured by quantitative determination with uricase, plasma total homocysteine (tHcy) levels were measured by the circulating enzyme method, and serum creatinine levels were determined by the Jaffe method using an automatic biochemical analyzer (Hitachi 7080). tHcy levels ≥15 μmol/L was defined as HHcy, according to the Guidelines for Prevention and Treatment of Hypertension in China (2018 Revised Edition) (9). Total cholesterol (TC) and fasting blood glucose (FBG) were measured enzymatically with an autoanalyzer (Hitachi 7080).

Assessment of Ideal CVH Metrics

The CVH metrics adopted in this study were consistent with the CVH metrics proposed by the AHA, which include seven health behaviors and factors: healthiness of diet, blood pressure (BP), physical activity, BMI, smoking status, TC, and FBG (10). According to the AHA guidelines (10), CVH metrics are classified as ideal, intermediate, or poor. The details of ideal, intermediate, and poor CVH metrics as defined by the AHA and by our modified version are described in Supplementary Table 2. Because all of the subjects in the study were hypertensive patients, BP was not included.

A total CVH score was calculated by summing scores for each of the 6 CVH metrics, ranging from 0 to 6, with the highest score indicating a better CVH. Total CVH score was then categorized into grades 0, 1, 2, 3, 4, 5, and 6.

Outcome Assessment

December 2017 was the end point of the follow-up period. After 5.7 years of follow-up, face-to-face interviews, physical exams, and biochemical measurements were performed at the Shenzhen

Nanshan Center for Chronic Disease Control. The methods were consistent with those in the baseline survey.

The primary outcome was the first occurrence of IS. This outcome was confirmed by examination of medical records including reports of symptoms and examination results. The examinations included computed tomography (CT), magnetic resonance imaging (MRI), cerebral angiography, and transcranial doppler ultrasound, in agreement with World Health Organization (WHO) criteria (11), and were conducted by two cerebrovascular experts.

Transient ischemic attack (TIA) was also diagnosed as IS if the scan did not visualize an infarction or hemorrhage, but the patient had symptoms that met the WHO criteria for IS (12). Both stroke and TIA were defined as cerebralvascular accident (CVA) (13).

IS was coded as I63 and I64 according to the 10th Revision of the International Classification of Diseases (ICD-10) and classified IS into 5 subtypes: large artery atherosclerosis, cardioembolism, small-vessel occlusion, stroke of other cause, and stroke of undetermined cause according to the TOAST classification (14).

Statistical Analysis

Categorical variables were described as percentages (%) and were compared using the chi-square test. Continuous variables were described as mean ± SD and were compared using one-way analysis of variance. Variables with skewed distributions were log-transformed to normal distributions.

Person years of follow-up were calculated from the date of the initial baseline interview until the date when the participants were diagnosed with IS, the date of death, or the end of the follow-up period, whichever occurred first.

Multivariable Cox proportional hazards regression models were used to examine the association between the number of baseline ideal CVH metrics and the incidence of IS by calculating HRs and 95% CIs. Because only 35 (0.75%) participants had the highest CVH score of 6, we combined them with the participants who had a CVH score of five. We fitted three models to the data.

TABLE 1 | The HRs (95% CIs) of incident ischemic stroke by Cardiovascular Health Metrics and sex.

Characteristics*	Cardiovascular health metrics						*P* trend
	0	1	2	3	4	5–6	
MALES							
Cases	8	23	47	42	21	7	
Number of participants	84	348	647	700	401	147	
M0†	1 [Reference]	0.73 (0.32–1.64)	0.35 (0.16–0.75)	0.41 (0.19–0.88)	0.37 (0.16–0.85)	0.22 (0.07–0.64)	0.006
M1‡	1 [Reference]	0.70 (0.31–1.58)	0.32 (0.15–0.70)	0.38 (0.18–0.83)	0.34 (0.15–0.79)	0.21 (0.07–0.63)	0.005
M2§	1 [Reference]	0.80 (031–2.07)	0.37 (0.14–0.94)	0.45 (0.17–1.17)	0.37 (0.12–1.08)	0.22 (0.06–0.81)	0.019
FEMALES							
Cases	4	31	52	60	34	11	
Number of participants	38	284	598	741	506	168	
M0†	1 [Reference]	0.47 (0.16–1.36)	0.42 (0.15–1.17)	0.35 (0.12–0.99)	0.33 (0.11–0.95)	0.31 (0.10–1.00)	0.036
M1‡	1 [Reference]	0.47 (0.16–1.36)	0.41 (0.14–1.14)	0.35 (0.12–0.98)	0.32 (0.11–0.93)	0.31 (0.09–1.00)	0.033
M2§	1 [Reference]	0.42 (0.14–1.29)	0.30 (0.10–0.88)	0.25 (0.08–0.77)	0.25 (0.08–0.75)	0.25 (0.07–0.86)	0.020
TOTAL							
Cases	12	54	99	102	55	18	
Number of participants	122	632	1,245	1,441	907	315	
M0†	1 [Reference]	0.57 (0.30–1.07)	0.39 (0.21–0.71)	0.37 (0.20–0.69)	0.36 (0.19–0.67)	0.28 (0.13–0.59)	<0.001
M1‡	1 [Reference]	0.55 (0.29–1.04)	0.37 (0.20–0.68)	0.36 (0.19–0.66)	0.34 (0.17–0.64)	0.27 (0.13–0.58)	<0.001
M2§	1 [Reference]	0.62 (0.31–1.25)	0.37 (0.19–0.74)	0.37 (0.18–0.74)	0.34 (0.16–0.71)	0.28 (0.12–0.63)	<0.001

BMI, body mass index.

**All non-pregnant participants older than 20 years with available cardiovascular health metrics were included.*

† Not adjusted.

‡ Adjusted for age, sex.

§ Adjusted for age, sex, education, alcohol consumption, diabetes mellitus, depression, family history of stroke, years of hypertension, antihypertensive medication, low density lipoprotein, systolic blood pressure, diastolic blood pressure, uric acid, triglyceride, total homocysteine and creatinine.

Model 0 (M0) was not adjusted. Model 1 (M1) was adjusted for age and sex. Model 2 (M2) was further adjusted for education level, alcohol consumption, depression, years of hypertension, antihypertensive drug use, diabetes, family history of stroke, SBP, DBP, low density lipoprotein (LDL), uric acid (UA), triglyceride (TG), tHcy, and creatinine.

We tested the interactions between CVH metrics and age (<60 years vs. ≥60 years), sex, hypertension duration (<5 years vs. ≥ 5 years), BP (<130/80 mmHg vs. ≥130/80 mmHg) and tHcy (<15 μmol/L vs. ≥15 μmol/L) after controlling for the aforementioned covariates.

We also analyzed the risk of IS events, according to the combined ideal CVH metrics, among participants with HHcy compared with participants with normal tHcy levels. To test the *P* trend, we assigned a numerical value to the number of ideal CVH metrics and analyzed it as a continuous variable using the PHREG process in SAS.

The data were analyzed using SAS 9.3 (SAS Institute, Cary, NC). Two-sided $p < 0.05$ were considered statistically significant.

RESULTS

Baseline Characteristics

This prospective study initially enrolled 5,935 participants, of whom 447 who presented with IS and CHD were excluded (Supplementary Figure 1). After an average follow-up period of 5.7 years, 826 of the remaining 5,488 participants were lost to follow-up (15.05%), 348 refused to continue, 206 returned to their hometowns, 164 changed their telephone numbers, 73 left the country, and 35 died. Finally, 4,662 participants were successfully followed and included in the prospective study.

Supplementary Table 1 presents the baseline characteristics of the participants according to the number of ideal CVH metrics. The mean (SD) age of the study participants was 41.3 (7.4) years, and 49.9% of the participants were male. Of the 4,662 participants, only 0.75% ($n = 35$) had 6 ideal CVH metrics; most of them (57.61%, $n = 2{,}686$) had 3–4 ideal CVH metrics. The participants with a greater number of ideal CVH metrics were more likely to be female, to be younger, to have higher education, to consume less alcohol, and to have experienced hypertension for only 2–5 years.

Comparison of Baseline Characteristics Between Participants Lost and Not Lost to Follow-Up

Supplementary Table 3 summarizes the characteristics of the participants lost and not lost to follow-up. Our analysis showed that fruit intake, age, and SBP differed significantly between the two groups (all $P < 0.05$).

Association Between the Number of Ideal CVH Metrics and Incident Ischemic Stroke Risk

After an average follow-up period of 5.7 years, a total of 340 IS cases were identified. Unadjusted and adjusted HRs (95% CIs) of the incidence of IS by both CVH metrics and sex are presented in **Table 1**. Multivariable adjusted Cox proportional hazards models indicated that the number of ideal CVH metrics were inversely associated with the incidence of IS after controlling for the aforementioned covariates. Compared with the participants with 0 ideal CVH metrics, the HRs (95% CIs) for the participants with scores of 5–6 ideal CVH metrics among all of the participants was 0.28 (0.12–0.63). For males, it was 0.22 (0.06–0.81), and for females it was 0.25 (0.07–0.86).

The Kaplan-Meier curves presented in Supplementary Figure 2 indicate that a graded decrease in the risk of IS is associated with an increased number of ideal CVH metrics (log-rank $P = 0.002$).

The associations between each CVH metric and IS risk are shown in **Table 2**. After adjustments for the aforementioned covariates, the ideal healthy diet score (7–9 points) was independently associated with a decreased risk of incident IS, with a HR (95% CI) of 0.53 (0.33–0.86). The ideal FBG (<5.5 mmol/L) was also associated with a decreased risk of incident IS, with a HR (95% CI) of 0.32 (0.17–0.62).

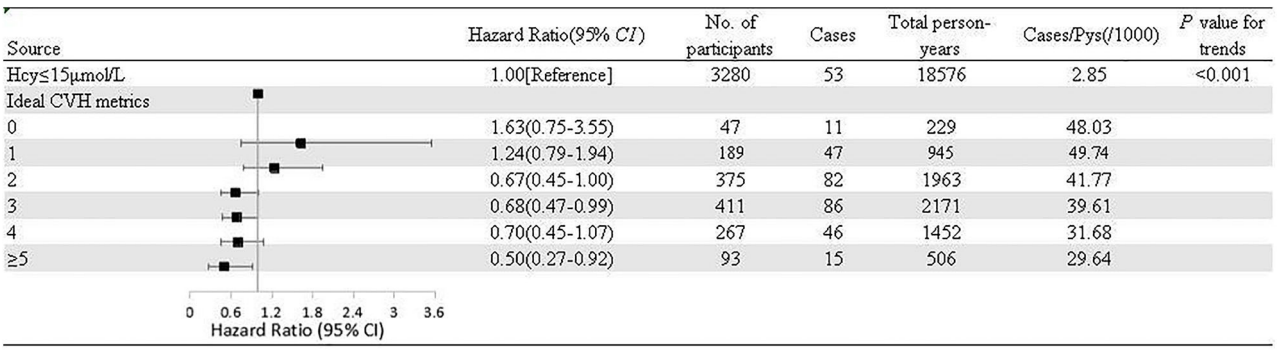
The HR (95% CI) of incident IS risk in relation to the number of ideal CVH metrics among the participants with HHcy,

TABLE 2 | The HRs (95% CIs) of incident ischemic stroke by each cardiovascular health metrics.

Characteristics	*β*	*HR**	95% *CI*	*P*
Smoking status				
Ideal	−0.13	0.88	0.60–1.29	0.523
Intermediate	0.08	1.08	0.69–1.67	0.727
Poor	Reference			
physical activity				
Ideal	−0.12	0.88	0.67–1.16	0.371
Intermediate	0.08	1.08	0.69–1.68	0.733
Poor	Reference			
BMI				
Ideal	−0.17	0.84	0.58–1.22	0.375
Intermediate	−0.16	0.85	0.59–1.21	0.380
Poor	Reference			
Healthy diet score				
Ideal	−0.63	0.53	0.33–0.86	0.010
Intermediate	−0.43	0.65	0.42–0.99	0.047
Poor	Reference			
Total serum cholesterol				
Ideal	0.05	1.05	0.62–1.76	0.861
Intermediate	−0.06	0.94	0.63–1.41	0.771
Poor	Reference			
Fasting blood glucose				
Ideal	−1.12	0.32	0.17–0.62	<0.001
Intermediate	−0.94	0.39	0.23–0.66	<0.001
Poor	Reference			

BMI, body mass index.

**Adjusted for age, sex, education, alcohol consumption, diabetes mellitus, depression, family history of stroke, years of hypertension, antihypertensive medication, low density lipoprotein, systolic blood pressure, diastolic blood pressure, uric acid, triglyceride, total homocysteine and creatinine.*



Source	Hazard Ratio(95% *CI*)	No. of participants	Cases	Total person-years	Cases/Pys(/1000)	*P* value for trends
Hcy≤15μmol/L	1.00[Reference]	3280	53	18576	2.85	<0.001
Ideal CVH metrics						
0	1.63(0.75-3.55)	47	11	229	48.03	
1	1.24(0.79-1.94)	189	47	945	49.74	
2	0.67(0.45-1.00)	375	82	1963	41.77	
3	0.68(0.47-0.99)	411	86	2171	39.61	
4	0.70(0.45-1.07)	267	46	1452	31.68	
≥5	0.50(0.27-0.92)	93	15	506	29.64	

FIGURE 1 | Adjusted hazard ratios and population attributable fractions of ischemic stroke events in relation to number of ideal cardiovascular health metrics among participants with hyperhomocysteinemia compared with normal homocysteine. Pys, Person years; Hcy, homocysteine; CVH, cardiovascular health metrics.

compared with those with normal tHcy levels, is presented in **Figure 1**. After being adjusted for the aforementioned covariates, the HR (95% CI) of incident risk of IS was 0.50 (0.27–0.92) for the participants with HHcy who had 5–6 CVH metrics, compared with the participants with normal tHcy levels.

Test for Interactions

Sensitivity analysis was performed for age (<60 years and ≥60 years), hypertension duration (<5 years and ≥5 years), controlled BP (SBP < 130 mmHg or DBP < 80 mmHg and SBP ≥ 130 mmHg or DBP ≥ 80 mmHg), and tHcy levels (<15 and ≥15 μmol/L). There were no significant interactions between the number of ideal CVH metrics and these variables in relation to IS risk (**Table 3**).

DISCUSSION

This community-based prospective cohort study revealed that hypertensive patients with a greater number of ideal CVH metrics had a lower incidence of IS during an average follow-up period of 5.7 years. An ideal healthy diet score (7–9 points) and ideal FBG (<5.5 mmol/L) were independently associated with decreased risk of IS. The participants with HHcy who had 5–6 ideal CVH metrics were 50% less likely to develop IS than those with normal tHcy levels.

Consistent with previous studies in China (15, 16), our study found that only 0.75% of all of the subjects met all ideal metrics, and that 26.2% met 4–6 ideal CVH metrics. These findings suggest that the distribution of CVH metrics among hypertensive patients are similar to those in the general population and are not well-controlled. The low prevalence of ideal CVH metrics among the participants in this study might be explained by the relatively ineffective primary care for hypertensive patients provided by CHSCs in Shenzhen (17). Another possible explanation for these findings is the high prevalence of older adults among our participants (49.6% of the participants were >60 years old), the prevalence of healthy behaviors and positive health factors are known to decrease with aging (2). This finding is consistent with previous results showing that the proportion of older participants who met 5 or more ideal CVH metrics was 2–3 times lower than that of younger participants (18).

We noted that the Kaplan-Meier curves for outcomes for metrics 1–3 and 4–6 merged toward the end (at about month 80), which indicated despite the incident risk of IS among participants with 4–6 metrics was lower than those among participants with 1–3 metrics during the most of the time of the follow-up, the incident risk for those with 4–6 metrics were higher at the end of the follow-up.

The prevalence of HHcy in Chinese populations (19), particularly in those with hypertension, is very high (approximately 80.3%) (4), besides, compelling evidence have demonstrated that the risk of IS faced by hypertensive patients with HHcy was 2.18 times higher than that of hypertensives with normal Hcy (3), thus it is very significant to study the CVH based on the stratification of Hcy in China. Importantly, we found that hypertensive patients with HHcy who had 5 or more ideal CVH metrics exhibited a significantly lower risk of IS events than those with normal tHcy levels, which is the major finding of this study. It' s reported that hypertension with HHcy may just result in higher incidence rate of IS events (20), which may explain why hypertensives with HHcy more likely to prevent IS if they had 5–6 ideal CVH metrics, even compared with hypertensives with normal Hcy. Furthermore, another study suggested that lower tHcy levels are significantly associated with a reduction in first stroke risk in Chinese adults with hypertension (21). As a result, in addition to general lifestyle interventions to improve CVH metrics, such as promoting a healthy diet, physical activity, and non-smoking (22), the Hypertension Group, Cardiology Branch of the Chinese Medical Association recommended that supplementation with folic acid, which is abundant in green leafy vegetables and fruits (23), can significantly reduce the risk of first IS among hypertensive patients with HHcy (4). However, in contrast with Western countries, China has not made the folic acid fortification of grain products mandatory, and Chinese populations have a relatively low dietary and supplemental folate intake (24). Therefore, our finding strongly support the promotion of the prevalence of ideal CVH metrics, particularly increasing the intake of vegetables and fruits among hypertensive patients with HHcy in China.

TABLE 3 | The HRs (95% CIs) of incident ischemic stroke by Cardiovascular Health Metrics and age, hypertension duration, controlled blood pressure, and homocysteine.

Characteristics*	Cardiovascular health metrics						*P* trend†	*P* for interaction
	0	1	2	3	4	5–6		
Age, years‡								0.506
<60	1.00	0.82 (0.24–2.84)	0.36 (0.10–1.27)	0.46 (0.13–1.56)	0.39 (0.11–1.42)	0.14 (0.03–0.74)	0.020	
≥60	1.00	0.52 (0.23–1.17)	0.33 (0.15–0.71)	0.31 (0.14–0.68)	0.31 (0.13–0.70)	0.35 (0.14–0.89)	0.025	
Hypertension duration, years‡								0.578
<5	1.00	1.52 (0.51–4.54)	0.83 (0.30–2.29)	0.56 (0.20–1.57)	0.77 (0.25–2.36)	0.32 (0.09–1.12)	0.009	
≥5	1.00	0.32 (0.13–0.76)	0.18 (0.08–0.41)	0.20 (0.08–0.49)	0.15 (0.06–0.38)	0.17 (0.06–0.53)	0.006	
Controlled blood pressure, mmHg‡								0.508
SBP<130 or DBP<80	1.00	0.99 (0.34–2.95)	0.51 (0.17–1.48)	0.58 (0.20–1.67)	0.60 (0.20–1.78)	0.41 (0.12–1.38)	0.108	
SBP≥130 or DBP≥80	1.00	0.32 (0.12–0.85)	0.21 (0.08–0.52)	0.18 (0.07–0.48)	0.16 (0.06–0.47)	0.21 (0.07–0.67)	0.007	
Homocysteine, μmol/L‡								0.412
<15	1.00	0.21 (0.09–0.49)	0.11 (0.05–0.24)	0.12 (0.05–0.27)	0.11 (0.05–0.26)	0.13 (0.05–0.35)	0.025	
≥15	1.00	1.21 (0.38–3.84)	0.87 (0.28–2.65)	0.65 (0.19–2.15)	0.61 (0.17–2.19)	0.30 (0.06–1.40)	0.012	

SBP, systolic blood pressure; DBP, diastolic blood pressure.

** All non-pregnant parti5cipants older than 20 years with available cardiovascular health metrics were included.*

† Trends were analyzed by logistic regression model with adjustment for age and sex.

‡ Adjusted for age, sex, education, alcohol consumption, diabetes mellitus, depression, family history of stroke, years of hypertension, antihypertensive medication, low density lipoprotein, systolic blood pressure, diastolic blood pressure, uric acid, triglyceride, total homocysteine and creatinne.

In our study, ideal healthy diet score was significantly associated with lower IS risk among hypertensive patients. Previous studies have indicated that salt reduction (25), intake of fruit and vegetables (5), whole-grain consumption (26), and moderate intake of meat and eggs (26), which make up our ideal healthy diet, were all related to a lower risk of stroke incidence among hypertensive patients. Although the association between Chinese cooking oils (mainly vegetable oils, including rapeseed oil, peanut oil, and soybean oil) (27) and IS among hypertensives is unclear, it is likely that the high omega-6/omega-3 fatty acid ratio in these oils is highly prothrombotic and proinflammatory and contributes to the prevalence of atherosclerosis, diabetes, and obesity (28, 29), which are all risk factors for IS among hypertensives (24, 30). Given that poor eating habits, such as high fat and sucrose intake, insufficient whole-grain consumption, and high salt levels, were still severe among Chinese populations from 1982 to 2012, according to the results of four Chinese national nutrition surveys (31), a healthy diet is essential for Chinese hypertensive patients to reduce the incidence of IS.

The ideal FBG (<5.5 mmol/L) was inversely associated with the risk of IS, which is consistent with prior reports that a baseline FBG concentration of ≥7.0 mmol/L, or diabetes, is a significant and independent predictor of stroke in Chinese hypertensive patients (24). Moreover, the findings of previous studies have demonstrated that diabetes not only independently multiplied the risk of stroke among hypertensives (32), but was itself also an independent risk factor for stroke (33). Therefore, together with existing evidence of the high prevalence of diabetes among Chinese hypertensive patients (34, 35), our findings suggest that the primary mode of preventing IS in Chinese hypertensive patients should be maintaining an ideal FBG.

Strengths

The strengths of our study include its prospective design, rigorous ascertainment of IS outcomes, and validation of diagnostic methods and biochemical indices at both baseline and at the end of follow-up. To the best of our knowledge, this is also the first study to reveal that the number of ideal CVH metrics is inversely associated with the risk of IS among hypertensive patients. Randomized clinical trials evaluating the effects on IS of multifactorial interventions in hypertensive patients have been sparse and yielded mixed results (23). Observational studies with regard to stroke effects among hypertensives have mainly assessed biochemical indices (36). In this study, taking advantage of the AHA's ideal CVH metrics, we analyzed a comprehensive IS risk factor profile among hypertensive patients to ascertain the association of CVH metrics with IS risk. More importantly, the consideration of the combination of Hcy and hypertension differentiates the current study from other similar studies on ideal CVH.

Limitations

Nevertheless, several limitations need to be considered. First, the novelty of our study might be limited, as there have been multiple studies about CVH metrics and various vascular diseases risk since 2012 (6, 16, 18), however, considering the higher prevalence of HHcy among hypertensive patients in China, our study have advantages in promoting healthy behaviors in those populations. Second, the sample resource of single center and single district, as well as loss of more than 15% of participants during follow-up might lead to the selection bias, thus the findings of our study should be interpreted with caution. Also, our findings are not generalizable to the general population due to the inclusion of only a subset of the Chinese population from Shenzhen. Besides, because Shenzhen is an immigrant city with a large population mobility, only local residents who had lived in Shenzhen for ≥6 months were included in our study, however, this might lead to selection bias, because the selected population would clearly be different from those who do not have these habitat characteristics. Third, the assessments of diet score, smoking status, and physical activity were based on self-reporting, so the exposure levels of those variables might not completely accurate. Moreover, although we modified definitions of smoking status, BMI, and a healthy diet from the AHA, these metrics may still be less valid and reproducible in Chinese populations. Fourth, the metrics of healthy behavior were evaluated only at baseline, so changes in these metrics over time could not be accounted for in this study. Furthermore, although we adjusted for the fruit intake, age, and SBP metrics that differed significantly between the participants lost and not lost to follow-up in the multivariable regression, there were still follow-up biases. Finally, we could not completely rule out all of the residual confounders that have been shown to be associated with the risk of IS, such as income level (37). Moreover, the use of medications (anti-diabetics, lipid-lowering, aspirin, nitrates) were not available in our study, thus limited the further analysis.

CONCLUSIONS

In this Chinese hypertensive population-based prospective study, we found that the number of ideal CVH metrics was inversely associated with the incidence of IS. Specifically, the participants with HHcy who had five or more ideal CVH metrics exhibited a lower IS risk than the participants with normal tHcy levels. In addition, an ideal healthy diet score (7–9 points) and ideal FBG (<5.5 mmol/L) were significantly related to a lower risk of IS. Our findings provide guidance for promoting healthy behaviors and health factors to prevent IS among populations with hypertension, especially those with HHcy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors on reasonable request, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Shenzhen Nanshan Center for Chronic Disease Control. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YY, LH, FK, and SL conceived and designed the study. SL collected the data. LH and CW organized the database. YY and FK executed the statistical analysis and drafted the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Acute Changes in Carotid-Femoral Pulse-Wave Velocity Are Tracked by Heart-Femoral Pulse-Wave Velocity

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Stone K, Fryer S, Faulkner J, Meyer ML, Zieff G, Paterson C, Burnet K, Kelsch E, Credeur D, Lambrick D and Stoner L (2021) Acute Changes in Carotid-Femoral Pulse-Wave Velocity Are Tracked by Heart-Femoral Pulse-Wave Velocity. Front. Cardiovasc. Med. 7:592834. doi: 10.3389/fcvm.2020.592834

Background: Carotid-femoral pulse-wave velocity (cfPWV) is the reference standard measure of central arterial stiffness. However, it requires assessment of the carotid artery, which is technically challenging, and subject-level factors, including carotid artery plaque, may confound measurements. A promising alternative that overcomes these limitations is heart-femoral PWV (hfPWV), but it is not known to what extent changes in cfPWV and hfPWV are associated.

Objectives: To determine, (1) the strength of the association between hfPWV and cfPWV; and (2) whether change in hfPWV is associated with change in cfPWV when central arterial stiffness is perturbed.

Methods: Twenty young, healthy adults [24.0 (SD: 3.1) years, 45% female] were recruited. hfPWV and cfPWV were determined using Doppler ultrasound at baseline and following a mechanical perturbation in arterial stiffness (120 mmHg thigh occlusion). Agreement between the two measurements was determined using mixed-effects regression models and Bland-Altman analysis.

Results: There was, (1) strong (ICC $>$ 0.7) agreement between hfPWV and cfPWV (ICC $=$ 0.82, 95%CI: 0.69, 0.90), and, (2) very strong (ICC $>$ 0.9) agreement between change in hfPWV and cfPWV (ICC $=$ 0.92, 95%CI: 0.86, 0.96). cfPWV was significantly greater than hfPWV at baseline and during thigh occlusion (*both* $P < 0.001$). Inspection of the Bland-Altman plot, comparing cfPWV and corrected hfPWV, revealed no measurement magnitude bias.

Discussion: The current findings indicate that hfPWV and cfPWV are strongly associated, and that change in cfPWV is very strongly associated with change in hfPWV. hfPWV may be a simple alternative to cfPWV in the identification of cardiovascular risk in clinical and epidemiological settings.

Keywords: arterial stiffness, measurement, vascular risk, Doppler ultrasound, pulse-transit time

INTRODUCTION

Pulse wave velocity (PWV) is the gold standard assessment of arterial stiffness and is widely used in epidemiological research to estimate cardiovascular disease (CVD) risk (1). Carotid-femoral PWV (cfPWV), a measure of aortic stiffness, is the reference standard, being a strong independent predictor of CVD risk in both general (2, 3) and patient populations (4). Assessments of cfPWV can be performed with accuracy and precision using tonometric (5), oscillometric (6, 7) or Doppler ultrasound technologies (8–10). However, regardless of the approach, applanation or imaging of the carotid artery is required. This can be technically challenging in certain populations, including persons who are obese and those with advanced carotid artery atherosclerosis (11) and this likely limits its clinical use (12). Also, the carotid artery is not consistent with the path of blood flow for the region of interest - the aortic-illiac pathway. As such it is assumed that the timing of the heart to carotid pressure wave is the same as that from the heart to the descending aorta when calculating cfPWV (13). Finally, cfPWV does not encompass the proximal aorta (14, 15), the distensibility of which is a predictor of CVD risk and mortality (16). One promising alternative measure of central arterial stiffness that overcomes these limitations is heart-femoral PWV (hfPWV).

PWV is calculated by measuring the pulse transit time (PTT) for the arterial waveform to pass between two points of a measured distance. For, hfPWV, PTT can be calculated as the time between the R wave of an electrocardiogram (ECG) and the foot of the femoral pressure waveform. The hfPWV approach confers a number of potential advantages over cfPWV: (i) it is simpler to conduct, as the measurement is not dependent on assessment of the carotid artery; (ii) the measurement path is consistent with the blood flow path; and, (iii) it incorporates the proximal aorta. Although measures of hfPWV have previously been used as a clinical or epidemiological cardiovascular monitoring tool (17–22), only two studies have determined its association with the criterion, cfPWV (13, 23). Both studies reported strong correlations ($r = 0.81$–0.83) between hfPWV and cfPWV in community dwelling healthy middle-aged (13) and older adults (23). However, what has not been determined is to what extent change in hfPWV corresponds to change in cfPWV when central arterial stiffness is perturbed. Whilst hfPWV is valid, to be of utility in clinical or epidemiological environments it must also be demonstrated to accurately track changes in central arterial stiffness and therefore CVD risk.

Central arterial stiffness can be artificially manipulated, including through the use of exercise (24), meal consumption (25) or pharmacological means (26). But these interventions have systemic affects and induce changes in heart rate (HR) which confound cfPWV (27, 28). A simple, non-invasive model that minimizes systemic hemodynamic changes and has been used by others (29) is the inflation of a tourniquet to a low-moderate pressure distal to the arterial segment of interest. This external compression obstructs forward pressure wave propagation, modifies pulse-wave reflection morphology, and alters blood flow profiles, distorting arterial stiffness in upstream vessels (29, 30). This model can be used to confirm whether hfPWV and cfPWV are able to similarly track changes in central arterial stiffness. The aims of this study were to determine: (1) the strength of the association between cfPWV and hfPWV; and (2) whether change (baseline *vs.* cuff inflation) in cfPWV is associated with change in hfPWV.

METHODOLOGY

This study is reported in accordance with CONSORT (Consolidated Standards of Reporting Trials) guidelines (31). Ethical approval was obtained from the University of North Carolina at Chapel Hill Institutional Review Board (17-0745), and all participants provided written informed consent prior to participating in the study.

Participants

To identify the upper limit for agreement between test and criterion measures, a homogeneous cohort of young (18–40 years of age) healthy adults were recruited to participate in the study. Participants were excluded if they reported any known cardiometabolic disorders, were taking medications known to affect cardiovascular function or reported cigarette smoking. To account for potential influences of hormonal status on study outcomes, premenopausal women were studied during the early follicular phase of their menstrual cycle or during the placebo phase of oral contraceptive use.

Experimental Design

A single-visit, two condition design, in which cfPWV and hfPWV were determined at rest and during thigh cuff inflation. The order of conditions was not randomized to prevent the impact of cuff inflation on baseline measures. However, the analyst was blinded to the order of testing. After familiarization, participants were tested in an environmentally controlled room (temperature, 22 $\pm$ 1°C; relative humidity, 51 $\pm$ 2%). All participants had fasted for 12 h and were asked to avoid strenuous physical activity, caffeine and alcohol for 24 h before measurements. After a 20-min rest period in a supine position, right sided cfPWV and hfPWV were determined using Doppler ultrasound. A rapid inflation cuff (SC10; Hokanson Inc., Bellevue, WA, USA), placed immediately proximal to the right knee, was then inflated to a pressure of 120 mmHg. Following a 5-min stabilization period all assessments were repeated. Pilot study [$n = 5$, 31.0 (SD: 4.4) years, 20% female] findings revealed that external compression of 120 mmHg permitted the greatest impact on lower-extremity arterial blood flow, without full occlusion. Ultrasound was used to image the right posterior tibial artery to ensure blood flow was maintained. In each condition, oscillometric pressure waveforms were recorded on the left upper arm, from which central haemodynmic measurements were derived. Stroke volume (SV), cardiac output (CO) and total peripheral resistance (TPR) were also determined.

Experimental Measures

Pulse Wave Velocity

A LOGIQ P6 ultrasound device equipped with an 11–2 mHz linear array probe (GE Healthcare, Wauwatosa, USA) was used

to sequentially scan and obtain ECG-gated pulse-wave Doppler waveforms at the right common carotid artery (CCA) and right superficial femoral artery (SFA) by a single trained operator. The CCA was imaged 2 cm proximal to the carotid bulb and the SFA was imaged 2 cm distal to the bifurcation from the common femoral artery. The US beam was placed at mid-vessel and was angled at $\leq 60^{\circ}$ relative to the longitudinal axis of the artery to permit simultaneous measurement of brachial diameter and blood velocity. An effort was made to ensure that the vessel clearly extended across the entire (un-zoomed) imaging plane to minimize the risk of skewing the vessel walls. Ultrasound global (acoustic output, gain, dynamic range, gamma and rejection) and probe-dependent (zoom factor, edge enhancement, frame averaging and target frame rate) settings were standardized. Three 10 s video recordings were obtained at each position during which participants were asked to hold their breath to avoid any respiratory noise.

An image was obtained from the beginning, middle and end of each video (i.e., 3 images × 3 videos for each measurement site). Images were analyzed offline using ImageJ (Version 1.51q, National Institutes of Health, Bethesda, USA) (32) by a single blinded analyst. The interval between the r-wave of the QRS complex and the foot of the systolic upstroke in the Doppler spectral envelope was measured and averaged over at least three consecutive cardiac cycles for each image at carotid and femoral sites (8, 33). The foot of the Doppler waveform was identified by the intersection of the upstroke of the wave with the baseline of zero frequency. ECG gated pulse-wave Doppler waveforms at both carotid and femoral arterial sites were used to determine carotid-femoral PTT (cfPTT), which represented the interval of time between the foot of the carotid and femoral arterial waveforms and calculated as heart-femoral PTT minus heart-carotid PTT (**Figure 1**). The cfPWV arterial path length (D) was estimated by measuring the linear distance from the suprasternal notch to the mid-point of the probe at the SFA and subtracting the distance from the suprasternal notch to the mid-point of the probe at the CCA. Path length was directly measured, using a custom device to bypass body contours. The r-wave to the foot of the femoral Doppler waveform interval represented the heart-femoral PTT (hfPTT) and estimated D was the linear distance from the suprasternal notch to the mid-point of the probe at the SFA only. Pulse-wave velocity was calculated as: PWV = D/PTT.

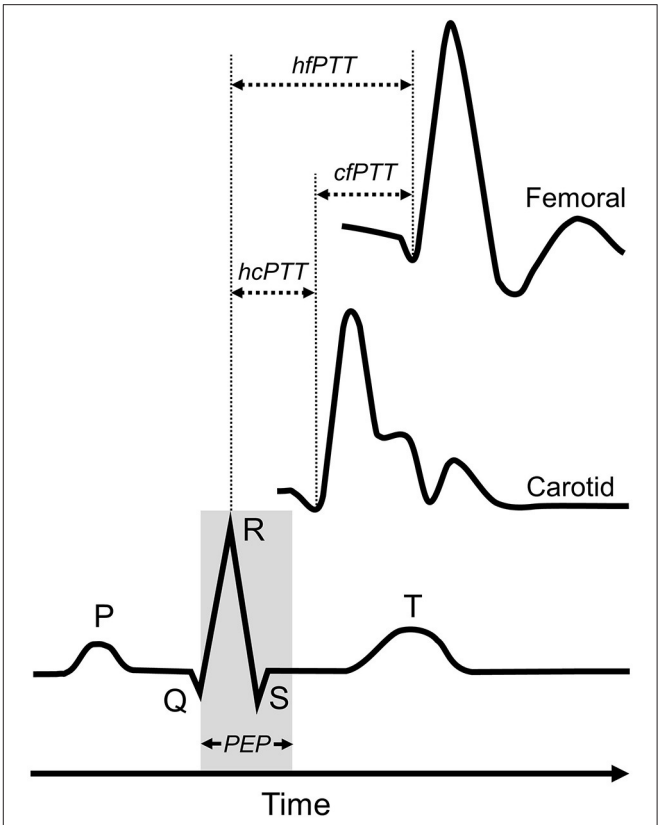

FIGURE 1 | Determination of pulse-transit time (PTT) for heart-femoral pulse-wave velocity and carotid-femoral pulse-weave velocity measures. Carotid-femoral PTT (cfPTT) was calculated as heart-femoral PPT (hfPTT) minus heart-carotid PTT (cfPTT). PEP, pre-ejection period.

Mechanistic Explanatory Variables

It is recognized that HR, mean arterial pressure (MAP) and arterial wave reflection morphology can impact central arterial stiffness. Accordingly, in order to determine the systemic effects of cuff inflation and their potential contribution to changes in central arterial stiffness, (i) central pressure and indices of wave reflection were determined using pulse wave analysis (PWA), and, (ii) CO, SV and TPR were determined using plethysmography. For PWA, oscillometric pressure waveforms were recorded on the left upper arm by a single observer using the SphygmoCor XCEL device (AtCor Medical, Sydney, Austrailia), following standard manufacturer guidelines (34). Each single measurement cycle consisted of a 60 s brachial blood pressure recording followed by a 10 s sub-systolic recording. A corresponding aortic pressure waveform was then generated using a validated transfer function (35), from which central systolic blood pressure (cSBP), augmentation index (AIx), augmentation pressure (AP), forward aortic pressure (Pf) and backward aortic pressure (Pb) were derived. Beat-to-beat CO, SV and TPR were monitored non-invasively at the finger (Human NIBP Nano System, Finapres Medical Systems B.V., Netherlands) and sampled at 200 Hz via PowerLab systems (LabChart 8, ADIinstruments, Australia). Presented data represents the mean of three 1-min periods at the start, middle and end of ultrasound data collection.

Sample Size

Sample size estimates were conducted using G*Power 3. For calculating the association between measures, we elected to use a mixed model to increase statistical power by enabling repeated measures to be nested within each subject. Using this approach, a sample of 37 data points can detect a 1-tailed correlation of 0.4 (moderate) with 80% power and a 5% chance of a type 1 error. For a mixed model-based approach to correlation, the degrees of freedom are equal to N – 1, where N is the total number of participants (36). For a sample size of 20, with two

repeated measures, the degrees of freedom (number of required participants) is 40 – 1 = 39.

Statistical Analysis

Statistical analyses were performed using RKWard (2019, Version 0.7.1), a frontend to the R statistical package. The significance level was set a *priori* for all statistical procedures at $\alpha = 0.05$. Raw data are presented as mean [standard deviation (SD)] and mixed model data are presented as mean [95% confidence interval (95%CI)]. The corresponding author (KS) had full access to the data in the study and was responsible for the integrity of the data set and the data analysis.

Paired *t*-tests were used to compare baseline and cuff inflation hemodynamic responses, as well as measures of cfPWV and hfPWV within conditions. Effect sizes (ES) were calculated as Cohen's *d* where <0.2 was defined as trivial, 0.2–0.3 as small, 0.4–0.8 as moderate, and >0.8 as large. The association between the criterion and test measurements was determined using nested mixed-effects regression models. This approach allowed us to maximize statistical power while accounting for the correlated error variances (repeated measures) and the condition (base, cuff) structure. Specifically, the test measure was regressed against the criterion measure and nested within subject and condition (base, cuff inflation). Subject and cuff intercepts were specified as random effects and used to estimate the between-subject (σ2s), between-condition (σ2c) and residual (σ2r) variance. Subsequently, the between-measurement ICC was calculated as σ2s/(σ2s + σ2r) and the between condition ICC as σ2s/(σ2c + σ2r). To adjudicate the strength of association we used existing criteria for Pearson product-moment correlation (*r*). Although there is no universal criterion, in general, r value estimates of <0.2, 0.2–0.4, 0.4–0.7, 0.7–0.9, and >0.9 indicate negligible, weak, moderate, strong, and very strong agreement, respectively (37). To ensure transparency in our statistical approach, the supplement also reports associations between measurements for each condition using the standard Pearson product moment correlation, and between measurement correlations rmcorr package for R. As hfPWV was found to be on a different scale to cfPWV, to permit direct comparison and visual analysis of the uniformity of error over the range of participant measurement values (38), hfPWV was corrected (hfPWVc) using the mixed model regression equation and subsequently used to generate Bland–Altman plots with hfPWVc and cfPWV on comparable scales (38).

RESULTS

All participants were included in analyses and there was no missing participant data. Participant descriptive characteristics are presented in **Table 1**.

TABLE 1 | Descriptive characteristics of study participants.

n = 20	Mean	(SD)
Age (years)	24	(3.1)
Female (%)	45	
Height (m)	1.7	(7.3)
Weight (kg)	70.2	(11.4)
BMI (kg/m^2)	23.9	(2.5)
PA (avg/mins/wk)	65.0	(21.1)
PA Sessions (avg/wk)	4.6	(1.4)

BMI, body mass index; PA, physical activity.

Blood Pressure and PWV Response to Cuff Inflation

Blood pressure and PWV responses to cuff inflation are reported in **Table 2**. None of the peripheral or central haemodynamic measures were significantly impacted by cuff inflation (all *P* >0.05). There was large effect (ES > 0.8) decrease in hfPWV (−0.10 m/s, 95%CI: −0.07, −0.14) and a moderate effect (ES 0.4–0.8) decrease in cfPWV (−0.22 m/s, 95%CI: −0.06, −0.38) with cuff inflation.

Measurement Comparison

Figure 2 presents the comparison between hfPWV and cfPWV. There was strong between-measurement agreement (ICC: 0.82, 95%CI: 0.69–0.90) and very strong between-condition agreement (ICC: 0.92, 95%CI: 0.86–0.96) for hfPWV and cfPWV comparison. Measures of cfPWV were significantly higher than hfPWV at baseline (mean difference: 1.9 m/s, 95%CI: 1.72–2.09, $P < 0.001$) and cuff inflation (mean difference: 1.78 m/s, 95%CI: 1.6–1.97, $P < 0.001$). Mean bias for Bland-Altman analysis comparing cfPWV and hfPWVc was 0.02 m/s (95%CI: −0.18 to 0.21) at baseline and −0.04 m/s (95%CI: −0.24 to 0.14) at cuff inflation. Inspection of the plots indicated no measurement magnitude bias at baseline or cuff inflation (**Figures 3A,B**).

Ancillary Analysis

Ancillary analysis is provided in the supplement. Pulse transit time and arterial path length values for the determination of cfPWV and hfPWV are presented in **Supplementary Table 1**. To ensure full transparency, Bland-Altman plots generated using uncorrected hfPWV and cfPWV are presented in **Supplementary Figure 1**. The associations between measurements for each condition are reported using standard Pearson product moment correlations in **Supplementary Figure 2**. To further interrogate intra-individual associations between change (cuff vs. baseline) in cfPWV and change in hfPWV repeated measures correlation was determined (**Supplementary Figure 3**).

DISCUSSION

This study investigated, (1) the strength of the association between cfPWV and hfPWV; and (2) whether change in cfPWV is associated with change in hfPWV. Our main findings show that there is a strong (ICC > 0.7) agreement between hfPWV and cfPWV, and that change (baseline vs. cuff inflation) in hfPWV and change in cfPWV demonstrated very strong agreement (ICC > 0.9).

TABLE 2 | Blood pressure, wave reflection, carotid-femoral pulse wave velocity (cfPWV) and heart-femoral pulse wave velocity (hfPWV) responses to cuff inflation ($n = 20$).

	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (beats min-1)	cSBP (mmHg)	AP (mmHg)	AIx (%)	Pb (mmHg)	Pf (mmHg)	SV (ml)	CO (l min-1)	TPR (mmHg.s)	hfPWV (m/s)	cfPWV (m/s)
Mean														
Rest	117	69	81	55	102	1	3	12	26	57	3.3	4.0	2.97	4.87
120 mmHg	118	70	81	57	102	0	1	13	26	54	3.2	3.8	2.87	4.65
Standard Deviation														
Rest	12	5	6	9	10	3	10	3	5	10	0.6	1.0	0.24	0.45
120 mmHg	13	6	9	10	11	7	10	5	5	11	0.6	0.7	0.20	0.45
Condition Effect														
β	1.28	1.25	0.15	1.35	0.70	−1.55	−2.00	0.80	−0.40	−2.80	−0.12	−0.29	−0.10	−0.22
P	0.747	0.494	0.954	0.659	0.827	0.382	0.552	0.556	0.812	0.393	0.562	0.287	<0.001	0.013
ES	0.05	0.11	0.01	0.07	0.03	−0.14	−0.09	0.09	−0.04	−0.14	−0.09	−0.17	−0.84	−0.43

MAP, mean arterial pressure; HR, heart rate; cSBP, central systolic blood pressure; AP, aortic pressure; AIx, augmentation index; Pb, aortic backward wave pressure; Pf, aortic forward wave pressure; SV, stroke volume; CO, cardiac output; TPR, total peripheral resistance; ES, Cohen's d effect size.

Limitations and Strengths

The limitations and strengths of this study are addressed to best contextualize the findings. Firstly, a relatively homogeneous group of young, healthy participants were recruited to ascertain the upper limit of agreement between changes in cfPWV and hfPWV measures, thus limiting the overall generalizability of our findings to populations of varying age and health states. Prior to clinical use, it is imperative to identify whether any inherent error, bias or variability is caused by the technique itself and not a consequence cardiovascular pathology. A major strength is that this is the first study to directly compare changes in the reference standard measure of central arterial stiffness, cfPWV, and a novel, simpler alternative hfPWV.

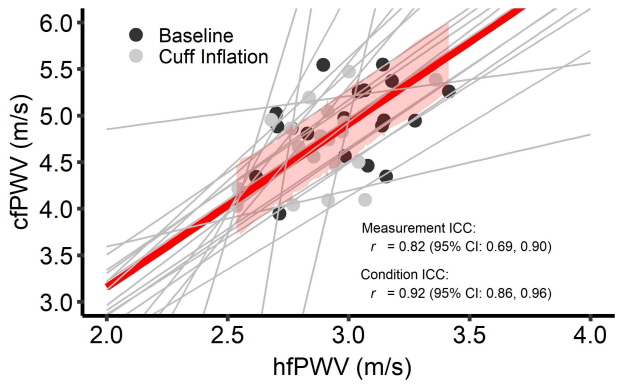

FIGURE 2 | Intra-class correlations (ICC) for between-measurement (i.e., overall comparison between criterion and test measure) and between-condition [i.e., change (cuff – base) in the test measure *vs.* change in the criterion measure] carotid-femoral pulse wave velocity (cfPWV) and heart-femoral pulse wave velocity (hfPWV) comparisons. $n = 20$. Red line and red shading depict overall between-measurement agreement and standard error, respectively. Gray lines depict between-condition agreement.

Comparison to Literature

In the only other studies to directly compare cfPWV and hfPWV measures (13, 23), comparable associations ($r = 0.81$–0.83) were observed to that reported in the present study (ICC = 0.82). However, in contrast to Stoner et al. (23) who reported significant bias at higher PWVs, we observed no measurement magnitude bias between cfPWV and corrected hfPWV measures. This contrast may be due to the inclusion of young, healthy individuals in the present study, whereas Stoner et al. (23), studied a large population of older adults of varying health and disease states. Higher PWV values in older adults may be accompanied by greater carotid plaque prevalence, which could influence local arterial mechanics and vessel elasticity (39), potentially leading to greater error variance. Unlike cfPWV, hfPWV measures do not require carotid artery assessment and are therefore not confounded by the presence of plaque.

The present study also extends previous findings by being the first to report that following a perturbation to acutely manipulate central arterial stiffness, changes in cfPWV and hfPWV also demonstrate very strong agreement (ICC = 0.92). In the intended absence of systemic changes, the reduction in central arterial stiffness is likely a consequence of lower limb blood pooling, induced by venous outflow occlusion, leading to a dampening of wave reflection and an attenuation of central pressure (40). Of note, Stoner et al. (23) did report that sex impacted the agreement between hfPWV and cfPWV; with a superior agreement in women, but a greater error variance at higher PWV's. It is recognized that vascular structural and functional properties differ between sexes (41, 42) and there are divergent relationships between HR and cfPWV (43). Accordingly, whilst the present study was not adequately powered to examine sex differences, future studies should identify if the agreement between changes in cfPWV and hfPWV is impacted by sex.

Measures of cfPWV were significantly higher than hfPWV measures; a difference that may have been driven by several sources. Firstly, there is an intrinsic difference between hfPWV and cfPWV in the start point from which PTT is measured (see **Figure 1**). cfPTT is determined as the interval between the foot of the upstroke of carotid and femoral waveforms. Hence, PTT is inversely proportional to PWV, being influenced by the

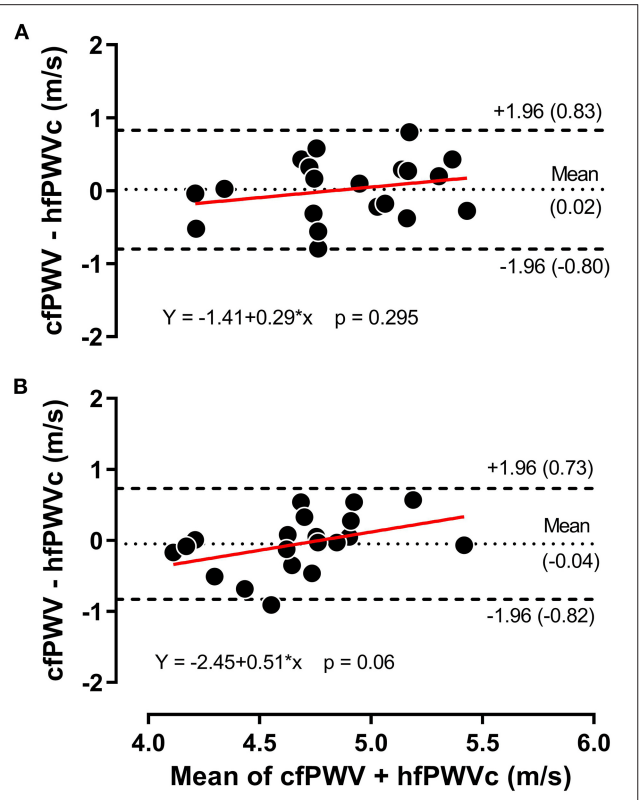

FIGURE 3 | Bland-Altman plot for carotid-femoral pulse wave velocity (cfPWV) vs. corrected heart-femoral pulse wave velocity (hfPWVc) at baseline **(A)** and during 120 mmHg thigh cuff inflation **(B)**, $n = 20$.

same arterial and haemodynamic factors. In contrast, hfPTT, or pulse arrival time, is determined as the interval between the r-wave of the QRS complex and the foot of the upstroke of the femoral waveform (8, 33). As such, hfPTT includes the pre-ejection period (PEP), which is the electromechanical delay associated with the conversion of electrical signal into mechanical pumping force and isovolumetric contraction to open the aortic valves (44, 45). hfPWV may therefore not represent a true PWV, consequently overestimating aortic PTT. One potential solution is to measure PTT from the beginning of the S segment of the ECG, thereby reducing the impact of PEP on hfPWV measures. However, in comparison to the prominent R-wave, the S-wave is susceptible to motion and measurement artifact and is likely less reliable. Alternatively, isovolumetric contraction time, and thus PEP, can be estimated by analyzing the heart to carotid waveform and using cfPWV as a surrogate for ascending aorta PWV (46). But whilst this promising deductive approach demonstrated good agreement with echocardiography derived PEP, a number of assumptions are made, including; (i) ascending aortic length, and (ii) that the electro mechanical delay of PEP is constant and equal to the QR interval (46). These assumptions may not always be appropriate, particularly within patient populations. Secondly, cfPWV measures do not encompass the proximal aorta (13–15). cfPWV assessment employs the carotid artery, which is not consistent with the path of blood flow for the region of interest - the aortic-illiac pathway. To adjust for this, the arterial segment from the ascending aorta to the carotid artery is omitted (subtracted) from the effective path lengths used for cfPWV calculations (14). In doing so, it is also assumed that the time the forward pressure wave takes to travel from the heart to the carotid artery is the same time it takes a simultaneous wave to travel the same distance from the heart to the descending aorta (13, 14). Therefore, cfPWV actually represents the PWV from the descending aorta to the femoral artery. Omission of the highly elastic proximal aorta inherently leads to cfPWV measures being higher than hfPWV measures, as the latter includes the whole aorta. Hickson et al. (47) demonstrated that exclusion of the aortic arch from global aortic PWV measures determined using magnetic resonance imaging reduced the difference between (up to 0.8 m/s) and improved the agreement with cfPWV determined using both tonometric and oscillometric technologies. Although the inclusion of PEP and the ascending aorta collectively contribute to causing greater divergence, hfPWV and cfPWV are consistently proportionate to each other, hence their strong agreement, as reported in the present study and by others (13, 23).

Implications

Despite cfPWV being a strong independent predictor of CVD risk (2–4), it is not widely used in clinical practice. Technical challenges associated with the assessment of the carotid artery, which can be difficult to perform in certain populations, likely limits it clinical use. Furthermore, although not widely recognized, the inherent omission of the proximal aorta may overlook important pathophysiological information (16). hfPWV is a simpler alternative which is less likely to be confounded by subject-level factors, including the presence of carotid plaque, and encompasses the predictive capacity of the whole aorta. Previously cfPWV and hfPWV measurements have demonstrated good baseline agreement. The current study extends the scant hfPWV literature by reporting that cfPWV and hfPWV demonstrate strong agreement when tracking acute changes in central arterial stiffness.

Measures of hfPWV are inherently lower than cfPWV. It may be possible to correct hfPWV to ensure greater comparability with cfPWV, however such corrections may limit measurement precision. Although there is some divergence in absolute PWV values, hfPWV and cfPWV are proportionate to one another. It is recognized that hfPWV measures still require assessment of the femoral artery. Like the carotid artery, femoral artery assessments can also be challenging and may require a highly trained operator to ensure precision, particularly when using ultrasound or tonometry technologies. One emerging technique which is operator independent and has demonstrated moderate agreement with cfPWV ($r = 0.64$) is the determination of hfPWV using an oscillometric thigh cuff to detect femoral waveforms (22). Further exploration of this technology is required, but the simplicity of the oscillometric-based hfPWV measures may encourage the clinical adoption of central arterial stiffness phenotyping as a marker of arterial aging and CVD risk. Regardless of approach, future work should seek to identify the repeatability of hfPWV measures and if hfPWV and cfPWV

similarly track central arterial stiffness changes in older adults with CVD progression.

CONCLUSIONS

The current findings indicate that cfPWV and hfPWV are strongly associated, and that change in cfPWV is very strongly associated with change in hfPWV following the acute perturbation of central arterial stiffness. Accordingly, hfPWV may be a simple, viable alternative to cfPWV in the tracking of CVD risk within clinical and epidemiological environments.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of North Carolina at Chapel Hill Office of Human Research Ethics (17-0745). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KS, SF, JF, and LS contributed to the conception and design of the experiment, data collection, analysis, interpretation of the data, and the drafting of the manuscript. GZ, CP, KB, and EK contributed to data collection and drafting of the manuscript. MM, DC, and DL contributed to data interpretation and the drafting of the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved and persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2020.592834/full#supplementary-material

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Serum Uric Acid Revealed a U-Shaped Relationship With All-Cause Mortality and Cardiovascular Mortality in High Atherosclerosis Risk Patients: The ASSURE Study

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Background: Previous studies have demonstrated an association between hyperuricemia and cardiovascular disease (CVD). The Framingham study confirmed that patients with high atherosclerotic risks (HARs) had worse prognoses. However, after adjusting for confounding factors, the association between serum uric acid (SUA) and all-cause mortality and cardiovascular mortality remains unclear, especially for HAR patients.

Objective: The aim of this study was to reveal the relationship of SUA with all-cause and cardiovascular mortality in HAR patients.

Methods: This multicenter cohort study enrolled 3,047 participants, and the follow-up was 68.85 ± 11.37 months. Factors related to cardiovascular and all-cause mortality were tested by multivariate Cox regression analysis. Restricted cubic splines (RCSs) with knots were used to explore the shape of the dose–response relationship with SUA and the hazard ratio (HR) of all-cause and CVD mortality. SUA transformed by RCS was added to the Cox regression model as an independent variable, and all-cause and CVD mortality scores were calculated. Survival receiver operating characteristic curves were produced using a regression model predicting the score.

Results: SUA demonstrated a "U-shaped" relationship with all-cause and cardiovascular mortality. SUA predicted all-cause and CVD mortality, with cutoff values of values of >370.5 μmol/L for males and >327.65 μmol/L for females and <180.5 μmol/L for males and <165.7 μmol/L for females, respectively. The survival ROC curve indicated that SUA is able to predict all-cause and CVD mortality, with areas under the curve of 0.702 and 0.711, respectively. The HRs of all-cause mortality (male and female) with hyperuricemia and hypouricemia were 2.08 and 2.01 and 2.04 and 1.98, respectively, and the HRs of CVD mortality (male and female) were 2.09 and 1.79, and 2.02 and 1.89, respectively.

Conclusion: Abnormal SUA levels were significant and independent risk factors for all-cause and CVD mortality. Hyperuricemia and hypouricemia increased mortality in both males and females. Routine SUA evaluation and intensive management are needed for HAR patients.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier: NCT03616769.

Keywords: serum uric acid, all-cause mortality, cardiovascular mortality, framingham risks, atherosclerosis risks

BACKGROUND

Previous studies in humans have demonstrated an association between hyperuricemia, arterial stiffness, and endothelial dysfunction (1), and the serum uric acid (SUA) level has been suggested to be an important modulator of the inflammatory process (2). Several studies have reported that SUA serves as a marker of an underlying pathophysiological process (3, 4), and some evidence shows that elevated SUA concentrations are associated with a higher risk of hypertension and cardiovascular disease (CVD) (5–7). However, the relationship between SUA level and CVD mortality remains controversial because established cardiovascular risk factors are complex and may be considered a confounding factor (8). Although previous studies have indicated that hyperuricemia plays a role in atherogenesis in the development of CVD (9–11), there is little research available on the relationship between hypouricemia and hypouricemia with all-cause and CVD mortality.

In addition, the Framingham Heart Study found that patients with high atherosclerotic risk (HAR) have worse CVD prognosis (12–14). However, the synergistic effect of SUA combined with HAR on prognosis is still unclear. Although a few multicenter cohort studies have also focused on abnormal SUA levels combined with HAR, most studies were from Western countries and lack Asian data, especially in Chinese patients. Therefore, the aim of this research was to explore relationships among SUA levels, all-cause mortality, and cardiovascular mortality in HAR patients.

METHODS

Study Population

The ASSURE study (ClinicalTrials.gov identifier NCT03616769) is a multicenter prospective cohort study. The first cross-sectional survey was conducted in 2011. Eligible participants were followed up from November 2011 to June 2018 (mean follow-up was 68.71 ± 11.35 months). During the follow-up period, 76 subjects had missing data, and 97 were not compliant. Thus, the study sample comprised 3,047 eligible participants (1,625 male and 1,422 female participants) older than or equal to 35 years (mean age 60.2 ± 10.4 years). A total of hospitalized subjects were consecutively enrolled from the Cardiology Department of Beijing University Affiliated and of Shanghai Tongji University–affiliated hospitals. All subjects were under treatment for CVD. The inclusion criterion was HAR. The exclusion criteria were severe congestive heart failure and severe renal failure. Severe congestive heart failure was defined as greater than or equal to cardiac functional classification 3 formulated by the New York Heart Association (NYHA). Severe renal failure was defined as an estimated glomerular filtration rate (eGFR) <30 mL/min per 1.73 m^2 (**Figure 1**). All participants provided written informed consent for this study, which was approved by the Ethics Committee of Tongji University.

Cardiovascular Event Definitions

Hospitalized myocardial infarction (MI) was classified as definite or probable based on chest pain symptoms, cardiac enzyme levels, electrocardiographic (ECG) findings, or angioplasty. Coronary heart disease (CHD) was determined to be present if there was (1) ECG evidence of a prior MI, (2) prior coronary artery bypass surgery or angioplasty, (3) coronary angiography showing CHD, (4) symptoms of angina and ECG revealing myocardial ischemia performance or laboratory tests showing increased cardiac enzymes and excluding other types of disease, or (5) a self-reported history of a physician-diagnosed heart attack. Coronary artery disease (CAD) death was classified as "definite" based on chest pain symptoms, hospital records, and medical history.

Assessment of Cardiovascular Events and Identification of Death From All Causes and CVD

Cardiovascular events are composed of cardiac events, including non-fatal MI, unstable angina, and coronary revascularization procedures, during the follow-up period. The exclusion criteria were stable angina (>6 months), revascularization procedure for CAD (>6 months), and MI (>6 months).

In this study, cardiovascular death was the only cardiac event death considered. Medical records and death certificates for all patients who had an event were obtained and validated by a cardiologist. Death was confirmed from hospital records or by

Abbreviations: CVD, cardiovascular disease; SUA, serum uric acid; ABI, ankle–brachial index; HAR, high atherosclerotic risk; RCS, restricted cubic splines; BMI, body mass index; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; DM, diabetes mellitus; HR, hazard ratios; FRS, Framingham risk score; PAD, peripheral arterial disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; IS, ischemic stroke; CRI, chronic renal insufficiency; DL, dyslipidemia; eGFR, estimated glomerular filtration rate; CHD, coronary heart disease; PCI, percutaneous coronary stent implantation; CABG, coronary artery bypass grafting; MI, myocardial infarction; Cr, serum creatinine; ROC, receiver operating characteristic; AUC, area under the curve.

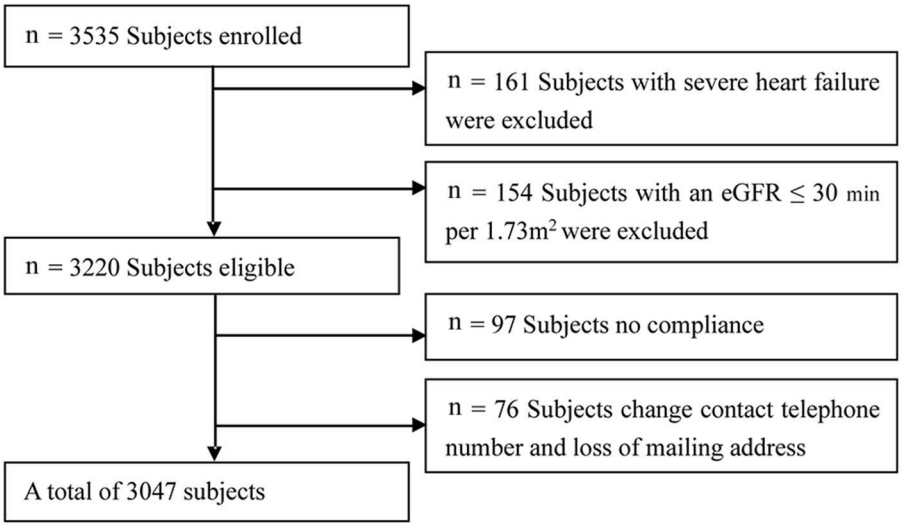

FIGURE 1 | Study flowchart.

contact with participants and their families. All materials were reviewed independently by five senior physicians of the cohort study to confirm the cause of death.

Hyperuricemia and Hypouricemia Definitions

Hyperuricemia refers to consuming a normal-purpurin diet, with two determinations on different days: SUA ≥420 μmol/L or 7 mg/dL (male), ≥ 357 μmol/L or 6 mg/dL (female) (15). Hypouricemia refers to consuming a normal-purpurin diet, with two determinations on different days: SUA ≤178 μmol/L or ≤3 mg/dL (male) and SUA ≤149 μmol/L or ≤2.5 mg/dL (female) (16, 17).

Framingham Risk Score and High Atherosclerosis Risk

The Framingham risk score (FRS) was calculated based on coronary risk factors, including age, sex, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), hypertension, and smoking status, according to the National Cholesterol Education Program Adult Treatment Panel III algorithm (18). The calculated total scores were used to estimate the 10-year CHD risk in participants without previous CVD and when an FRS > 20% or between 10 and 20% was considered HAR (18).

Framingham Risk Factors

The diagnostic criteria for hypertension were as follows: patients taking antihypertensive medication or systolic blood pressure (SBP) ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg (1 mm Hg = 133 kPa) or both. The criterion for hypertension was a single hypertensive disease.

Similarly, the criterion for dyslipidemia was a single dyslipidemic disease. The definition of dyslipidemia is abnormalities in serum levels of lipids, including overproduction or deficiency. Abnormal serum lipid profiles include high TC, high triglycerides (TGs), low HDL-C, and elevated LDL-C.

Measurement of Ankle and Arm Blood Pressures

Qualified ultrasonographers measured ankle and brachial SBPs and Doppler ultrasound (Nicolet Vascular, Elite 100R, USA) was used to measure SBP in the bilateral brachial, tibial, and dorsal pedal arteries. The ankle–brachial index (ABI) has been shown to be a powerful independent marker of cardiovascular risk, with predictive ability similar to that of the Framingham criteria (19, 20).

A questionnaire was designed to collect from all participants information about general characteristics, diagnosis, medical history and related factors, medical treatment, and biochemical examination.

Baseline Measurements

Factors were assessed for all subjects, including daily habits, medical histories, and blood samples. These blood samples were used to measure TC, TGs, HDL-C, LDL-C, serum creatinine (Cr), SUA, glucose, and fasting plasma glucose. Blood samples were drawn from an antecubital vein into a Vacutainer using a 19-gauge needle, and serum concentrations were assessed with commercially available kits. The GFR was calculated as GFR (mL/min per 1.73 m^2) = 186 × creatinine$^{-1.154}$ × age$^{-0.203}$ and GFR (mL/min per 1.73 m^2) = 142 × creatinine$^{-1.154}$ × age$^{-0.203}$ for males and females, respectively. Glucose was measured using a Hitachi 717 analyzer (Roche) with enzymatic reagents from Roche. The presence of symptomatic peripheral arterial disease (PAD) was evaluated by the Rose questionnaire (21). A previous MI or ischemic stroke was documented by hospital records. Physical examination data included body mass index (BMI), blood pressure, and ABI. Severe congestive heart failure was defined as greater than or equal to cardiac functional

TABLE 1 | Comparison of subjects' baseline characteristics of sex categories according to serum uric acid level.

Characteristics	Male					Female				
	Quartile of SUA levels, μmol/L				*P*-value	Quartile of SUA levels, μmol/L				*P*-value
	0–242.0	242.1–312.0	312.1–386.75	>386.75		0–242.0	242.1–312.0	312.1–386.75	>386.75	
Age (years)	67.3 ± 11.3	65.8 ± 11.7	65.7 ± 11.8	68.0 ± 11.5	0.002	65.6 ± 10.8	67.3 ± 10.5	67.7 ± 9.7	70.4 ± 9.9	<0.001
Diabetes, *n* (%)	198 (41.8)	174 (35.9)	154 (31.8)	159 (32.9)	0.006	310 (43.7)	189 (42.3)	134 (45.1)	99 (38.2)	0.372
DM duration (years)	2.8 ± 5.0	3.0 ± 6.0	2.3 ± 4.8	2.5 ± 5.0	0.173	3.6 ± 6.1	3.8 ± 6.4	3.8 ± 7.1	3.6 ± 6.9	0.925
Hypertension, *n* (%)	309 (65.2)	324 (66.8)	343 (70.7)	380 (78.7)	<0.001	479 (78.7)	347 (78.7)	252 (84.8)	216 (83.4)	<0.001
HT duration (years)	8.5 ± 11.5	8.9 ± 11.2	9.7 ± 12.2	10.8 ± 11.3	0.012	8.3 ± 10.8	10.5 ± 11.6	12.1 ± 11.8	12.6 ± 13.1	<0.001
Dyslipidemia, *n* (%)	134 (34.6)	152 (38.0)	185 (46.7)	171 (40.8)	0.005	271 (46.5)	161 (42.5)	117 (50.0)	98 (44.1)	0.297
DL duration (years)	1.3 ± 0.31	1.8 ± 0.45	1.8 ± 0.41	2.1 ± 0.46	0.069	1.9 ± 0.39	1.9 ± 0.43	2.0 ± 0.42	1.6 ± 0.34	0.532
Smoking, *n* (%)	310 (65.4)	307 (63.3)	325 (67.0)	320 (66.3)	0.648	57 (8.0)	48 (10.7)	27 (9.1)	29 (11.2)	0.318
Smoking duration (years)	20.7 ± 1.86	20.0 ± 1.81	21.4 ± 1.85	21.3 ± 1.91	0.552	2.7 ± 1.02	3.4 ± 1.08	2.7 ± 0.97	4.3 ± 1.32	0.218
MI history, *n* (%)	87 (18.4)	83 (17.1)	94 (19.4)	102 (21.1)	0.439	60 (8.5)	51 (11.4)	34 (11.4)	27 (10.4)	0.307
Diuretics, *n* (%)	105 (22.2)	100 (20.7)	132 (27.2)	195 (40.5)	<0.001	140 (19.8)	116 (26.0)	106 (35.8)	111 (43.0)	<0.001
PTCA history, *n* (%)	56 (11.8)	70 (14.4)	69 (14.2)	64 (13.3)	0.631	59 (8.3)	42 (9.4)	24 (8.1)	18 (6.9)	0.721
CABG history, *n* (%)	18 (3.8)	16 (3.3)	19 (3.9)	20 (4.1)	0.918	9 (1.3)	7 (1.6)	7 (2.4)	5 (1.9)	0.634
IS history, *n* (%)	182 (38.4)	163 (33.6)	161 (33.2)	167 (34.6)	0.315	212 (29.9)	139 (31.1)	83 (27.9)	83 (32.0)	0.719
CRI history, *n* (%)	31 (6.8)	29 (6.3)	38 (8.1)	83 (17.6)	<0.001	56 (8.1)	29 (6.6)	32 (11.0)	53 (21.1)	<0.001
TG, mmol/L	1.4 ± 0.80	1.5 ± 0.90	1.6 ± 1.00	1.8 ± 1.50	<0.001	1.6 ± 1.10	1.8 ± 1.3 0	2.0 ± 1.40	2.0 ± 1.20	<0.001
HDL-c, mmol/L	1.2 ± 0.40	1.2 ± 0.3 0	1.1 ± 0.40	1.1 ± 0.50	0.106	1.3 ± 0.40	1.2 ± 0.30	1.3 ± 0.40	1.2 ± 0.40	0.001
LDL-c, mmol/L	2.5 ± 0.80	2.7 ± 0.80	2.7 ± 0.90	2.6 ± 0.80	<0.001	2.8 ± 0.90	2.9 ± 0.80	3.0 ± 0.90	2.8 ± 1.00	0.052
CRE	93.6 ± 7.39	103.2 ± 8.06	109.2 ± 8.55	138.2 ± 13.17	<0.001	79.0 ± 6.73	84.9 ± 6.83	91.2 ± 6.53	143.0 ± 15.90	<0.001
Blood glucose	6.8 ± 0.30	6.4 ± 0.27	6.0 ± 0.24	6.1 ± 0.26	<0.001	6.7 ± 0.32	6.6 ± 0.28	6.6 ± 0.28	6.6 ± 0.32	0.816
BMI, kg/m^2	23.2 ± 3.4	24.7 ± 3.3	24.6 ± 3.4	24.9 ± 3.7	<0.001	23.8 ± 3.6	24.3 ± 3.8	25.1 ± 3.5	24.9 ± 4.0	<0.001
ABI	1.01 ± 0.20	1.02 ± 0.20	0.99 ± 0.20	0.99 ± 0.20	0.066	0.99 ± 0.20	0.97 ± 0.20	0.95 ± 0.20	0.91 ± 0.30	<0.001
FRS†, *n* (%)	454 (24.7)	459 (25.5)	461 (25.3)	462 (25.1)	0.910	408 (21.9)	446 (24.8)	296 (16.7)	258 (13.6)	<0.001
FRS††, *n* (%)	357 (24.1)	379 (25.5)	375 (25.3)	373 (25.1)	0.647	488 (27.2)	424 (23.1)	287 (15.6)	247 (12.4)	<0.001

SBP, Systolic blood pressure; DBP, diastolic blood pressure; HT, hypertension; BMI, body mass index; TC, total cholesterol; TG, triglycerides; FPG, fasting plasma glucose; HDL-c, high-density lipoprotein; LDL-c, low-density lipoprotein; Cr, serum creatinine; SUA, serum uric acid; DM, diabetes mellitus; MI, myocardial infarction; IS, ischemic stroke; CRI, chronic renal insufficiency; DL, dyslipidemia; CVD, cardiovascular disease; PAD, peripheral arterial disease; ABI, ankle–brachial index; PTCA, percutaneous coronary angioplasty; HR, hazard ratio; CABG, coronary artery bypass grafting; FRS, Framingham risk score. †FRS analysis of participants with FRS 10–20%. ††FRS analysis of participants with FRS; >20% was identified as high risk for 10-year coronary heart disease.

classification 3 formulated by the NYHA. Severe renal failure was defined as an eGFR <30 mL/min per 1.73 m^2.

Follow-Up Methods

For follow-up, participants were contacted by physicians of the cohort study at annual intervals. Outcomes were obtained based on the annual phone interviews, 6-year follow-up examinations, hospital records, and death records. The primary clinical event endpoints of this study were estimated all-cause mortality and cardiovascular mortality. The secondary endpoints were CHD and CHD risk equivalent, including PAD, stroke, diabetes mellitus (DM), and CVD. Follow-up time was the number of years from the baseline (first) visit. For subjects who had more than one event, all clinical events were considered in the analysis.

Statistical Analysis

All analyses were performed using the R statistical package (version 3.6.2) [http://www.r-project.org (22)]. Continuous variables are expressed as the mean ± SD and categorical variables as percentages. Continuous and categorical variable difference comparisons were made by independent-samples ANOVA (analysis of variance) and the χ^2 test, as appropriate. The Kruskal–Wallis test was used to evaluate non-normally distributed continuous variables. A $P < 0.05$ was considered statistically significant. Due to a skewed distribution, TC, TG, HDL-C, LDL-C, Cr, and SUA were logarithm-transformed (log) for analyses. Crude death from all-cause and CVD was assessed by SUA stratification. Cumulative event rates were estimated with Kaplan-Meier survival curves, and probability values were calculated with the log-rank test. Cox proportional hazard analyses were performed to test the association of SUA and death from all causes or CVD. A Cox regression model was adjusted for potential confounders, including age, sex, duration of hypertension, smoking status, dyslipidemia history, chronic renal insufficiency history, DM history, percutaneous coronary angioplasty (PTCA) history, coronary artery bypass grafting (CABG) history, PAD history, MI history, ischemic stroke history, hypertension, ABI, FRS, eGFR, diuretics, center, and year of screening examination. Potential confounding variables with $P < 0.10$ were adjusted for multivariate analysis.

TABLE 2 | Adjusted hazard ratio for all-cause mortality and cardiovascular disease (CVD) mortality by Cox regression models according to serum uric acid level.

Variable	SUA				*P* for difference
Characteristic SUA level, μmol/L	**Quartile 1**	**Quartile 2**	**Quartile 3**	**Quartile 4**	
	0–242.0	**242.0 < SUA ≤ 312.0**	**312.0 < SUA ≤ 386.75**	**386.75 < SUA**	
All-cause mortality	973	789	672	613	
No. of deaths	238 (24.5%)	151 (19.1%)	154 (22.9%)	220 (35.9%)	
Multivariable adjustment					
Model 1	2.13 (1.45–3.09)	1	1.94 (1.23–2.92)	2.20 (1.73–3.17)	<0.001
Model 2	2.10 (1.21–3.12)	1	1.90 (1.19–2.98)	2.17 (1.70–3.09)	<0.001
Model 3	2.06 (1.35–2.90)	1	1.86 (1.54–2.89)	2.12 (1.63–3.17)	<0.001
Model 4	2.05 (1.35–2.90)	1	1.85 (1.54–2.76)	2.11 (1.59–3.07)	<0.001
CV mortality	973	789	672	613	
No. of deaths	119 (12.2%)	85 (10.8%)	90 (13.4%)	112 (18.3%)	
Multivariable adjustment					
Model 1	2.21 (1.32–3.04)	1	1.82 (1.36–2.94)	2.57 (1.77–3.32)	<0.001
Model 2	2.18 (1.43–2.97)	1	1.81 (1.32–2.93)	2.53 (1.71–3.30)	<0.001
Model 3	1.98 (1.32–2.94)	1	1.71 (1.08–2.85)	2.45 (1.67–3.22)	<0.001
Model 4	1.95 (1.29–2.90)	1	1.70 (1.05–2.81)	2.42 (1.61–3.12)	<0.001

Model 1 was adjusted for age and sex, estimated glomerular filtration rate (eGFR), and diuretic use. Model 2 was adjusted for model 1 covariates and smoking, alcohol drinking, use of diuretics, a history of heart failure, a history of diabetes, a history of renal insufficiency, a history of metabolic syndrome, and a history of stroke. Model 3 was adjusted for model 2 covariates ABI (ankle–brachial index) and FRS (Framingham risk score). Model 4 was adjusted for model 3 covariates central effect, year of screening examination.

Restricted cubic splines (RCSs) with knots were used to further explore the shape of the dose–response relationship between SUA level and the hazard ratio (HR) of all-cause mortality and CVD mortality. Knots were at the 5th, 50th, 90th, and quartile of the SUA distribution. Missing values were handled by K-means clustering imputation. All *P*-values were 2-tailed, and a value < 0.05 was considered significant. The optimized prognostic cutoff value of SUA was analyzed with X-Tile Software (version 3.6.1, https://medicine.yale.edu/lab/rimm/research/software). SUA transformed by RCS was added to the Cox regression mode as an independent variable, and all-cause CVD mortality scores were calculated. A survival receiver operating characteristic (ROC) curve was used to determine the predictive effect of SUA level and its variability on all-cause mortality and CVD mortality. The ROC curve and area under the curve (AUC) were implemented with a regression model predicting the score.

RESULTS

Baseline Characteristics

A total of 3,220 eligible participants with available baseline data were enrolled. Among the participants, 76 individuals had missing follow-up data because of changing the telephone number or family address during follow-up; 97 subjects were not compliant (**Figure 1**). Therefore, the study sample actually comprised 3,047 participants. Based on careful calculation, the missing participants did not significantly affect the results. Our research showed hyperuricemia and hypouricemia prevalences of 18.1 and 16.7%, respectively, and the average SUA for the entire cohort was 322.65 ± 33.12 μmol/L (standard deviation). According to SUA level, values were subdivided into 0–242.0, 242.0–312.0, 312.0–386.75, and more than 386.75 μmol/L subgroups. As shown in **Table 1**, among all variables examined, abnormal SUA was associated with hypertension, a higher proportion of male subjects, older age, a higher level of Cr, diuretic use, and chronic renal insufficiency. Regarding subjects with HAR, there was no significant difference for ischemic stroke, MI history, CABG history, PTCA history, DM, dyslipidemia, and smoking. Of note, significant differences were observed for female participants with FRS risk stratification. However, FRS risk stratification by subgroup was not significant in male participants. In contrast with the reference group, the participants were more likely to be treated with ACEI.

All-Cause and CVD Mortality Rates in SUA Quartile Groups

After multivariable adjustment, Cox regression models revealed that compared with the SUA (242.0–312.0) subgroup, all-cause mortality was 24.5, 19.1, 22.9, and 35.9% in the SUA (0–242.0), SUA (312.0–386.75), and SUA >386.75 μmol/L subgroups, respectively (**Table 2**); CVD mortality rates were 12.2, 10.8, 13.4 and 18.3%, respectively. HRs of all-cause mortality and cardiovascular mortality were 2.05 (95% CI = 1.35–2.90), 1.85 (95% CI = 1.54–2.76), and 2.11 (95% CI = 1.59–3.07) and 1.95 (95% CI = 1.29–2.90), 1.70 (95% CI = 1.05–2.81), and 2.42(95% CI = 1.61–3.12), respectively, for the above subgroups.

Survival Analysis of SUA Quartile Groups

Figures 2A,B illustrate the relationship among the SUA quartile categories of all-cause mortality and CVD mortality, respectively.

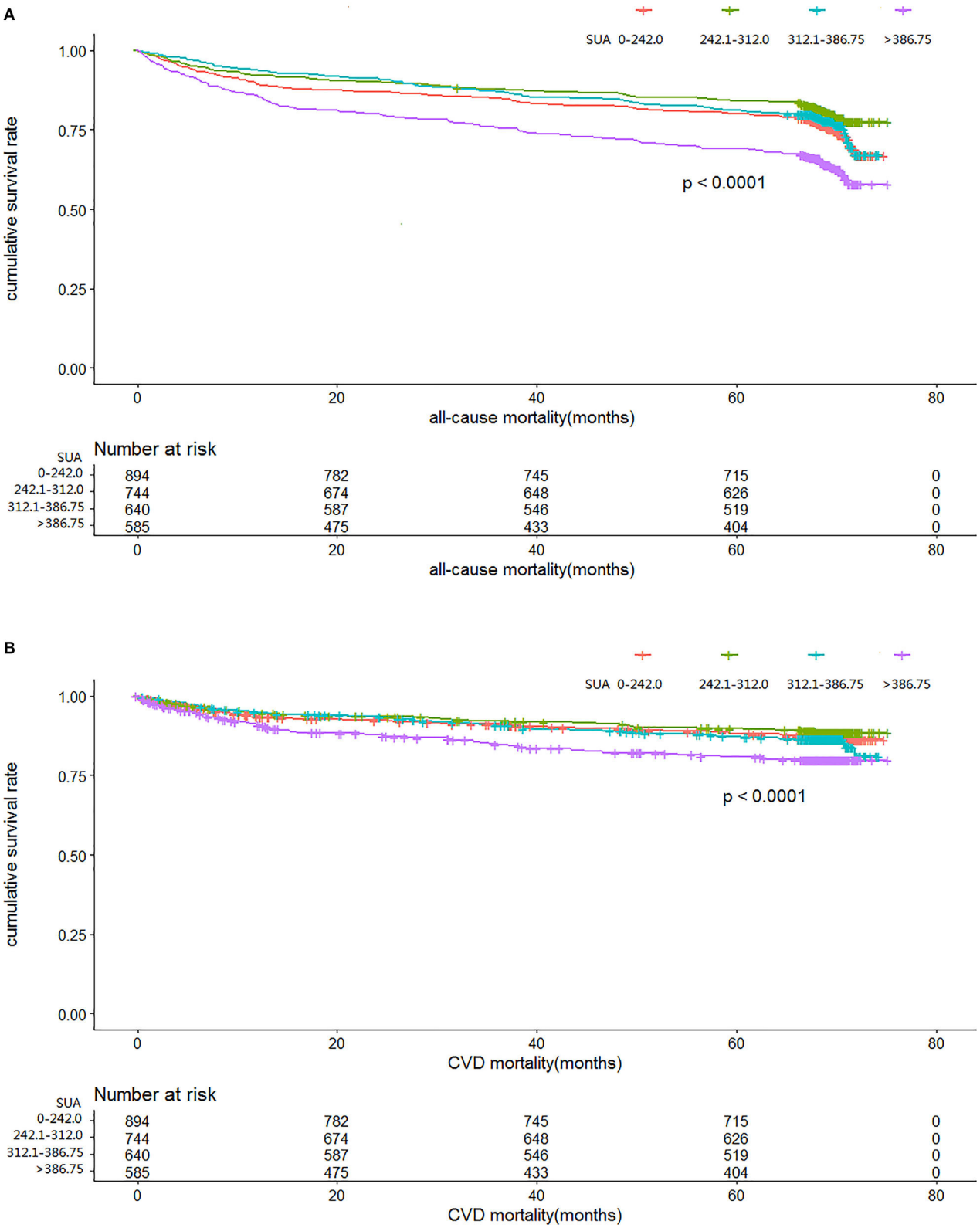

FIGURE 2 | Time course to death from all causes **(A)** and cardiovascular disease (CVD) **(B)** according to serum uric acid level in the cohort study during 6-year follow-up.

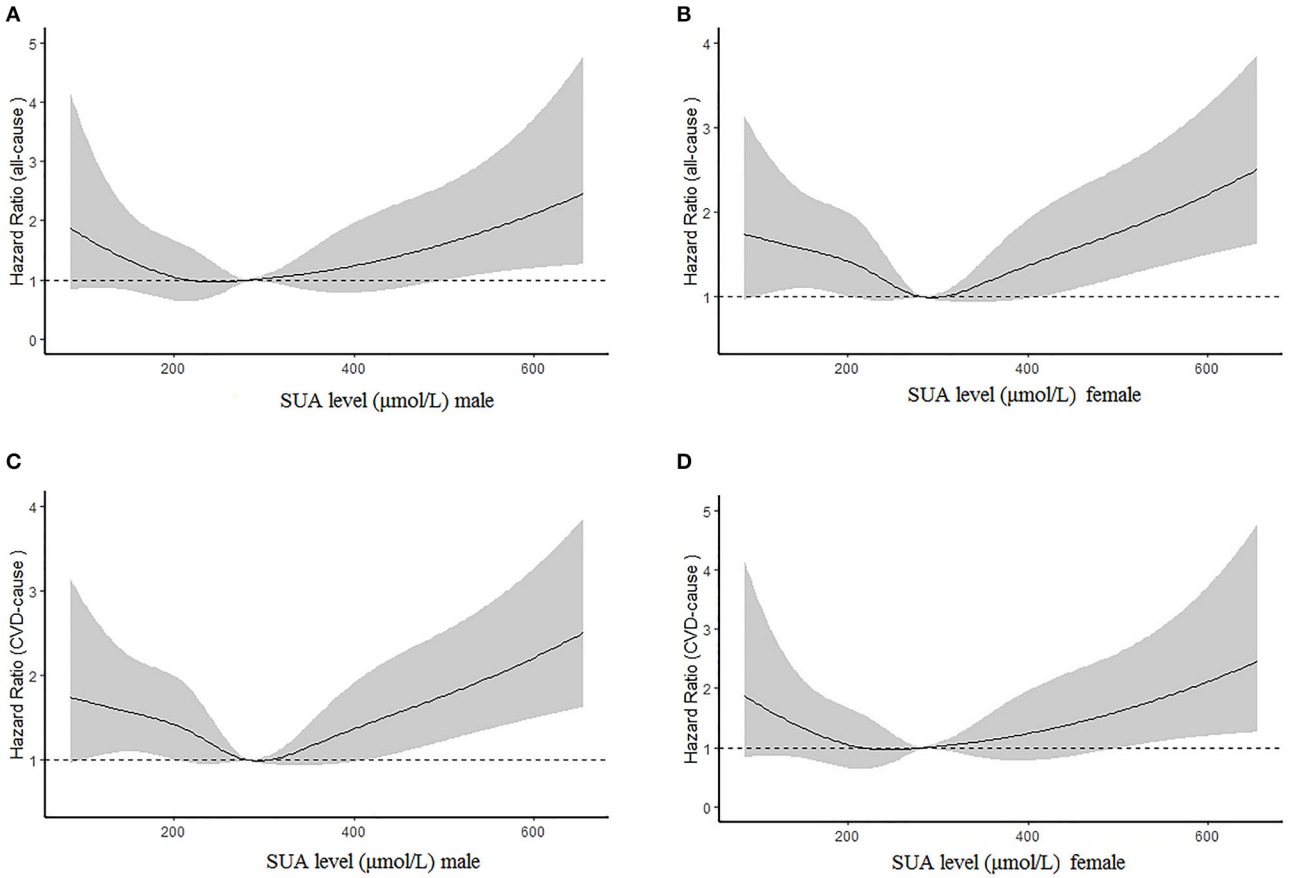

FIGURE 3 | Multivariable adjusted cubic spline models for the association between serum uric acid (SUA) level and hazard ratios (HRs) for all-cause mortality among males **(A)** and females **(B)** and cardiovascular disease (CVD) mortality between males **(C)** and females **(D)**.

Kaplan–Meier curves of survival showed the highest all-cause mortality and cardiovascular mortality for the SUA >386.75 μmol/L subgroup, followed by the SUA 0–242.0 and 312.0–386.75 μmol/L subgroups. The SUA 242.0–312.0 μmol/L subgroup had the least mortality. As depicted in **Figure 3**, after multivariable adjustment, cubic spline models showed a U-shaped association between SUA level with all-cause mortality (A, B) and CVD-cause mortality (C, D) among males and females, respectively.

Survival Analysis of SUA Values According to ROC

The results of age- and sex-adjusted survival ROCs (**Figure 4**) indicated that SUA level can predict all-cause mortality, with an AUC of apparent prediction of 0.706 (95% CI = 0.696–0.727; $P < 0.001$); when adjusted for age and sex, the AUC was 0.702 (95% CI = 0.692–0.725, $P < 0.001$). Meanwhile, SUA levels predicted CVD-cause mortality, and the AUC of apparent prediction was 0.716 (95% CI = 0.701–0.747, $P < 0.001$); when adjusted for age and sex, the AUC was 0.711 (95% CI = 0.697–0.742, $P < 0.001$).

HR of All-Cause and Cardiovascular Mortality According to SUA Cutoff Value

Figure 5 shows the adjusted proportional HR of mortality according to SUA cutoff value. Compared with the SUA 180.5–370.5 μmol/L subgroup, HRs for all-cause mortality and cardiovascular mortality in males in the SUA 0–180.5 and SUA >370.5 μmol/L subgroups were 2.01 (95% CI = 1.35–2.56) and 2.08 (95% CI = 1.42–2.57) and 1.79 (95% CI = 1.45–2.57) and 2.09 (95% CI = 1.47–2.62), respectively, after adjusting for age, hypertension, stroke, MI history, smoking status, chronic renal disease, PAD history, PTCA history, and CABG history. In females, HRs of all-cause and CVD mortality were 1.98 (95% CI = 1.25–2.53) and 2.04 (95% CI = 1.39–2.61), and 1.89 (95% CI = 1.31–2.67) and 2.02 (95% CI = 1.43–2.74), respectively.

Other Mortality Risk

Based on **Figures 6A,B**, Cox regression models also demonstrated that factors for risk of mortality included sex, age, hypertension, stroke, metabolic syndrome, DM, MI history, PAD history, hyperlipidemia history, smoking status, PTCA history, CABG history, and low eGFR. Of note, among these risk factors,

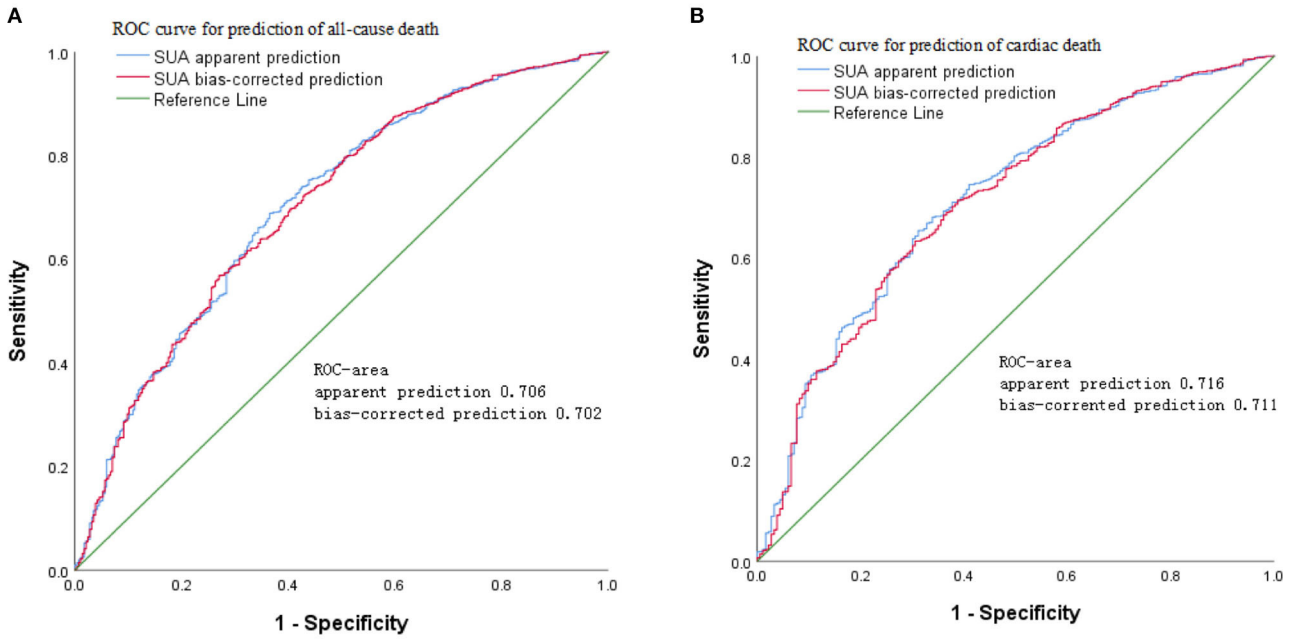

FIGURE 4 | Age- and sex-adjusted receiver operating characteristic (ROC) curves for predicting **(A)** all-cause mortality and **(B)** cardiovascular disease (CVD) mortality according to serum uric acid (SUA) in the cohort study during 6-year follow-up.

age, hypertension, stroke, DM, lower eGFR, abnormal ABI, MI history, and CABG history occupied major positions.

DISCUSSION

Because of the close relationship between SUA level, dietary structure, and obvious diet differences among northern and southern China, we selected in-hospital patients from Beijing and Shanghai to represent these areas. In this cohort study, after further adjusting for potential confounders, the main findings according to SUA levels suggest a U-shaped, independent relationship, instead of a dose–response relationship, between SUA level and all-cause mortality and CVD mortality in both males and females. Furthermore, the findings indicate that long-term average SUA levels >370.5 μmol/L or 6.22 mg/dL (male) and >327.65 μmol/L or 5.50 mg/dL (female) significantly increase the risk of all-cause mortality and CVD mortality. Moreover, long-term low-level SUA <180.5 μmol/L or 3.03 mg/dL (male) <165.7 μmol/L or 2.78 mg/dL (female) was an independent risk factor for all-cause mortality and CVD mortality.

According to normal hyperuricemia and hypouricemia definitions, the prevalences of hyperuricemia and hypouricemia were 18.1 and 16.7%, respectively. Previous studies have indicated that sex affects SUA metabolism and the normal value range. Therefore, sex stratification analysis was carried out. Considering that FRS was a potential confounding factor, the Cox regression model was adjusted for FRS between males and females. The results revealed that the second quartile of SUA level (242.0–312.0 μmol/L) had the least mortality compared with other quartiles. In addition, RCSs and Kaplan–Meier survival estimation indicated high and low SUA to be undesirable.

Because we enrolled HAR patients, baseline characteristics indicated no significant difference in stroke, DM, MI history, PTCA history, CABG history, hyperlipidemia, or smoking status. Nonetheless, we found that the use of diuretics and low eGFR correlated substantially with SUA; after controlling for eGFR and diuretic use, HR estimates suggested only marginal changes, and the data only demonstrated some collinearity among eGFR, diuretics, and SUA. Consequently, the results revealed an independent association between SUA, all-cause mortality, and CVD mortality.

The relationship between SUA and mortality has been explored in previous studies. Similarly, several studies including a longitudinal Taiwanese cohort study (23), US adult cohort study (24), Korean study (25, 26) and Chinese chronic kidney disease study (17) reported U-shaped associations between SUA and all-cause mortality as well as cardiovascular mortality. Nevertheless, the different patient populations recruited might affect the occurrence of ending events to affect different SUA cutoff values and ranges of variation. Our study assessed values providing better prediction by X-Tile Software and ROC curves. After adjusting for confounding factors, this research revealed that the lowest mortality for a range of SUA of 180.5–370.5 μmol/L or 3.03–6.22 mg/dL (male) and 165.7–327.65 μmol/L or 2.78–5.50 mg/dL (female). Although early studies, including the Framingham Heart Study (27), Atherosclerosis Risk In Communities study (28), and Vorarlberg Health Monitoring and Promotion Program (29), reported a relationship between

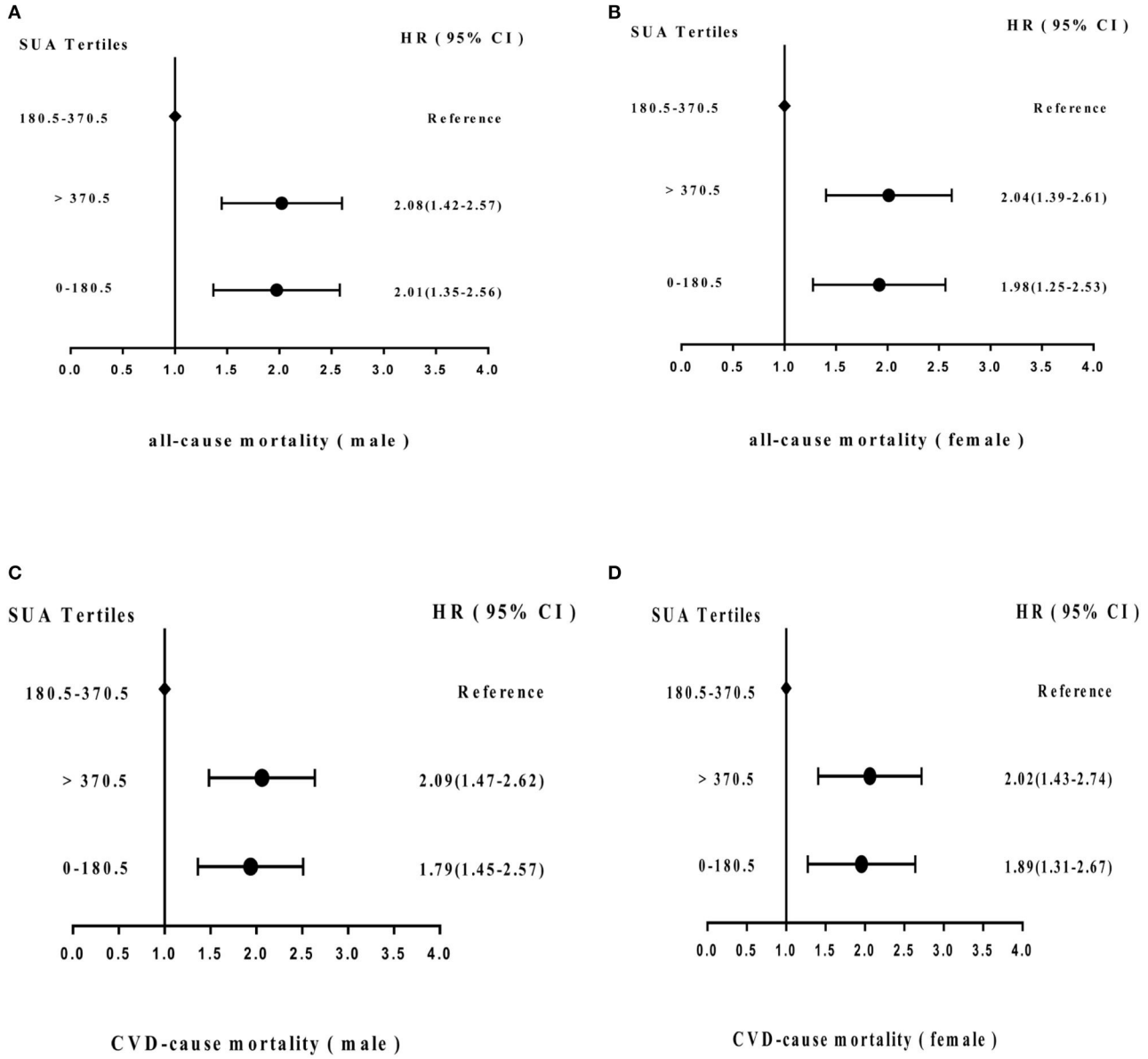

FIGURE 5 | Adjusted hazard ratios (HRs) for **(A)** all-cause mortality (male) and **(B)** (female) and **(C)** cardiovascular disease (CVD) mortality (male) and **(D)** (female) according to cutoff values of SUA level in the cohort study during 6-year follow-up. CI, confidence interval.

SUA and CHD and death, no U-curve distribution was observed because the studies mainly focused on patients with hyperuricemia. Recently, the *Journal of Hypertension*, *High Blood Pressure*, and *Cardiovascular Prevention* all published cutoff points (30, 31). Notably, the URRAH Project investigating hyperuricemia correlations with all-cause mortality and CVD mortality reported the best cutoff values of 4.7 and 5.6 mg/dL, respectively (31). Although our findings were similar, AUCs were smaller than the URRAH Project's results. The reason may be that ROC curves only assess statistical performance for single-trend variables, and the statistical efficiency is insufficient to demonstrate a U-shaped curve. For this reason, our study involved adding transformed SUA variables by RCS to a Cox regression model and calculating predictive all-cause and CVD-cause mortality scores. Then, the optimum predictive effect of the SUA level was determined using ROC analysis. This may explain why the AUC was slightly smaller but the HR higher than in the URRAH Project.

From the perspective of pathophysiology, previous studies have revealed that hyperuricemia may have detrimental effects on the endothelium and functions of platelets (32), induce oxidative stress and activate the local renin–angiotensin system in cultured vascular smooth muscle cells (33), lead to attenuated nitric oxide bioavailability, and promote the proliferation of vascular smooth

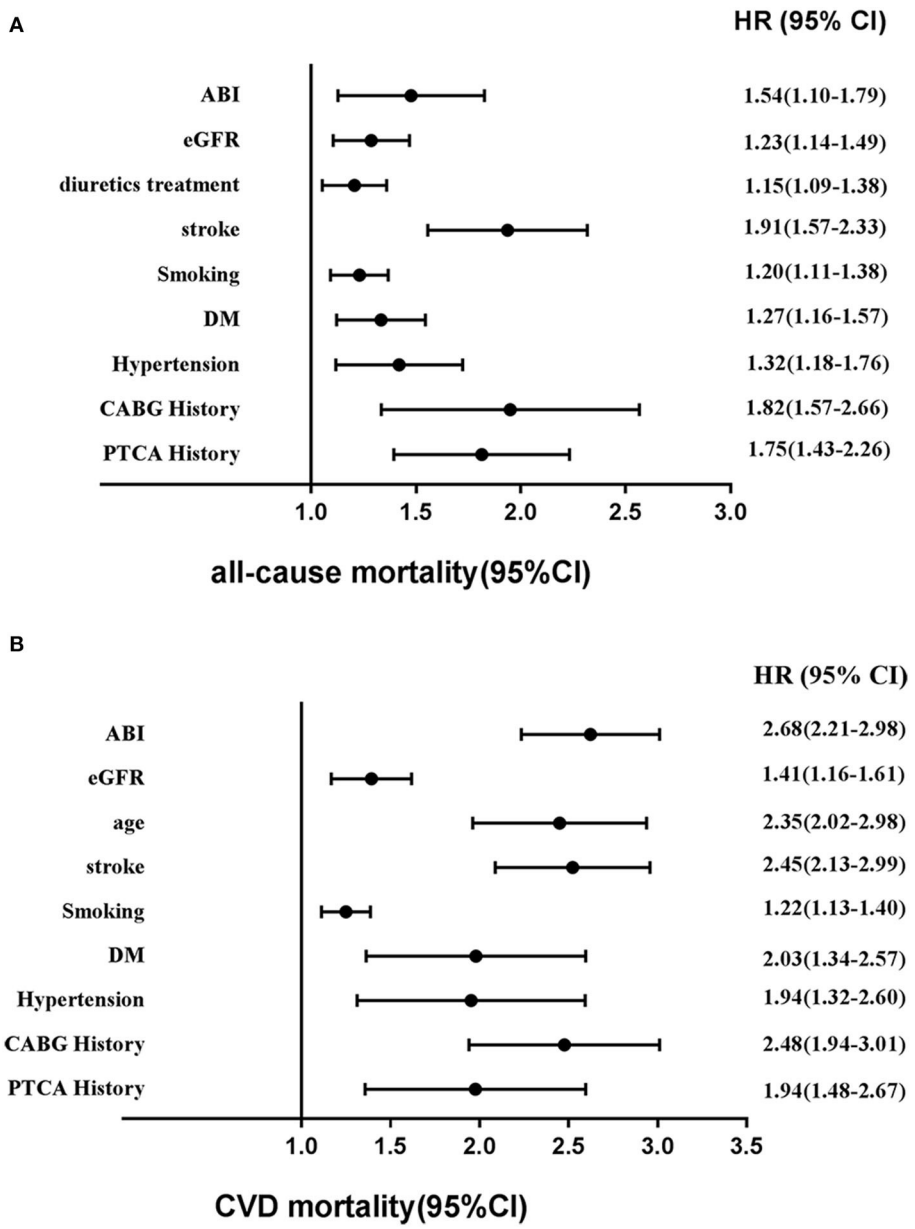

FIGURE 6 | Adjusted other hazards ratios for **(A)** all-cause mortality and **(B)** CVD mortality in the cohort study during 6-year follow-up. CVD, cardiovascular disease; HR, hazards ratio; CI, confidence interval; eGFR, estimated glomerular filtration rate; ABI, ankle–brachial index.

muscle (32, 34, 35). Further studies have reported that reduced SUA levels may contribute to the treatment of hyperuricemia-associated diseases (36, 37).

Notably, hypouricemia was also found to be associated with all-cause mortality and cardiovascular mortality. Recently, several studies, including USA Adults Study, reported similar results (24). At present, there is no recognized standard for the diagnosis of hypouricemia. According to the definition of hypouricemia in most previous studies (16, 17, 38, 39), the reference limit was defined as a level of SUA $<$ 149 μmol/L or 2.5 mg/dL. A possible etiology by which hypouricemia increases all-cause mortality may be caused by malignancy (40, 41), DM, and concomitant medication (42, 43). Nevertheless, these studies were limited by the absence of important covariates, such as smoking status and BMI. Recently, growing evidence suggests that SUA plays an important role in immune regulation and tumor inhibition (43). Therefore, the increase in mortality due to hypouricemia is partly owing to cancer incidence (44). Meanwhile, malignant tumors, as a kind of consumptive disease, have repeatedly appeared later in hypouricemia (45). These details may partly explain why hypouricemia patients tend to have higher

all-cause mortality. SUA also acts as an antioxidant that can interact with hydrogen peroxide and hydroxy radicals to effectively scavenge free radicals in the body, thus protecting vascular endothelial cells (12). This research also indicates that patients with hypouricemia have increased all-cause and CVD mortality.

In addition to hyperuricemia and hypouricemia, age, smoking status, hypertension, ischemic stroke, MI, PAD, BMI, and lower eGFR were independent mortality risk factors in this study.

In conclusion, the findings reveal a U-shaped relationship between SUA level and all-cause mortality and cardiovascular mortality in HAR patients, regardless of sex. The results indicate that hyperuricemia and hypouricemia increased mortality and that SUA may serve as an easily tested index to predict mortality. Therefore, more intensive management regarding SUA is needed in clinical practice, especially in HAR patients. In addition, well-designed clinical trials focusing on SUA and its concomitant changes are needed to prevent all-cause and cardiovascular mortality.

Study Limitations and Strengths

First, subjects with HAR were enrolled to assemble a study population as homogeneous as possible, as HAR patients have a poor prognosis. Thus, the results cannot be extended to the entire population. Second, as follow-up participants were contacted by annual phone interviews, our results may have information bias. In addition, some patients had poor compliance, and withdrawal bias may exist. Finally, the follow-up time was not long compared with Western country prospective cohort studies. Hence, the data from this research are not comprehensive, and additional studies are needed.

The strengths of our study include its prospective design and reliable assessment of mortality and cardiovascular events. Second, this research was a multicenter prospective cohort registration study with little heterogeneity. This cohort study also had a longer follow-up time and larger sample size than other Chinese studies.

CONCLUSION

After adjusting for sex and other covariates, this study revealed that SUA level shows a U-shaped relationship with all-cause mortality and cardiovascular mortality. Abnormal SUA values correlated strongly, independently, and inversely with all-cause and cardiovascular mortality. Long-term average SUA levels >370.5 μmol/L or 6.22 mg/dL (male) and >327.65 μmol/L or 5.50 mg/dL significantly increased the risk of all-cause mortality and CVD mortality in females. Meanwhile, low-level SUA <180.5 μmol/L or 3.03 mg/dL (male) and <165.7 μmol/L or 2.78 mg/dL (female) was an independent risk factor for all-cause mortality and CVD mortality. Age- and sex-adjusted survival ROC curve analyses indicated that SUA level can predict all-cause mortality, with an AUC of 0.702 (95% CI = 0.692–0.725, $P < 0.001$), and CVD mortality, with an AUC of 0.711 (95% CI = 0.697–0.742, $P < 0.001$). After adjusting for confounding factors, compared with the SUA 180.5–370.5 μmol/L subgroup, HRs for all-cause mortality and cardiovascular mortality were 2.01 (95% CI = 1.35–2.56) and 2.08 (95% CI = 1.42–2.57) and 1.79 (95% CI = 1.45–2.57) and 2.09 (95% CI = 1.47–2.62), respectively, in males in SUA (0–180.5) and SUA >370.5 μmol/L subgroups and 1.98 (95% CI = 1.25–2.53) and 2.04 (95% CI = 1.39–2.61), and 1.89 (95% CI = 1.31–2.67) and 2.02 (95% CI = 1.43–2.74), respectively, in females in SUA (0–180.5) and SUA >370.5 μmol/L subgroups. In addition to hyperuricemia and hypouricemia, age, smoking status, hypertension, ischemic stroke, MI, PAD, BMI, and lower eGFR are independent mortality risk factors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Tongji University (NCT03616769). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YC had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. YC: study concept and design, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, and statistical analysis. YC and SX: acquisition of data. YC and YX: study supervision. All authors contributed to the article and approved the submitted version.

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Correlation Between CHA_2DS_2-VASc Score and Left Atrial Size in Patients With Atrial Fibrillation: A More Than 15-Year Prospective Follow-Up Study

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Background: Left atrial (LA) size represents atrial fibrillation (AF) burden and has been shown to be a predictor for AF stroke. The CHA_2DS_2-VASc score is also a well-established predictor of AF stroke. It is unknown to cardiologists whether these two risk scores are correlated, whether both are independent prognostic predictors and complimentary to each other, or whether one of them is a major determinant of stroke risk for AF patients.

Method: A total of 708 patients from the National Taiwan University Atrial Fibrillation Registry were longitudinally followed up for more than 15 years. Left atrial size was measured by M mode of echocardiography. Adverse thromboembolic endpoints during follow-up were defined as ischemic stroke or transient ischemic attack.

Results: The mean age was 72.1 ± 12.9 years, with 53% men. Both LA size and CHA_2DS_2-VASc score were associated with the risk of stroke in univariate analyses. There was a weak but significant positive correlation between LA size and CHA_2DS_2-VASc score ($r = 0.17$, $P < 0.0001$). Patients with higher CHA_2DS_2-VASc scores had a higher mean LA size ($P < 0.01$ for trend). When combining LA size and CHA_2DS_2-VASc score in the multivariable Cox model, only CHA_2DS_2-VASc score remained statistically significant [HR 1.39 (1.20–1.63); $P < 0.001$].

Conclusion: LA size is not an independent predictor of AF stroke, and calculation of CHA_2DS_2-VASc score may be an alternative to measurement of echocardiographic LA size when evaluating the risk of stroke for AF patients.

Keywords: left atrial size, CHA_2DS_2-VASc score, stroke, atrial fibrillation, Asia

INTRODUCTION

The left atrium (LA) of human heart functions to act as a contractile pump that delivers 15 to 30% of the left ventricular (LV) filling, as a reservoir that collects pulmonary venous return during LV systole, and as a conduit for the passage of stored blood from the LA to the LV during early LV diastole (1). LA enlargement has been proven to be associated with increased cardiovascular events, such as stroke, congestive heart failure, cardiovascular death, and atrial fibrillation (AF) (2). Patients with enlarged LA are more prone to AF, and an enlarged LA is more likely to maintain AF. On the other hand, AF has also been known to affect LA remodeling and geometry. Regardless of whether LA enlargement is a cause for or a consequence of AF, LA structure, and function represent AF burden and duration, conferring a risk for stroke and systemic embolization (3).

Several studies have investigated the relationship between the LA size and stroke in general populations, showing increased risk of stroke when LA was enlarged (4–11). Given that LA enlargement represents increased burden and duration of AF, it is proposed as an independent predictor of risk for stroke among AF populations (11). Therefore, the more the AF burden is, the higher the risk of cardiac embolic stroke is. However, performing echocardiography is an expensive modality and is less feasible in large population screening. Instead, the CHA_2DS_2-VASc score has been demonstrated to be another good scoring scheme to predict stroke risk in patients with AF and could be easily calculated and used in routine clinical practice (12). The CHA_2DS_2-VASc score is based on a point system in which two points are assigned for a history of stroke or transient ischemic attack (TIA) and age more than 75 years, and one point is assigned for presence of hypertension, diabetes, congestive heart failure, and vascular diseases or a female gender.

Therefore, both LA size and CHA_2DS_2-VASc score have been proposed as the predictors of AF-related stroke. However, echocardiography may not be immediately available in all clinics. Calculation of CHA_2DS_2-VASc score is an easier tool to use in clinics to predict stroke risk for patients with AF. The aims of this study were to assess whether LA size and the CHA_2DS_2-VASc score were correlated and to determine which one was the independent predictor of AF-related stroke in a long prospective AF follow-up cohort in Taiwan.

METHODS

Patient Population and Follow-Up

The National Taiwan University Atrial Fibrillation Registry (NTUAFR) was first established in Jan 1998, and so far the patients had been followed up for more than 15 years. The NTUAFR includes data on inpatients and outpatients with incident AF. The enrollment was staggered, and details of selection of AF patients have been described previously and according with current guidelines (13–16). The presence of AF was determined by taking the patient's history, serial ECG, and/or ambulatory ECG monitoring. A total of 1,233 patients were recruited. Patients with palpitations without ECG documentation were excluded from the registry (16). Patients with hyperthyroidism were excluded because AF in these patients might be curable. Patients who refused to join the study or were lost to follow-up were also excluded. Finally, the study population consisted of 708 consecutive adult AF patients from the NTUAFR with detailed echocardiographic data. The mean age was 72.1 $\pm$ 12.9 years, and 53% (375/708) were men. The study protocols were reviewed and approved by the institutional review committee, and all patients agreed to participate in the study.

Clinical and Outcome Assessments

At enrollment, the medical history was recorded, and transthoracic echocardiographic data of left atrial and left ventricular dimensions, left ventricular ejection fraction, and severity of valvular heart disease were also recorded. The LA size was determined as the M-mode measurement of the anteroposterior diameter of LA in parasternal long-axis view. We defined the cutoff value of LA enlargement as LA diameter more than 50 mm, as LA diameter more than 50 mm had been previously reported to be associated with a higher risk of stroke and systemic embolization in patients with AF (11). Besides, in our study the LA size was not controlled for body size because it did not significantly affect the results and it has been reported that LA size not controlled for body size was associated with an increased risk of stroke in patients with AF (17).

All the baseline characteristics were collected as previously described (13–15). The medical diagnoses of hypertension (blood pressure consistently above 140/90 mmHg or treated with hypertension medication), heart failure (admission for congestive heart failure), diabetes mellitus (DM) (fasting glucose $>$125 mg/dL or treatment with oral hypoglycemic agent and/or insulin), and vascular disease (peripheral arterial disease, coronary artery disease, and myocardial infarction) are also searched in the medical records (12).

The CHA_2DS_2-VASc scores were calculated and recorded as measures of stroke risk. The CHA_2DS_2-VASc score is based on a point system in which two points are assigned for a history of stroke or TIA and age more than 75 years, and one point is assigned for presence of hypertension, diabetes, congestive heart failure, and vascular diseases or a female gender (12). We categorized the patients into 3 groups (CHA_2DS_2-VASc score $\leqq$2, 3–5 and $\geq$6), because CHA_2DS_2-VASc score $\leqq$2 is associated with a lower risk of stroke but CHA_2DS_2-VASc score $\geq$6 is associated with a significantly higher risk of stroke (16, 18, 19).

Adverse thromboembolic endpoints during follow-up were defined as incident episodes of ischemic stroke or TIA. Ischemic stroke was defined as sudden-onset and focal or global neurological deficits that were not explained by other origins, with supporting evidence from imaging studies. Hemorrhagic stroke was excluded from the study because it was generally a complication of use of anticoagulant and not related to AF *per se*.

Abbreviations: AF, atrial fibrillation; AUC, area under curve; DM, diabetes mellitus; ECG, electrocardiography; LA, left atrium; LV, left ventricle; NTUAFR, National Taiwan University Atrial Fibrillation Registry; ROC, receiver operator characteristics; TIA, transient ischemic attack.

Statistical Methods

Continuous variables are presented as means ± standard deviation and categorical variables as percentages. The comparison of data was performed by using the chi-squared test (categorical variables) or the Student's *t*-test (continuous variables). The time to first episode of stroke event were depicted with the Kaplan–Meier estimate of the survival function. Difference between the survival curves was tested by the log-rank statistics. The independent effect of variables to predict thromboembolic events was calculated using a Cox proportional hazards regression model. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated accordingly. To test the accuracy of different risk factors in predicting the occurrence of thromboembolic events, the receiver operator characteristic curves (ROC) were constructed.

The ROC analyses gave an estimate of the overall discriminate ability of risk predictor by the area under the curve (AUC) statistic or *c* statistic. We calculated sensitivity and specificity across the range of possible cutoff values of risk predictor scores. AUCs and associated 95% CIs were calculated. The correlation between CHA_2DS_2-VASc score and LA size was determined by calculating the Pearson's correlation coefficient (*r*). The Cochran–Armitage trend test was used for the trend of increase of LA size with different groups with increasing mean CHA_2DS_2-VASc score. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using STATA version 7.0 for Windows (STATA, Inc., Texas, USA).

RESULTS

Baseline Characteristics

Baseline characteristics of study patients are summarized in **Table 1**. The mean age was 72.1 ± 12.9 years, and 53% (375/708) were men. Among the study subjects, 44.9% (318/708) aged over 75 years, 59.9% (424/708) had history of hypertension, 25.8% (183/708) had history of DM, 10.6% (75/708) had history of congestive heart failure, and 32.6% (231/708) had vascular diseases. The mean $CHADS_2$-VASc score was 3.02 ± 1.53.

Regarding the echocardiographic data, 21.3% of the study subjects had LA dimension more than 50 mm. The mean left ventricular ejection fraction was 64.1 ± 12.7 %. The mean LV end-systolic dimension (LVESD) was 58.9 ± 16.5 mm. The mean LV end-diastolic dimension (LVEDD) was 33.7 ± 11.9 mm.

TABLE 1 | Baseline characteristics of the patients at enrollment ($n = 708$).

Age, y	72.1 ± 12.9
Age ≧ 75 yrs	44.9% (318/708)
Male	53.0% (375/708)
Congestive heart failure	10.6% (75/708)
Diabetes mellitus	25.8% (183/708)
Hypertension	59.9% (424/708)
Current smoking	27.0% (191/708)
Vascular diseases (CAD or AMI or PAOD)	32.6% (231/708)
$CHADS_2$-VASc score	3.02 ± 1.53
Echocardiographic parameters	
Left atrial dimension, mm	43.8 ± 9.4
LVEDD, mm	58.9 ± 16.5
LVESD, mm	33.7 ± 11.9
LVEF, %	64.1 ± 12.3
LV mass, g	245 ± 84

AMI, acute myocardial infarction; CAD, coronary artery disease; LVEDD, left ventricular end diastolic dimension; LVESD, left ventricular end systolic dimension; LVEF, left ventricular ejection fraction; PAOD, peripheral arterial occlusive disease.

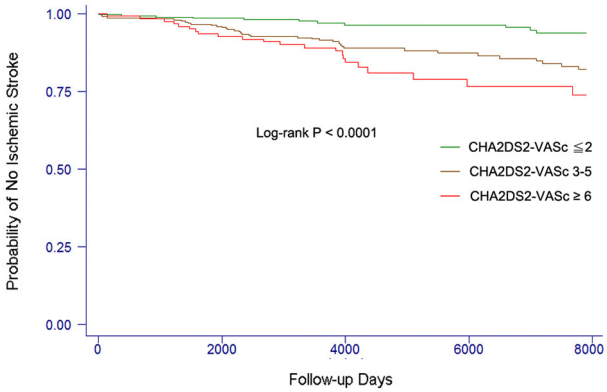

FIGURE 1 | Kaplan–Meier curves showing the development of ischemic stroke among patients with different levels of CHA_2DS_2-VASc score. The log-rank analysis showed significant difference ($P < 0.001$).

CHA_2DS_2-VASc Score as a Predictor of Incident Stroke Events

At the end of the follow-up, 70 AF patients developed ischemic stroke or TIA. We first investigate whether CHA_2DS_2-VASc score was associated with risk of stroke in our cohort (8, 9). As expected, CHA_2DS_2-VASc score was incrementally associated with the risk of stroke. Using the univariate Cox model, we found that there was a 42% increase of risk of stroke with a one-point increase of CHA_2DS_2-VASc score in this very long follow-up cohort (HR 1.42, 95% CI 1.22–1.66, $P < 0.001$).

The event-free survival from stroke comparing patients with different levels of CHA_2DS_2-VASc score is shown in **Figure 1**. The cut-point CHA_2DS_2-VASc score for each category was defined to make the case number similar in each group. Patients with a higher CHA_2DS_2-VASc score were more likely to develop ischemic stroke or TIA than those with lower CHA_2DS_2-VASC score (log-rank $P < 0.0001$).

Increase of Incident Stroke Event Risk When LA Size Increases

In the present study, we also investigated whether the risk of ischemic stroke or TIA was increased when the LA size increased (3, 8, 9, 11). We found that LA size was incrementally associated with the risk of stroke. Using the univariate Cox model, there was a 30% increase of risk of stroke with a 1-mm increase of LA size (HR 1.30, 95% CI 1.04–1.62, $P = 0.019$). The event-free survival from stroke comparing patients with different LA size is shown in **Figure 2**. Patients with LA size >50 mm were more likely to

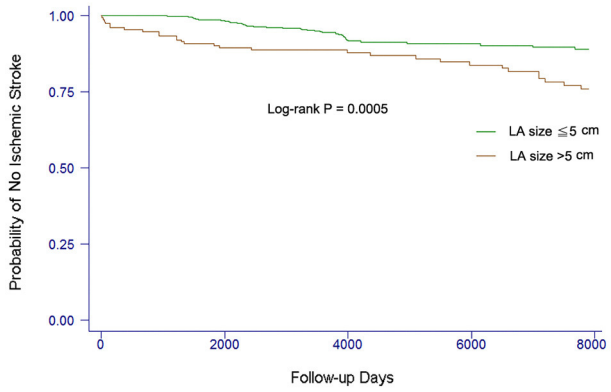

FIGURE 2 | Kaplan–Meier curves showing the development of ischemic stroke among patients with different LA sizes. The log-rank analysis showed significant difference ($P < 0.001$).

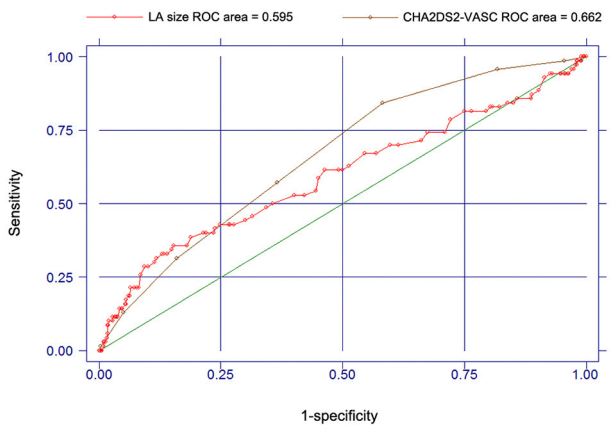

FIGURE 3 | Receiver operating characteristic curves and c-statistics for CHA_2DS_2-VASC score and LA size in predicting the occurrence of stroke or TIA.

develop ischemic stroke or TIA than those with LA size ≦50 mm (log-rank $P = 0.0005$).

Since both CHA_2DS_2-VASC score and LA size were associated with the risk of stroke for patients with AF, we also use ROC and *c* statistic (AUC statistic) to test the accuracy of CHA_2DS_2-VASC score and LA size in predicting the occurrence of stroke or TIA (**Figure 3**). For the CHA_2DS_2-VASC score, the AUC or *c* statistic for stroke events was 0.662 (95% CI, 0.601–0.723). For the LA size, the AUC or *c* statistic for stroke events was 0.595 (95% CI, 0.516–0.674). The *c* statistic of CHA_2DS_2-VASC score was larger than that of LA size, but not statistically significant ($P = 0.187$; **Figure 3**).

Correlation Between LA Size and $CHADS_2$ VASC Score

In the present study we showed both CHA_2DS_2-VASc score and LA size could predict the risk of stroke for patients with AF (20, 21). We further hypothesized that the CHA_2DS_2-VASc score predicted the risk of stroke through its association with LA size. To address this issue, we first investigated whether CHA_2DS_2-VASc score correlated with LA size, which had never been addressed before our study.

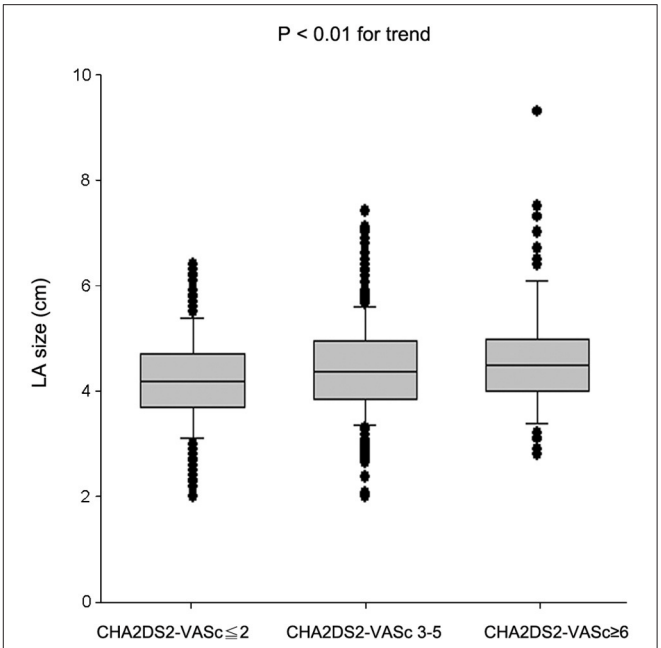

FIGURE 4 | Mean left atrial size for different levels of CHA_2DS_2-VASc score.

Interestingly, we found a positive correlation between LA size and CHA_2DS_2-VASc score ($r = 0.17$, $P < 0.0001$). There is an increase of mean LA size when the mean CHA_2DS_2-VASc score increases ($P < 0.01$ for trend; **Figure 4**). It indicates that AF patients with a higher CHA_2DS_2-VASc score may have a larger LA size.

The Role of CHA_2DS_2-VASc Score and LA Size in Determining the Stroke Risk for Patients With AF

We have shown a positive correlation between the CHA_2DS_2-VASc score and LA size, and both CHA_2DS_2-VASc score and LA size are predictors of stroke risk for patients with AF. It is logical to speculate that the CHA_2DS_2-VASc score predicts the risk of stroke through its association with LA size since LA size represents AF burden. To determine if CHA_2DS_2-VASc score predicted the risk of stroke through its association with LA size or if CHA_2DS_2-VASc score and LA size are independent predictors, we ran a multivariable Cox model.

Unexpectedly, when both CHA_2DS_2-VASc score and LA size were incorporated simultaneously into the Cox model, only CHA_2DS_2-VASc score remained statistically significant (HR 1.39; 95% CI 1.20–1.63; $P < 0.001$; **Table 2**).

DISCUSSION

The present study showed that LA enlargement was significantly associated with an increased risk of stroke in a very long-term

TABLE 2 | Univariate and multivariable analyses in Cox models.

Mode of analysis	Hazard ration (95% confidence interval)	*P*-value
Univariate analysis*		
$CHADS_2$-VASc score	1.42 (1.22–1.66)	<0.001
Left atrial size	1.30 (1.04–1.62)	0.019
Multivariable analysis*		
$CHADS_2$-VASc score	1.39 (1.20–1.63)	<0.001
Left atrial size	1.20 (0.96–1.48)	0.106

**In univariate analyses, single covariate, such as $CHADS_2$-VASc score or left atrial size was put into the Cox model. In the multivariate analysis, both $CHADS_2$-VASc score and left atrial size were put into the Cox model.*

AF follow-up cohort, but the association disappeared if the CHA_2DS_2-VASc score was also included in the multivariable model. The LA size was positively correlated with the CHA_2DS_2-VASc score. These results indicate that the association of LA size with incident stroke in patients with AF is through its correlation with the CHA_2DS_2-VASc score and in evaluating the risk of stroke for patients with AF, calculating the CHA_2DS_2-VASc score is enough without the need for LA size measurement.

LA Enlargement, AF Burden, and Risk of Stroke

A recent large-scale, prospective study with 2-year follow-up has shown that LA enlargement is independently associated with an increased risk of stroke and systemic embolization (11). However, another substudy of Atrial Fibrillation Follow-Up Investigation of Rhythm Management (AFFIRM) Study with a 3.5-year follow-up has shown that larger LA size is not associated with increased risk of stroke (22). Finally, sub-analyses of three clinical trials with a 1.6-year follow-up have also shown that left ventricular dysfunction is an independent predictor of stroke while LA size is not (23). In these studies, the follow-up periods were short, and the relationship between CHA_2DS_2-VASc score and LA size was not addressed. In the present study, with the longest follow-up of AF patients, we showed that LA size was positively and linearly correlated with CHA_2DS_2-VASc score but did not provide additional information in the risk stratification of stroke.

Clinical Implications of Positive Correlation Between LA Size and CHA_2DS_2-VASc Score

LA enlargement could promote blood stasis, which in turn predisposes thrombus formation, especially in the left atrial appendage (24). Many studies have shown that LA dimension could reflect the AF burden, which has been reported to be associated with the risk of stroke in several device-detected AF trials (25–27). Therefore, since AF burden is difficult to measure in patients without cardiac implanted electronic devices, it has been proposed that LA dimension could be used as a good surrogate marker of AF burden and duration. However, while measurement of LA dimension may add additional information in AF patient care, echocardiography is a less feasible modality and not recommended for broad screening of unselected populations in the current guidelines and trials (28, 29). Our study first showed the positive correlation between LA size and the CHA_2DS_2-VASc score. This result further increases the simplicity of quantification of AF burden and duration for screening a large number of AF patients. Application of the CHA_2DS_2-VASc score seems useful in assessing the AF burden and duration in large AF population screen without the need to perform echocardiography or implantation of cardiac electronic device.

Initially, we expected that echocardiographic LA size was a good surrogate measurement of AF burden and LA size was the ultimate independent predictor of stroke risk for patients with AF. However, echocardiography may not be immediately available in all clinics. Calculation of CHA_2DS_2-VASc score is an easier tool to use in clinics to predict stroke risk for patients with AF, and we hypothesized that CHA_2DS_2-VASc score was effective to predict stroke risk through its correlation with LA size. However, unexpectedly, we found that CHA_2DS_2-VASc score, but not LA size, was the only independent predictor of AF stroke risk.

CHA_2DS_2-VASc Score Is More Important Than LA Size in AF Stroke Risk Assessment

LA size is a surrogate measurement of AF burden, but whether AF burden is directly related to AF stroke risk remains debated (30–32). A significant proportion of cause of stroke in AF patients may be atheroemboli or atherosclerotic occlusion from carotid arteries, aorta, or other sources. This atheroembolic burden is related to atherosclerosis burden. Interestingly, all of the components of CHA_2DS_2-VASc score are risk factors of systemic atherosclerosis. Our results imply the possibility that a large proportion of cause of stroke in AF patients is atheroemboli or atherosclerotic occlusion from the arterial circulating system. To sum up, application of the CHA_2DS_2-VASc score still remain the mainstream to assess the stroke risk in large AF populations.

In addition to the possibility that the cause of stroke in AF patients is atheroemboli or atherosclerotic occlusion from the arterial circulating system, another possibility is that the cause of stroke is the thrombus in the left atrial appendage and the mechanism of thrombus formation in the left atrial appendage is a pathological process in the appendage endocardium, which is similar to vascular endothelial dysfunction or an atherosclerosis process, but not enlarged LA or left atrial appendage. It is logical to speculate that risk factors of endothelial dysfunction or atherosclerosis, such as the components of CHA_2DS_2-VASc score, are also risk factors of appendage endocardial pathology.

Our study first shows a positive correlation between CHA_2DS_2-VASc score and LA size. Regarding the plausible explanation of this correlation, all of the components of CHA_2DS_2-VASc score are predisposing factors of AF attack and LA remodeling. Patients with a higher CHA_2DS_2-VASc score may have more predisposing factors of AF attack and suffer from more AF episodes, which may increase their LA size.

Limitations

The major limitation of the present study is no measurement of the LA volume. Because this is a large cohort with standard cares for AF patients, LA volume is not a routine measurement in our cohort. LA diameter measurement is more widely employed in daily practice. Furthermore, it has been shown that LA volume can be well-estimated from LA diameter using a non-linear equation with an elliptical model (33). Finally, a recent large-scale AF follow-up study also used echocardiographic M-mode measurement of the anteroposterior diameter of LA in parasternal long-axis view but not LA volume as one of their study parameters (11).

CONCLUSION

In conclusion, the present study shows a positive correlation between echocardiographic LA size and CHA_2DS_2-VASc score. LA size is not an independent predictor of AF-related stroke but provides a diagnostic value to predict AF-related stroke risk through its association with CHA_2DS_2-VASc score. Calculation of the CHA_2DS_2-VASc score may be an alternative to measure echocardiographic LA size when evaluating the risk of stroke for patients with AF.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s. The corresponding author has full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Taiwan University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR'S NOTE

The content of this manuscript has been presented at the ESC Congress 2019.

AUTHOR CONTRIBUTIONS

C-FT and P-SH: study design, analyses, conducting, and manuscript preparation. J-JC, S-NC, and L-PL: study design. F-CC: analyses. T-TL: study design and analyse. J-JH: study design and planning. C-TT: study design, analyses, and manuscript preparation. All authors contributed to the article and approved the submitted version.

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Development and Validation of a Nomogram of In-hospital Major Adverse Cardiovascular and Cerebrovascular Events in Patients With Acute Coronary Syndrome

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Background and Objective: This study aims to develop and validate a nomogram for the occurrence of in-hospital major adverse cardiovascular and cerebrovascular events (MACCE) in acute coronary syndrome (ACS) patients.

Methods: A total of 1,360 ACS patients admitted between November 2014 and October 2019 from Zhongda Hospital and Yancheng Third People's Hospital were included. Patients admitted in Zhongda Hospital before 2018 were split into the training cohort ($n = 793$). Those admitted after 2018 in Zhongda Hospital and patients from Yancheng Third People's Hospital were split into the validation cohort ($n = 567$). Twenty eight clinical features routinely assessed including baseline characteristics, past medical history and auxiliary examinations were used to inform the models to predict in-hospital MACCE (all-cause mortality, reinfarction, stroke, and heart failure) in ACS patients. The best-performing model was tested in the validation cohort. The accuracy and clinical applicability were tested by the area under the receiver operating characteristic curve (AUC), calibration plots, and decision curve analyses (DCA).

Results: The in-hospital MACCE occurred in 93 (6.83%) patients. The final prediction model consists of four variables: age, Killip grading, fasting blood-glucose (FBG) and whether percutaneous coronary intervention (PCI) was performed at early stage. A nomogram was used to present the final result. Individualized nomogram exhibited comparable discrimination to the Global Registry of Acute Coronary Events (GRACE) score [AUC: 0.807 (95% CI 0.736–0.878) vs. 0.761 (95% CI 0.69–0.878)], $P = 0.10$) and a better discrimination than the Evaluation of the Methods and Management of Acute Coronary Events (EMMACE) score [AUC: 0.807 (95% CI 0.736–0.878) vs. 0.723(95% CI 0.648–0.798), $P = 0.01$] in predicting the risk of in-hospital MACCE in ACS patients. A good prediction performance was maintained in the validation cohort (AUC =0.813, 95% CI 0.738–0.889). The prediction model also exhibited decent calibration ($P = 0.972$) and clinical usefulness.

Conclusion: The nomogram may be a simple and effective tool in predicting the occurrence of in-hospital MACCE in ACS patients. Further longitudinal studies are warranted to validate its value in guiding clinical decision-making and optimizing the treatment of high-risk patients.

Keywords: ACS, nomogram, prediction model, high-risk patients, MACCE

INTRODUCTION

Coronary heart disease (CHD) is the most common cause for high mortality in the world (1), accounting for more than 40% deaths in China (2). Acute coronary syndrome (ACS) is the most severe type of CHD. The major adverse cardiovascular and cerebrovascular events (MACCE) after ACS include all-cause mortality, non-fatal myocardial infarction, non-fatal stroke, heart failure, and unplanned revascularization. Risk stratification of patients is potentially important in guiding clinical decision making and optimizing care and treatment (3–5).

There are many parameters that predict adverse outcomes after ACS, including serum biomarkers and risk scores. However, some of the serum biomarkers are not routinely tested in hospitals due to their high cost and difficulty in obtaining. In addition, sensitivity and prediction performance of these parameters are unstable in different studies (6–9). Numerous risk scores have been developed for risk stratification in patients with ACS. Commonly used risk scores include GRACE score (10), TIMI score (11) and PURSUIT score (12), etc. However, most risk scores were developed when only limited percentage of patients underwent percutaneous intervention (PCI) (13–16). With the establishment of larger number of chest pain centers across the country and current advanced medical treatments, the application of PCI has been more prevalent (17), so the risk score should be updated accordingly. Secondly, ACS has an urgent onset and the variables contained in risk scores should be available at early medical contact to all ACS patients admitted to hospital. Thirdly, many risk scores are not commonly used in clinical practice, probably because of the heavy clinical workload and the cumbersome process of using risk scores. The more streamlined the model, the better, in order to make it more user-friendly. Therefore, a contracted and updated risk score that is fitting of current clinical practice is necessitated to supplement the use of previous scoring system.

This study aimed to develop and validate a nomogram that can predict the occurrence of in-hospital MACCE in patients with ACS by using the most recent data from the Improving Care for Cardiovascular Disease in China-Acute Coronary Syndrome (CCC-ACS) project. The model incorporated the demographic, auxiliary examination, serum biomarker, past medical history, pharmacological treatment and angiographic parameters of patients. This article is written according to the TRIPOD reporting checklist (18).

METHODS

Data Source and Participants

The baseline characteristics were registered in CCC-ACS project, a collaborative study of the American Heart Association and the Chinese Society of Cardiology launched in 2014, aimed to improve the quality of care of patients with ACS in China. Institutional review board approval was granted for this research with a waiver for informed consent by the local ethics committee. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki.

The population included was patients admitted for ACS and registered in the CCC-ACS project. The data in Zhongda Hospital from November 2014 to October 2019 and in Yancheng Third People's Hospital from May 2015 to December 2016, which were two tertiary hospitals in China, were finally included in this study. According to the time of enrollment, patients admitted in Zhongda Hospital from November 2014 to December 2017 were serve as the training cohort ($n = 793$). Those admitted after 2018 in Zhongda Hospital and patients from Yancheng Third People's Hospital were split into the validation cohort ($n = 567$). ACS was defined as: (1) ST-segment elevation myocardial infarction (STEMI): patients with acute chest pain and persistent (>20 min) ST-segment elevation; (2) non-ST-segment elevation ACS (NSTE-ACS): patients with acute chest discomfort but no persistent ST-segment elevation exhibit ECG changes that may include transient ST-segment elevation, persistent or transient ST-segment depression, T-wave inversion, flat T waves, or pseudo-normalization of T waves; or the ECG may be normal (19, 20). Patients younger than 18 years old or with missing important variables were excluded from the analysis.

Observation Indexes and Outcomes

Baseline clinic data, including age, gender, body mass index (BMI), clinical conditions at admission, medical history, diagnose, auxiliary examination, pre-hospital, and in-hospital treatment was derived from CCC-ACS project. From our initial list, missing data were filled by querying the hospital's electronic medical records and variables in the dataset with >30% missing values were excluded. Under the assumption that the rest of the missing data were missing-at-random, we use the multiple interpolation method for missing values. Meanwhile, we calculated the admission GRACE score. A total of 28 parameters obtained on admission were shown in **Table 1**. All indicators were available within 24 h of admission. The primary outcome of the study was the occurrence of MACCE, which was

TABLE 1 | Baseline characteristics of ACS patients with or without in-hospital MACCE.

Characteristic	Training cohort			Validation cohort		
	MACCE = 0 (*n* = 737)	MACCE = 1 (*n* = 56)	*p*	MACCE = 0 (*n* = 530)	MACCE = 1 (*n* = 37)	*p*
Male, *n* (%)	551 (74.8)	30 (53.6)	<0.01	373 (70.4)	21 (56.8)	0.08
Age, years	65 ± 13	75 ± 11.2	<0.01	64.2 ± 12.9	75.1 ± 8.5	<0.01
BMI, kg/m^2	24.8 ± 3.1	23.7 ± 2.5	0.02	25.5 ± 3.3	24.6 ± 2.9	0.13
Clinical conditions at admission						
HR, beats/min	80 ± 16	88 ± 20	<0.01	80 ± 16	86 ± 22	0.02
SBP, mmHg	132 ± 22	130 ± 28	0.63	136 ± 25	124 ± 26	<0.01
Killip, *n* (%)			<0.01			<0.01
I	537 (72.9)	21 (37.5)		449 (84.7)	15 (40.5)	
II	143 (19.4)	13 (23.2)		64 (12.1)	12 (32.4)	
III	34 (4.6)	7 (12.5)		9 (1.7)	4 (10.8)	
IV	23 (3.1)	15 (26.8)		8 (1.5)	6 (16.2)	
Cardiogenic shock, *n* (%)	24 (3.3)	12 (21.4)	<0.01	6 (1.1)	3 (8.1)	<0.01
AHF, *n* (%)	62 (8.4)	25 (44.6)	<0.01	20 (3.8)	16 (43.2)	<0.01
Medical history, *n* (%)						
Smoke	309 (41.9)	14 (25.0)	0.01	236 (44.5)	14 (37.8)	0.43
Hypertension	478 (64.9)	43 (76.8)	0.07	317 (59.8)	21 (56.8)	0.71
COPD	12 (1.6)	4 (7.1)	<0.01	6 (1.1)	2 (5.4)	0.03
DM	226 (30.7)	24 (42.9)	0.06	122 (23.0)	11 (29.7)	0.35
History of MI	55 (7.5)	5 (8.9)	0.69	30 (5.7)	1 (2.7)	0.44
History of PCI	108 (14.7)	8 (14.3)	0.94	38 (7.2)	1 (2.7)	0.3
Renal failure	18 (2.4)	3 (5.4)	0.19	9 (1.7)	2 (5.4)	0.11
Diagnosis, *n* (%)			<0.01			<0.01
NSTEMI	277 (37.6)	33 (58.9)		144 (27.2)	9 (24.3)	
STEMI	427 (57.9)	18 (32.1)		294 (55.5)	28 (75.7)	
UAP	33 (4.5)	5 (8.9)		92 (17.4)	0 (0.0)	
Auxiliary examination						
ST-segment deviation, *n* (%)	570 (77.3)	46 (82.1)	0.41	400 (75.5)	36 (97.3)	<0.01
LVEF, %	57 ± 12	54 ± 10	0.1	57 ± 9	50 ± 10	<0.01
TnI, ng/ml	6.5 ± 15.1	6.4 ± 16.3	0.97	7.0 ± 25.6	15.0 ± 21.8	0.07
Hemoglobin, g/dl	13.6 ± 2.1	12.3 ± 2.5	<0.01	13.8 ± 1.9	12.4 ±2.4	0.27
Scr, umol/L	97 ± 84	156 ± 145	<0.01	87 ± 79	115 ± 69	0.03
FBG, mmol/L	7.0 ± 2.8	10.7 ± 6.7	<0.01	6.9 ± 2.8	7.7 ± 4.1	0.1
HDL, mmol/L	1.1 ± 0.3	1.0 ± 0.3	0.12	1.1 ± 0.3	1.2 ± 0.4	0.18
LDL, mmol/L	2.9 ± 0.9	2.7 ± 0.9	0.32	2.8 ± 1.0	2.4 ± 0.9	0.02
In-hospital treatment, *n* (%)						
PCI	574 (77.9)	25 (44.6)	<0.01	351 (66.2)	20 (54.1)	0.13
DAPT	710 (96.3)	49 (87.5)	<0.01	431 (81.3)	31 (83.8)	0.71
Pre-hospital treatment, *n* (%)						
β-blocker	60 (8.1)	3 (5.4)	0.46	31 (5.8)	4 (10.8)	0.23
ACEIs or ARBs	103 (14.0)	9 (16.1)	0.66	71 (13.4)	3 (8.1)	0.36
GRACE score	153 ± 34	192 ± 43	<0.01	139 ± 33	192 ± 38	<0.01

Data are presented as n (%) or mean ± SD.

MACCE, major adverse cardiovascular and cerebrovascular events; BMI, body mass index; HR, heart rate; SBP, systolic blood pressure; AHF, acute heart failure;COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; MI, myocardial infarction; PCI, percutaneous coronary intervention; STEMI, non-ST segment elevation myocardial infarction; STEMI, ST-segment elevation myocardial infarction; UAP, unstable angina pectoris; LVEF, left ventricular ejection fraction; TnI, troponin I;Scr, serum creatinine; FBG, fasting blood-glucose; LDL, low-density lipoprotein cholesterol; DAPT, Dual antiplatelet therapy; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor inhibitor.

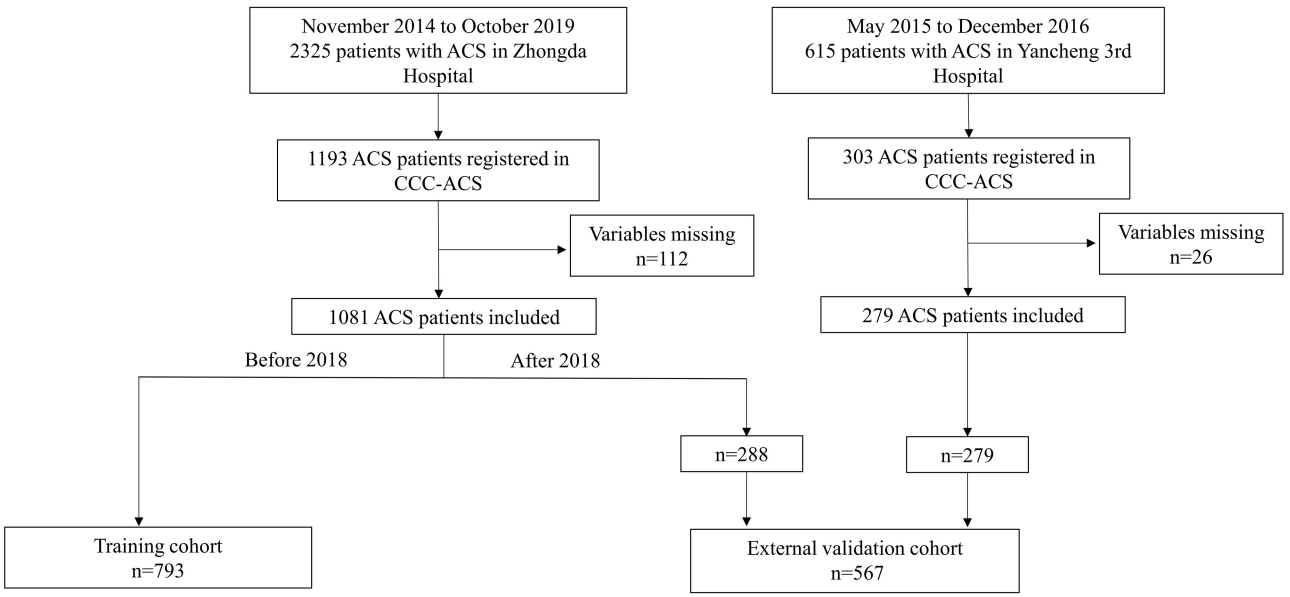

FIGURE 1 | Study flow chart. Patients from Zhongda Hospital and Yancheng Third People's Hospital were included in the study. The enrolled study population was divided into a training cohort and a validation cohort. ACS, acute coronary syndrome; CCC, Improving Care for Cardiovascular Disease in China.

defined as all-cause death, reinfarction, stroke, and heart failure during hospitalization.

Statistical Analysis

Continuous variables were expressed as mean ± standard deviation and categorical variables were described as counts and percentages. Differences between two groups for continuous variables were compared by simple *t*-test and categorical variables were compared by exact Fisher's exact test.

Firstly, a univariate analysis was performed based on the variables available in the database to derive risk factors for the occurrence of MACCE in patients with ACS. Then, combining previously reviewed literature and clinical experience, possible risk factors were selected. A total of 28 candidate predictor variables were included to construct the prediction model. Through the Least absolute shrinkage and selection operator (LASSO) analysis, a regression penalty was applied to all variables so that the coefficients of relatively insignificant variables been excluded from the model, the independent variables that had a greater impact were selected and the corresponding regression coefficients were calculated, resulting in an optimal logistic model. The final model built to predict in-hospital MACCE in ACS patients was presented by nomogram for ease of use. The risk model was then test on the validation cohort. Model performance was assessed according to the area under the receiver operating characteristic curve (AUC), calibration plot and decision curve analyses (DCA).

All statistical tests were two-sided, and $P < 0.05$ were considered significant. Statistical analyses were completed using Stata 15.0 for Windows (StataCorp Texas, USA) and R software version3. 6. 1 (R Foundation for Statistical Computing).

RESULT

Participants

A total of 1,496 patients (1,193 from Zhongda Hospital and 303 from Yancheng Third People's Hospital) were consecutively registered in CCC-ACS project between November 2014 to October 2019. Patients with missing data in the variables (n = 138, 112 in Zhongda Hospital, 26 in Yancheng Third People's Hospital) were excluded (**Figure 1**), resulting in a population of 1,360 patients were included in the final analysis.

Clinical Characteristics

Baseline characteristics of ACS Patients with or without in-hospital MACCE in the training and validation cohort are summarized in **Table 1**. As shown, variables significantly different between the MACCE group and non-MACCE groups were: gender, age, BMI, heart rate, Killip grading, cardiogenic shock and acute heart failure at admission, smoke, past history of chronic obstructive pulmonary disease (COPD), Hemoglobin, serum creatinine, fasting blood-glucose (FBG), early-stage percutaneous coronary intervention (PCI), and dual antiplatelet therapy (DATP) at admission. When comparing the GRACE scores, it was found that the GRACE scores in the MACCE group were significantly higher than the non-MACCE values ($p < 0.01$, **Table 1**).

Outcomes

During hospitalization, 137 MACCE occurred, and a total of 93 people had MACCE, giving a MACCE incidence of 6.84%, with 40 in-hospital deaths and a mortality of 2.94%. The mean hospitalization period in training cohort was 9 ± 6 days. The

TABLE 2 | Outcome of ACS Patients in training cohort and validation cohort.

Outcome	Training cohort ($n = 793$)	Validation cohort ($n = 567$)	*p*
Hospital stays, days	9 ± 6	9 ± 16	0.39
MACCE, *n* (%)	56 (7.1)	37 (6.5)	0.7
Mortality, *n* (%)	28 (3.5)	12 (2.1)	0.13
In-hospital HF, *n* (%)	21 (2.6)	25 (4.4)	0.08
Stroke, *n* (%)	10 (1.3)	2 (0.4)	0.08
Infarction again, *n* (%)	1 (0.1)	1 (0.2)	0.81

Data are presented as n (%) or mean ± SD.
MACCE, major adverse cardiovascular and cerebrovascular events; HF, heart failure.

incidence of MACCE did not differ between the training and validation cohort ($P = 0.7$, **Table 2**).

Training Model and Screening Variables

For nomogram development, we first performed univariate analysis and a systemic literature review of existing ACS risk models, aiming to identify candidate predictor variables. Then, the available clinical and laboratory data of patients in the CCC-ACS database were reviewed. We initially selected 28 candidate variables available at 24 h of admission (**Table 1**). These candidate variables were reduced to four potential predictors on the basis of 793 patients in the training cohort (**Figure 2**). The four features were: age, Killip grading, FBG and whether PCI was performed at early medical contact and coefficients of these features in the LASSO logistic regression model were not zero. A logistic regression analysis identified the age, Killip grading, FBG and whether PCI was performed at early medical contact as independent predictors (**Table 3**). The model that incorporated the above predictors was presented as a nomogram (**Figure 3**).

Model Prediction Ability Evaluation

The AUC of the nomogram was 0.807 (95% CI 0.736–0.878, **Table 4** and **Figure 4A**) in the training cohort, and the AUC of the GRACE score and EMMACE score were 0.761(95% CI 0.69–0.878) and 0.723 (95% CI 0.648–0.798). In the validation cohort, the nomogram yielded an AUC of 0.813 (95% CI 0.738–0.889, **Table 4** and **Figure 4B**), and the AUC of the GRACE score and EMMACE score were 0.851(95% CI 0.786–0.916) and 0.675(95% CI 0.585–0.764), proving the discriminatory capacity of the nomogram was comparable with the GRACE score (training $P = 0.10$, validation $P = 0.28$) and superior to the EMMACE score (training $P = 0.01$, validation $P < 0.01$).

Depending on whether the ST segment elevated or not, ACS patients can be divided into STEMI and NSTE-ACS cohort. The subgroup analysis showed consistent results as the overall population. The nomogram performed better in the NSTE-ACS patients than in the STEMI cohort with an AUC of 0.913 (95% CI 0.851–0.975, **Table 4**), higher than the AUC of 0.852 (95% CI 0.68–1.000) of the GRACE score, but the difference was not statistically significant ($P = 0.4$). In the STEMI cohort, the nomogram yielded an AUC of 0.801 (95% CI 0.712–0.890, **Table 4**) and the AUC of the GRACE score was 0.836(CI 0.712–0.890, 0.760–0.912), the difference was not statistically significant ($P = 0.33$). The nomogram performed better than the EMMACE score, whether validated in the ACS population or separately in subgroups, with a statistically significant difference.

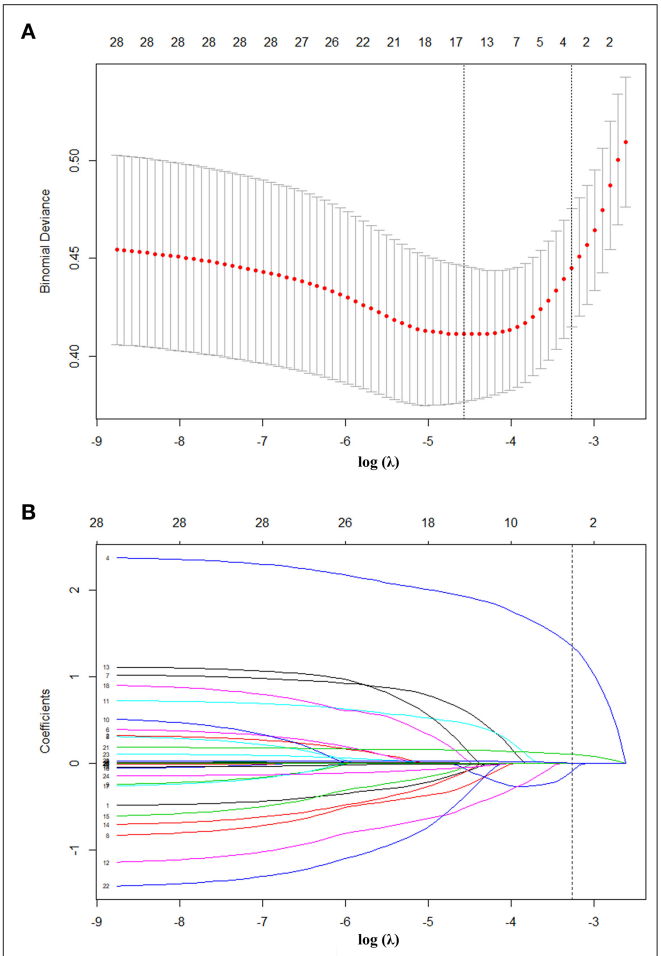

FIGURE 2 | Texture feature selection using the LASSO binary logistic regression model. **(A)** Tuning parameter (λ) selection in the LASSO model used 10-fold cross-validation via minimum criteria. The AUC curve was plotted vs. log (λ). Dotted vertical lines were drawn at the optimal values by using the minimum criteria and the 1 standard error of the minimum criteria (the 1-SE criteria). A λ value of 0.038, with log (λ), 3.273 was chosen (1-SE criteria) according to 10-fold cross-validation. **(B)** LASSO coefficient profiles of the 28 texture features. A coefficient profile plot was produced against the log (λ) sequence. Vertical line was drawn at the value selected using 10-fold cross-validation, where optimal λ resulted in 4 non zero coefficients. AUC, area under the receiver operating characteristic; LASSO, least absolute shrinkage and selection operator.

Good calibration was observed for the probability of MACCE both in the training and validation cohort. The calibration plot yielded a non-significant statistic (training, $p = 0.847 > 0.05$; validation, $p = 0.972 > 0.05$, **Figures 4C,D**), revealed a proper consistency between nomogram predictions and actual observations.

TABLE 3 | Logistic regression model for predicting in-hospital MACCE in ACS.

Variable	β	Odds Ratio (95% CI)	*P*
Age	0.346	1.413 (1.069–1.867)	0.015
Killip	0.707	2.027 (1.517–2.710)	<0.01
PCI	−0.648	0.522 (0.275–0.995)	0.048
FBG	0.161	1.174 (1.1–1.252)	<0.01
Intercept	−7.177	-	-

Age, per 10 years; FBG, mmol/L.

Clinical Application

A DCA for nomogram and GRACE score was performed to determine their clinical validity by quantifying the net benefit at different threshold probabilities in the validation cohort (**Figures 4E,F**).

The decision curve showed that if the threshold probability of a patient is between 2 and 80%, the net benefit of applying this nomogram is significantly higher than the two extreme cases, where all patients are considered to be at high risk or all at low risk. The net benefit of the nomogram was comparable to the GRACE score, but the nomogram has a much wider range of benefits. This model has good clinical usefulness of and of great value for evaluating the clinical prognosis of clinical patients with ACS.

DISCUSSION

In this study, a clinical prediction nomogram was developed and validated for predicting in-hospital MACCE in patients with ACS. The nomogram comprises four variables: age, Killip grading, FBG and whether early-stage PCI was performed during hospitalization. All indicators were available within 24 h of admission, and laboratory tests were routinely performed on patients admitted to the hospital. So that this model can predict the occurrence of in-hospital MACCE in ACS patients and provide risk stratification of patients at early medical contact without additional economic burden on patients.

Various indicators have been reported to predict prognosis of ACS: NLR (21), the hs-cTnT and hs-cTnI (22, 23), plasma ceramides (24), and progenitor cells (25), etc. Among them, some of the serological markers are not frequently tested-in hospital and demand additional time and cost. Moreover, the sensitivity and fitting degree of predicting MACCE by a single serum marker are poor and found instable in different studies. Meanwhile, several risk scores for risk assessment have been proposed, including the Evaluation of the Methods and Management of Acute Coronary Events (EMMACCE) (26), TIMI (27), PURSUIT (28), and the Global Registry of Acute Coronary Events (GRACE) risk score (29, 30). These risk scores were derived from randomized controlled trials or multinational registries and each has its own strengths. The objective of EMMACE is to evaluate care of patients admitted with acute myocardial infarction. The EMMACE risk model contains the fewest number of variables and uses three variables–age, SBP and HR on admission to predict 30-day mortality for STEMI (26). The PURSUIT risk model predicts 30-day death and the composite of death or AMI in ACS patients without persistent ST-segment elevation (28). The TIMI scores differ slightly for the different populations of NST-ACS and STEMI. The endpoint events for the NST-ACS population are composite endpoints, including mortality, myocardial infarction and emergency revascularization within 2 weeks of ACS (31). The score for STEMI patients predicts the risk of death within 30 days (11). The GRACE risk score included patients with ACS and predicts all-cause in-hospital and 6-month death (29, 30, 32). The TIMI score does not compare favorably with other scores in the prediction of NST-ACS and the prediction of prognosis in STEMI patients is comparable to that of the GRACE score (33). However, the GRACE score is more commonly used in clinical practice based on the fact that it contains more clinically relevant evaluation indicators and includes all patients with ACS (34). In these scores, the most frequently occurring indicators with the highest predictive effectiveness are age, SBP and heart rate on admission. The similarities and differences in the variables included were detailed in **Supplementary Table 1.**

Although these risk scores have been externally validated and some have even been recommended by guidelines for clinical practice, when applied to a "real world" population, there are still some limitations. Firstly, many risk scores are derived from randomized controlled trials that may exclude many high-risk patients or are validated only in specific populations. The mortality in this CCC-ACS cohort is higher than that in the derivation of GRACE and PURSUIT scores, which reveals the difficulties in generating "real world" data from trial populations owing to exclusions of higher-risk individuals from clinical trials. This should be considered when applying risk stratification methods to the general population (35). Secondly, the risk scores were created long ago and incorporated populations from about 20 years ago. The greater use of newer therapies for ACS has altered model discriminative performances with time. Coronary angiography and PCI were performed more widely since <30% of patients underwent PCI two decades ago when the GRACE studies conducted (10). At the same time, the application of medical treatments has changed, such as dual antiplatelet therapy, ACEIs/ARBs and β-blockers. All these changes may induce previous risk scores not being applicable to today's ACS population. Additionally, most of the patients included in the GRACE score were from Europe and The United States, among which the sample size of China or Asia was definitely limited. Whether the GRACE score is applicable in the Chinese population in the new era of widespread PCI remains to be tested. Thirdly, in clinical practice, ACS is often too urgent for risk assessment, so coronary angiography is performed directly. Moreover, many indicators included in the risk score are difficult to obtain at early medical contact. For example, testing for serum creatinine in the GRACE score takes extra time, and waiting for its results may delay the patient's condition. The high number of variables included in risk scores and the cumbersome evaluation process are also among the reasons that limit their application. Fourthly, there is currently controversy about whether risk scores can truly improve patient prognosis. A recent prospective study confirmed that the use of the GRACE

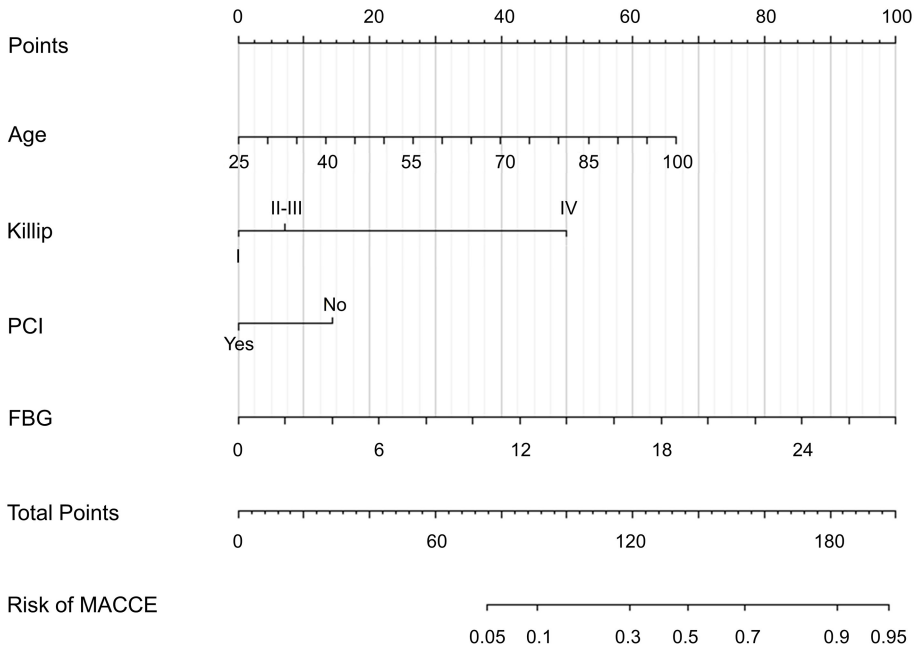

FIGURE 3 | Developed in-hospital MACCE prediction nomogram in patients with ACS. ACS, acute coronary syndrome; FBG, fasting blood-glucose; MACCE, major adverse cardiovascular and cerebrovascular events; PCI, percutaneous coronary intervention.

TABLE 4 | The AUC (95% CI) of different models in training cohort and validation cohort.

Cohort		Nomogram	GRACE score	EMMACE model	P_1	P_2
Training cohort		0.807 (0.736–0.878)	0.761 (0.69–0.878)	0.723 (0.648–0.798)	0.10	0.01
Validation cohort	All	0.813 (0.738–0.889)	0.851 (0.786–0.916)	0.675 (0.585–0.764)	0.28	<0.01
	NSTE-ACS	0.913 (0.851–0.975)	0.852 (0.683–1.000)	0.733 (0.598–0.869)	0.41	<0.01
	STEMI	0.801 (0.712–0.890)	0.836 (0.760–0.912)	0.73(0.608–0.813)	0.33	0.03

P_1, Nomogram compared with GRACE score; P_2, Nomogram compared with EMMACE model.

risk score to guide treatment decisions in ACS increased the rate of early dumping, but does not improve the 1-year risk of death or the risk of recurrent infarction (36). This study suggests that the implementation of routine GRACE risk assessment based on guideline adherence at higher levels of cardiac centers does not benefit though may be associated with a low event rate and premature study termination. This reveals a well-recognized difficulties in generating "real world" data from trial populations and the conflict between theory and reality suggests that more exploration of risk scores is needed.

In the newly developed prediction model, age and Killip classification coincide with the evaluation indicators in the GRACE score and TIMI score (27, 29). It also emphasized on the patient's vital signs at admission, because it is closely related to the severity of the disease and the prognosis of the patients. As it is evident from the prediction model, higher FBG was risk factors for MACCE, which can also be seen from the nomogram and was the most highly co-relatable with outcomes, weighting highest among four variables included in the model. FBG is of great value in the prognosis assessment of ACS patients. Previous studies have shown that patients with hyperglycemia have a higher risk of in-hospital and long-term MACCE regardless of whether they have diabetes, which relates high FBG with the stress state and the release of inflammatory factors (37). In this study, as shown, early PCI treatment is considered to be an important protective factor for in-hospital outcomes in ACS patients. In this dataset, which is current and up-to-date, the prevalence of PCI is about 70%, which is significantly higher than 20 years ago and close to the incidence of PCI at present, demonstrating that the sample in this study represents the current population (10, 38, 39). More research is needed to explore whether early PCI can provide greater benefit to all ACS patients. The mortality rate in this study was 2.94 %, which is at a median level compared to previous studies (39, 40). The incidence of MACCE was 6.84 %, which was slightly higher than that in previous studies (39, 41), which may corelates with broader definition of MACCE in this study

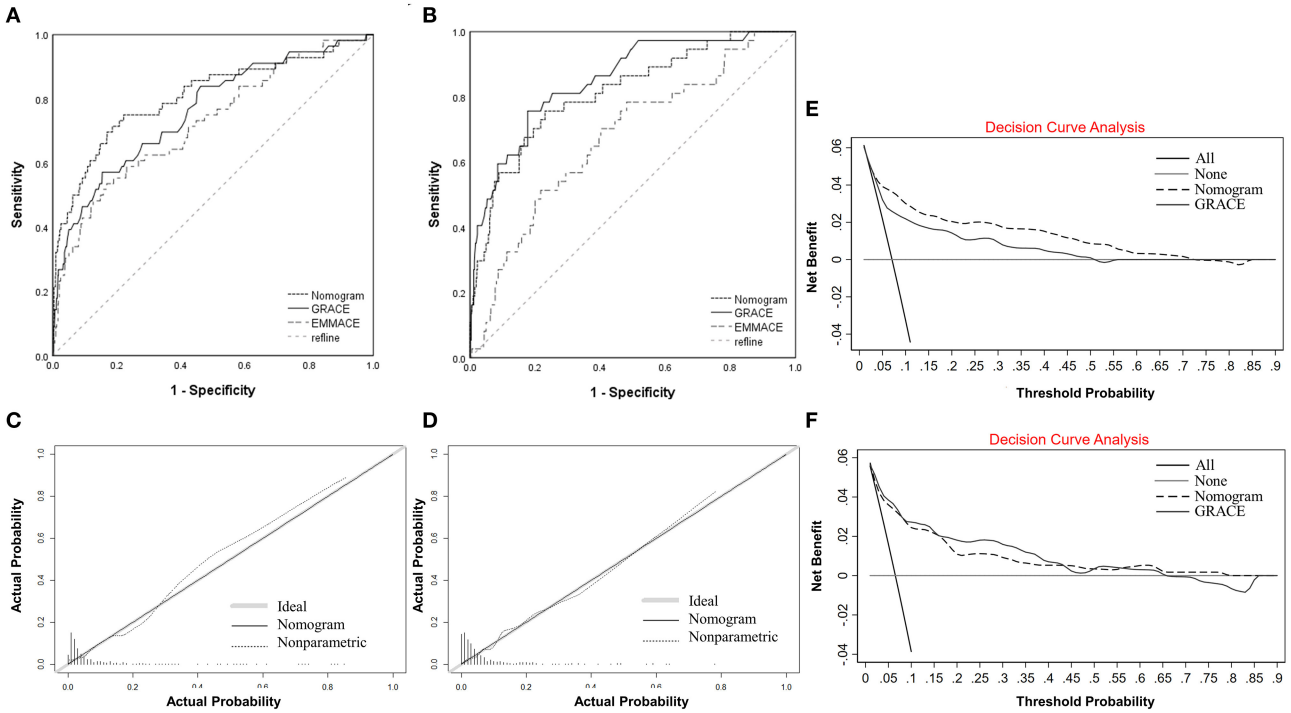

FIGURE 4 | Comparison of ROC curves for nomogram with GRACE score and EMMACE score for predicting in-hospital MACCE in patients with ACS in the training cohort. **(A)** Comparison of ROC curves for the in-hospital MACCE nomogram with GRACE score and EMMACE score in the training cohort. **(B)** Comparison of ROC curves for the in-hospital MACCE nomogram with GRACE score and EMMACE score in the validation cohort. **(C)** Calibration plot of the in-hospital MACCE nomogram in the training cohort. **(D)** Calibration plot of the in-hospital MACCE nomogram prediction in the validation cohort. The x-axis represents the predicted in-hospital MACCE risk in ACS. The y-axis represents the actual diagnosed in-hospital MACCE. The diagonal line represents a perfect prediction by an ideal model. The solid line represents the performance of the nomogram, in which a closer fit to the diagonal line represents a better prediction. **(E)** Decision curve analysis for the in-hospital MACCE nomogram and GRACE score in the training cohort. **(F)** Decision curve analysis for the in-hospital MACCE nomogram and GRACE score in the validation cohort. The y-axis measures the net benefit. The black dotted line represents the in-hospital MACCE nomogram and the black zone line represents the GRACE score. The gray solid line represents the assumption that all patients are ACS complicated with MACCE. The black solid line represents the assumption that none of the patients used MACCE in ACS. The decision curve analysis showed that if the threshold probability of a patient is between 2 and 80%, using this in-hospital MACCE nomogram prediction in the current study to predict in-hospital MACCE risk adds more benefit than the "intervention-for-all" patient scheme or the "intervention-for-none". ACS, acute coronary syndrome; EMMACE, Evaluation of the Methods and Management of Acute Coronary Events; GRACE, Global Registry of Acute Coronary Events; MACCE, major adverse cardiovascular and cerebrovascular events; ROC, receiver operating characteristic.

and patients included were from tertiary hospitals with chest pain center, indicating severe underlying conditions in patients compared to previous studies.

While developing a prediction model, the data set partition is usually necessary before its construction. Common methods include external validation from different centers, completely random grouping i.e., internal validation and external out time validation. The external validation had the highest level of evidence among all validation methods, but some of the external validation did not yield good results due to the possible genetic, environmental, and lifestyle effects of the data from different centers and the way data distributed. Dodson et al. (42) developed a prediction model for readmission within 30 days for elderly AMI patients using a completely randomized approach. Although the study included 3,006 patients from 94 hospitals in the United States, a very representative sample as a multicenter study, a completely randomized approach was used in assigning the sample. Although almost equal C-index could be obtained in the training and validation cohort statistics, it does not provide strong evidence of good predictive efficacy of this prediction model, as the completely randomized grouping may have made the characteristics of the data more similar between the two groups, reducing the variation in components. Recently, a study to predict the risk of patients with diabetic kidney disease initiating renal replacement in 3 years were grouped according to the chronological order of the data (43). In our study, a comprehensive method was used. Data from 2014 to 2017 in Zhongda Hospital was set into the training cohort, mixed data from Zhongda Hospital after 2018 years and Yancheng Third People's Hospital between 2015 and 2016 was set into the validation cohort. It was also an external validation. We did not use the data only from the Yancheng Third People's Hospital as external validation because it contained too small a sample size and an even smaller number of endpoint events, so the validation results were not representative. Also, dividing the data according to time has certain problems. As time progresses, the treatment of the disease and people's awareness of the disease may also change, which may make the two data sets differ in some characteristics. However, the comprehensive method prevented us from artificially selecting models that contribute to the test set

data, making the prediction results closer to the actual clinical results. The robust and stable performance in the validation cohort of the nomogram also proved the reliability of the model.

The nomogram may serve as a complement to previous risk scores with variables that are routine available at early medical contact. Firstly, it exhibited good discrimination ability, which is comparable with the GRACE score. However, the model contains only four variables, which is easier to use than the GRACE score. Compared to the EMACCE score, the number of variables included in the model increased by only one, but the predictive power was greatly improved. Secondly, we only excluded patients with missing data, and the population included in this study was between 2014 and 2019, so the study population was the latest and closer to the real-world than other registries. This nomogram may have potential applications. It can be used to stratify patients at risk much easier and to accurately treat and care for the patient accordingly. Variables included in this model that are the same as in other models need to be highlighted in the risk stratification of patients, and predictors like FBG and early-stage PCI may provide useful information for updating other models. With the advancement of diagnosis and treatment technology, more new models are likely to emerge in the future.

LIMITATION

There are some limitations of this study. Firstly, the data in this study was from a web-based data collection platform and there may be a selective bias. Although the study included data from only two centers, we did external validation in Yancheng Third People's Hospital and Zhongda Hospital has 65 group hospitals and involves patients from many parts of Anhui and Jiangsu, so the results are somewhat representative. Secondly, in the process of statistical analysis, variables with more than 30% of missing values were removed, which may lead to a certain bias in the results of statistical analysis, and to some extent, affect the objectivity and correctness of the study conclusions. For missing data, the multiple fill method was used, which to a certain extent avoided the possibility that the confidence interval range of the effect indicators might be underestimated when filling in the missing values, but the data processing techniques could only be infinitely close to the real data and could not be fully equivalent. The implementation process and data management stage of clinical trials should prevent the generation of missing data as much as possible, strengthen data collection, and avoid cases shedding missing visits, so as to ensure the integrity and validity of the data. Thirdly, the present study included a relatively modest sample size. However, the validation of the model in an internal cohort somewhat mitigates the sample size concern. In the future, more detailed studies with larger sample sizes and external verifications should be designed to further improve and confirm the accuracy of this model.

CONCLUSION

Risk stratification of patients is potentially important in guiding clinical decision making and optimizing care and treatment. Here we proposed a simple and user-friendly method to objectively and accurately predict the possibility of in-hospital MACCE in ACS patients, and analysis in the validation cohort and subgroups also confirmed its forecast robustness and reliability. Although there is no evidence that it improves prognosis, more prospective observational studies are needed to confirm its clinical value.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Institutional Review Board of Zhongda Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XB and LC had full access to all data used in this study and take responsibility for their integrity and the accuracy of the data analysis. XB and YL participated in data cleaning, interpreted the data, statistical analysis, developed the predictive model, prepared figures and/or tables, and wrote the first draft of the manuscript. LC and MY participated in critically revising the manuscript. ZL and YZ were responsible for filling in the clinical data of participants. All authors participated in concept and design of the present study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.699023/full#supplementary-material

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REVIEW
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A Primer on Repeated Sitting Exposure and the Cardiovascular System: Considerations for Study Design, Analysis, Interpretation, and Translation

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Sedentary behavior, particularly sitting, is ubiquitous in many contemporary societies. This is a major societal concern considering the evidence for a strong association between sitting behavior and cardiovascular disease morbidity and mortality. Unsurprisingly, leading public health agencies have begun to advocate "reduction" in sitting behavior. Though, the guidelines are typically vague and non-specific. The lack of specific guidelines for prolonged sitting is attributable to the absence of available evidence to facilitate guideline development. To inform policy, well-designed randomized controlled trials are required to test the efficacy of specific and translatable sitting reduction strategies. To guide the design of randomized controlled trials, this review postulates that several gaps in the literature first need to be filled. Following a general discussion of the importance of sitting behavior to contemporary societies, each of the following are discussed: (i) acute sitting exposure and systems physiology; (ii) recommendations for a systems physiology toolbox; (iii) study design considerations for acute sitting exposure; and (iv) translation of sitting-focused research.

Keywords: biological plausibility, ecological validity, external validity, internal validity, methodology, sedentary behavior, arterial stiffness, endothelial function

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INTRODUCTION

Sedentary behavior (SB), particularly sitting, is ubiquitous in many contemporary societies (**Figure 1**) (1). This is a major societal concern in light of the evidence for a strong association between SB and cardiovascular disease (CVD) mortality. However, while leading public health agencies, including the World Health Organization (WHO), have begun to advocate "reduction" in SB, the guidelines are typically vague and non-specific (2, 3). To inform SB policy, well-designed cohort studies and randomized controlled trials (RCTs) are needed (see **Figure 2**) to test the efficacy of specific and translatable SB reduction strategies. To assist in the design of RCTs, this review postulates that several gaps in the literature first need to be filled. Following a general discussion of the importance of SB to contemporary societies, each of the following are discussed:

(i) Acute SB exposure and systems physiology, i.e., how and why SB increases CVD risk.
(ii) Systems physiology toolbox, i.e., methods to investigate the effects of acute SB exposure on systems physiology. The flow-mediated dilation (FMD) and pulse wave velocity (PWV) test are used as exemplars to provide context to the key considerations that need to be made.
(iii) Study design consideration, i.e., how to design an acute SB study with forethought to internal validity, external validity, and ecological validity. The FMD and PWV tests are further used to provide context.
(iv) Translation, i.e., steps to increase the likelihood of SB-focused research informing policy.

While some basic context is provided, it is not the intention of this review to comprehensively discuss the historical and socio-cultural considerations, or to comprehensively discuss potential mechanistic pathways. For clarity, the intention of this review is to provide a primer for important considerations to assist in the design of acute SB studies. **Table 1** presents a glossary of terms, provided to assist in the interpretation of concepts specific to the sitting literature and discussed within this review. This review should be viewed as an addition to existing research design guidelines, including the Consolidated Standards of Reporting Trials (CONSORT) (4).

IMPORTANCE OF SEDENTARY BEHAVIOR TO SOCIETY

Since the mid-20th century, societal changes have led to increased SB, particularly sitting (5). Today, the average

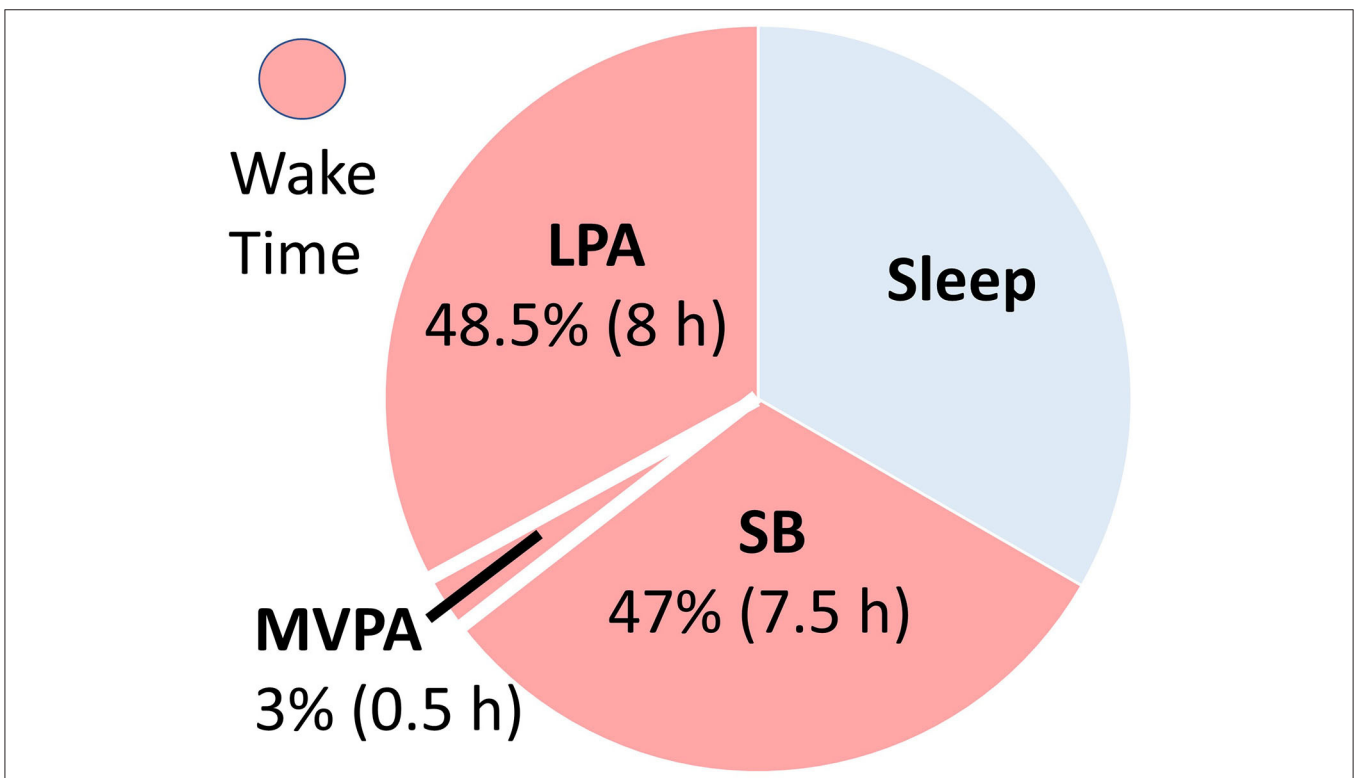

FIGURE 1 | Example of a typical 24-h activity cycle for individuals in modernized economies. The average adult engages in sedentary behaviors (SB) ~half their waking hours, with the majority of the remaining waking hours engaged in light physical activity (LPA). Time spent in moderate-to-vigorous physical activity (MVPA) is typically minimal (~3% of the day), even for adults meeting recommended levels.

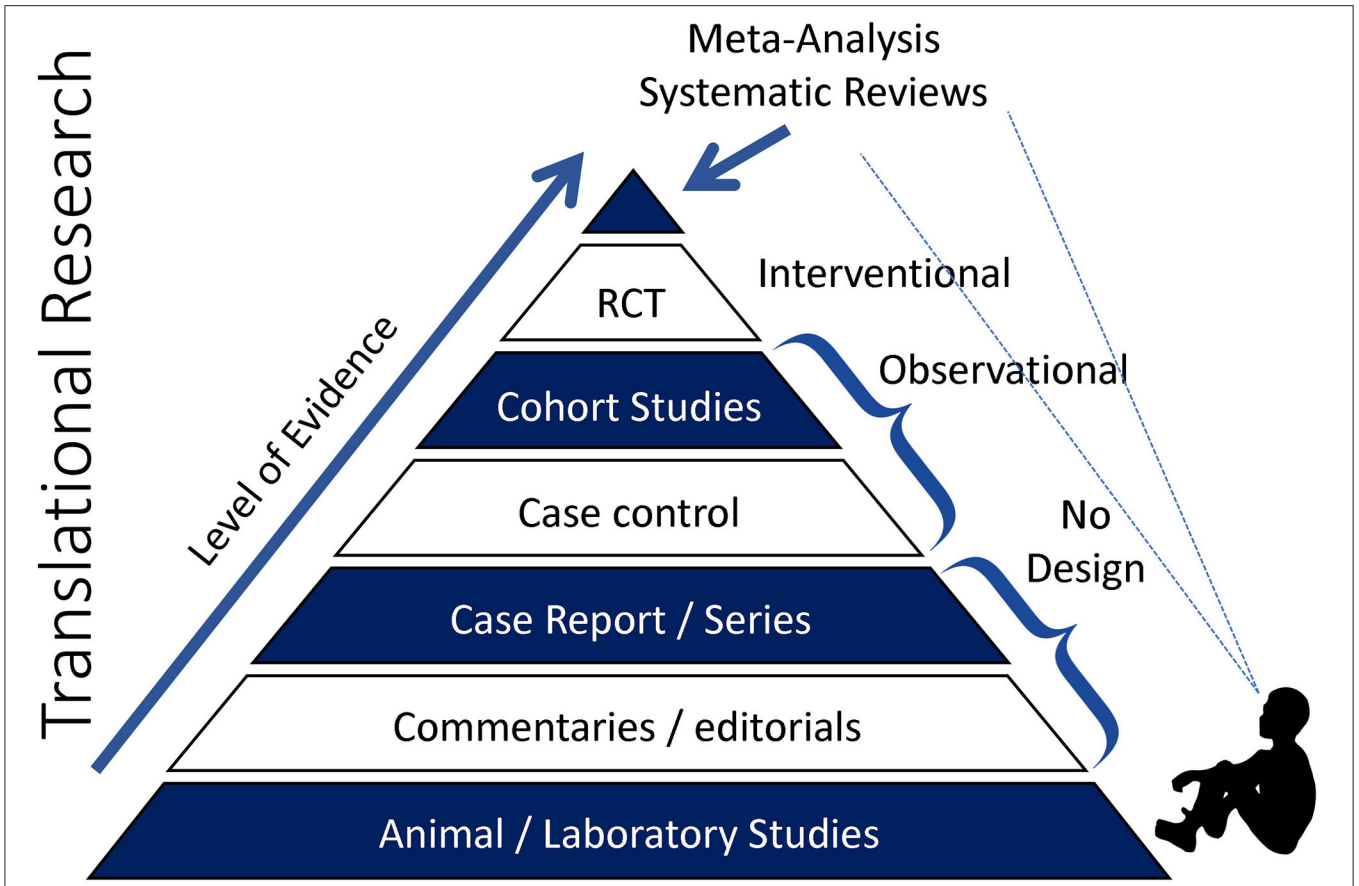

FIGURE 2 | Pyramid of clinical evidence. With respect to sedentary behavior-focused research and the capacity to develop sedentary behavior-reduction policy, we are currently sitting somewhere near the bottom of the pyramid peering up.

American spends 55% (7.7-h/day) of their waking time sedentary, with much of that time spent sitting, and inactive (1). The terms "physical inactivity" and "sedentary behavior" have been used interchangeably. However, physical inactivity refers to not meeting moderate-to-vigorous physical activity (MVPA) guidelines, and SB refers to very low intensity behaviors ($\leq$1.5 metabolic equivalents) in a seated, reclined, or supine posture (6). SB is a distinct CVD risk factor (1, 7), and interrupting SB with light intensity physical activity has health benefits independent from MVPA (8, 9). Despite SB being a large part of our daily behavior (see **Figure 1**), public health programs and policies have primarily focused on other modifiable health behaviors, such as diet, smoking, and MVPA. Little is known about optimal prescription for reducing SB (2).

Using MVPA as an exemplar, the 2018 Physical Activity Guidelines provide a clear public health message: "For substantial health benefits, adults should do at least 150 min per week…of moderate-intensity…aerobic physical activity" (2). These guidelines, which are based on the FITT (frequency, intensity, time, and type) principle, are driven by numerous RCTs and subsequent meta-analyses exploring the health benefits of physical activity (2). Such RCTs and meta-analysis data are unavailable for SB. However, it should be acknowledged that, while the first physical activity study was published in 1953 (10), SB-focused research is in its infancy. As such, the 2018 Physical Activity Guidelines do recommend sitting less, based on the "strong" overall and dose-response association between SB and CVD mortality, but they do not provide specific guidelines for sitting substitution (2). The absence of specific guidelines can be attributed to the fact that very few studies, mostly cross-sectional, have investigated the physiological mechanisms of SB and its association with CVD. This incomplete mechanistic understanding limits the ability to identify informed sitting-interruption strategies. The next part of this review will briefly discuss plausible physiological mechanisms, specifically as they relate to sitting behavior, followed by an overview of available tools to investigate these mechanisms.

SYSTEMS PHYSIOLOGY AND ACUTE EXPOSURE TO PROLONGED SITTING

To provide context to the sections that follow, this section briefly outlines a plausible working model for the relationship between acute prolonged sitting exposure and negative cardiovascular responses. The primary outcome of this model, depicted in

TABLE 1 | Glossary of terms.

Term	Definition
24-h activity cycle	Activities conducted over a 24-h period including, sleep, sedentary behavior, light-intensity physical activity, and moderate-to-vigorous physical activity.
Accuracy (Validity)	Deviation of a measure from its true value, including systematic (constant bias) and random error (variable) components.
Arterial stiffness	Fundamental mechanical behavior or rigidity of the material properties of the artery wall, determined by both structural and functional components.
Biological plausibility	Proposal of a causal association (or relationship between a cause and outcome) that is consistent with existing biological and medical knowledge.
Cardiovascular disease (CVD)	A general term for pathologies affecting the heart or blood vessels, including cerebrovascular disease, myocardial infarction, coronary heart disease, and peripheral artery disease.
Exercise	A subset of physical activity that is planned, structured, and repetitive.
Ecological validity	The degree to which results obtained from research or experimentation are representative of conditions in the wider world.
Endothelial function	The ability of the endothelium to adequately perform its physiological roles in maintaining homeostasis.
External validity	The degree to which causal relationships can be generalized to different measures, persons, settings, and times.
Intermediate outcome	A surrogate measure (e.g., pulse wave velocity) used to measure the effect of a treatment that may correlated with a real clinical endpoint (e.g., cardiovascular disease).
Internal validity	Degree to which a study establishes the cause-and-effect relationship between the treatment and the observed outcome.
Metabolic equivalent (MET)	Unit used to describe the absolute intensity of physical activity. A ratio of your working metabolic rate relative to your resting metabolic rate.
Physical activity	Any bodily movement produced by skeletal muscles that requires energy expenditure.
Physical inactivity	An insufficient level of moderate-vigorous physical activity level to meet present physical activity recommendations.
Policy	A course or principle of action adopted or proposed by an organization or individual.
Precision (Reliability)	Dispersion of a measurement or degree to which a measure contained on one occasion is repeated on a second occasion, including intra-rater (test-retest) and inter-rater components.
Randomized controlled trial (RCT)	People are randomly assigned to two (or more) groups, typically including one control and one or more experimental groups.
Sedentary behavior (SB)	Any waking behavior characterized by an energy expenditure $\leq$1.5 METs, while in a sitting, reclining or lying posture.
Translation	The use of scientific evidence by decision makers to inform and generating health policy.

Figure 3, is aortic arterial stiffness, a measure explained in further detail below and that we have reported to increase in response to uninterrupted prolonged sitting (11–14). The elastic aorta is directly proximal to the heart and is responsible for dampening the speed and amplitude of retrograde pressure waves that increase the heart's workload during systole (myocardial burden) (15). This measure is particularly appealing because arterial stiffness is a multifactorial measure of central hemodynamics, dependent on blood pressure and intrinsic arterial structural and functional properties (15). Therefore, an increase in aortic arterial stiffness not only signifies increased myocardial burden and target organ damage susceptibility, but also reflects the stress being placed on the aorta and the potential for detrimental changes to the vessel.

Our working hypothesis is that acute sitting-induced increases in arterial stiffness are likely driven by local hemodynamic changes and compounded by a number of detrimental autonomic, hormonal, and metabolic factors. With respect to hemodynamics, we have reported that hydrostatic pressure and lack of muscle pump activity led to lower extremity venous blood pooling, the slower transit time of blood through the venous circulation, which decreased venous return and was inversely associated with stroke volume (16). This decreased stroke volume in turn reduces shear stress (17, 18), the primary regulator of endothelial function (19). Decreased shear stress promotes oxidative stress, endothelial dysfunction, and acutely increases aortic arterial stiffness (20–24). As mentioned above, the increased aortic stiffness may exacerbate myocardial burden as a function of the increased speed and amplitude of retrograde pressure waves. Changes to peripheral vessels, particularly in the lower-limbs, may compound the retrograde pressure waves. In support, we reported endothelial dysfunction (25) and increased arterial stiffness in the legs (26). Lower-limb leg endothelial dysfunction is induced by the pro-atherogenic shear stress profile as a result of decreased stroke volume and alterations in vascular resistance near tortuous vessels (27–29).

Autonomic and hormonal changes further elevate aortic arterial stiffness by increasing systemic vascular resistance (30). The autonomic changes are driven by reduced pressure along the arterial baroreceptors, and the hormonal changes by reduced blood pressure along the kidneys, which stimulates the renin-angiotensin aldosterone system (31). Metabolically, lack of muscle contractile stimulation suppresses lipoprotein lipase activity—required for triglyceride uptake and high-density lipoprotein production—and glucose uptake (32, 33). Elevated plasma glucose promotes higher circulating insulin concentrations, which contribute to arterial stiffness *via* a combination of enothelin-1 release from mitogen-activated protein kinase activation, and centrally-mediated adrenergic vasoconstriction (34).

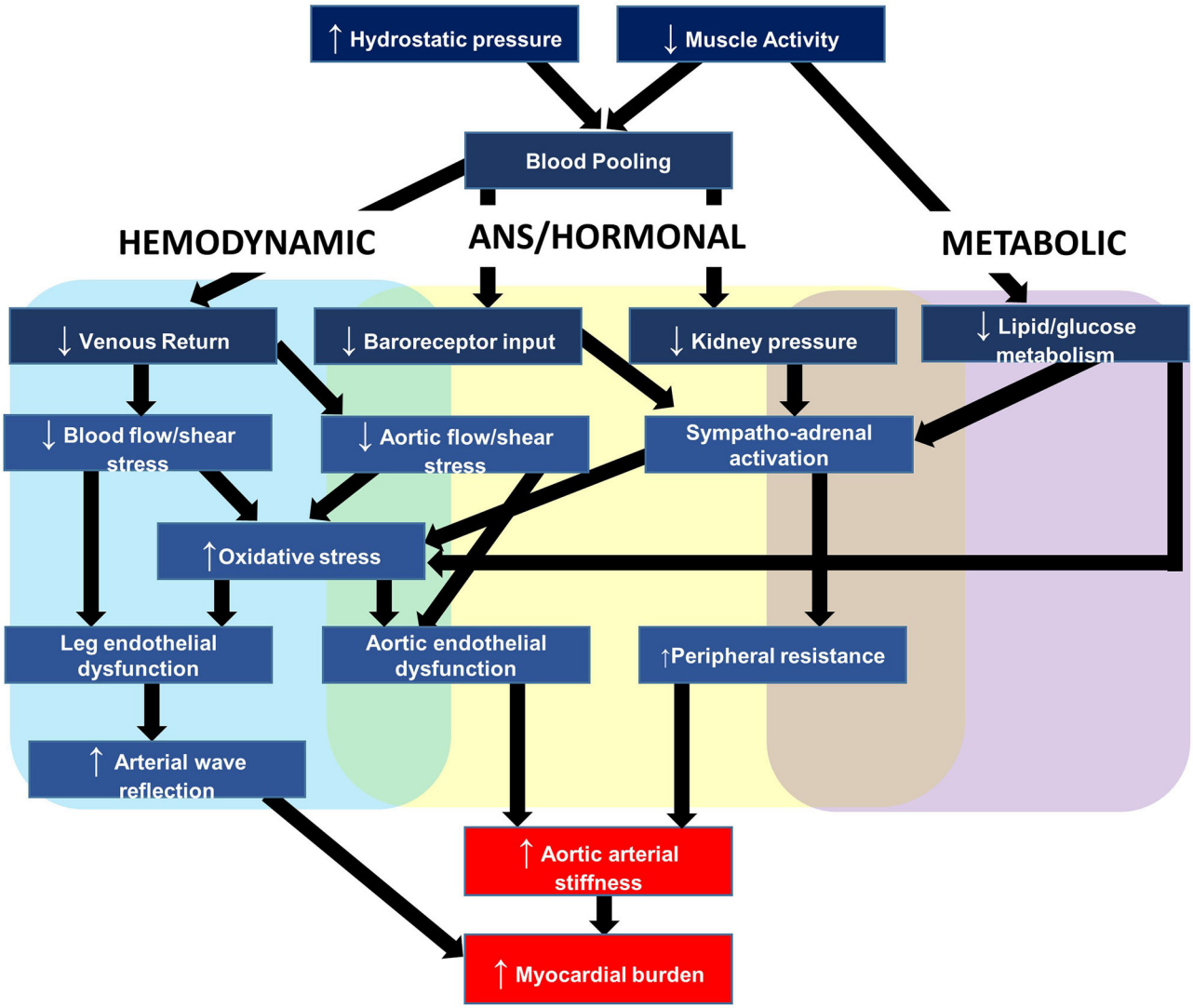

FIGURE 3 | Hypothesized mechanisms linking repeated prolonged sedentary behavior (sitting) exposure to cardiovascular disease.

The hemodynamic, autonomic, hormonal, and metabolic changes briefly described in this section lead to acutely increased aortic arterial stiffness. However, we hypothesize that repeated increases in arterial stiffness leads to structural remodeling of vessel walls (15), and this helps to explain, in part, the positive association between chronic SB and aortic arterial stiffness (35), and between SB and CVD (2, 3).

SITTING TOOLBOX

Table 2 outlines example tools available to acute sitting-focused researchers and relates these tools to the working model outlined in **Figure 3**. At the end of this section, we provide further details of two commonly used tools, FMD and PWV, which will subsequently be further discussed in terms of internal validity in the proceeding section Internal Validity Considerations. As outlined in **Table 3**, when selecting appropriate tools, a number of considerations need to be made, starting with "what is your research question?" Is the purpose to elucidate the mechanisms by which acute prolonged sitting exposure leads to increased myocardial burden? If so, which tool(s) can reflect the myocardial burden, and which physiological systems are contributing to this burden? Additionally, are these physiological changes systemic, such as hormonal changes, or are the physiological changes more local, such as increased arterial stiffness in the lower extremities? If the physiological changes are more local, what caused said physiological changes and how do these local changes influence systems physiology? For example, what mechanisms contribute to decreased endothelial function in the lower extremities, and how does this endothelial function contribute to myocardial burden. Answering these questions requires appropriate tool selection.

TABLE 2 | Research toolbox for investigating the effect of acute sitting exposure on the cardiovascular system.

Outcome			Cost		Test			Rationale
Construct	**Measure**	**Technology**	**Instrument**	**Consumable**	**Time (min)**	**Skill**	**Reliability**	
Hemodynamic								
Arterial stiffness	PWV	Oscillometry or tonometry	>$15 k	Negligible	5	Med	High	Reflects stress placed on arterial wall.
BP (Peripheral)	Peripheral	Oscillometry	<$1 k	Negligible	5	Low	High	Force exerted on a vessel.
BP (Central)	PWA	Oscillometry	>$15 k	Negligible	5	Low	High	Reflects myocardial load.
Blood flow	i) Aorta ii) Brain iii) Peripheral	i) CW-US ii) TCD iii) Doppler US	>$40 k	Negligible	<5	High	Med	↓blood flow-induced shear stress induces endothelial dysfunction.
Blood pooling	Deoxy[heme]	NIRS	>$10 k	Negligible	5	Med	High	Reflects blood pooled in venous compartment.
Endothelial Function	FMD	US	>$40 k	Negligible	10	High	Med	Represents macrovascular dysfunction.
Microvascular Function	Reactive hyperemia	US	>$40 k	Negligible	8	High	Med	Represents microvascular dysfunction
SVR	CO/ MAP	Finger PPG	>$30 k	Negligible	1	Low	High	Moderates blood pooling and venous return.
Wave reflection	PWA	Oscillometry	>$15 k	Negligible	5	Low	High	Wave reflection increases myocardial load.
Autonomic								
PNS	HRV	ECG	>$10 k	Negligible	5	Low	High	Reflects change in PNS activity.
SNS	Catecholamines	ELISA		$500/kit	5	Med	High	Reflects change in SNS activity.
Hormonal								
CACs	Venous blood	Flow cytometry	>$20 k	$65/person	5	Med	High	Reflect endothelial repair.
EMPs	Venous blood	Flow cytometry	>$20 k	$50/person	5	Med	High	Reflect endothelial damage.
Insulin	Venous blood	ELISA		$500/kit	5	Med	High	May ↑ SNS activity.
RAAS	Venous blood	ELISA		$500/kit	5	Med	High	↑BP.
Metabolic								
Glucose	Venous blood	ELISA		$500/kit	5	Med	High	Reflects glycemic control.
Lipids	Venous blood	ELISA		$500/kit	5	Med	High	May impair vascular function.

BP, blood pressure; CACs, circulating angiogenic cells; CO, cardiac output; CVD, cardiovascular disease; CW-US, continuous-wave ultrasound; deoxy[heme], deoxygenated heme concentration; ECG, electrocardiogram; ELISA, enzyme-linked immunosorbent assay; EMPs, endothelial micro-particles; FMD, flow-mediated dilation; HRV, heart rate variability; MAP, mean arterial pressure; NIRS, near infrared spectroscopy; PNS, parasympathetic nervous system; PPG, photo-plethysmography; PWA, pulse wave analysis; PWV, pulse wave velocity; RAAS, renin-angiotensin aldosterone system; SB, sedentary behavior; SNS, sympathetic nervous system; SVR, systemic vascular resistance; TCD, transcranial Doppler; US, ultrasound.

Once viable tools have been identified, additional questions need to be asked. For example, how frequently do the measurements need to be made to capture the anticipated physiological response? If the frequency of measurements is too high, and/or the measurements are too disruptive to the subject, the subsequent increase in subject arousal may influence the physiological systems of interest. That is, the measurements themselves may confound internal validity (further discussion in Internal Validity Considerations.). For some measurements, this is not a concern, as they can be continuously captured without burdening the subject. For other measurements, such as blood pressure derived using oscillometry, there will be a burden to the subject. Ideally, measurements which are potentially disruptive to the subject are made at baseline and then at the end of the experiment. However, this brings an additional consideration: which posture should the measurements made in? Some of the measurements listed in **Table 2** have been validated for use in the supine posture only and may be less precise in the seated posture. Measurement posture will be further considered in section Methodological Considerations.

Endothelial Function: Flow-Mediated Dilation

Endothelial dysfunction is a pivotal, yet potentially reversible step that precedes and predicts overt CVD (36). This is because the vascular endothelium is responsible for governing several aspects of vascular homeostasis, including regulating lipoprotein permeability, platelet aggregation, and vascular tone (37). A popular non-invasive test of endothelial function is FMD, which is quantified as the vasodilatory response to transiently increased shear stress during reactive hyperemia (**Figure 4**) (38). The vasodilatory (expressed as the percentage change in vessel diameter) and shear stress response can be measured using commercial duplex Doppler ultrasound coupled with dedicated image analysis software (e.g., FMD Studio, QUIPU; or Vascular Tools, Medical Imaging Applications). An important consideration, and one that will be expanded upon in the Internal

TABLE 3 | Measurement considerations for studies investigating the effect of acute sitting exposure on the cardiovascular system.

Consideration	Example(s)	Recommendation(s)
Physiological		
Research question	Which physiological systems are relevant to the question?	Ensure question is clear and addressable.
Multi-system	Prolonged sitting impacts multi systems, how can these changes be accounted for?	Do you need to control or monitor for potential confounders?
Central vs. local	Will local physiological changes impact systems physiology?	Consider interactions between local and systems physiology and how this can be controlled/measured.
Stimulus time	What duration needed for desired physiological response?	Ensure prolonged sitting bout is sufficient for inducing desired physiology change.
Toolbox selection		
Research question	Will the tools answer your question?	Will the tools measure change in the desired construct?
Level of invasiveness	Will invasive measurements arouse the participant?	Limit measurement of disruptive/invasive measurements.
Subject burden	Will repeated measurements arouse the participant?	Limit measures to those pertinent to research question.
Measurement time	How long will each measurement take?	Consider subject burden and impact of measurement on subsequent measures.
Measurement frequency	Too frequent measurement may act as a stimulus.	Limit measure frequency to minimize potential carry-over effect.
Measurement posture	Are the measurements validated for use in the measurement posture?	Measure under recommended/standardized conditions.
Methodological		
Study design	Parallel group or randomized cross-over?	Provide a rationale for design choice.
Single vs. multi-day	Time needed for physiological response?	Determine whether a single sitting bout is sufficient to address question.
Randomization process	Which process, and who does it?	Third party, blinded to testing conditions, should complete using established software.
Blinding	Should/can the participant and operator be blinded?	Blind subjects, researchers, and statisticians where feasible.
Adherence to protocol	Will poor adherence reduce the treatment impact?	Measure and report protocol adherence. Report missing data.
Power calculations	Is the study powered to address the question with respect to the primary outcome (under proposed testing condition, and specific to lab/observer)?	Should be pre-planned. Clearly describe calculations.
Statistical	Address dropouts, confounders, and clinical inference.	Describe statistical procedures in sufficient detail to replicate.
Internal validity		
Regression	If subjects are selected on basis of extreme scores, will the distribution regress to mean with repeated testing?	Describe rationale for population selection.
Treatment randomization	How will the intervention be given from one participant to the next?	Randomize treatment allocation
Selection	What should be the appropriate control group condition?	The control group/condition should be appropriately matched to the experimental group/condition.
History	Is there a carry-over or period effect?	Provide rationale for wash-out period and statistically test.
Drop-outs	Are those who remain different?	Consider intention-to-treat framework or decide strategy for drop-outs *a priori*.
Pre-assessment control	Consider physical activity and sleep, caffeine, medications and supplements?	Control and standardize as much as possible. Consider food diaries, accelerometers and other tools for monitoring.
During assessment control	Consider environment (temperature, humidity, noise), meal consumption, fluid replacement, and fidgeting?	Standardize and control environmental conditions.
Instrumentation	Consider precision, accuracy, single-observer, measurement posture, signal interpretation, and covariates?	Ensure choice of instrumentation is appropriate for research question(s), technicians, participants, and protocols.
External validity		
Internal validity	Will internal validity compromise external validity?	Balance robust methods with the need for generalizability.
Sampling	Random sampling and allocation?	Provide clear inclusion/exclusion criteria in the methods.
Sex	Control for menstrual cycle?	Consider whether changes in the menstrual cycle are relevant to your research question(s) and whether participants need to be in a particular phase of the cycle or whether this limits generalizability.
Race	Will race moderate the effect of prolonged sitting on the outcomes?	Provide clear rationale for race stratification and statistical analysis.
Age	Will the same sitting interruption strategies work for young and older populations?	Consider feasibility of strategy in older age groups.
Clinical and Special Populations	Will the findings generalize to clinical (e.g., Type II diabetes) and special (e.g., spinal cord injury) population?	Consider whether tested mechanism(s) or sitting reduction strategies need to be adjusted.

(Continued)

TABLE 3 | Continued

Consideration	Example(s)	Recommendation(s)
Ecological validity		
Feasibility	Will people do the sitting interruption strategy?	Consider the balance between mechanistically optimal vs. feasible sitting interruption strategies.
Time of day	Will testing in the morning (to standardize) conditions limit the findings?	Consider whether the effects of sitting on body systems differ across the day.
Interaction with other behaviors	What other behaviors do people engage while sitting?	Consider whether co-occurrence of lifestyle behaviors impact sitting response

Validity Considerations: Flow-Mediated Dilation section, is that the FMD test was validated for use in the brachial artery, and the vasodilatory responsiveness of this artery is thought to be a barometer of systemic endothelial function (38, 39).

Meta-analytic findings indicate that, after adjusting for confounding risk factors, a chronic increase of 1% (e.g., improving FMD from 6 to 7%) in brachial artery FMD equates to a 13% (95% confidence interval [CI]: 9–17%) reduction in the risk of future cardiovascular events (39). Our group recently conducted a meta-analysis (22 trials, $n = 269$) to summarize the effect of acute uninterrupted sitting exposure on FMD% (25). Uninterrupted prolonged sitting acutely decreased FMD% (weighted mead difference [WMD] = −2.14 %, 95% CI: −2.69 to −1.59), and sub-group analysis revealed that uninterrupted decreased lower- but not upper-limb FMD%. Additionally, we found that sitting interruption increased lower-extremity FMD% (beneficial) compared to uninterrupted sitting (WMD = 1.91%, 95% CI: 0.40–3.42). Subgroup analysis revealed a moderate but non-significant effect for aerobic interventions (WMD = 2.17, 95% CI: −0.34 to 4.67) and simple resistance activities (WMD = 2.40, 95% CI: −0.08 to 4.88) and a trivial effect for standing interruptions (WMD = 0.24, 95% CI: −0.90 to 1.38). In Internal Validity Considerations: Flow-Mediated Dilation section we will explore the internal validity of these findings.

Arterial Stiffness: Pulse-Wave Velocity

Arterial stiffness is dependent on the functional and structural characteristics of a vessel (15, 22, 23). Functionally, acute changes in endothelial function influence arterial stiffness (22, 23). Structurally, arterial stiffness is dependent on the vessel wall extracellular matrix, including elastin and collagen (15). Decreased endothelial function and structural remodeling adversely affect pulsatile hemodynamics, leading to increased pulsatile stress toward end-organs, including the brain (40, 41), and greater arterial wave reflection with a resultant increase in myocardial load (15). A number of approaches are available for measuring arterial stiffness, of which the most widely used is the PWV between the carotid and femoral arteries (cfPWV, **Figure 5**). PWV is the speed at which the forward pressure wave is transmitted between two arterial segments, with faster PWV indicative of increased arterial stiffness. The PWV is traditionally assessed using tonometry, though more recently oscillometric-based devices have simplified the measurement procedure. Commercial oscillometric-based devices automate PWV calculations, confer excellent reliability (intra-class correlation coefficient [ICC]: 0.98), and compare the outcome to known reference values (**Figure 5**) (42).

A meta-analysis of 17 longitudinal studies ($n = 15{,}877$, mean follow-up 7.7 years) reported that for a 1 m/s increase in cfPWV, the risk of future cardiovascular events, cardiovascular mortality, and all-cause mortality increased by 14, 15, and 15%, respectively (43). Our group has reported that uninterrupted prolonged sitting acutely increases cfPWV by 0.2–0.4 m/s (11–14). These changes may seem small in relation to a 1 m/s clinical cut point, but that cut-point is for chronic and not acute changes in cfPWV. The chronic changes in cfPWV are likely driven predominantly by changes in vessel structural, whereas the acute changes in cfPWV are mostly likely driven vessel function. In support, our group and others have reported that acute decreases in endothelial function are associated with increased arterial stiffness (20–22). The importance of these acute—and likely regular—SB-induced increases in cfPWV warrants further research. Additionally, further research is warranted to understand the pathophysiological implications of the 62% reduction if cfPWV we have reported when participants stand 30-min once/h decreased cfPWV by 62% vs. sitting only (11).

METHODOLOGICAL CONSIDERATIONS

An example timeline for an acute sitting study is shown in **Figure 6**. This is for a single-day, uninterrupted sitting study design. Whether or not a single day is sufficient is dependent on the research question, the proposed mechanism(s), and the primary outcome. If a multiple-day protocol is required, consideration should be given to adherence to protocol, including the ability to measure adherence (i.e., *via* use of accelerometry). In the **Figure 6** example the primary outcome, cfPWV, is part of the non-continuous battery of assessments (**Figure 6B**). These measurements are ordered with consideration to the chance of one measurement confounding the next. Continuous measurements may include blood pressure *via* volume-clamp plethysmography and heart rate *via* electrocardiogram. In this example, the non-continuous measurements are made pre- and post-sitting in the supine position, as well as each hour during the sitting bout. Though in accordance with recommendations for cfPWV assessments, hypothesis testing is based on measurements made in the supine position (44, 45). Measurements made in the seated position are used for monitoring trends, though repeated testing should be

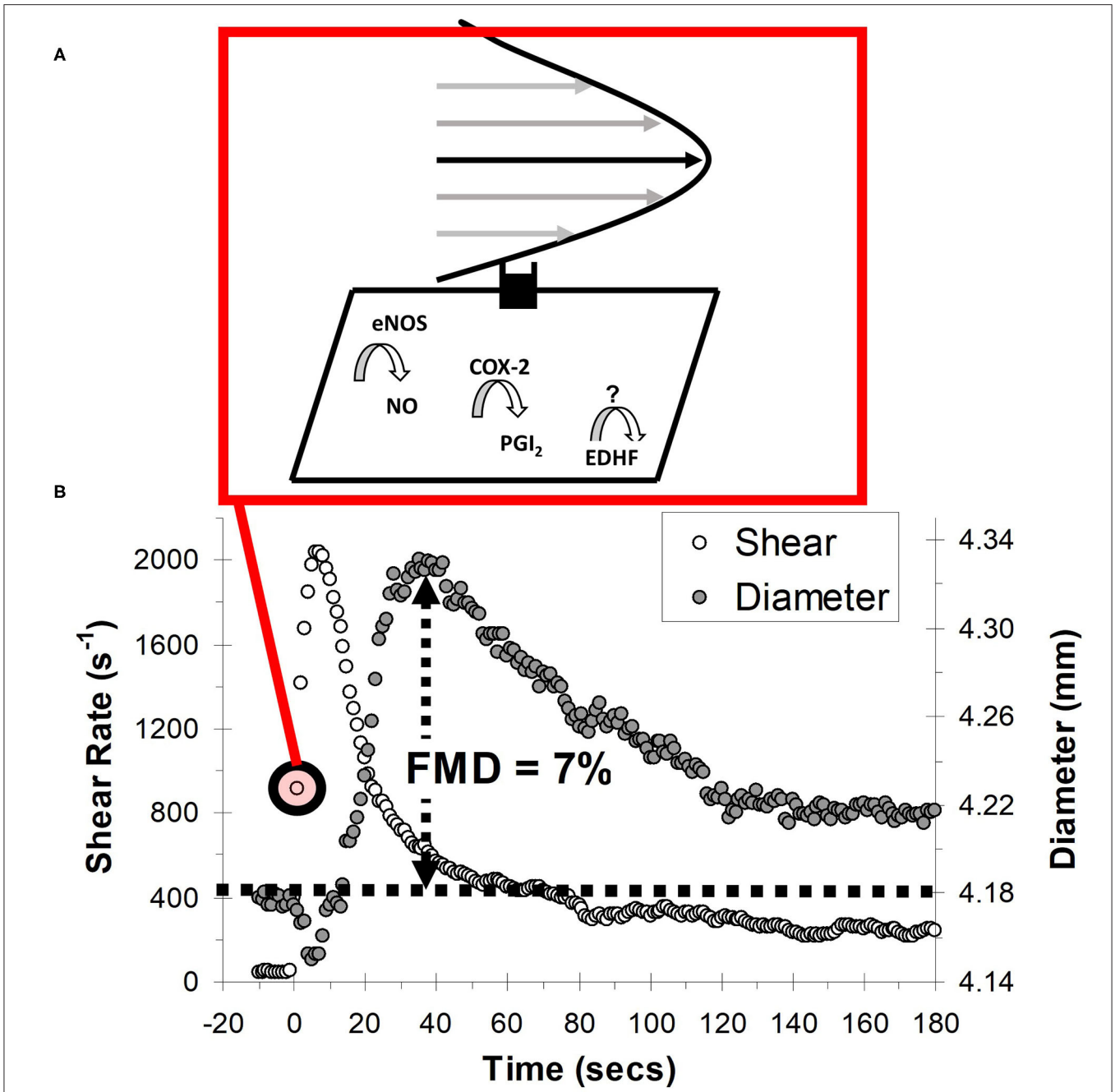

FIGURE 4 | Schematic of the flow-mediated dilation (FMD) test. **(A)** Shear stress created by red blood cells flowing past the vessel wall is detected by mechanoreceptors in the endothelium, triggering a signal cascade whereby vasodilators, nitric oxide (NO) prostacyclin (PGI_2) and endothelial hyperpolarizing factor (EDHF) are produced. Vasodilators subsequently diffuse to the vascular smooth muscle cells, prompting vasodilation. **(B)** Vessel diameter and shear rate responses before and after 5-min ischaemic stimulus. eNOS, endothelial nitric oxide synthase; COX-2, cyclooxygenase-2.

carefully considered as the measurement itself may confound the study outcomes (discussed further below).

The timeline shown in **Figure 6** indicates one condition, uninterrupted sitting. However, it is highly likely that a researcher will want to compare this uninterrupted sitting condition to another condition, such as a strategy to interrupt sitting. For this scenario a decision has to be made as to whether a parallel group RCT design or cross-over study design is used. For practical reasons an RCT may not be feasible, as such designs require large sample sizes, and it is likely that—considering the time requirement—a given laboratory can test only one subject per day. For this reason, cross-over trials have commonly been

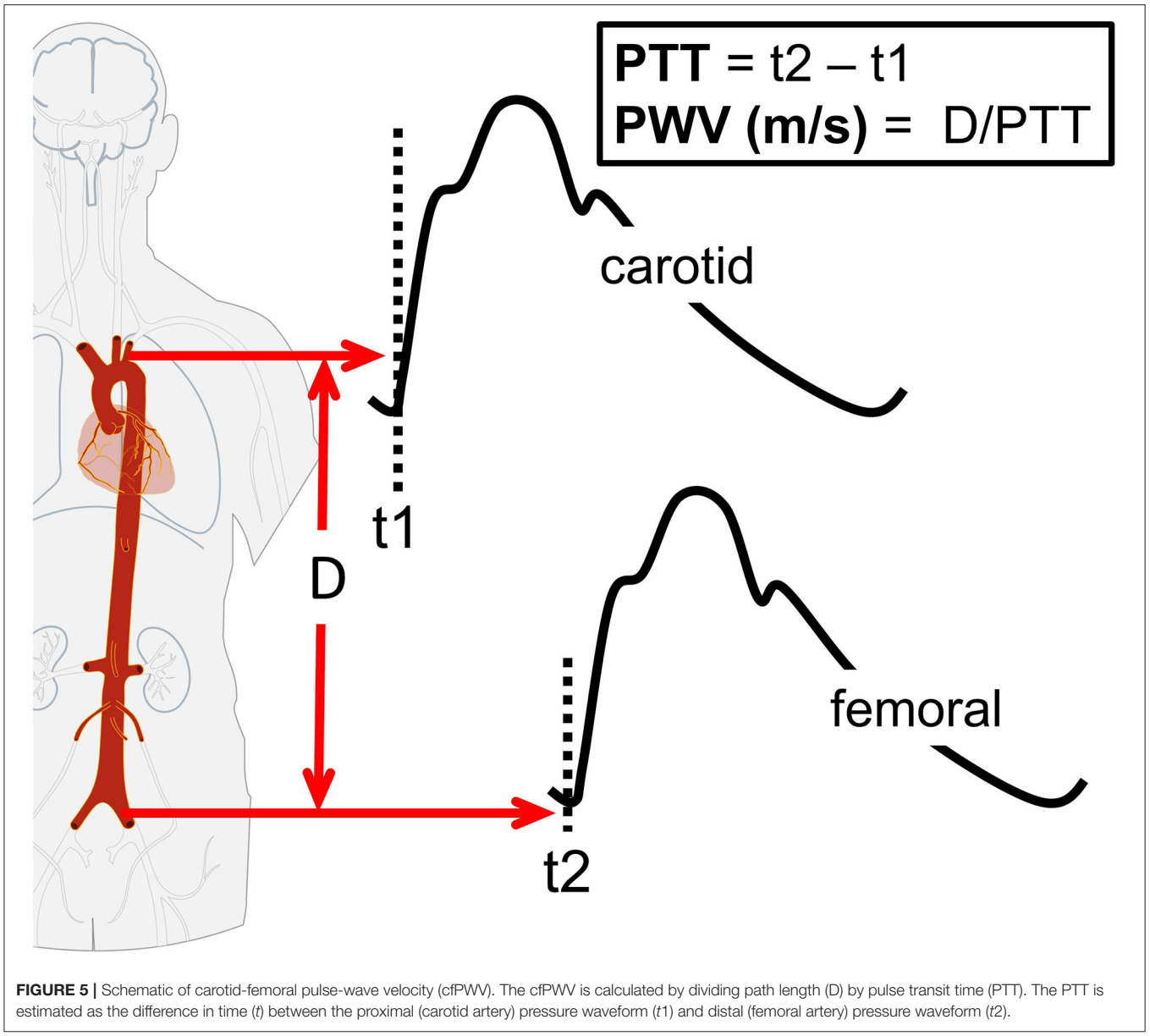

FIGURE 5 | Schematic of carotid-femoral pulse-wave velocity (cfPWV). The cfPWV is calculated by dividing path length (D) by pulse transit time (PTT). The PTT is estimated as the difference in time (*t*) between the proximal (carotid artery) pressure waveform (*t*1) and distal (femoral artery) pressure waveform (*t*2).

used in the literature (11–14). This design permits each subject to serve as their own control, thereby eliminating between-subject variation as a statistical consideration and substantially increasing statistical efficiency. However, as will be discussed in Internal Validity the potential for a carry-over effect needs to be factored into the time between testing sessions. For both types of study design, consideration also needs to be given to treatment randomization. Randomizing subjects to control and treatment groups ensures that allocation to exposure is not determined by a third variable, which itself may influence the outcome. Ideally, consideration is given to both random allocation and random sampling, where random allocation is used to increase internal validity (see section Internal Validity) and random sampling increases generalizability (see section External Validity) to the population of interest. For acute sitting-based studies it is difficult, if not impossible, to blind the subject and research technician to the order of testing. However, the subjects and researcher technicians can be blinded to the condition until each day of testing, and the statistician and any technicians processing outcome data should be blinded.

Cross-over designs pose some additional statistical challenges. One challenge is loss to follow-up, and an additional challenge is accounting for confounders. With respect to loss to follow-up, this can occur when a given subject does not complete all testing days. A confounder is a variable that influences both the dependent variable and independent variable, causing a spurious association. For example, mean arterial pressure (MAP) is an important determinant of PWV (45). A change in MAP during and between each experiment testing day may confound the relationship between the sitting condition(s) and

A

Time	-60	-40	-15	0	10	60	120	180	220	240	245	260
Meal	X											X
Void Bladder		X										X
Bloods	X			X		X		X		X		
Non-cont. measures			X		X		X		X		X	
Cont. Measures			X		X		X	X		X	X	

B

Measure	Leg BF	Carotid BF	Carotid AS	Aortic AS	Leg AS	CBP
Description	1 x 30 s	1 x 30 s	3 X 10s	3 X 10s	3 X 10s	3 X 1 min
Device	US	US	US	PWV	PWV	PWA
Task Time (mins)	1	1	1	2	2	3
Running Time (mins)	1	2	3	5	7	10

FIGURE 6 | Example experimental protocol for an acute sitting study. **(A)** Indicates the time for a 3-h sitting study. Upon arrival to the laboratory the subject consumes a standard small meal/snack and venous blood is collected. The subject then rests quietly in the supine posture for 20-min prior to passive transfer to the seated posture. At the end of the 3-h sitting bout the subject is transferred back to the supine posture. Primary outcomes are collected in the supine posture. The cross marks indicate the measurement timepoints. **(B)** Indicates example non-continuous outcomes, including ultrasound (US)-derived leg blood flow (BF) and carotid arterial stiffness (AS), pulse wave velocity (PWV)-derived aortic (carotid-femoral)- and leg (thigh-ankle)-AS, and pulse wave analysis (PWA)-derived central blood pressure (CBP).

PWV. Collectively, these examples illustrate that the statistical strategy should be able to cope with missing data as well as time varying covariates. For these reasons mixed models have gained popularity.

While the statistical model is important, careful consideration should also be given to clinical inference. Clinical inference refers to whether a "significant" result is clinically beneficial or harmful. A *p*-value provides insight into whether or not the data are statistically significant within the context of the target population, i.e., the probability that the observed magnitude of difference for the sample is different than that of the intended target population, but does not provide information about clinical significance, i.e., whether the findings are likely to be meaningful with regards to physiology or pathophysiology (46). The practice of including statistical and clinical significance allows scientists to better infer the clinical and practical viability of study findings, which in turn will assist in translating the findings.

The magnitude of a clinically significant difference should be considered when making the study power calculation, i.e., the minimum sample size required to detect a true effect. Additional considerations include the importance of type I error (alpha level), which refers to incorrectly rejecting the null hypothesis, and type II errors (beta level), which refers to incorrectly accepting the null hypothesis. Lastly, the precision of the instrument selected to measure the primary outcome should be considered. This precision should be specific to the laboratory setting and observer, and specific to the testing conditions—particularly if measurements are made under non-standard conditions. For example, if FMD and PWV are measured in the seated posture, the precision estimates determined in the supine posture may not be appropriate. Additionally, as will be discussed in the next section, when measurements are made in the seated posture the device assumptions may be violated and measurement accuracy may be reduced.

INTERNAL VALIDITY

The term "validity" is most commonly used in the context of the accuracy of a measurement tool, e.g., does a particular PWV device truly reflect the arterial stiffness of the segment of interest. However, validity also has a related meaning when it comes to the design of a study. There are two classes of threat to the

validity of a study—internal validity and external validity. In the next section, we will discuss external validity. Here we focus on internal validity, which is concerned with the rigor (degree of control) of the study design. Controlling for confounding variables minimizes the potential for an alternative explanation for treatment effects and provides more confidence that any effects are due to the independent variable. For example, a study with high internal validity will control for the factors that may confound the true effect of acute prolonged sitting (independent) on the cardiovascular system (dependent variable).

The first set of threats to internal validity pertain to population. First, there is the threat of *regression to the mean*, which occurs when subjects have been selected on the basis of extreme scores, e.g., low and/or high FMD scores. With repeated testing, extreme (low and high) scores in a distribution tend to move closer to the mean, i.e., they regress (47). The second threat is treatment allocation. There should be an equal chance of allocation to each experimental condition, which can be controlled using *randomization*. In the case of cross-over trials, the order of conditions should be randomized. This process will help to control the confounding impact of the third threat, the "carry-over effect," i.e., treatment one influencing the outcome for treatment two. However, while condition randomization will help to minimize this threat, attention also needs to be given to the minimum wash-out period to reduce any carry-over effect. Depending on the intervention conditions, it is likely that any carry-over effects can be "washed-out" with a minimal number of days. Third, consideration should be given to the *control group*. Control groups strongly affect inferences drawn from a study by ensuring that the interventional effects are beyond normal variation. In the case of a parallel group design, the control group should be appropriately matched to the experimental group(s). For cross-over designs the primary consideration is the *control condition*. That is, what is the appropriate control condition when investigating the effects of acute prolonged sitting on the cardiovascular system? Fourth, consideration needs to be given to *drop-out*, especially if those who drop-out from a study are different from those who remain in the study. This is a particular concern if a parallel group design is used, as drop-out may result in the groups no longer being matched.

The second set of threats pertain to extraneous factors that may confound the relationship between the variables of interest. These threats can be controlled through the appropriate employment of pre-assessment guidelines, and consideration to the environmental conditions during-assessment. Since vascular function is influenced by circadian and diurnal variation, it is typical to control experimental time of day with further consideration to the minimum number of awake hours prior to testing (48, 49). Additionally, changes in lifestyle behaviors can influence vascular function, including sleep, prior physical activity, and diet. While sleep has not traditionally been considered a component of pre-assessment control, arterial stiffness has been reported to increase following acute sleep deprivation (50). Therefore, in addition to establishing a minimum number of awake hours it may be beneficial to ask subjects to go to bed at the same time between visits. Dietary control may include providing a standardized meal for the night prior to testing and asking the subjects to report for testing having fasted. The fasted state is a typical requirement for vascular health testing as different macro- and micro-nutrients can greatly affect vascular function (51, 52). However, for studies incorporating long testing-day conditions, the fasted state may not be feasible. Additionally, having subjects fast may be unethical for certain population groups, such as type II diabetics who are at risk for hypoglycemia. For the aforementioned scenarios, careful consideration needs to be given to the timing and composition of the meal provided prior to testing. Ideally, to ensure consistency between testing sessions, the pre-assessment control factors should be actively monitored, including through the use of accelerometry to monitor physical activity and sleep, and diaries and continuous glucose monitors to monitor diet.

During-assessment, it is important to control environmental conditions, including ambient noise and temperature. Ambient noise may raise arousal, whilst core temperature and skin temperature have both been reported to affect vascular function (53). Fluid replacement should also be considered. Over a prolonged period, there will be insensible water loss, which could lead to a drop-in blood pressure, activate renin-angiotensin aldosterone system and confound the vascular outcome. As such, it important to estimate predicted water loss and put in place a strategy for water replacement. Lastly, considerations should be given to what subjects do and how they behave during the study. In terms of what they do, should they be permitted to engage in their typical desk work (e.g., homework assignment), or should they watch a non-stimulatory documentary to keep the subject awake while minimizing arousal? In terms of behavior, consideration needs to be given to bathroom breaks and how this is controlled for between study visits, as well as incidental movement (e.g., fidgeting) during the study. Ideally an accelerometer is worn on the ankle during the study so that movement can be used as a statistical covariate if needed.

The final set of threats pertains to instrumentation. To investigate the relationship between an independent and dependent variable, an instrument should be able to capture changes in the dependent variable with acceptable accuracy (validity) and precision (reliability). Moreover, it is important that the observer can report acceptable accuracy and precision in their laboratory setting, under the clearly articulated study conditions. For example, if measurements are made in the seated posture the accuracy and precision of measurements in said posture should be reported and used to determine statistical power. We have previously reported that both the seated posture and the non-fasted state decrease the precision of blood pressure and arterial wave reflection readings (54, 55). Additionally, we have reported that changing arm position relative to heart level impacted the generalized transfer function used for pulse wave analysis (56). Lastly, if measurements are made under non-standard conditions (i.e., with respect to guidelines, operating manual, and/or previous validation studies), it is important to understand the device assumptions and whether the non-standard conditions violate these assumptions. For example, pulse wave analysis is commonly used to measure augmentation index, an estimate of arterial wave reflection. One determinant of arterial wave reflection is arterial stiffness, and it may be expected

that increased arterial stiffness during a bout of prolonged sitting would increase arterial wave reflection. However, we have reported a paradoxical decrease in augmentation index during prolonged sitting (12, 14). We rationalized that the decrease was a function of blood pooling in the lower-extremities, which would dampen the pressure wave.

Flow-Mediated Dilation

As noted above, there has been interest on the effects of acute prolonged sitting on FMD as a measure of endothelial function. Our recent meta-analysis found that uninterrupted prolonged sitting decreased lower- but not upper-limb FMD (25). However, in interpreting these findings several considerations should be made, including accuracy, precision, the test assumptions, and measurement posture. Recall that accuracy refers to the closeness of a measurement to a known construct. The FMD test was originally validated by Celermajer et al. (57) for use in the brachial (upper-arm) artery, and shortly thereafter brachial artery FMD was validated against invasively assessed endothelial function in the coronary arteries (58). Subsequently, brachial FMD has been shown to be regulated by endothelium-derived molecules, most notably nitric oxide (NO) (59). As such, we have a good idea as to what brachial artery FMD is telling us (i.e., its validity). However, we know little about the accuracy of lower-limb FMD. Further, we know little about the precision of lower-limb FMD. One study has estimated the precision of popliteal artery FMD, reporting an ICC of 0.25 (60). To put this into context, we calculated the standard error of measurement from this aforementioned study to be 1.74%, meaning that for an average baseline FMD of 4.1%, a relative change exceeding 42% (i.e., 4.1–6.0%) would have to be observed to detect a true effect (61).

Test assumptions and measurement posture will be considered collectively. A major assumption is that the predominant stimuli regulating the FMD response (i.e., change in vessel tone) is the increase in shear stress during reactive hyperemia (38, 62). Shear stress is determined by red blood cells moving close to endothelial cells (see **Figure 4B**). As the fluid particles "travel" through the lumen, their velocity increases from zero at the vessel wall to a maximum value at the center of the vessel. This leads to the establishment of a gradient, which is defined as shear stress. Shear stress therefore acts at a tangent to the wall to create a frictional force at the surface of the endothelium. The endothelial cells are equipped with mechanosensors to detect this stress, enabling vascular tissues to respond with acute adjustments in vascular tone (59). This vasodilatory response reflects alterations in the rate of production of endothelial-derived mediators, including NO (59). NO performs a myriad of anti-atherogenic functions and is considered the most important molecule governing endothelial function and health (59). Therefore, it is assumed that FMD acts as a barometer of the bioavailability of these anti-atherosclerotic NO molecules. Ideally though, the vasodilatory response is adjusted for the shear stress stimulus. Fortunately, the duplex Doppler ultrasound equipment used for FMD can also estimate shear stress as the product of the shear rate and blood viscosity, where shear rate is estimated using an equation based on Poiseuille's law (38, 62). A major assumption of Poiseuille's law is that the velocity profile is parabolic, as depicted in **Figure 4B**. The velocity profile will generally not develop to a full parabola as a consequence of flow unsteadiness and short vessel entrance lengths, but the underestimation is likely not pronounced when resting in the supine position (63). However, in the seated posture, the shear stress profile in the lower-extremities (e.g., femoral or popliteal artery) may become turbulent during reactive hyperemia as a function of increased vessel tortuosity and hydrostatic pressure (64).

An alternative to taking FMD measurement in the seated posture is to take the measurements in the supine posture. Measurements in the supine posture are likely to be more accurate and precise, not only because of greater likelihood of capturing the true shear stress stimulus, but also because the measurements are less technically challenging. However, the caveat is that following the transition from the seated to supine posture, a short period of time, e.g., 10 min, will be required to ensure hemodynamic stability prior to FMD assessment. This may be particularly problematic for sitting interruption studies, as it will extend the time between the last interruption and the FMD assessment and, will likely mask the true interaction effect between prolonged sitting and the sitting interruption strategy. Conversely, by assessing FMD within 10 min of the final interruption (65), it could be argued that the subsequent elevation in shear stress as a consequence of the interruption will also mask the true effect of prolonged sitting. The optimal conditions for assessing the FMD response to prolonged sitting require careful consideration.

Pulse-Wave Velocity

As with FMD assessments, accepted guidelines recommend measuring PWV in the supine posture (45). Additionally, as with any methodology, we should consider accuracy—what is the measurement reflecting? PWV, which is an estimate of arterial stiffness, is dependent on the functional and structural characteristics of a vessel (15, 22, 23). With prolonged sitting it is likely that changes in PWV reflect changes in vessel function. However, it should be considered that the device algorithm was validated in the supine posture (66), and that any changes in PWV in the seated posture may be confounded by changes in autonomic balance. During orthostasis there is a propensity for blood to pool in the sub-diaphragmatic venous system (67). To compensate for blood pooling, and attempt to ensure adequate venous return, the baroreflex response leads to increased lower extremity vascular tone (68). The increase in vascular tone leads to the fixed vessel mass being squeezed into a smaller space, which would increase PWV. To control for this potential confounding PWV should be adjusted for within-condition changes in MAP. However, it should be considered that the adjustment for MAP may not completely control for the confounding as it is a systemic rather than local measure. MAP does increase with vascular tone, but vascular tone is regulated by both autonomic and local factors, and that regulatory factors may differ by vessel segment.

Our group has compared PWV measures in the supine and seated posture (in review). The cfPWV was measured in the

seated and supine postures prior to and following 3-h sitting in 18 young adults (mean age: 23 years, standard deviation [SD: 3] years). We found a posture effect, with higher cfPWV ($\beta = 1.7$ [SD: 0.14] m/s, $p = 0.001$) in the seated posture. However, no posture by time interaction effects were observed, with cfPWV changing similarly over time measures across postures. Across postures, we calculated an agreement of ICC = 0.77 (95%CI: 0.63–0.86) for cfPWV measurements made in supine and seated postures. In a separate study, carotid-radial (cr) PWV and carotid-ankle (ca) PWV were measured in the seated and supine postures prior to and following 7.5 h of sitting in 25 middle-aged adults (42 [SD: 12] years) (69). We again found a posture effect, with higher crPWV ($\beta = 0.43$ m/s, standard error [SE: 0.13], $p = 0.001$) and caPWV ($\beta = 2.04$ [SE: 0.17] m/s, $p = 0.001$) in the seated posture, but no posture by time interaction effect. Similarly, in 20 young adults (26 [SD: 8] years) we compared supine and seated caPWV and crPWV response to a caffeine perturbation, and found posture effects for caPWV ($\beta = 0.55$ m/s, $p < 0.001$) and caPWV ($\beta = 3.59$ m/s, $p < 0.001$), but no posture by time interaction effects (70). We also reported that crPWV (ICC: 0.77 vs. 0.64) and caPWV (ICC: 0.76 vs. 0.36) were more precise in the seated vs. supine posture (70), but that femoral-ankle PWV (faPWV) had acceptable precision in the supine (ICC: 0.83), but not seated posture (ICC = 0.29) (61). A poorer faPWV in a seated posture may be a consequence of orthostasis induced blood pooling compromising waveform detection. In summary, assessment posture does impact absolute PWV measures, but appears not to affect the observed response to prolonged sitting.

The standard measure of PWV is cfPWV, which represents the aorto-illiac pathway (15). This measure is particularly appealing because cfPWV is strongly associated with the risk of cardiovascular events (43), and international reference norms have been established for population, age, and risk factor strata (71). However, cfPWV assessments typically require applanation of the carotid artery. This can be technically challenging in certain populations, including persons who are obese and those with advanced carotid artery atherosclerosis (72), and accuracy may be compromised. Additionally, applanation of the carotid artery can result in a pressor reflex, which can become problematic with repeated measurements as the measurement itself may confound the study outcomes. An alternative PWV measure is heart-femoral PWV, here the "heart" portion can be determined from an electrocardiogram and/or a phonocardiogram. We recently found good (73) ($r > 0.75$) agreement between hfPWV and cfPWV ($r = 0.83$) in 4,133 older-aged (75 [SD: 5] years) adults) (74), and we also found that these measures responded similarly ($r = 0.92$) to an orthostatic challenge (modified tilt-table test), a perturbation known to impact aortic arterial stiffness (75). An additional alternative is brachial-femoral PWV (bfPWV), which can be obtained using oscillometric cuffs placed around the upper arm and upper thigh. Recently, we assessed bfPWV and cfPWV in the seated and supine postures prior to and following 3 h of sitting in 18 young adults (23 [SD: 3] years) and found acceptable overall agreement between the two measures (ICC: 0.74, 95%CI: 0.69–0.84) (in review).

EXTERNAL VALIDITY

External validity refers to whether your study results, obtained from a sample of the population of interest, can be generalized to the population at large. There is a reciprocal relationship between the external and internal validity, in that controlling for one often comes at the cost of the other. For example, with respect to acute sitting-based studies, the research setting is typically constrained to maximize internal validity. For example, it is common to control for population age, sex, time of day, diet, prior physical activity, and activities permitted during the study (e.g., use of the arms and legs). These factors are controlled so that the underlying causes of a phenomena can be established. However, in the real-world setting, the controlled variables may impact the relationship between prolonged sitting and the particular outcome of interest. Therefore, it is recommended that once a particular phenomenon has been established, subsequent consideration should be given to understanding whether said phenomena persists when extended to more presentative populations and settings. Two additional factors, which should be considered irrespective of the desired balance between internal validity and external validity are population sampling and population demographics.

As discussed above, the randomization component of an RCT is typically synonymous with the process through which subjects are allocated to groups. However, this process controls internal validity. To ensure external validity the population of interest should be randomly sampled (47). A number of sitting-based studies have used convenience sampling. That is, they have conducted the study using readily available higher education students. This is not surprising given the resources required to conduct acute sitting studies and the costs required for random sampling. However, with convenience sampling, individuals may not be representative of the larger population, thus, affecting both internal and external validity. To enable the reader to discern the generalizability of findings, the methods section should provide clear details about sampling and group allocations as well as inclusion and exclusion criteria, the results section should clearly articulate the sampling process and demographic characteristics, and the discussion section should contextualize who the study findings can be generalized to Slack and Draugalis (47).

Demographic considerations include age, race, and sex. Older adults (aged > 65 years) exhibit greater SB, with a recent meta-analysis indicating that older people are sedentary for 65–80% of their waking time (76). However, it is currently unknown whether the cardiovascular effects of acute prolonged sitting exposure differ in older compared to younger adults, and whether specific sitting interruption strategies are required to mitigate any unique negative cardiovascular effects. Similarly, we know that CVD mortality rate differs by race (77), but we do not know whether race interacts with the effects of repeated prolonged sitting exposure on the cardiovascular system. An additional consideration is sex. Men and women have different trajectories for the acquisition of CVD over the life course (78, 79). However, little is known about the effects of acute prolonged sitting exposure on the cardiovascular system in men vs. women (80). Further, because fluctuations in estrogen

can affect cardiovascular measures, women are typically studied during the first 1–7 days of their menstrual cycle, or during the placebo week of contraceptive use. Controlling the stage at which women are tested increases internal validity, but at the expense of external validity. Although challenging experimentally, since larger sample sizes and/or more testing sessions will be required, it is important to understand the effects of repeated prolonged sitting exposure across the menstrual cycle so that we can better understand any unique sex effects.

To this point, we have discussed general population sampling and demographic issues, but attention should also be paid to clinical populations. For example, CVD is a major concern in people with Type II diabetes, and it is likely that this population will benefit from the hemodynamic effects of sitting interruption strategies. However, special attention needs to be given to glycemic control. Compared to fasting plasma glucose or glycated hemoglobin levels, hyperglycemic spikes are more strongly associated with CVD in this population (81), and the greatest hyperglycemic spikes occur following the consumption of each daily *ad libitum* meal (82). These spikes trigger oxidative stress, elevate inflammatory cytokines, reduce NO bioavailability, and induce endothelial dysfunction (32, 81, 83). As such, it may be particularly important to plan sitting interruption strategies around meal times in this group. Further, if glycemic control is the key regulatory mechanism, sitting interruption strategies should specifically target reductions in post-prandial glucose.

Recently, the WHO released the first global physical activity and SB guidelines for people living with disability (84). With respect to SB, similar to guidelines published for the general population (2, 3), the advice for reducing SB is vague and non-specific. When designing acute SB studies to address the gaps in knowledge, we need to consider that certain disabilities may preclude these individuals from interrupting SB through conventional means, and that these individuals may be at heightened risk for CMD. For example, one study using data from the Canadian Community Health Survey reported that the prevalence of heart disease (odds ratio [OR]: 2.7, 95%CI: 1.9–3.8) and stroke (OR: 3.7, 95%CI: 2.2–6.2) is much higher among those with a spinal cord injury (SCI) compared to those without (85). We have previously reported that the legs in in individuals with SCI are particularly susceptible to poor vascular health (86). A likely contributing factor is that persons with SCI may not be able to voluntarily contract skeletal muscle in their lower limbs, which limits hemodynamic stressors in this region, including decreased shear stress. To help lessen or reverse these regional abnormalities novel strategies will be required, of which regular sitting interruption may be a viable option. However, the strategies used to interrupt sitting behavior in able-bodied individuals are unlikely to be a suitable option. One potential alternative strategy is neuromuscular electrical stimulation to engage the lower-extremity musculature (87), and an additional is intermittent pneumatic compression therapy. The latter therapy, which is typically used to treat patients with peripheral arterial disease, can be used to raise lower extremity vascular shear stress. We recently reported that a single 60 min bout of intermittent pneumatic compression therapy acutely increased posterior tibial artery FMD by 31% (88).

ECOLOGICAL VALIDITY

Ecological validity tells us whether or not our findings can be generalized to real-world or naturalistic settings. To put this concept into perspective, it is important to understand the mechanisms linking repeated prolonged sitting exposure to increased CVD risk, so that optimal sitting interruption prescription can be identified with respect to the FITT (frequency, intensity, time, and type) principle. However, the optimal FITT prescription will do little to lessen CVD risk if there is poor adherence to the prescription. As such there needs to be some compromise between what is considered to be the physiological optimal prescription vs. what people will actually do. For example, it is common practice to standardize the time of day when conducting an acute sitting-based study. This standardization is important to internal validity as circadian and diurnal variation can affect vascular outcomes (48, 49). In practice, this means our knowledge about the effects of prolonged sitting on the cardiovascular system are limited to effects that occur during the morning. We know little pertaining to whether these cardiovascular responses differ across the day, or whether sitting interruption strategies need to differ across the day to mitigate varying cardiovascular responses. Further, it is unclear if people will adhere to sitting interruption across the day, especially if these interruption strategies need to be varied.

A final consideration is the interaction with additional lifestyle behaviors. When people exercise, they tend to just exercise. This means that an optimal FITT prescription can be applied with limited consideration to other lifestyle behaviors. The same cannot be said for sitting behavior. People tend to engage in other behaviors while they are sitting, including consuming meals and performing stressful activities. This complicates both our physiological understanding and how we prescribe sitting interruption strategies. For example, we recently found that, in young healthy men (n = 13, 22 [SD: 2] years), combining prolonged sitting (3 h) with the consumption of a high-fat meal led to a greater increase in cfPWV (0.59 m/s, 95%CI: 0.29–0.89, Cohen's d = 1.12) when compared to a low-fat meal (0.14 m/s, 95%CI: 0.05–0.34, Cohen's d = 0.44) (in review). Additionally, in young, healthy adults (n = 18, 22.6 [SD: 3.1] years, 33% female) we investigated the effects of prolonged sitting (3 h) combined with the consumption of high- or low-glycemic index meal (26). Global PWV (a measure incorporating cf-, bf- and fa-PWV) increased by 0.29 m/s (95%CI: 0.14–0.45, d = 0.25), with a non-significant time effect. However, glucose variability was significantly correlated with bfPWV (r = 0.52, 95%CI: 0.19–0.71). In sum, these limited data indicate that special attention to concomitant lifestyle behaviors may be required when prescribing sitting interruption strategies.

TRANSLATION

Ultimately, the goal of sitting-based research should be to inform policy that benefits the health of our societies. To provide context to this section, here we detail the considerations applied by the US Preventive Services Task Force (USPSF) when developing policy. The target audience of the USPSF is primary care clinicians,

and its focus is conditions that cause a large burden of suffering to society and that also have available a potentially effective preventive service (89). In developing policy, the USPTF utilize a process similar to that used by many evidence-based groups, which is to systematically review the research literature and judge the strength of the evidence. The USPSTF assigns a grade ranging: from A (strongly recommended) to D (recommend against), or I (insufficient evidence) (89). This grade is assigned following three strata of admissible evidence, as follows: (i) individual studies (study design, internal validity); (ii) the linkage between each key question (aggregate internal validity, aggregate external validity, and coherence/consistency); and (iii) whether the evidence is adequate to determine the existence and magnitude of a causal connection between the preventive service and health outcomes. With respect to (i) individual studies, it is common for evidence-based groups to adjudicate the strength of individual studies based on a hierarchical grading system similar to that presented in **Figure 2**. This grading systems typically assigns the highest grade to RCTs (89). The USPSTF has extended their appraisal system to also consider RCTs cohort studies, case-control studies, and diagnostic accuracy studies (89). When appraising these studies, the USPSTF now separately rates the study design as well as internal and external validity (89). With respect to (ii) the linkage between each key question, the USPSTF considers aggregate internal- and external-validity, and the coherence of the body of evidence. Last, the USPSTF considers (iii) whether the evidence is adequate to determine the existence and magnitude of a causal connection between the preventive service and health outcomes. This includes assessing the magnitude of net benefit, in either words do the benefits outweigh potential harms? Additionally, in accordance with other agencies, the USPSTF considers the likelihood of a biologically plausible mechanism between cause and effect (89–91).

One such policy developed by the USPSTF is: "Healthful Diet and Physical Activity for Cardiovascular Disease Prevention in Adults With Cardiovascular Risk Factors: Behavioral Counseling" (92). The physical activity recommendation received a B grade—a recommendation that the service is routinely provided. Now consider that the first physical activity study was conducted in the 1950s (10), and since this time many well-controlled (internally-valid) RCTs have been conducted across numerous populations, and there is ample evidence to support net benefit to cardiovascular health (2). The sitting-based literature is currently void of well-designed cohort and RCTs that test mechanism-informed sitting interruption strategies. A major reason for this is a lack of true understanding of the effects of repeated exposure to uninterrupted prolonged sitting on the cardiovascular system. We need studies with high internal validity to assess mechanisms (biological plausibility) and to identify sitting interruption strategies that directly tackle the mechanisms of action. Establishing biological plausibility will facilitate the design of well-controlled RCTs that can be replicated across populations. Using the Pyramid of Clinical Evidence (**Figure 2**), we are currently *sitting* somewhere toward the bottom of the pyramid looking up. Accumulating the necessary evidence will take time and resources; physical activity did not receive such a lofty status over-night, but such status does shine a path.

CONCLUSIONS

While leading public health agencies have begun to advocate a "reduction" in sitting behavior, these guidelines are typically vague and non-specific. The lack of specific guidelines for interrupting prolonged sitting bouts is attributable to the lack of available robust evidence to facilitate guideline development. To inform policy, well-designed RCTs are required to test the efficacy of specific and translatable sitting reduction strategies. To assist in the design of RCTs, this review postulated that several gaps in the literature first need to be filled, including the design of acute prolonged sitting-based studies that are internally valid and help to establish biological plausibility. The establishment of biological plausibility will assist the development of RCTs that target the mechanisms through which repeated sitting exposures stress the cardiovascular system. At present, we are some ways from developing specific sitting interruption guidelines. However, if, as a research community we consider the steps necessary to design well-controlled sitting exposure studies, while contemplating the criteria used to aid policy development, we can accelerate guideline development.

AUTHOR CONTRIBUTIONS

LS conceptualized the first draft. All authors contributed to revisions and approved for submission.

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Impact of Chronobiological Variation in Takotsubo Syndrome: Prognosis and Outcome

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Background: A considerable amount of evidence has shown that acute cardiovascular diseases exhibit specific temporal patterns in their onset.

Aim: This study was performed to determine if takotsubo syndrome (TTS) shows chronobiological variations with short and long-term impacts on adverse events.

Design: Our institutional database constituted a collective of 114 consecutive TTS patients between 2003 and 2015.

Methods: Patients were divided into groups defined by the onset of TTS as per time of the day, day of the week, month and quarter of year.

Results: TTS events were most common afternoon and least common in the night, indicating a wave-like pattern ($p = 0.001$) of manifestation. The occurrence of TTS events was similar among days of the week and weeks of the month. TTS patients diagnosed in the month of November and subsequently in the fourth quarter showed a significantly longer QTc interval. These patients also revealed a significantly lower event-free-survival over a 1-year follow-up. In a multivariate Cox regression analysis, TTS events occurring in the fourth quarter of year (HR 6.8, 95%CI: 1.3–35.9; $p = 0.02$) proved to be an independent predictor of lower event-free-survival.

Conclusions: TTS seems to exhibit temporal preference in its onset, but nevertheless this possibly coincidental result needs to be analyzed in a large multicenter registry.

Keywords: takotsubo, chronobiology, heart failure, outcome, complication

INTRODUCTION

The exact pathophysiological mechanism for selective wall motion abnormality in the absence of significant coronary artery stenosis remains unknown in Takotsubo syndrome (TTS). TTS patients may suffer from complications e.g., acute heart failure, ventricular tachyarrhythmias, thromboembolic events and significant mitral valve regurgitation similar to acute coronary syndrome (ACS). Patients often complain about chest pain and/or dyspnea (1–5). TTS has been firstly described 1991. TTS related complications may cause a similar mortality rate as ACS.

Chronobiology is the biomedical science aimed at the study of biological rhythms. Defined by the cycle length, biological rhythms may be divided into three main types: ultradian (period <24 h e.g., hours), circadian (24 h period e.g., days), infradian (>24 h e.g., weeks, months). The natural predilection of the cardiovascular system is oriented to function in an oscillatory circadian order, both in conditions of health or disease. The occurrence of cardiovascular events is not evenly distributed in time but shows peculiar temporal patterns that vary with time of the day, day of the week, and month of the year (6, 7).

There have been reports suggesting a chronobiological difference and circadian variation to the occurrence of TTS (8, 9). However, a description detailing the short and long-term impact of time onset of TTS on adverse events or complications is constrained by the lack of scientific data.

The primary hypothesis of the present study is that circadian variant may have a different impact on the outcome of TTS patients.

METHODS

We retrospectively studied a collective of 114 consecutive patients diagnosed with TTS between January 2003 and September 2015 at our institution, the Medical Faculty of Mannheim. Patients were diagnosed as per the Mayo Clinic Criteria (10), which outlines the clinical features associated with TTS, and the results were reviewed by two independent experienced cardiologists to reaffirm this diagnosis. Patients with a concomitant coronary artery disease were not excluded, but we excluded only patients with a significant coronary plaque, which explains the reason for wall motion abnormality. Eighteen patients with uncertain TTS (due to absence of coronary angiogram and/or follow-up echocardiogram) were excluded from this study.

This study was conducted in compliance with the Declaration of Helsinki concerning investigations in human subjects and the study protocol was approved by the Ethics Committee of the Medical Faculty Mannheim, University of Heidelberg. The need for informed consent was not required by the ethics committee. All methods were performed in accordance with the relevant guidelines and regulations.

Screening for TTS was done prospectively at the TTS event. The assessment of complications associated with TTS was conducted at index-event and at the subsequent follow-up after 1 year. The clinical outcome of patients was assessed by chart review and/or telephone review. If medical records, treating physicians or relatives were unable to substantiate information identifying the circumstances of death, it was defined as death due to an unknown cause.

All patients included in this study were grouped in a standardized fashion as per the timing of their initial 12-lead ECG recordings on admission to the emergency department reflecting the event onset and diagnosis of TTS. This was used to determine if the occurrence of this syndrome was consistent with circadian dependence. The day was spitted into 6-h blocks beginning at 06:00 a.m. The week was divided into 7 days and the year into 12 months. The year was divided into 3-month blocks: spring including March to May, summer including June to August, autumn including September to November, and finally winter including December to February.

The end-point of our study was a composite of thromboembolic events, life-threatening arrhythmias, all-cause mortality, re-hospitalization due to heart failure, stroke, myocardial infarction, and recurrence of TTS as assessed by chart review and/or telephone review over a mean follow-up for 1 year. The selection of endpoint components was based on previous results which have described these complications to influence the prognosis of TTS patients (11). The follow-up was 1 year.

STATISTICS

Our data are presented as means ± SD for continuous variables with a normal distribution, median (interquartile range) for continuous variables with a non-normal distribution, and as frequency (%) for categorical variables. Student's *t*-test and the Mann–Whitney U-test were used to compare continuous variables with normal and non-normal distributions. The chi-squared-test and Fisher's exact test were used to compare categorical variables. Additionally, with the use of the chi-squared "Goodness of Fit" test, observed vs. expected numbers were compared to evaluate whether TTS events are evenly distributed across the day, months, or quarter of year. The log-rank test was used to compare the survival curves between groups. Factors with $p < 0.10$ in univariate analysis were subjected to the Cox multivariate regression analysis to define independent risk factors for the adverse outcome. Data was analyzed in an exploratory manner to generate new hypotheses and therefore we did not adjust the *p*-values with the number of all performed analyses. The IBM SPSS 23.0 or GraphPad InStat were used for statistical calculations. In all analyses, $p < 0.05$ (two-tailed) was taken to indicate statistical significance, $0.05 > p \leq 0.10$ was taken to indicate significance by tendency.

RESULTS

Clinical Features of TTS Patients

The mean age of patients presenting with TTS was 67 ± 11 years, with our data skewing toward a female preponderance (83%), **Table 1**. The most common clinical symptom was chest pain (50.8%), followed by dyspnea (37%). An ST-segment elevation on ECG was observed in 30% and inverted T-waves in 89.5%. A detailed patient history revealed emotional stress in 29% and physical stress in 56% of the patients. LV-function at the time of admission, as measured by transthoracic echocardiography and laevo-cardiography, was moderately reduced (EF 38.3%). The involvement of the right ventricle was documented in 22.8% of the patients. An apical type TTS was observed in 72% ($n = 82$) of patients, whereas the non-apical ballooning form could be diagnosed in 28% ($n = 32$). The most frequent complications associated with TTS were life-threatening arrhythmias (11.4%), pulmonary congestion with need for invasive respiratory support (20%), thromboembolic

TABLE 1 | Baseline characteristics of patients presenting with TTS in the fourth quarter of year.

Variables	All quarters ($n = 114$)	Quarter 1–3 ($n = 81$)	Quarter 4 ($n = 33$)	p-value*
Demographics				
Age, mean ± SD	67 ± 11	67.3 ± 10.9	66.8 ± 12.1	0.86
Female, *n* (%)	95 (83)	65 (80.24)	30 (90.90)	0.16
Symptoms, *n* (%)				
Dyspnoe	58 (50.8)	34 (41.97)	9 (27.27)	0.14
Chest pain	43 (37)	41 (50.61)	17 (51.51)	0.93
Clinic parameter				
Systolic BP, mmHg	131.8 ± 33	134.07 ± 35.10	124.38 ± 22.03	0.17
Diastolic BP, mmHg	76.7 ± 17.5	78.07 ± 18.42	72.22 ± 14.42	0.22
Heart rate, bpm	100.2 ± 27.2	102.19 ± 27.03	96.59 ± 27.53	0.34
ECG Data, *n* (%)				
ST-segment elevation	34 (30)	21 (25.92)	13 (39.39)	0.15
Inversed T-Waves	102 (89.5)	73 (90.12)	29 (87.87)	0.21
PQ-interval	160.5 ± 20.1	160.39 ± 30.65	160.92 ± 25.13	0.93
QTc (ms), mean ± SD	474 ± 69	472.96 ± 48.26	494.59 ± 59.59	0.05
Stress factor, *n* (%)				
Emotional sress	30 (26.3)	24 (29.62)	6 (18.18)	0.20
Physical stress	64 (56.1)	46 (56.79)	18 (54.54)	0.82
None	25 (21.9)	16 (19.75)	9 (27.27)	0.37
Laboratory values, mean ± SD				
Troponin I (U/L)	3.6 ± 5.2	4.17 ± 5.86	2.70 ± 3.79	0.21
Creatine phosphatkinase (U/L)	640 ± 2,609	802.99 ± 3,129.13	288.16 ± 406.62	0.35
CKMB	35.8 ± 59.3	42.48 ± 70.82	22.17 ± 14.48	0.24
C-Reactive protein (mg/l)	49 ± 78.4	55.5 ± 88.2	33.7 ± 48.5	0.21
Hemoglobin	12 ± 2	12.20 ± 2.07	12.01 ± 1.86	0.65
Creatinine (mg/dl)	1.1 ± 0.7	1.21 ± 0.81	1.03 ± 0.40	0.23
GFR < 60 ml/min	32 (28.0)	24 (29.6)	8 (24.2)	0.58
Echocardiography data, *n* (%)				
LV EF%	38.3 ± 9.4	39 ± 10	37 ± 9	0.49
Apical ballooning	82 (71.9)	58 (71.60)	24 (72.72)	0.78
Non-apical ballooning	30 (26.3)	22 (27.1)	8 (24.2)	0.79
Mitral regurgation	60 (52.6)	42 (51.85)	18 (54.54)	0.79
Tricspid regurgation	49 (43)	33 (40.74)	16 (48.48)	0.44
RV-Involvement	26 (22.8)	17 (21)	9 (27.3)	0.47
Medical history, *n* (%)				
Smoking	36 (31.5)	27 (33.33)	9 (27.27)	0.52
Diabetes mellitus	26 (22.8)	19 (23.45)	7 (21.21)	0.79
BMI>25 kg/m²	31 (27.2)	24 (29.62)	7 (21.21)	0.53
Hypertension	66 (57.9)	48 (59.25)	18 (54.54)	0.64
COPD	22 (19.2)	18 (22.22)	4 (12.12)	0.21
Atrial fibrillation	21 (18.4)	16 (19.75)	5 (15.15)	0.56
Coronary artery disease	22 (19.2)	17 (20.98)	5 (15.15)	0.47
History of malignancy	16 (14.0)	10 (12.34)	6 (18.18)	0.41
Drugs on admission, *n* (%)				
Beta-blocker	35 (30.7)	22 (27.16)	13 (39.39)	0.14
ACE inhibitor	35 (30.7)	24 (29.62)	11 (33.33)	0.60
Aspirin	29 (25.4)	18 (22.22)	11 (33.33)	0.17
Anticoagulation	7 (6.1)	5 (6.17)	2 (6.06)	0.98

**p-values for the comparison between group 1 and group 2; SD, Standard deviation; ECG, Electrocardiogram; EF, Ejection fraction; BMI, body-mass-index, COPD, Chronic obstructive pulmonary disease; ACE, Angiotensin-convetring-enzyme.*

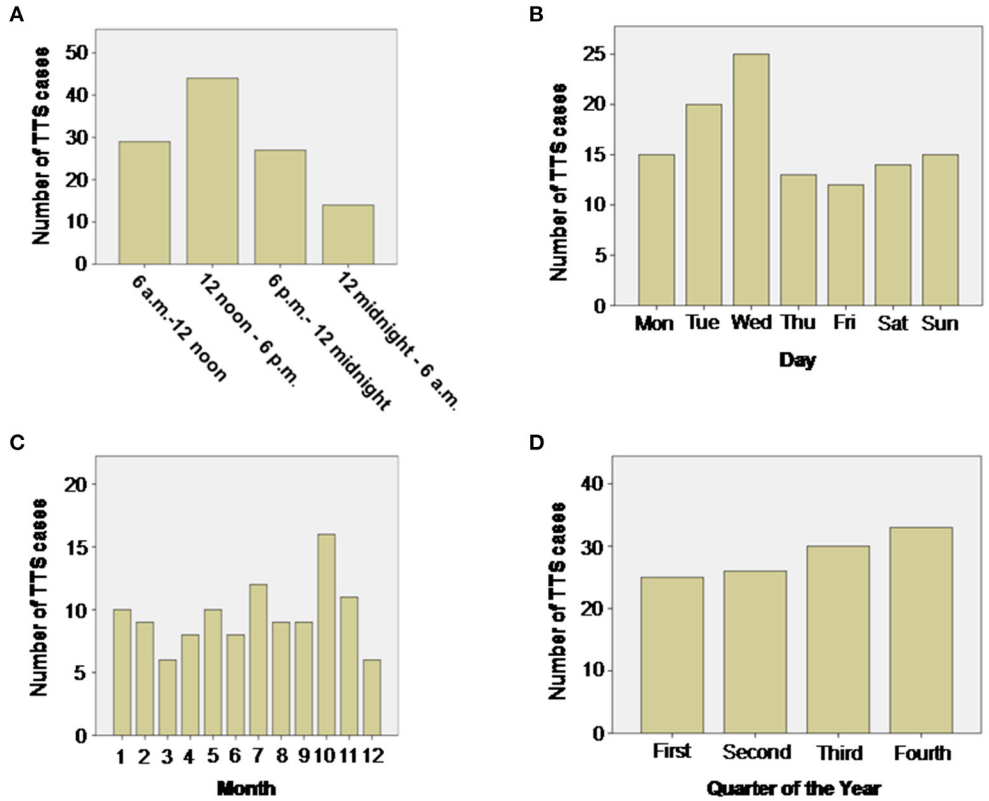

FIGURE 1 | Distribution of TTS events during the day **(A)**, week **(B)**, month **(C)** and quarter of year **(D)**. During the day the number of TTS events show a wave-like pattern with most TTS events taking place in the afternoon between noon and 6 p.m. ($p = 0.001$ Chi-Squared Goodness of Fit Test). No significant differences could be detected by evaluating weeks, months or quarters of year.

events (12.2%), cardiogenic shock (19.2%) and in-hospital death (7.9%).

Descriptive Circadian-, Day-, Month-, and Seasonal- Analysis

The role of diurnal variations influencing the onset of TTS was evaluated by dividing the day into four periods: 6 a.m. to 12 noon, 12 noon to 6 p.m., 6 p.m. to 12 midnight, 12 midnight to 6 a.m. TTS events were most common between 12 noon and 6 p.m. ($n = 44$, 38.5%) and least common between 12 midnight and 6 a.m. ($n = 14$, 12.2%), respectively, depicting a wave-like pattern of occurrence. This distribution was significantly different from the expected equal distribution of $n = 28.5$ TTS cases per 6 h unit (114/4), $p = 0.001$ (Chi-Squared Goodness of Fit Test), **Figure 1A**.

Although the onset of TTS events over a week-long period was statistically insignificant with an expected equal distribution of $n = 16.3$ TTS cases per day ($p = 0.25$), **Figure 1B**, most events were recorded on a Wednesday ($n = 25$, 21.9%) and least on Friday ($n = 12$, 10.5%).

A monthly distribution of TTS events revealed that most cases took place in October ($n = 16$, 14%) and the least in March and December ($n = 6$, 5.5%). However, this distribution was not significantly different from the expected equal distribution with $n = 9.5$ TTS cases per month ($p = 0.67$), **Figure 1C**.

TTS events, grouped quarterly, increased continuously in number with a maximum number of cases diagnosed at the last quarter of the year ($n = 33$, 28.9%), however, this distribution was also not significantly different from the expected equal distribution with $n = 28.5$ TTS cases per quarter of year ($p = 0.70$), **Figure 1D**.

Impact of Time Onset of TTS-Event on the Outcome

TTS events taking place during the four 6 h units of the day were not significantly different in their association with the composite endpoint after 1 year, log-rank $p = 0.76$, **Figure 2A**. The composite endpoint included thromboembolic events, life-threatening arrhythmias, all-cause mortality, re-hospitalization due to heart failure, stroke, myocardial infarction and recurrence of TTS. Time onset of TTS during the day of the week also did not impact the outcome (log-rank; $p = 0.80$), **Figure 2B**. However, patients with TTS diagnosed in the fourth quarter of the year showed a lower event-free survival rate as compared to the other patients (quarter

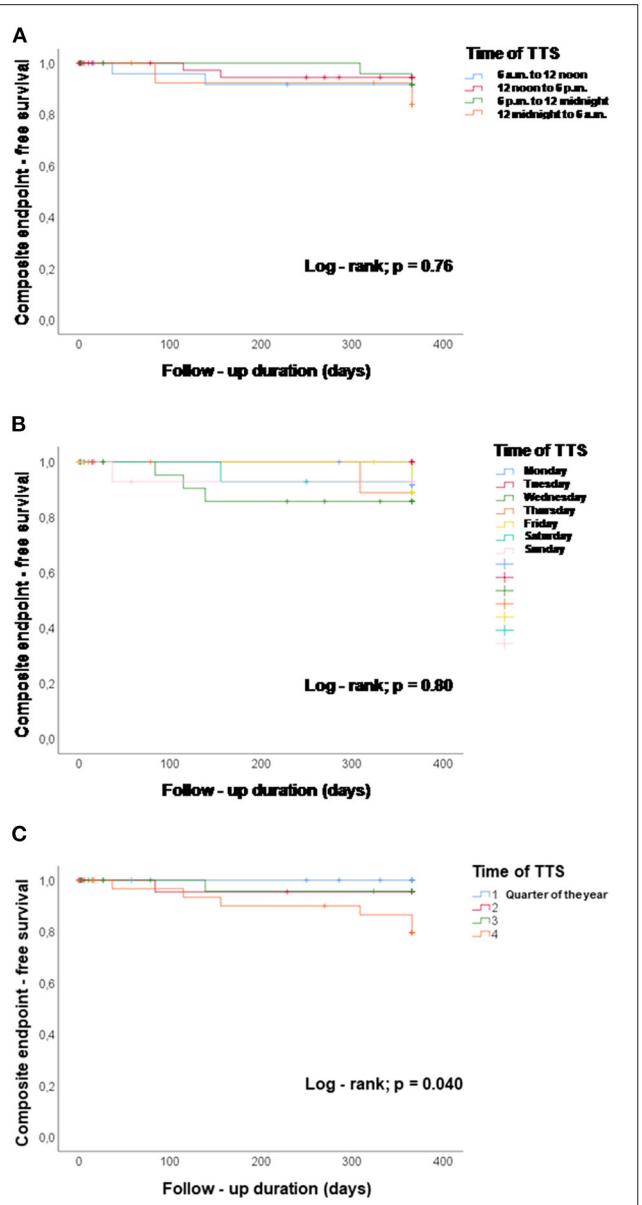

FIGURE 2 | Chronobiological distribution of TTS events and event-free survival (events are defined by the composite endpoint consisting of thromboembolic events, life-threatening arrhythmias, all-cause mortality, re-hospitalization due to heart failure, stroke, myocardial infarction and recurrence of TTS rate). **(A)** TTS events taking place during the four 6 h units of the day were not associated with the event-free survival during 1 year, log-rank $p = 0.76$. **(B)** Time onset of TTS during the day of the week did not impact the event-free survival, log-rank $p = 0.80$. **(C)** Patients with TTS in the fourth quarter of the year showed a lower event-free survival rate compared with the other patients, $p = 0.04$, quarter of year 4 vs. quarters of year 1–3.

of year 4 vs. quarters of year 1–3; $p = 0.04$), **Figure 2C**. Regarding seasonal variations of disease incidences, average temperature in each month was presented together with the monthly incidence of TTS. No significant correlation has been found, **Figure 3**.

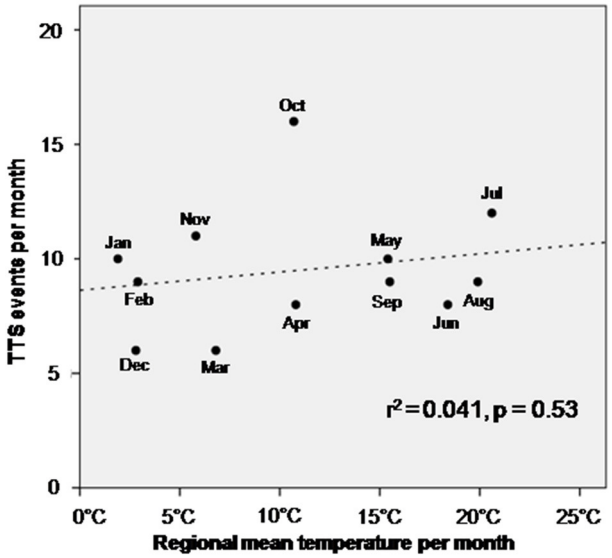

FIGURE 3 | Correlation of temperature per month with the incidence of TTS. No significant association has been observed.

Comparison of November With Other Months

Baseline characteristics of patients diagnosed with TTS in the last quarter were compared to TTS patients from other groups, due to the increased incidence of adverse outcomes in the former.

The values of blood pressure as well as heart rate were not significantly different. ECG-changes like ST-segment elevation and inverted T-waves were present in both groups, without significant difference. However, it was noticed that the QTc interval was significantly longer in TTS patients admitted in the fourth quarter as compared to those patients diagnosed in other months and other quarters (494.59 ± 59.59 ms vs. 472.96 ± 48.26 ms; $p = 0.05$), **Table 1**. The echocardiographic criteria were also similar in both groups. Additionally, in-hospital complications such as life-threatening arrhythmia, cardiogenic shock, in-hospital death, resuscitation and thromboembolic events were similar in both groups, **Table 2**.

In a Cox univariate analysis of TTS events in the fourth quarter of year (HR 3.04, 95%CI: 1.1–8.3; $p = 0.03$), right ventricular involvement (HR 3.22, 95%CI: 0.8–12.9; $p = 0.09$) and glomerular filtration rate <60 ml/min (HR 3.63, 95%CI: 0.8–16.2; $p = 0.09$) were associated with lower event-free survival rate with the composite endpoint. A multivariate Cox regression analysis was performed with adjustment for the known risk factors, to highlight an independent association with a lower-event free survival rate. This analysis revealed that TTS taking place only in the fourth quarter (HR 6.8, 95%CI: 1.3–35.9; $p = 0.02$) was an independent predictor of lower event-free survival rate, **Table 3**.

TABLE 2 | In-hospital events in TTS patients with events in the fourth quarter of year.

Variables	All quarters ($n = 114$)	Quarter 1–3 ($n = 81$)	Quarter 4 ($n = 33$)	*p*-value*
In-hospital events				
Life-threatening arrhythmia	13 (11.4)	8 (9.87)	5 (15.15)	0.42
Resuscitation	9 (7.8)	7 (8.64)	2 (6.06)	0.64
Admission to ICU, length of stay	4.4 ± 6.3	4.4 ± 6.7	4.5 ± 5.3	0.86
In-hospital death	9 (7.9)	8 (9.87)	1 (3.03)	0.21
Acquired long QTs	73 (64.0)	48 (59.25)	25 (75.75)	0.02
Cardiogenic shock	22 (19.2)	16 (19.7)	6 (18.1)	0.94
Treatment strategy				
NPPV and intubation	39 (34.2)	28 (34.56)	11 (33.33)	0.90
Inotropic agents	21 (18.4)	16 (19.75)	5 (15.15)	0.56
Defibrillator-implantation	1 (0.8)	1 (1.23)	0 (o)	0.52
VA-ECMO	1 (0.8)	1 (1.2)	0 (0)	1.00

**p-values for the comparison between group 1 and group 2; NPPV, Non-invasive positive pressure ventilation; VA-ECMO, Veno-arterial extracorporal membrane oxygenation; ICU, Intermediate care unit.*

TABLE 3 | Univariate and multivariate Cox regression analysis of TTS patients reveal TTS events in the fourth quarter of year as an independent predictor with elevated risk for the composite endpoints during 1 year follow up.

	Univariate analysis			Multivariabe analysis		
	HR	95%CI	*P*-value	HR	95%CI	*P*-value
Male	0.84	0.1–6.9	0.87			
Age	1.02	0.9–1.1	0.42			
GFR < 60 ml/min	3.63	0.8–16.2	0.09	0.24	0.05–1.2	0.08
Cardiogenic shock	2.24	0.4–11.6	0.33			
Fourth quarter	3.04	1.1–8.3	0.03	6.83	1.3–35.9	0.02
EF ≤ 35%	2.83	0.6–11.8	0.15			
Right ventricular involvement	3.22	0.8–12.9	0.09	1.51	0.3–7.3	0.60
Inotropic drugs	2.03	0.4–10.0	0.38			
DM Typ II	0.42	0.0–3.4	0.42			
Hypertension	1.19	0.3–4.9	0.80			
Apical ballooning	2.83	0.3–23.0	0.33			
Smoking	0.66	0.1–3.3	0.61			

The composite endpoints consisted of thromboembolic events, life-threatening arrhythmias, all-cause mortality, re-hospitalization due to heart failure, stroke, myocardial infarction and recurrence of TTS rate.
HR, hazard ratio; EF, ejection fraction, CRP, C-reactive protein; GFR, glomerular filtration rate.

DISCUSSION

Our retrospective clinical investigation of these 114 consecutive TTS patients, has helped us summarize the following; (i) The occurrence of TTS exhibits a circadian rhythm; (ii) the event-free survival rate is significantly lower in TTS patients admitted in the fourth quarter of year; (iii) TTS patients presenting with TTS in fourth quarter of the year show a significant QTc prolongation in comparison to the general TTS population.

Mechanisms dictated by the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic adreno-medullary system maintain biological homeostasis and protect living organisms from internal and external stresses during environmental and physiological challenges. The HPA axis is activated by corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus, stimulating production of glucocorticosteroids (12). The central biological clock controls the HPA, with cortisol levels maintaining a robust daily rhythm, while circulating levels exhibit an early morning peak.

ACS seems to exhibit some temporal preference in its onset, and is characterized by diurnal variations, variations as per day of the week as well as monthly variations, albeit with analogies and differences. Each of these temporal frames has been discussed in detail (9, 13–19).

A meta-analysis extrapolating data from 30 studies on ACS and 19 studies on sudden cardiac death estimated that nearly 27.7% of morning cases of ACS and 22.5% of sudden cardiac deaths were attributable to an increased risk at that time of day. Recent studies give credence to the hypothesis that there exists a Monday preference for the occurrence of ACS (20). In cases

of TTS, however, this data is controversial. Our study suggested a wave-like pattern of occurrence throughout the day with a predominance of TTS events in the afternoon between noon and 6 p.m. and fewer events at night/early morning between midnight and 6 a.m. This data correlates with studies by Sharkey et al. (9, 18, 21) from USA. In that study, the day was divided in 4 h rhythm beginning at 12.00 a.m. In this study the TTS rate was higher presented on Tuesday compared to a higher presence on Wednesday as illustrated in the present study. Additional data from Japan, France, Korea and Italy have presented higher TTS event rates in the morning (8, 9, 18, 21, 22). Kurisu et al. showed a higher presentation of TTS from 06.00 to 06.00 p.m. In that study, the day was divided into six periods in Japan. Song et al. divided the day into four periods and presented a similar result in Korea. Previtali et al. showed also a consistent data of the study group in Japan and Korea. In a study from Italy, the day was also divided into four periods. Data of the present study presenting an afternoon peak of TTS events and its difference to other studies may reflect different habits in different countries. The recruitment of patients in all these studies was in particular similar including patient chart review with retrospective and/or prospective character.

A Monday preference for TTS events has been suggested in studies from Italy and Korea, although our data did show a possible Wednesday preference for occurrence of TTS (increasing from Monday to Wednesday but not reaching significance), more like data reported from studies conducted in the USA (9, 22, 23). Sharkey et al. presented a study suggesting an increased frequency of TTS events occurring in winter. In contrast, studies form Italy and Korea suggest a predominance of TTS events occurring in summer (19, 22). Our data suggested a possible increase in TTS cases reported from the first quarter of year to the fourth with predominance in autumn but without significance. These differences might be linked to differences of the weather in each country.

Increases in plasma catecholamines and cortisol, heart rate, blood pressure, coronary vasomotor tone and platelet aggregation predispose to coronary artery occlusion and this has been correlated to the early morning circadian peak characteristic of ischemic heart disease–related events (24–26). In contrast, the afternoon peak shown here in TTS is consistent with the importance that environmental factors play as triggers in this condition. The predilection of TTS events for early afternoon hours reported here offers some potential insights into the pathophysiology of this condition.

It has been suggested that diurnal variations may have an impact on the clinical outcome of ACS patients, because several fatal cases were diagnosed in the morning, independent of patient age and infarction site or extension (27). A correlate establishing chornobiological variations similarly influencing the outcome of TTS patients can be drawn based on this existing data. TTS patients admitted in the fourth quarter of the year showed a significantly decreased event-free survival rate after a follow-up of 1 year. This finding may support the evidence that the pathophysiology of TTS is still not clearly defined.

Our data offers a detailed analysis of the chronobiological association of TTS events in a German population for the first time, and additionally, detail its influence on the reported adverse outcomes.

This study had a few limitations; firstly, this was a single-center retrospective case series study including patients admitted over the period of 13 years without a comparative control group and therefore there is a need to evaluate results in large multicenter registries. Secondly, data was analyzed in an exploratory manner to generate new hypotheses concerning time-dependency of TTS events and associated adverse events. Therefore, we cannot rule-out random or accidental findings. The timing of onset of TTS may vary because some patients who had a symptom at night might come to the hospital on the next day.

CLINICAL PERSPECTIVES AND TRANSLATIONAL OUTLOOK

TTS seems to exhibit temporal preference in its onset. The chrono-biological distribution of TTS may influence the outcome of patients. These novel findings may help to identify high risk TTS patients with a need of subsequent follow-ups.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: original data are available under request according to the ethical committee. Requests to access these datasets should be directed to ibrahim.el-battrawy@medma.uni-heidelberg.d.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by MA 2018/KA. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IE-B, AA, SL, XZ, UA, MB, and IA designed the research and wrote the paper. IE-B, IA, and TG performed the clinical research. IE-B, SL, AM, XZ, NU, TG, and IA analyzed data. All authors contributed to the article and approved the submitted version.

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Social Jetlag and Cardiometabolic Risk in Preadolescent Children

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Objective: Childhood cardiometabolic disease risk (CMD) has been associated with short sleep duration. Its relationship with other aspects of sleep should also be considered, including social jetlag (SJL) which represents the difference between a person's social rhythms and circadian clock. This study investigated whether childhood CMD risk is associated with sleep duration, sleep disturbances, and SJL.

Study Design: The observational study included 332 children aged 8–10 years (48.5% female). The three independent variables were sleep duration, sleep disturbances, and SJL. SJL was calculated as the variation in hours between the midpoint of sleep during free (weekend) days and work/school days. Eleven cardiometabolic biomarkers were measured, including central blood pressure, lipids, glycated hemoglobin, arterial wave reflection, and glucose. Underlying CMD risk factors were identified using factor analysis.

Results: Four underlying CMD risk factors were identified using factor analysis: blood pressure, cholesterol, vascular health, and carbohydrate metabolism. Neither sleep disturbances nor sleep duration were significantly associated with any of the four CMD factors following adjustments to potential confounders. However, SJL was significantly linked to vascular health ($p = 0.027$) and cholesterol ($p = 0.025$).

Conclusion: These findings suggest that SJL may be a significant and measurable public health target for offsetting negative CMD trajectories in children. Further studies are required to determine biological plausibility.

Keywords: circadian clock, social clock, activity behavior, sleep duration, vascular, social jetlag (SJL), metabolic

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INTRODUCTION

Cardiometabolic disease (CMD) etiology is insidious, with the trajectory beginning during childhood or potentially pre-birth (1–4). Childhood is important because biological systems are highly malleable, responding to the interplay between genetic and environmental cues (3, 4). Environmental cues include exposure to the activity behaviors of sleep, sedentary time and physical activity (2–4). The importance of these activity behaviors during childhood has been emphasized through shifts in public health policy. For example, the recent Canadian 24-h Movement Guidelines

advised on the amount of time children should engage in each activity behavior across the day in a digestible format (5). With respect to sleep, the guidelines recommend that children and youth (aged 5–13 years) should sleep for 9–11 h each night. However, while insufficient sleep length is related to increased CMD risk in children (6–9), other aspects of sleep, such as social jetlag (SJL), should be considered. Social jetlag is defined as the discrepancy between an individual's biological and social rhythms and, in children, is calculated as the total difference in the midpoints of sleep on weekdays vs. weekend days (10, 11).

Physiological processes, including glucose metabolism and blood pressure, display a circadian rhythm that is controlled by the internal circadian biological clock (12, 13). For children living in today's society, social obligations, particularly school schedules, can lead to SJL and disruption of circadian rhythms. Studies in children and early adolescence have reported associations between SJL and measures of body composition (14, 15). One of the studies found a statistically non-significant association between SJL and CMD risk (15). CMD risk was calculated as the linear sum of z scores for systolic blood pressure (SBP), high-density lipoprotein cholesterol (HDL), triglycerides (TG), waist circumference, and homeostatic model of insulin resistance (HOMA). This linear combination approach, which assumes equal weighting for each variable, is prone to multicollinearity and fails to recognize that some risk factors cluster (16). Alternatively, a data driven approach, such as factor analysis, can determine the appropriate weighting for each predictor, eliminate the problem of multicollinearity, and identify risk factor clusters (17).

This study investigated whether CMD risk in preadolescent children is associated with sleep duration, sleep disturbances, and SJL. Novel and conventional CMD risk stratification biomarkers were used in factor analysis to classify CMD risk factor clusters. We evaluated the hypothesis that SJL, independent of sleep duration and sleep disturbances, would be associated with CMD risk factor clusters.

METHODOLOGY

This registered cross-sectional study (ACTRN12614000433606) meets the conditions of the New Zealand Health and Disability Ethics Committees (HDEC: 14/CEN/83), and accords with STROBE reporting guidelines (18).

Participants

Between April 2015 and April 2016, children (aged 8–10 years) were recruited from schools within three regions (Canterbury, Otago, Wellington) across New Zealand. Schools were classified according to a decile system based on the predominant socioeconomic status (SES) of attending students. We categorized schools as low SES (Deciles 1–5) or high SES (Deciles 6–10), and randomly sampled from these strata. All children were eligible to participate, unless they had experienced orthopedic injury in the previous 3 months or had been prescribed cardiovascular medications. An information packet was provided to eligible children, and parental/guardian consent and child assent were obtained prior to data collection.

Experimental Design

Data collection took place at the participating schools. Cardiometabolic measurements were made on a single day (Monday–Thursday) between the 9:00 and 12:00 o'clock hours. Children were asked to refrain from exercise for 24 h prior to measurement, be adequately hydrated, and have fasted for at least 3 h. The primary caregiver was asked to complete a questionnaire at home, which provided demographic and sleep habits data. Only children with complete sleep data and cardiometabolic health information were included in the analyses.

Independent Variables

The three assessed sleep variables were sleep duration, sleep disturbances, and SJL. The participant's caregiver was asked to report the times their child typically rested and woke on both school days and free (weekend) days. The mean of sleep duration was calculated using a ratio of two weekend days to five weekdays. Social jetlag was estimated as the total discrepancy between the midpoints of sleep on weekend days vs. weekdays (10). Single items of customary school/weekday sleep display sufficient concurrent validity with actigraphy and diary records (19).

The Children's Sleep Habits Questionnaire (CSHQ) was utilized to estimate sleep disturbances, for which adequate test–retest reliability, discriminant validity, and internal consistency have been reported (20). The questionnaire consisted of 33 questions on a 7-point Likert-type scale ranging from Always (1) to Never (7) with higher scores indicative of greater sleep disturbances. The sum of all 33 scored CSHQ questions (with a potential range of 33–99), was used to calculate a Total Sleep Disturbance score. A Total Sleep Disturbance score >41 was applied to signify troubled sleep, as this cutoff has been revealed to precisely identify 80% of children with a clinically detected sleep disorder. Only the Total Sleep Disturbance score was used for this study (20).

Dependent Variables

Eleven cardiometabolic variables were measured: central hemodynamics [augmentation index (AIx), central systolic blood pressure (cSBP), heart rate (HR)], peripheral hemodynamics [diastolic blood pressure (DBP) and SBP], cardiac blood markers [HDL, TG, total cholesterol (TC), low density lipoproteins (LDL)], metabolic blood markers [glycated hemoglobin (HbA1c) and fasting blood glucose (FBG)].

Peripheral and Central Hemodynamics

Pulse wave analysis (BP+, Uscom, Sydney, Australia) was applied to gauge all central hemodynamic variables. Oscillometric

Abbreviations: AIx, augmentation index; β, beta; cSBP, central systolic blood pressure; CSHQ, Children's Sleep Habits Questionnaire; DBP, diastolic blood pressure; FBG, fasting blood glucose; HbA1c, glycated hemoglobin; HDL, high density lipoproteins; HR, heart rate; LDL, low density lipoproteins; mmHg, mmol/L, millimoles per liter; %, percentage; mmHg, millimeters of mercury; *n*, sample size; SBP, systolic blood pressure; SD, standard deviation; SES, socioeconomic scale; SJL, Social jetlag; TC, total cholesterol; STROBE, Strengthening the Reporting of Observational Studies in Epidemiology; TG, triglycerides; WD, weekday; WE, weekend.

pressure waveforms were recorded by a single operator on the left upper arm following 20 min of undisturbed supine rest (21). Each measurement cycle was ∼40 s, comprising a 10 s suprasystolic recording, followed by a brachial blood pressure recording. Utilizing a validated transfer function, an aortic pressure waveform was generated from which cSBP was estimated. Arterial wave reflection (AIx) was estimated from the suprasystolic waveform by means of the formula AIx = (P3 – P0)/(P1 – P0), where P0 denotes the pressure at the onset of the pulse, P1 the peak pressure of the incident wave, and P3 the peak pressure of the reflective wave. This index explains the relative height of the reflected pressure wave when compared to the incident waveform. The recordings with a quality signal were the only ones accepted (sign to noise ratio of >3dB). Two measurements were recorded, with a third if blood pressures differed by >5 mmHg or the AIx by >4%. Our group has reported acceptable between-day reliability for cSBP (ICC: 0.90), cSBP (ICC: 0.94) and AIx (ICC: 0.71) (22).

Cardiac and Metabolic Blood Markers

Capillary blood was collected to measure fasting HbA1c (A1CNow+, PTS Diagnostics) (23), and TG, FBG, TC, HDL and LDL (CardioChek PA, PTS Diagnostics) (23).

Sample Size

Factor analysis was utilized to calculate the sample size, for which two dissimilar methods have been advocated based on: (i) the ratio of participants to variables, or (ii) a minimum total sample size (24). (ii) The recommended minimum participant to item ratio ranges from 5:1 to 10:1. For eleven variables, a minimum of 110 participants would be required using the 10:1 ratio. Minimum sample sizes, ranging from 50 to 400 have been recommended. Comfrey and Lee state that, "the adequacy of sample size might be evaluated very roughly on the following scale: 50–very poor; 100–poor; 200–fair; 300–good; 500–very good; 1000 or more–excellent" (24). The $n = 332$ for the current study meets Comfrey and Lee's "Good" criteria (24).

Statistical Analysis

The Jamovi (v1.1.3.0) platform was used for statistical analysis. Participant demographic data were reported as mean and standard deviation. Regression outcomes are reported as beta (β) with 95% confidence intervals (95% CI). The significance level was set *a priori* for all statistical procedures at $\alpha = 0.05$. The corresponding author (NC) had full access to the data in the study and was responsible for the integrity of the dataset. Anonymized data will be shared upon reasonable request.

CMD risk factors were identified using factor analysis (17). Factor analysis is a data-driven approach that can be used to identify how cardiometabolic variables cluster together. The assumption is that for a collection of observed variables there is a set of latent variables (factors) that can explain the interrelationships among those variables. This data-driven approach confers several advantages beyond simply summing *Z* scores: (i) accurate determination of predictor weighting; (ii) control for collinearity; and (iii) provision of additive information by the factors, beyond the individual components (17). The number of factors (i.e., latent variables) was determined by the minimum eigenvalue principle of greater than one for a factor analysis of the correlation matrix. The implication being that if an eigenvalue is greater than one the derived dimension captures less variability in the data than any single variable. Following orthogonal varimax rotation, the correlation between the derived factors and the underlying variables were used to interpret (label) each factor. We used a loading of >0.40 to interpret the factor pattern.

The associations between CMD risk and the three sleep measures were examined using linear regression analyses. To account for the clustered data (students within schools), we used Gaussian family generalized estimating equations with robust standard errors (25). Following the assessment of model assumptions, separate univariate analyses examined the association between each independent (average sleep duration, SJL, sleep disturbances) and each dependent variable (CMD risk factors), followed by a multivariable analysis. Two multivariable models were specified. Model 1 included the three sleep variables without further adjustments. Model 2 was additionally adjusted for school decile rating, ethnicity, age, and sex.

RESULTS

Participants

Complete sleep and cardiometabolic data were available for $n = 332$ (**Figure 1**). These children were not discernibly different from the full 392 participants (9.6 ± 1.1 years, 48.5% female, 29.2% overweight, 51.5% low decile school). The demographic data are presented in **Table 1**. Most of the participants (92.8%, $n = 308$) slept for at least 9 h nightly, and the mean of the total sleep duration did not differ for the week compared to the weekend (10.1 vs. 10.1 h, $p = 0.453$). Less than half (39.2%) the students reported disturbed sleep. All but two of the participants that reported going to bed later on the weekend (mean: 42 min, 95% CI: 44, 50), and 97% ($n = 330$) also reported waking later on the weekend (42 min, 95% CI: 37, 46). Consequently, the average SJL was 44.4 ± 31.7 min, and 36.1% had >1-h SJL.

Sleep and Cardiometabolic Disease Risk

The outcomes from factor analysis are reported in **Table 2**. The table displays the association of each variable with the four factors. The factors are labeled, in order of variance explained by the model: Blood Pressure, Cholesterol, Vascular Health and Carbohydrate Metabolism (CHO-MET). For example, Factor 1, which explains 23% of the cumulative variance, is labeled Blood Pressure because the variables which load most strongly onto the factor are the blood pressures.

Table 3 displays the univariate models. Sleep disturbances and sleep duration were not significantly associated with any CMD risk factor. Social jetlag was associated with cholesterol (β: −0.110, 95% CI: −0.416, −0.008) and vascular health (β: 0.162, 95% CI: 0.103, 0.501). Multivariable models 1 and 2 are shown in **Table 3**. Following adjustments for potential confounders (Model 2), SJL retained significant associations with cholesterol (β: −0.126, 95% CI: −0.448, −0.030) and vascular health (β: 0.125, 95% CI: 0.027, 0.438) risk factors.

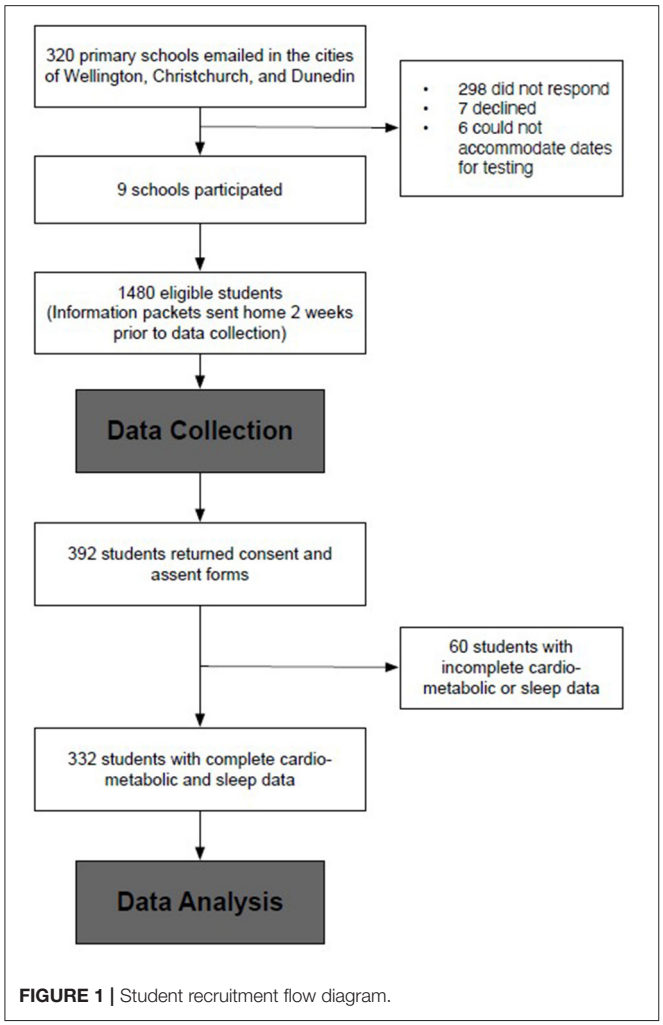

FIGURE 1 | Student recruitment flow diagram.

TABLE 1 | Participant demographics stratified by sex ($n = 332$).

	All	Male	Female
Categorical variables	***n* (%)**	***n* (%)**	***n* (%)**
n	332 (100.0)	171 (51.5)	161 (48.5)
Female	161 (48.5)	0 (0.0)	161 (100.0)
Ethnicity			
New Zealand European	269 (81.0)	143 (83.6)	126 (78.3)
Māori	37 (11.1)	16 (9.4)	21 (13.0)
Pacific	22 (6.6)	10 (5.8)	12 (7.5)
Not recorded	4 (1.2)	2 (1.2)	2 (1.2)
School year			
4 (7–8 years old)	72 (21.7)	35 (20.5)	37 (23.0)
5 (8–9 years old)	93 (28.0)	51 (29.8)	42 (26.1)
6 (9–10 years old)	105 (31.6)	54 (31.6)	51 (31.7)
7 (10–11 years old)	62 (18.7)	31 (18.1)	31 (19.3)
School decile			
Low (≤5)	171 (51.5)	89 (52.0)	82 (50.9)
High (>5)	161 (48.5)	82 (48.0)	79 (49.1)
Obesity status			
Overweight-obese	235 (70.8)	121 (70.8)	114 (70.8)
Non-overweight	97 (29.2)	50 (29.2)	47 (29.2)
Sleep			
>9 h of sleep	308 (92.8)	159 (93.0)	149 (92.5)
>1 h of SJL	120 (36.1)	60 (35.1)	60 (37.3)
Sleep disorder	130 (39.2)	65 (38.0)	65 (38.0)
Continuous variables	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
Sleep outcomes			
WD sleep duration, hours	10.1 (0.89)	10.1 (0.89)	10.1 (0.91)
WE sleep duration, hours	10.1 (1.03)	10.1 (0.99)	10.1 (1.08)
Sleep duration, hours	10.1 (0.84)	10.1 (0.83)	10.1 (0.86)
Social jetlag, hours	0.74 (0.53)	0.68 (0.50)	0.80 (0.55)
Cardiometabolic outcomes			
Age, years	9.55 (1.13)	9.54 (1.06)	9.57 (1.20)
Systolic blood pressure, mmHg	101 (7.44)	101 (7.44)	101(8.18)
Diastolic blood pressure, mmHg	61.8 (6.24)	61.2(6.16)	62.4 (6.35)
Central systolic blood pressure, mmHg	93.5 (7.73)	93.2 (7.19)	93.7 (8.27)
Total cholesterol, mmol/L	3.57 (0.61)	3.5 (0.64)	3.64 (0.57)
Low-density lipoprotein cholesterol, mmol/L	1.85 (0.51)	1.78 (0.48)	1.91(0.53)
High-density lipoprotein cholesterol, mmol/L	1.47 (0.36)	1.51 (0.39)	1.43 (0.32)
Augmentation index, %	55.8 (15.2)	55.5 (15.0)	56.1 (15.5)
Heart rate, bpm	74.9 (11.5)	72 (10.2)	77.8 (12.3)
Fasting blood glucose, mmol/L	5.05 (0.39)	5.13 (0.39)	4.96 (0.38)
Triglycerides, mmol/L	1.47 (0.36)	0.839 (0.47)	0.892 (0.38)
Glycated hemoglobin, %	5.12 (0.31)	5.11 (0.31)	5.12 (0.31)

WD, weekday; WE. weekend; n, sample size; mmHg, millimeters of mercury; mmol/L, millimoles per liter; %, percentage; SD, standard deviation, effect size (Cohen's d).

DISCUSSION

The major finding of this study is that neither sleep disturbances nor sleep duration significantly correlated with any of the four CMD risk factors. However, SJL was significantly associated with cholesterol and vascular health factors. These findings suggest that SJL may be an important sleep characteristic with respect to CMD risk in children.

Strengths and Limitations

The limitations and strengths of the current study are discussed here to provide context to the discussion that follows. First, we attempted to ensure the findings are generalizable to children across New Zealand by recruiting participants from three regions across the country. However, the findings may not generalize to populations outside of New Zealand. Second, this was a cross-sectional study and association is not causality. Longitudinal and intervention analyses are warranted to validate the results of the current study. Third, parent-report questionnaires were used to collect the sleep data rather than objective measures. While concurrent validity has been reported for the single item sleep surveys compared to actigraphy and diary data (19), future studies with objective assessments are warranted. Last, while our CMD risk model did include both FBG (short term glycemic

control) and HbA1c (chronic glycemic control), insulin was not measured. There is some evidence that, at least in obese children, hyperinsulinemia precedes impaired fasting glucose control (13, 26). Future CMD risk models may be improved through the addition of insulin. The key strengths included the use of a data-driven approach for characterizing CMD risk, the co-assessment of three sleep variables thought to be important to a child's health, and our relatively large and representative cohort of New Zealand-based preadolescents.

TABLE 2 | Factor analysis and eigenvalues ($n = 332$).

	Factor 1	Factor 2	Factor 3	Factor 4
	Blood pressure	Cholesterol	Vascular	CHO-metabolism
Systolic blood pressure	**0.90**	0.06	0.16	−0.01
Diastolic blood pressure	**0.89**	−0.01	0.04	−0.05
Central systolic blood pressure	**0.88**	−0.02	−0.08	0.04
Total Cholesterol	0.01	**0.92**	−0.01	0.07
Low density lipoproteins	0.02	**0.70**	0.03	0.03
High density lipoproteins	0.00	**0.55**	−0.14	**−0.45**
Augmentation index	0.18	−0.04	**−0.73**	0.02
Heart rate	0.27	0.10	**0.68**	0.02
Fasting blood glucose	0.04	−0.13	**0.51**	−0.05
Triglycerides	0.15	−0.10	−0.02	**0.73**
Glycated hemoglobin	−0.17	0.15	−0.10	**0.68**
Eigenvalue	2.5	1.7	1.3	1.2
% variance explained	23	16	12	11.0
Cumulative variance	23	39	51	62

Bold numbers represent variables with a factor loading 0.4.

Comparison to the Literature

A number of studies in adults have reported positive (i.e., negative) associations between CMD risk and SJL (13, 26, 27). Though only one known study has examined the association between CMD risk and SJL in children (15). Cespedes Feliciano et al. (15) reported a non-significant association between CMD risk *Z* scores and SJL for both girls ($\beta = 0.07$, 95% CI: −0.02 to 0.15) and boys ($\beta = 0.00$, 95% CI: −0.08 to 0.08). One potential explanation for the conflicting findings between the Cespedes Feliciano et al. study (15) and current study is the difference in sample characteristics, including the recruitment of older children (12–17 year vs. 8–10 years, respectively). However, considering CMD risk has been associated with SJL in various adult populations this explanation is unlikely. A more likely explanation pertains to methodological differences. For the current study, we measured a comprehensive and diverse mix of eleven cardiometabolic parameters and used factor analysis to identify risk factor clusters (17). Using this approach we found that SJL was associated with the factors we labeled "cholesterol" and "vascular health," but not "blood pressure" and "CHO-metabolism." Cespedes Feliciano et al. (15) divided their sample of 479 adolescents by sex (breakdown not provided) and characterized CMD risk using composite z scores for waist circumference, SBP, HDL, TG, and HOMA. This linear combination method would lack the sensitivity of the data-driven approach used in the current study as it assumes equal weighting for each variable, is prone

TABLE 3 | Linear associations between cardiometabolic risk factors and sleep measures ($n = 332$).

	Univariate ($n = 332$)					Multivariate (Model 1) ($n = 332$)					Adjusted (Model 2) ($n = 332$)				
	β	LCI	UCI	*P*	ES	β	LCI	UCI	*P*	ES	β	LCI	UCI	*P*	ES
Factor 1. Blood pressure															
Average sleep duration	0.002	−0.125	0.132	0.953	0.002	0.015	−0.113	0.147	0.793	0.012	0.026	−0.102	0.164	0.648	0.021
Sleep score	0.044	−0.011	0.025	0.425	0.263	0.040	−0.012	0.025	0.473	0.237	0.043	−0.011	0.025	0.442	0.254
Social jetlag	0.092	−0.029	0.377	0.093	0.049	0.091	−0.034	0.375	0.102	0.048	0.065	−0.086	0.332	0.251	0.501
Factor 2. Cholesterol															
Average sleep duration	−0.002	−0.132	0.127	0.969	−0.002	−0.013	−0.146	0.115	0.813	−0.010	0.019	−0.110	0.156	0.735	0.016
Sleep score	−0.034	−0.024	0.012	0.532	−0.205	−0.029	−0.023	0.013	0.604	−0.165	−0.022	−0.022	0.015	0.691	−0.130
Social jetlag	−0.110	−0.416	−0.008	0.041	−0.058	−0.111	−0.415	−0.005	0.045	−0.056	−0.126	−0.448	−0.030	0.025	−0.065
Factor 3. Vascular health															
Average sleep duration	−0.085	−0.226	0.027	0.122	−0.073	−0.069	−0.207	0.046	0.212	−0.058	−0.056	−0.197	0.065	0.321	−0.047
Sleep score	0.068	−0.007	0.029	0.215	0.415	0.048	−0.010	0.026	0.386	0.290	0.032	−0.013	0.032	0.557	0.196
Social jetlag	0.162	0.103	0.501	0.003	0.088	0.155	0.089	0.488	0.005	0.084	0.125	0.027	0.438	0.027	0.066
Factor 4. CHO-MET															
Average sleep duration	−0.058	−0.198	0.061	0.296	−0.048	−0.051	−0.192	0.069	0.357	−0.042	−0.026	−0.164	0.101	0.638	−0.022
Sleep score	0.017	−0.015	0.021	0.751	0.103	0.004	−0.018	0.019	0.948	0.022	−0.016	−0.021	0.015	0.776	−0.093
Social jetlag	0.097	−0.020	0.389	0.076	0.052	0.094	−0.027	0.384	0.089	0.049	0.031	−0.149	0.266	0.577	0.016

Univariate: specify the sleep measures (sleep duration, sleep disturbances, and social jet lag) separately (separate mode); Model 1: unadjusted multivariable (sleep duration, sleep disturbances, and social jet lag); Model 2: Model 1 adjusted for age, sex, ethnicity, socioeconomic status.
n, sample size; β, beta; LCI, lower confidence interval; UCI, upper confidence interval; P, p-value; ES, effect size (Cohen's d).

to multicollinearity, and fails to recognize that risk factors cluster (16).

SJL, but not sleep duration or sleep disturbances, was associated with CMD risk. This finding is particularly surprising considering that both sleep-related problems and short sleep duration have previously been associated with CMD risk (6–9). One potential explanation is the lack of co-consideration of SJL in previous studies. However, a more likely explanation is statistical in nature, particularly with respect to sleep duration. In the current study, 93% of children reported having slept for the recommended >9 h each night. While these data were self-reported, it remains highly likely that the number of short sleepers in the current study was low. The restricted range in the data likely limited the capacity to detect an association between sleep duration and CMD risk (28). Therefore, future pediatric sleep research should consider utilizing more objective measures of sleep in children (e.g., actigraphy).

We found that SJL correlated with vascular health and cholesterol factors, but not the blood pressure or CHO-metabolism factors. This finding is perhaps somewhat surprising when considering that SJL may lead to disruption of circadian rhythms in glucose metabolism and blood pressure, and have negative consequences for the regulation of these processes (12, 13). However, it should be acknowledged that while the vascular health factor was primarily driven by AIx, a measure of arterial wave reflection, FBG did load on to this factor. It is plausible that SJL disrupts metabolic pathways, and that these disruptions impair vascular function and hemodynamic parameters, including increased arterial wave reflection. Further research is warranted to determine biological plausibility for the relationship between SJL and CMD risk in children.

Implications

There has been a recent shift in public health policy to acknowledge childhood activity behaviors in the context of the entire 24 h day (5). With respect to sleep, it is recommended that children and youth sleep for 9–11 h each night. Even though short sleep duration is linked to increased CMD risk in children (6–9), there are other aspects of sleep that should also be considered in the context of 24 h activity behavior. In particular, findings from the current study suggest that SJL contributes to CMD in children. The current findings are strengthened by the fact that 93% of the children in the current study reported having slept the recommended minimum 9 h of sleep (5). Despite most children achieving an adequate duration of sleep, SJL remained associated with CMD risk. Considering that SJL is arguably a readily modifiable target, i.e., ensuring children maintain consistent and chronotype-driven sleep–wake cycles, further research is warranted to ascertain causality and to determine whether SJL-based interventions improve cardiometabolic outcomes. Another area that warrants future attention is the exploration of sex-specific effects. In the current study, we did not stratify our data by sex as the smaller sample would have been insufficient for the factor analysis. Our group and others have acknowledged sex to moderate the relationship between SJL and health outcomes, such as body composition (14, 15) and cardiorespiratory fitness (29). Hence, the causal relationship between SJL and CMD risk may differ by sex justifying the need for tailored sex-specific interventions.

CONCLUSIONS

This study examined the independent associations between sleep disturbances, sleep duration, and SJL with CMD risk in pre-adolescent children. We found that SJL, but not sleep duration or sleep disturbances, was associated with CMD risk. These results suggest that SJL may be a significant and quantifiable public health target for offsetting negative CMD trajectories in children.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by New Zealand Health and Disability Ethics Committee (HDEC: 14/CEN/83). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

NC, JF, SL, MW, and LSt conceived of the present idea and contributed to the design and implementation of the research. NC planned and performed the experiments. NC and LSt developed the theory and performed the computations. MH, LSt, and PS performed the numerical calculations for the suggested experiment and verified the analytical methods. NC, JD, and JB took the lead in writing the manuscript. All authors discussed the results and provided critical feedback to the writing of the manuscript.

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Comodulation of NO-Dependent Vasodilation by Erythroid Band 3 and Hemoglobin: A GP.Mur Athlete Study

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GP.Mur, a red blood cell (RBC) hybrid protein encoded by *glycophorin B-A-B*, increases expression of erythroid band 3 (Anion Exchanger-1, SLC4A1). GP.Mur is extremely rare but has a prevalence of 1–10% in regions of Southeast Asia. We unexpectedly found slightly higher blood pressure (BP) among healthy Taiwanese adults with GP.Mur. Since band 3 has been suggested to interact with hemoglobin (Hb) to modulate nitric oxide (NO)-dependent hypoxic vasodilation during the respiratory cycle, we hypothesized that GP.Mur red cells could exert differentiable effects on vascular tone. Here we recruited GP.Mur-positive and GP.Mur-negative elite male college athletes, as well as age-matched, GP.Mur-negative non-athletes, for NO-dependent flow-mediated dilation (FMD) and NO-independent dilation (NID). The subjects were also tested for plasma nitrite and nitrate before and after arterial occlusion in FMD. GP.Mur+ and non-GP.Mur athletes exhibited similar heart rates and blood pressure, but GP.Mur+ athletes showed significantly lower FMD (4.8 ± 2.4%) than non-GP.Mur athletes (6.5 ± 2.1%). NO-independent vasodilation was not affected by GP.Mur. As Hb controls intravascular NO bioavailability, we examined the effect of Hb on limiting FMD and found it to be significantly stronger in GP.Mur+ subjects. Biochemically, plasma nitrite levels were directly proportional to individual band 3 expression on the red cell membrane. The increase of plasma nitrite triggered by arterial occlusion also showed small dependency on band 3 levels in non-GP.Mur subjects. By the GP.Mur comparative study, we unveiled comodulation of NO-dependent vasodilation by band 3 and Hb, and verified the long-pending role of erythroid band 3 in this process.

Keywords: flow-mediated vasodilation (FMD), nitric oxide (NO), Band 3, GP.Mur, red blood cell (RBC), hemoglobin (Hb), nitrite, athletes

INTRODUCTION

Hemoglobin (Hb) modulates vascular tone by controlling nitric oxide bioavailability in the blood stream. The chemical reaction between Hb and NO metabolites is coupled with the dynamics of O_2 saturation and desaturation of Hb (1–5). While intraerythrocytic Hb is considered the most powerful NO scavenger in human body, its effect on NO-associated vasodilation is limited by the RBC membrane (6). Hb-mediated NO bioavailability has been proposed to require band 3, the main anion transporter on RBCs. The interaction between band 3 and deoxygenated Hb in the submembranous domain has been suggested to assist the transport of NO metabolites (e.g., nitrite and SNO) across the red cell membrane by unclear means (7–10). Currently, the role of band 3 in RBC-dependent NOx processing and hypoxic vasodilation is enigmatic.

GP.Mur (also known as Miltenberger subtype III or Mi.III), is a Southeast Asian special blood type with 1–10% prevalence among Filipinos, Vietnamese, Thai, and Taiwanese, but is very rare among Caucasian (0.0098%), northern Asian like Han Chinese and Japanese (0.006%) (11–14). Biochemically, GP.Mur is a hybrid protein originated from homologous gene recombination of the highly homologous *GPYB* and *GYPA* genes (15). As GP.Mur contains a segment of glycophorin A (GPA) and GPA acts as a chaperone to band 3 (16, 17), the consequence of the expression of GP.Mur is to support increased expression of band 3 on the erythrocyte surface (18). Band 3, a Cl^-/HCO_3^- antiporter, allows HCO_3^- permeation across the erythrocyte membrane, which is physiologically essential for intraerythrocytic CO_2/HCO_3^- conversion and postnatal CO_2 respiration (19–21). People with GP.Mur blood type express more band 3 on RBCs and show faster CO_2 respiration after a mild physical challenge (18, 22). Unexpectedly, from our previous human exercise study on Taiwanese adults aged up to 50 years old, GP.Mur+ subjects exhibit slightly higher blood pressure than the non-GP.Mur counterparts (22, 23).

The structural-functional hallmarks of GP.Mur+ red cells are enhanced band 3 expression (18) and altered band 3 complex structure with other membrane proteins on the red cell surface (21, 24–26). GP.Mur+ red cells present stronger band 3-AQP1 interaction (18, 26), contain reduced amount of RhAG and consequently altered band 3-Rh/RhAG macrocomplex organization (25, 27), and are more resilient physically in the osmolarity-fragility test (18). These presumably could affect band 3 interaction with submembranous deoxy Hb and metHb (28) and/or result in differential NOx processing, vasodilation, and blood pressure setting. To test this hypothesis, we examined NO-dependent FMD and NO-independent vasodilation primarily in a uniform cohort—Taiwanese elite college athletes who have been trained professionally for national and/or international sports competitions for approximately half of their lifetime. Together with blood measurements for band 3 and NOx changes during FMD, we identified co-modulation of FMD by erythroid band 3 and Hb.

MATERIALS AND METHODS

Subject Recruitment, GP.Mur Phenotyping, and Study Design

This human study protocol had been approved by MMH-Institutional Review Board (IRB registration: 19MMHIS081e). For uniform cohorts, we primarily recruited male elite athletes from top sports universities in Taiwan, where they enrolled with their competition scores from national/international games. Before college, all of them had professional sports training and competition experiences for at least 6 years. All subjects were free from major illness. To maintain eligibility for sports competitions, they were prohibited from taking most medication. We also recruited age- and BMI-matched, healthy male non-athletes as controls. All subjects had given written informed consents before participating in this study.

GP.Mur blood type was first determined by red cell serology using anti-Mur and anti-Mi^a antisera, and then confirmed by PCR-sequencing, as described (29). It has long been observed in Taiwan that the prevalence of GP.Mur among elite Taiwanese athletes is much higher. We pre-screened 129 elite male college athletes and found 32 of them with GP.Mur blood type (24.8%). Among the elite athletes, 51 of them agreed to participate in this study, and 19 of them bear GP.Mur phenotype (37.3%). As for the non-athlete subjects, only one out of 20 bear GP.Mur (5%), which is similar to the occurrence frequency of GP.Mur in general Taiwanese population.

The study design was illustrated in the flow chart in **Figure 1**. Briefly, each subject was tested on two separate days: 1 day for ultrasound measurements of FMD and then NID; the other day for assessments of the changes of blood plasma NO metabolites during FMD (blood withdrawal mostly completed within 1 min after cuff deflation). The subjects were not allowed intake of food or caffeinated drinks at least 2 h prior to FMD tests on both days. They were asked not to practice sports at least 6 h before testing.

Ultrasound Measurements for FMD and NID

We followed the FMD and NID protocols provided by the Hitachi-Aloka eTRACKING (echo-tracking) system (30). The Aloka eTRACKING program was installed in the Hitachi Arietta 60 (Ar60) ultrasound system equipped with a 12–2 MHz transducer (L441).

Before ultrasound examination, each subject was allowed to calm down in bed in a dark, quiet examination room, and his left wrist was wrapped with a pneumatic cuff. The L441 transducer locked in a probe holder was placed about 5–10 cm above his left elbow to image his left brachial artery. Under the FMD mode of eTRACKING, a selected region of his brachial artery was first focused and diameter changes in the focused brachial artery were recorded over time. The FMD recording began with 1-min baseline (stabilization), 5-min inflation of the wrist pressure to 50 mmHg above his systolic blood pressure (SBP), followed by deflation and 5 more minutes of monitoring the changes of the brachial arterial diameter. The peak of reactive hyperemia generally appeared within 30–90 s post-arterial occlusion, and the

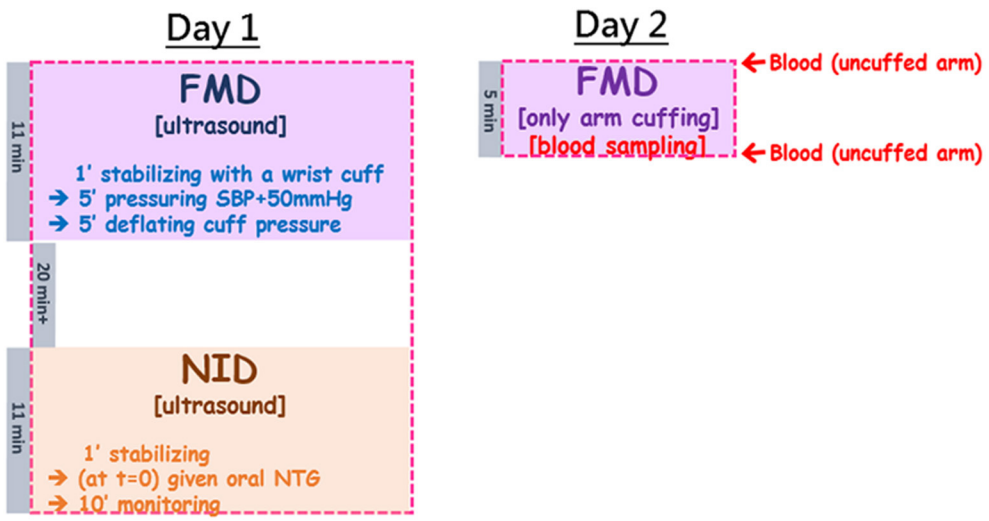

FIGURE 1 | The study flow chart combined ultrasound measurements of FMD and NID, and blood sampling before cuffing and right upon pressure deflation which allowed assessment of plasma NOx changes by arterial occlusion during FMD. The study was arranged in two separate days: Day 1 for ultrasound-based tests; Day 2 for blood sampling before and after 5-min brachial arterial constriction. In Day 1, the subject was started with the ultrasound measurement for FMD, followed by a minimal 20-min break to allow recovery of the tested brachial artery, and then the second ultrasound measurement for NID. FMD took ∼11 min (including 1 minute of stabilization, 5 min of cuff pressuring to 50 mmHg above the subject's SBP, and another 5 min of observation after wrist cuff deflation). The NID measurement also began with 1-min stabilization, followed by oral administration of NTG and then 10-min monitoring of the changes of the brachial arterial diameter. In Day 2, the setting was very similar to FMD in Day 1 (5 min of pressuring to 50 mmHg above the subject's SBP), but ultrasound was replaced by blood sampling before and after cuffing.

brachial arterial diameter generally returned to its baseline level within 2 min (see **Figure 2A**).

After FMD, the subject was given a 30-min break and then stabilized again in bed for ultrasound examination of NID on a similar region of the brachial artery as for FMD. NO-independent dilation recording by eTRACKING also began with 1-min baseline. The subject was then given 0.6 mg sublingual nitroglycerin (Nitrostat®, *Pfizer*), and his changes of the brachial arterial diameter were recorded for 10 min. These subjects generally reached maximal vasodilation by the 6th minute post-nitroglycerin. Nitroglycerin-induced hyperemia generally persisted till the end of the total 11-min recording (see **Figure 2A**).

Determination of Blood Plasma Nitrate and Nitrite Concentrations

To find out blood plasma NOx changes during FMD, on a separate visit the subject underwent a similar mechanical stimulus–5-min cuff inflation to 50 mmHg above his SBP on his right upper arm. Venous blood was sampled from his left median cubital vein at two timepoints: (1) before cuffing; (2) after deflation of the arm cuff. This allowed us to evaluate the consequence of regional ischemia and onset of NO-dependent vasodilation reflected by plasma NOx. Our blood sampling protocol for NOx during reactive hyperemia followed a report by Allen et al. (31), with slight modification.

Right after blood withdrawal into an ACD tube, the sample was immediately centrifuged to separate plasma from RBCs. Blood plasma was further filtered using an Amicon Ultra-4 Centrifugal unit with 10-kDa cutoff at 3,220 × *g* for 30 min at 4°C, and then aliquoted and stored at a −80°C freezer. Due to the short lifetime of nitrite, the handling process from blood withdrawal to sample storage in −80°C was limited to 45 min or less. Plasma NOx and NO_3^- were quantitated using the Nitrate/Nitrite colorimetric assay kit (Cayman Chemical, MI, USA); plasma NO_2^- was quantitated with the Nitrate/Nitrite fluorometric assay kit (Cayman).

Band 3 Quantitation

RBCs were hypotonically ruptured to obtain the membrane fraction (ghost). Total proteins in freshly lysed ghosts were quantified by Lowry protein assay (Bio-RAD, CA, USA), and band 3 levels measured by band 3 sandwich ELISA. The band 3 sandwich ELISA was developed in house. Briefly, anti-band 3 rat monoclonal BRAC 18 (International Blood Group Reference Laboratory [IBGRL], NHS, UK) was applied as the coating antibody (1:500 dilution) onto an ELISA plate overnight at 4°C. RBC ghost lysates were then reacted with the coated BRAC 18 for 30 min at 37°C, followed by PBST (0.05% Tween-20-supplemented PBS) washes and incubation with another anti-band 3 mouse monoclonal BRIC 170 (IBGRL; 1:500 dilution) for 2 h at 37°C. The signal of BRIC 170-sample binding was amplified by incubation with 1:5,000 HRP-conjugated, anti-mouse-specific secondary IgG (Jackson ImmunoResearch Laboratories, PA, USA); this secondary anti-mouse antibody had been immunoaffinity-purified to yield minimal cross reactivity with rat. The standard curve for absolute quantitation of band 3 in the sandwich ELISA used a custom-made band 3 peptide that contains the known epitope sequence of BRIC 170 (AA 368-382); the custom-made peptide sequence

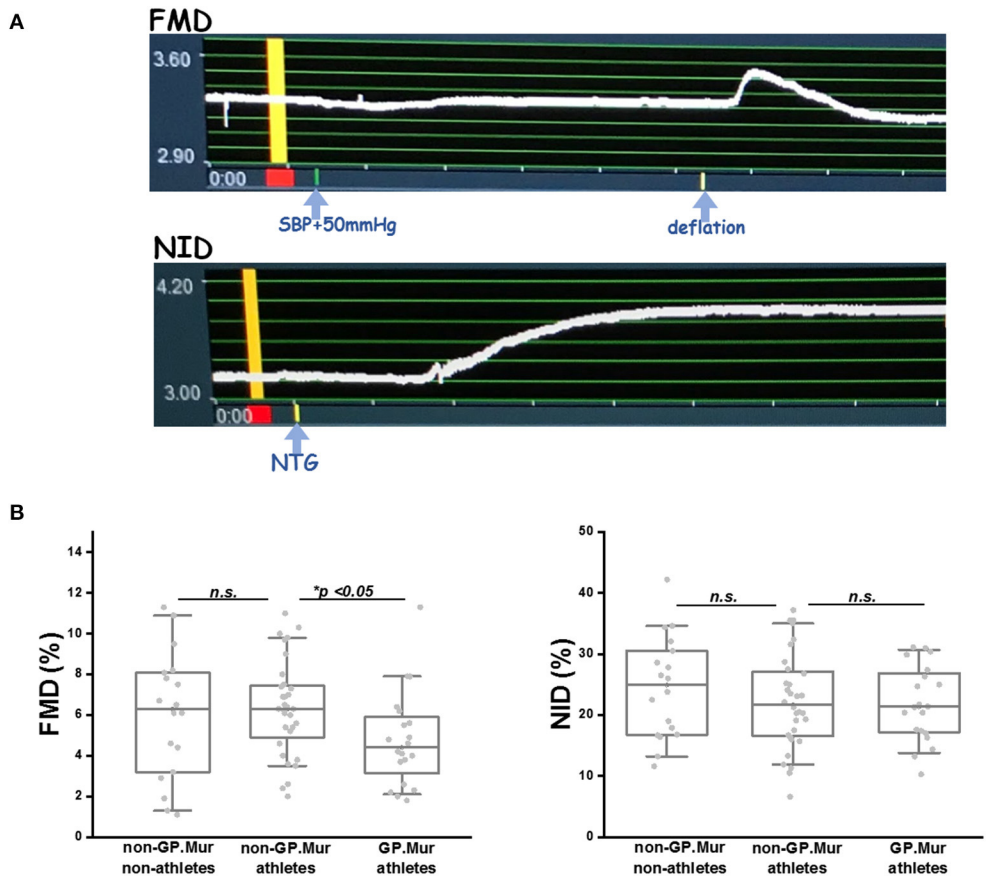

FIGURE 2 | FMD was significantly lowered in GP.Mur+ male elite athletes than GP.Mur-negative athletes or nonathletes, whereas NID was not affected by GP.Mur expression. **(A)** TOP: An example of FMD captured by the eTRACKING program of Hitachi-Aloka ARIETTA 60. The diameter of the region tracked is shown at the y-axis (unit: mm). The recorded time is shown at the x-axis (unit: minute). The yellow bar indicates the baseline of the vessel diameter that was to be compared with the maximally dilated diameter of the same region for %FMD or %NID. BOTTOM: An example of NO-independent vasodilation. **(B)** FMD was compared among the three groups of subjects—GP.Mur athletes, non-GP.Mur athletes, and non-athletes. Each dot represented the datum of a subject. The box covered data ranging 25–75%; the line inside a box indicated the median value. Data between the paired groups were compared by two-sample *t*-test (*$p < 0.05$). RIGHT: Comparison of NID (*n. s.*, not significant).

is NGGPDDPLQQTGQLFGGLVR (AA 365-384 of human band 3 ([NP_000333.1]). One's band 3 level relative to all RBC membrane proteins in his sample (w/w) was obtained by dividing the band 3 concentration by the ghost protein concentration and expressed in percentile (%).

RESULTS

To probe into the potential impacts of GP.Mur/band 3 on NO-dependent reactive hyperemia in a uniform cohort, this study recruited primarily male elite college athletes from top-ranking Taiwan sports universities where the prevalence of GP.Mur is much higher than the general Taiwanese population. The athlete subjects all have similar age, lifestyle, and enrolled in rigorous sports training programs aiming for national/international competitions for approximately half of their lifetimes (**Table 1**). They were divided into GP.Mur-positive (19/70) and GP.Mur-negative athlete groups (32/70). For comparison, twenty age- and BMI-matched non-athlete subjects were also recruited (only 1/20 GP.Mur+). Compared to the non-GP.Mur non-athletes (19/70), both groups of athletes showed similarly lower heart rates and slightly higher systolic blood pressuer (**Table 1**).

Lower FMD in GP.Mur Subjects

Compared to GP.Mur-negative athletes, FMD measured by ultrasound e-TRACKER was significantly lowered in GP.Mur+ athletes (4.6 ± 2.3% [GP.Mur+ athletes] vs. 6.3 ± 2.4% [non-GP.Mur athletes], *$p < 0.05$), despite that their blood pressure profiles were not different (**Figure 1**, **Tables 1**, **2**). Both GP.Mur+ and non-GP.Mur athlete groups exhibited similar degrees of NO-independent vasodilation triggered by nitroglycerin (**Figure 2**). The similar NID responses between GP.Mur+ and non-GP.Mur hint similar maximal functional capacities in their eNOS and smooth muscle cells (SMCs) (32). As for the GP.Mur-negative non-athletes, their FMD or NID were indistinguishable from either athlete groups (**Figure 2**, **Table 2**).

TABLE 1 | Subject characteristics.

Group (*N*)	Non-GP.Mur non-athlete (19)	Non-GP.Mur athlete (32)	GP.Mur athlete (19)	Difference between athletes with GP.Mur and without	Difference between nonGP.Mur athletes and non-athletes
Age (yrs)	23.6 ± 1.7	20.8 ± 3.0	22.2 ± 3.5	*n.s.*	*n.s.*
BMI	22.8 ± 2.6	24.7 ± 4.2	23.8 ± 2.8	*n.s.*	*n.s.*
% have smoked	5.3	31.3	26.3		
Athletic training yrs	0	10.4 ± 3.7	11.2 ± 3.6	*n.s.*	
Athletic training frequencies (hrs/wk)	0	15.0 ± 5.2	17.5 ± 9.9	*n.s.*	
Distribution of sports specialties		*71% mixed*	*67% mixed*		
		23% sprint	*22% sprint*		
		6% endurance	*11% endurance*		
Quiet heart rate (beats/min)	65.5 ± 10.8	59.5 ± 9.7	59.1 ± 6.8	*n.s.*	$p < 0.05$
SBP (mmHg)	120.2 ± 8.8	127.9 ± 9.3	123.8 ± 13.4	*n.s.*	$p < 0.05$
DBP (mmHg)	71.8 ± 8.1	66.7 ± 6.9	69.7 ± 8.3	*n.s.*	*n.s.*
Pulse pressure (mmHg)	48.4 ± 7.3	59.2 ± 8.4	54.1 ± 13.0	*n.s.*	$p < 0.001$
RBC (10^6/μL)	4.8 ± 0.7	5.0 ± 0.7	4.9 ± 0.4	*n.s.*	*n.s.*
Hb (g/dL)	15.1 ± 0.8	15.0 ± 1.0	14.9 ± 0.8	*n.s.*	*n.s.*
Hemotocrit (%)	45.1 ± 2.3	44.5 ± 2.6	44.2 ± 2.4	*n.s.*	*n.s.*
Platelets (10^3/μL)	243.0 ± 43.7	229.5 ± 72.7	236.3 ± 68.0	*n.s.*	*n.s.*

Data were shown in mean ± SD. BMI, body mass index. Mixed sports types here include judo, taekwondo, karate, soccer, basketball, wrestling, and soft tennis. The sprint athletes here are specialized in discus throw, shot put, hammer throw, javelin, high jump, weightlifting, 100/200 m run, and 50/100 m swim. The endurance athletes here are both mid-to-long distance runners. Data were shown in mean ± SD. Statistical differences were calculated by two-sample t-test; $p < 0.05$ was deemed significant; n.s., not significant.

TABLE 2 | FMD, NID, blood plasma NO metabolites, and band 3 levels on RBCs.

Group (*N*)	Non-GP.Mur non-athlete (19)	Non-GP.Mur athlete (32)	GP.Mur athlete (19)	*Difference between athletes with GP.Mur and without*	*Difference between nonGP.Mur athletes and non-athletes*
% FMD	6.0±3.1	6.3 ± 2.4	4.6 ± 2.3	$p < 0.05$	*n.s.*
% NID	24.5 ± 8.4	22.3 ± 7.9	21.4 ± 6.0	*n.s.*	*n.s.*
FMD baseline diameter (mm)	3.7 ± 0.5	3.9 ± 0.5	4.0 ± 0.4	*n.s.*	*n.s.*
NID baseline diameter (mm)	3.8 ± 0.6	3.8 ± 0.5	4.0 ± 0.4	*n.s.*	*n.s.*
Time to reach max diameter after deflation (s)	48.6 ± 10.6	57.0 ± 16.1	49.8 ± 15.7	*n.s.*	$p < 0.05$
Plasma NOx before cuffing (μM)	9.63 ± 6.70	5.66 ± 3.52	6.02 ± 4.73	*n.s.*	$p < 0.05$
Plasma NOx upon cuff deflation (μM)	9.13 ± 6.21	5.43 ± 3.64	5.87 ± 4.67	*n.s.*	$p < 0.05$
Plasma NO_2^- before cuffing (μM)	0.42 ± 0.15	0.47 ± 0.14	0.42 ± 0.16	*n.s.*	*n.s.*
Plasma NO_2^- upon cuff delfation (μM)	0.45 ± 0.18	0.53 ± 0.20	0.46 ± 0.17	*n.s.*	*n.s.*
Plasma NO_3^- before cuffing (μM)	9.21 ± 6.72	5.19 ± 3.55	5.60 ± 4.84	*n.s.*	$p < 0.05$
Plasma NO_3^- upon cuff delfation (μM)	8.68 ± 6.22	4.91 ± 3.70	5.41 ± 4.78	*n.s.*	$p < 0.05$
$\Delta[NO_2^-]_{cuffing}$ (μM)	0.03 ± 0.09	0.07 ± 0.08	0.05 ± 0.09	*n.s.*	*n.s.*
$\Delta[NO_3^-]_{cuffing}$ (μM)	−0.24 ± 0.58	−0.19 ± 0.40	−0.23 ± 0.33	*n.s.*	*n.s.*
% band 3 in RBC membrane proteins (w/w)	4.15 ± 1.89	4.30 ± 2.41	5.22 ± 1.69	$p < 0.05$	*n.s.*

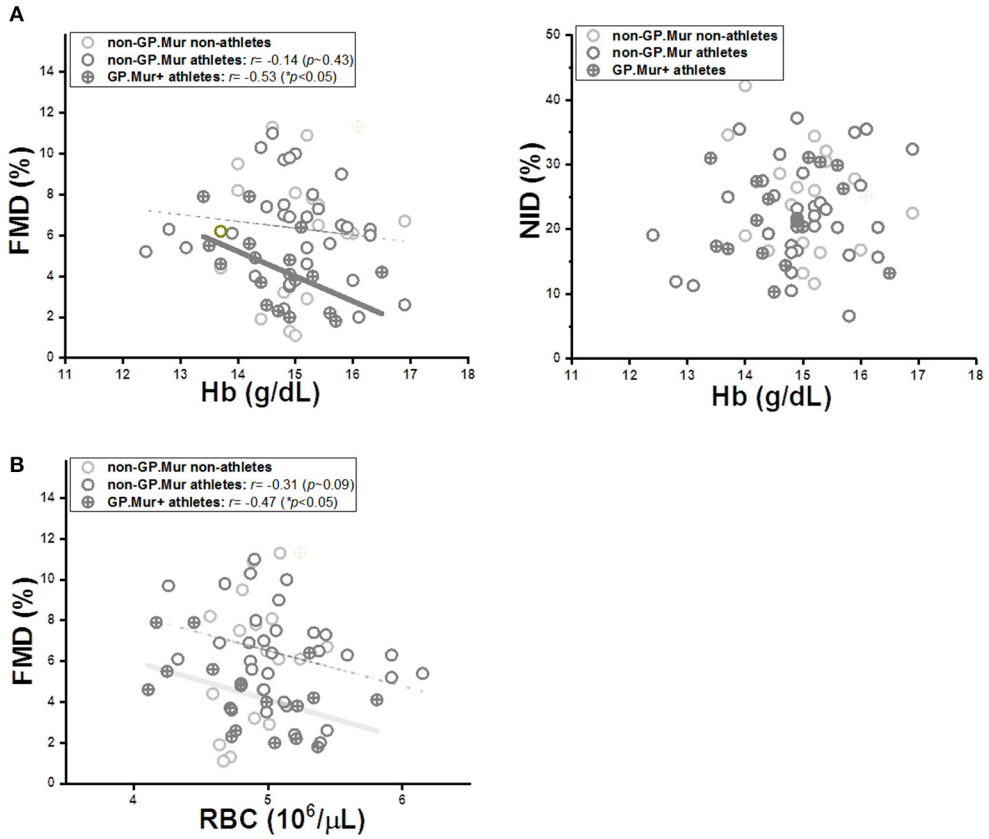

FIGURE 3 | The limiting effect of intraerythrocytic Hb on FMD was more significant in GP.Mur+ athletes (cross circular symbols) than in the non-GP.Mur control subjects (empty circular symbols). **(A)** LEFT: Linear regression analyses revealed a significant inverse correlation between Hb and FMD in GP.Mur+ athletes, which was less obvious in non-GP.Mur athlete and non-athlete subjects (GP.Mur: Pearson's $r = -0.53$ ($^*p < 0.05$); non-GP.Mur: weak correlation). RIGHT: Intraerythrocytic Hb exerted no specific effects on NID for GP.Mur+ or non-GP.Mur subjects. **(B)** The dependency of FMD on RBC count was similar for both GP.Mur+ and non-GP.Mur groups. Solid thick lines represent significant linear fitting; thin, dotted lines represent poor fitting ($p > 0.05$).

FMD Limited More by Hb in GP.Mur+ Than in Non-GP.Mur Subjects

In a study on hypertensive patients with high-altitude erythropoiesis, FMD in these patients was inversely correlated with their abnormally high Hb (>21 g/dL) and excessive number of circulating RBC cells (33). Here, our subjects were healthy young students with Hb and RBC count in the normal ranges. Their Hb and RBC count were not different because of GP.Mur (**Table 1**). But only in GP.Mur+ subjects was FMD significantly inversely correlated with Hb (**Figure 3A**: thick solid line). This trend would not be obvious in most non-GP.Mur people with normal Hb, as shown with the poor fitting in **Figure 3A** (33). If comparing the Pearson's correlation *r* between Hb and FMD, the correlation in GP.Mur was 4-fold that in non-GP.Mur (−0.53 [GP.Mur+] vs. −0.14 [non-GP.Mur]) (**Figure 3A**). From the data, Hb in GP.Mur+ RBCs was more effective in scavenging NO, and this likely involved distinct components in the GP.Mur red cell membrane. In contrast, the correlation between Hb and NID was poor for all three groups ($p > 0.05$), indicating that NID was not associated with Hb (**Figure 3A**, right). These results confirm the central role of Hb in NO-dependent vasodilation.

On the other hand, FMD correlation with RBC count was not much different between GP.Mur and non-GP.Mur (**Figure 3B**: *r* or the slopes for GP.Mur+ athletes, −0.47; for non-GP.Mur athletes, −0.31). From their similar dependency of FMD on RBC count, their drastically different FMD sensitivity to Hb had to be primarily triggered by their differences in the RBC membrane composition (i.e., GP.Mur/band 3 protein complexes).

Blood Plasma Nitrite Levels Dynamically Correlated With Band 3 Expression

Similar to the FMD/NOx study by Allen et al. (31), we observed that occlusion of the brachial artery followed by cuff deflation decreased systemic plasma nitrate and increased systemic plasma nitrite contents (**Table 2**, **Figure 4A**). But the concentrations of plasma nitrite, nitrate, or NOx before pressure cuff or right after cuff deflation were not different between GP.Mur+ and non-GP.Mur athletes (**Table 2**). The increase of nitrite or the decrease of nitrate by arterial occlusion and cuff release ($\Delta[NO_2^-]_{cuffing}$ & $\Delta[NO_3^-]_{cuffing}$) were also not significantly different between GP.Mur and non-GP.Mur (**Table 2**). The level of plasma nitrite is a balance between nitrite production

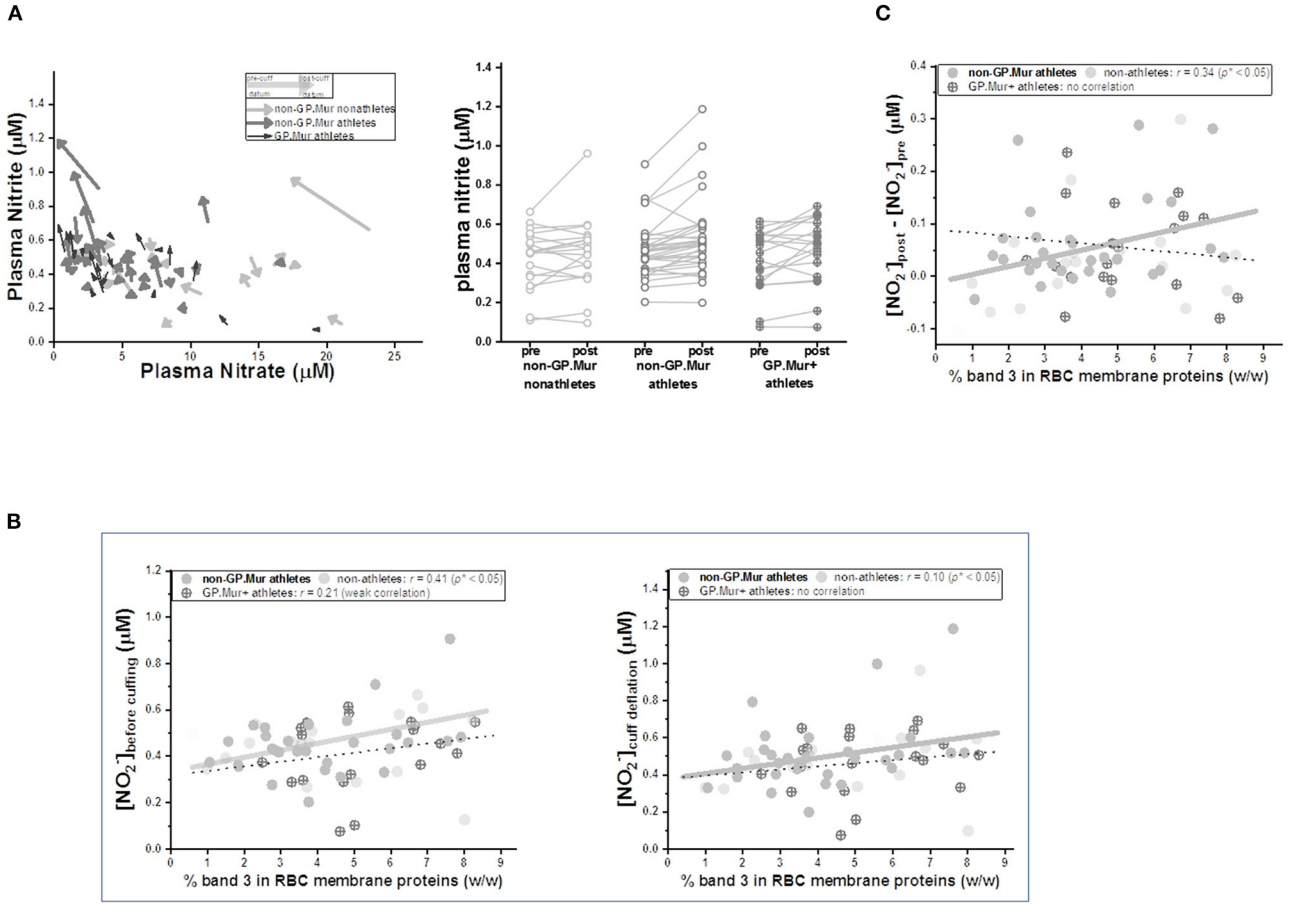

FIGURE 4 | Arterial occlusion following cuff deflation during FMD decreased plasma nitrate and increased nitrite, the latter of which was quantitatively directly correlated with individual erythroid band 3 levels. **(A)** LEFT: Plasma nitrite and nitrate levels before cuffing and upon deflation were compared. Their changes ($\Delta[NO_2^-]$ and $\Delta[NO_3^-]$) were generally inversely correlated. Each arrow represents a test subject: GP.Mur athletes in black color, non-GP.Mur athletes in dark gray color, and non-GP.Mur non-athletes in light gray color. The beginning of an arrow indicated concentrations of plasma nitrite and nitrate right before cuffing; the arrowhead indicated concentrations of plasma nitrite and nitrate upon release of the cuff pressure. RIGHT: Plasma nitrite generally increased upon cuff deflation following 5-min arterial occlusion. Each line connected the nitrite concentration before arm cuffing and the concentration upon pressure deflation for an individual subject. **(B)** The concentrations of blood plasma nitrite were directly proportional to individual band 3 levels. The thick gray line indicated significant linear fitting for non-GP.Mur (*$p < 0.05$); the thin dashed line indicated poor linear fitting for GP.Mur data (*n.s.*). LEFT: the concentration of plasma nitrite ($[NO_2^-]$) before cuffing. RIGHT: $[NO_2^-]$ upon cuff deflation following 5-min occlusion of the brachial artery. **(C)** For all non-GP.Mur subjects (athletes and non-athletes), their changes of blood nitrite concentrations by arterial occlusion during FMD ($\Delta[NO_2^-]_{cuffing} = [NO_2^-]_{pre-cuff} - [NO_2^-]_{upon\ deflation}$) was directly correlated with erythroid band 3 levels (*$p < 0.05$). This direct relation between band 3 and ($\Delta[NO_2^-]_{cuffing}$) was not apparent in GP.Mur+ subjects.

and nitrite consumption. Under physiologically oxygenated conditions, most nitrite is constitutively generated in tissue from oxygenation of NO produced by eNOS (34), and is consumed by reduction into NO primarily via deoxy Hb (35). Individual eNOS levels were expected to vary, and hence individuals with high $[NO_2^-]_{pre-FMD}$ generally showed high $[NO_2^-]_{upon\ deflation}$ (**Figure 4B**).

Band 3 complexes have been suggested to assist Hb in NO-dependent hypoxic vasodilation, either as a nitrite transporter (7) or/and as a mediator for permeation of NOx/SNO metabolites across the red cell membrane (8, 9). Band 3 expression was slightly higher in people with GP.Mur (**Table 2**) (18, 22, 36). We thus explored the possible involvement of band 3 in NOx changes during FMD. The concentrations of systemic plasma nitrite before cuff pressing and after deflation were both directly correlated to band 3 expression levels on the RBC membrane (**Figure 4B**). The correlation between band 3 and systemic nitrite was better before than after regional arterial occlusion (**Figure 4B**: larger Pearson's correlation *r* before cuffing [left] than after cuff deflation [right]). Notably, for GP.Mur+ athletes, their band 3-nitrite correlation was much smaller and diminished after cuff deflation. We calculated the extent of systemic nitrite changes induced by this regional hypoxia ($[NO_2^-]_{post}$ - $[NO_2^-]_{pre}$, or $\Delta[NO_2^-]_{cuffing}$), which was significantly correlated with erythroid band 3 levels but only in non-GP.Mur subjects (**Figure 4C**). This discrepancy suggests different impacts of GP.Mur+ and non-GP.Mur membrane structure on NOx metabolism, as seen in **Figure 3A**.

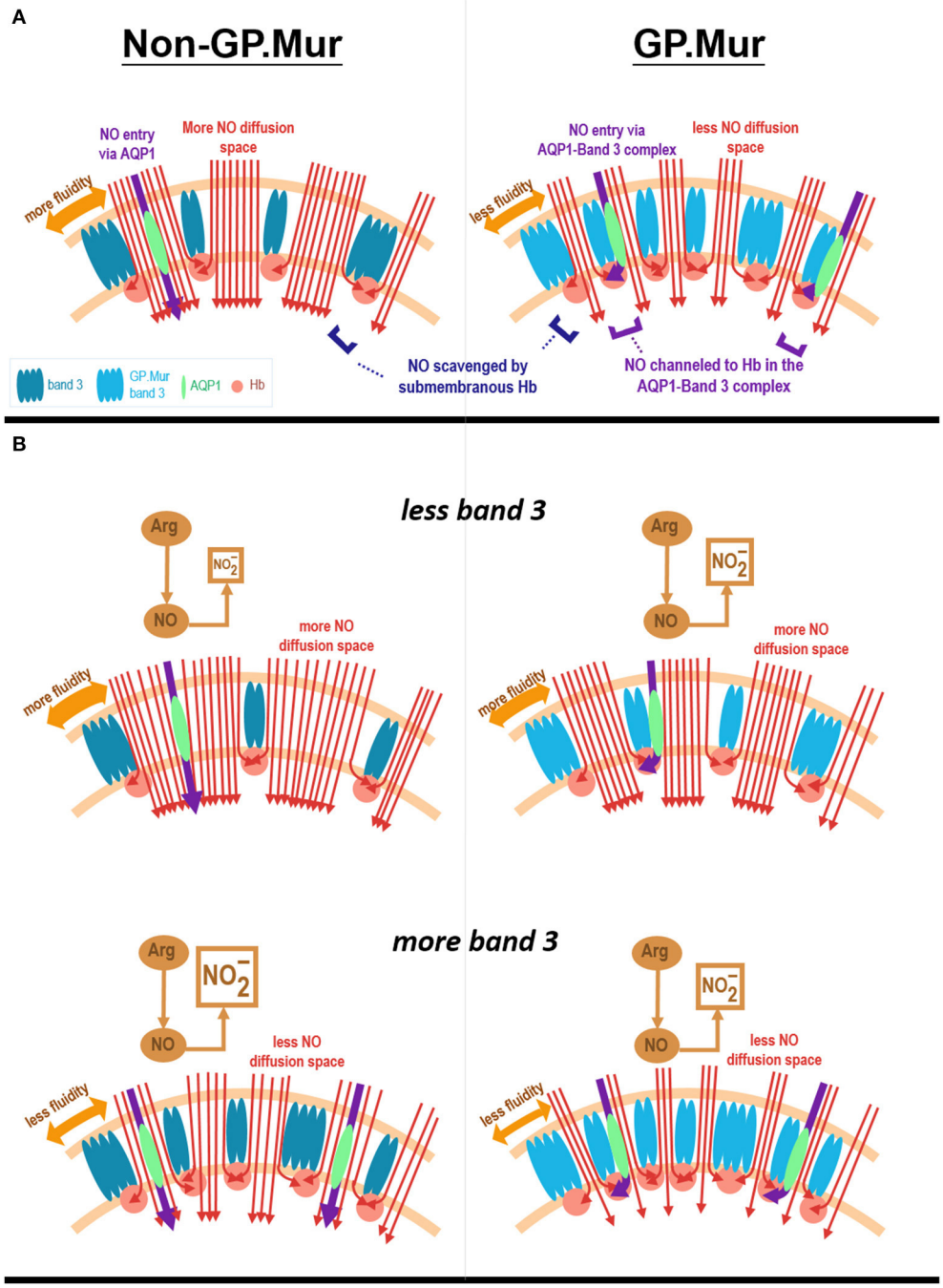

FIGURE 5 | Hypothesis. **(A)** A model depicts how Hb in GP.Mur+ RBCs could be more effective in scavenging NO than Hb in non-GP.Mur RBCs. GP.Mur band 3 dimers and tetramers differ from non-GP.Mur in higher expression and distinct structural organization (labeled with different shades of blue color). Cytoplasmic band 3 binds Hb (red balls), preferentially less O_2-saturated Hb. Because GP.Mur+ RBCs express more band 3, there is more Hb lining on the inner leaflet of the lipid bilayer. In terms of protein complex organization, AQP1 (green) is mobile in non-GP.Mur RBCs but is often associated with band 3 in GP.Mur+ RBCs. Their oligomerization on the GP.Mur+ membrane enables channeling of NO to Hb through the AQP1-band 3 complexes (shown in purple arrows). Both structural differences equip GP.Mur+ RBCs with a stronger suction for NO (red arrow lines). **(B)** A model depicts the relations between erythroid band 3 expression and plasma nitrite levels. Band 3 expression on the RBC membrane vary among individuals. The red cell membrane inserted with more band 3 protein tends to be less fluid and hence allows for less NO diffusion into the red cells (shown in red arrow lines). For non-GP.Mur (left panels), higher band 3 expression is associated with less NO entry and scavenge by erythrocytes; their plasma nitrite levels are thus higher. The content of plasma nitrite is complementary to the amount of NO scavenged by RBCs. For GP.Mur (right panels), NO diffusion into erythrocytes is not only affected by band 3 protein expression/membrane fluidity; GP.Mur+ RBCs also scavenge NO more efficiently (as explained in **A**). For GP.Mur, these two opposite effects conceivably even out and obscure the direct correlation between erythroid band 3 and plasma nitrite levels that was seen in non-GP.Mur.

DISCUSSION

Intraerythrocytic Hb is the major NO scavenger inside human body, but deoxy Hb as a nitrite reductase also generates NO in low O_2 during the respiratory cycle. The sources of NO for maintaining the vascular tone mainly come from constitutive conversion of L-Arginine by endothelial NO synthase (eNOS) and from reduction of NOx by heme reductase like deoxy Hb. NO production by deoxy Hb complements NO production by eNOS, which takes place preferentially in high O_2. This may explain, at least in part, why eNOS expression alone cannot fully reflect NO bioavailability or FMD (37–39).

Upon deflation of the cuff pressure in FMD, we observed decrease of plasma nitrate and increase of plasma nitrite for both GP.Mur and non-GP.Mur groups (**Table 2**, **Figure 4A**). The reaction of NO in oxygenated RBCs mainly forms nitrate; the reaction of NO in oxygenated tissues mainly forms nitrite. Thus, decreasing nitrate suggests lower NO and a need to increase NO/nitrite bioavailability from both tissues and RBCs. Nitrite synthesis was accelerated to increase NO bioavailability.

This study probed into the effects of GP.Mur/band 3 on NO-dependent vasodilation. Our age-matched, young elite athletes all had similar blood pressure, but GP.Mur+ athletes had already exhibited lower FMD than non-GP.Mur counterparts (**Figure 2**). Their Hb levels were all within the normal range, but Hb in GP.Mur+ RBCs was more effective in limiting NO-dependent vasodilation (**Figures 2**, **3**). This suggests that GP.Mur/band 3-associated structural features could further facilitate NO scavenge by intraerythrocytic Hb (**Figure 3A**). Band 3 protein complexes differ between GP.Mur and non-GP.Mur in two aspects: (1) there are more band 3 molecules on the GP.Mur+ RBCs; (2) GP.Mur+ band 3 complexes are structurally distinguishable from non-GP.Mur (18, 25–27). In particular, AQP1 and band 3 interact more strongly in GP.Mur+ than in non-GP.Mur RBCs (26). The water channel AQP1 is also a NO gas channel (40–42). Thus, we hypothesize that Hb in GP.Mur+ RBCs was more effective in scavenging NO for the two reasons: (1) more band 3 on the GP.Mur+ membrane could attract more Hb to the inner leaflet of the cell membrane; (2) their stronger AQP1-band 3 interaction could channel NO influx via AQP1 to band 3-bound Hb (**Figure 5A**). Though Hb was not different between GP.Mur+ and non-GP.Mur groups (**Table 1**), more Hb distributed on the inner leaflet would result in more efficient NO scavenge. Since NO gas diffuses into erythrocytes extremely rapidly (at a rate near its diffusion limit), the rate of NO scavenge by intracellular Hb is critical to NO bioavailability and NO-dependent vasodilation (as in **Figure 3A**).

We also found that erythroid band 3 levels were directly correlated with $\Delta[NO_2^-]_{FMD}$ in non-GP.Mur but not in GP.Mur+ subjects (**Figure 4**). Band 3 likely could support permeation of NO_2^- across the red cell membrane, as proposed by other groups (7, 8). Band 3 transports HCO_3^- at the rate of $\sim 10^4$/s. If band 3 transports NO_2^- at a similar rate as HCO_3^-, this is still much slower than the rate of NO diffusion through the plasma membrane. Thus, the different correlations between band 3 levels and $\Delta[NO_2^-]_{FMD}$ more likely involved different degrees of NO scavenge between GP.Mur and non-GP.Mur.

We hypothesize that for non-GP.Mur RBCs, a higher band 3 protein level shall decrease membrane fluidity and hence reduce NO diffusion into the red cells (**Figure 5B**, bottom). This is because the inner leaflet of the RBC membrane is mainly covered by metHb. Despite that metHb in RBCs is only 0.5–3%, it reacts with NO at a much slower rate than oxyHb or deoxyHb, and thus forms a barrier for NO diffusion into the red cells. Increase of band 3 expression in non-GP.Mur RBCs thus could reduce NO entry and scavenge; more plasma nitrite could then be observed (**Figure 5B**). On the other hand, the lack of the correlation between erythroid band 3 and plasma nitrite levels in GP.Mur+ RBCs suggest that factors other than membrane barriers to NO diffusion (i.e., metHb and fluidity) were involved. These other factors include more extensive lining of Hb with cytoplasmic GP.Mur band 3 in the inner leaflet, and unique channeling of NO to submembranous Hb via AQP1-band 3 complexes (**Figure 5A**). Because of these additional factors, higher band 3 levels in GP.Mur+ RBCs conceivably also promote NO scavenging. These effects were opposite to the decrease of NO entry in less fluid red cells (**Figure 5B**). These may explain why the direct correlation between plasma nitrite and band 3 levels seen in non-GP.Mur subjects was absent in GP.Mur (**Figures 4**, **5**).

A final interesting observation from this study is the lower plasma nitrate (but not nitrite) among the elite athletes, regardless of GP.Mur (**Table 2**). Skeletal muscles are the dominant nitrate reservoir over other organs or blood (43). It has been reported that the nitrate stores, particularly that in skeletal muscles, are essentially reduced to nitrite and NO for vasodilation during high-intensity exercise (43). Our athlete subjects were routinely trained at high intensity. Indeed, their nitrate store was more depleted than that in the non-athlete controls (**Table 2**). Thus, from our results, even young athletes will need nitrate supplementation more than non-athletes of similar age, not just for sports performance but also for their vascular health (43).

Limitation

This work nonetheless is limited by the small sample size for a FMD study (44). The main reason is the low prevalence of GP.Mur blood type in northern Taiwan (< 2%) where this study was conducted. To minimize variations among subjects, we recruited only male elite athletes from top sports universities in the region. But this type of study may be even harder to conduct in the future, as we have noticed gradual dilution of the *GYP.Mur* gene in Taiwan in the past half century.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by MMH-Institutional Review Board (IRB

registration: 19MMHIS081e). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KH, Y-YL, and W-CT conceived the project. W-CT and K-WT recruited the subjects. Y-YL, KH, and H-JL performed ultrasound experiments. C-YL supported ultrasound experiments. L-YC, H-LL, and H-JL performed blood tests and analyzed the data. K-TH helped conceptualize the data. H-IY edited the paper. KH analyzed the data and wrote the paper. All authors contributed to the article and approved the submitted version.

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Association Between Initiation, Intensity, and Cessation of Smoking and Mortality Risk in Patients With Cardiovascular Disease: A Cohort Study

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Objectives: To examine the effect of smoking status, smoking intensity, duration of smoking cessation and age of smoking initiation on the risk of all-cause and cause-specific mortality among cardiovascular disease (CVD) patients.

Design: A population-based prospective cohort study.

Setting: The National Health Interview Survey (NHIS) in the U.S. that were linked to the National Death Index (NDI).

Participants: 66,190 CVD participants ≥ 18 years of age who were interviewed between 1997 and 2013 in the NHIS linked to the NDI through December 31, 2015.

Outcome Measures: The primary outcome was all-cause mortality and the secondary outcome was cause-specific mortality including CVD mortality and cancer mortality.

Results: During the mean follow-up of 8.1 years, we documented 22,518 deaths (including 6,473 CVD deaths and 4,050 cancer deaths). In the overall CVD population, former and current smokers had higher risk of all-cause (Former smokers: hazard ratios (HRs), 1.26; 95% confidence interval (CI), 1.21–1.31, $P < 0.001$; Current smokers: HRs, 1.96; 95%CI, 1.86–2.07, $P < 0.001$), CVD (Former smokers: HRs, 1.12; 95%CI, 1.05–1.21, $P = 0.001$; Current smokers: HRs, 1.80; 95%CI, 1.64–1.97, $P < 0.001$) and cancer mortality (Former smokers: HRs, 1.49; 95%CI, 1.35–1.64, $P < 0.001$; Current smokers: HRs, 2.78; 95%CI, 2.49–3.09, $P < 0.001$) than never smokers. Furthermore, similar results were observed when the study subjects were stratified according to the type of CVD. Among current smokers, the risk for cancer mortality increased as the daily number of cigarettes increased, regardless of the specific type of CVD. However, the association of the risk for all-cause and CVD mortality with smoking intensity did not present a dose-response relationship. In participants with angina pectoris or stroke, smoking intensity was inversely associated with deaths from CVD. In addition, the risk for all-cause, CVD and cancer mortality declined as years of smoking cessation increased.

Finally, the relative risk of all-cause mortality was not significantly different in individuals with a younger age of smoking initiation.

Conclusions: CVD patients who are smokers have an increased risk of all-cause, CVD and cancer mortality, and the risk decreases significantly after quitting smoking. These data further provide strong evidence that supports the recommendation to quit smoking for the prevention of premature deaths among individuals with CVD.

Keywords: cardiovascular disease, smoking status, all-cause mortality, cardiovascular disease mortality, cancer mortality

STRENGTHS AND LIMITATIONS OF THIS STUDY

- The key strengths of this study include its prospective design, large sample size, long-term follow-up and a large number of deaths, which allowed the investigation of long-term survival and provided sufficient deaths for analyses of more detailed causes of death, and careful adjustments for a multitude of potential risk factors.
- Additionally, unlike previous studies that only included subjects with specific CVD diseases, we included the entire CVD population and examined the association of all-cause and cause-specific mortality risk with smoking status, the age of smoking initiation, the number of cigarettes smoked per day and years since quitting.
- The CVD diseases, smoking status were self-reported and these information were self-reported only at baseline.
- The information on what types of tobacco products were smoked were not available.
- Since the NHIS is a passive follow-up study, the cause of death may be misclassified and the estimated hazards of cause-specific mortality may have been affected.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality and has become a global public health crisis. The Global Burden of Disease (GBD) 2015 study estimated that 422.7 million individuals worldwide were affected by CVD in 2015 (1). Based on the most recent estimates from the GBD study, nearly 18 million individuals died from CVD, representing one-third of deaths globally (1, 2).

Smoking is recognized as a well-established major risk factor for CVD and the second leading attributable risk factor for death (3, 4). Even smokers who quit smoking for 10∼15 years still have a higher risk of CVD than non-smokers (5). Although compelling evidence has indicated that smoking has harmful effects on health, approximately 25.0% of men and 5.4% of women are still daily smokers worldwide (6). In particular, a sizeable proportion (10 to 50%) of patients continued to smoke even after the diagnosis of CVD (7–10).

To date, the adverse influence of smoking on the risk of CVD (such as ischaemic heart disease, acute myocardial infarction, cerebrovascular disease, peripheral arterial disease, heart failure or other heart condition/disease) (11, 12) and all-cause and specific-cause mortality have been extensively studied and analyzed in the general population (13–16). However, among individuals with CVD, who have a 2- to 4-times higher risk of mortality than the general population, evidence regarding the association of smoking status with health outcomes remains inconclusive and insufficient. Previous studies have revealed a phenomenon called the "smoker's paradox", implying that smokers who have developed myocardial infarction (MI), acute ischaemic stroke and acute heart failure had lower rates of adverse short-term outcomes, especially in-hospital mortality (17–19). Conversely, increasing evidence has recently indicated that smoking increases long-term adverse outcomes in patients with established CVD (7, 8, 20). However, the relationship between smoking status and adverse long-term outcomes in most of the above studies has so far been concentrated on patients with coronary artery disease, with limited evidence on the relation of smoking with long-term adverse outcomes among patients with different types of CVD. In addition, few studies have assessed the association of the age of smoking initiation, the number of cigarettes smoked per day and the duration since smoking cessation with long-term all-cause and cause-specific mortality, especially CVD and cancer mortality, among patients with established CVD.

Therefore, additional investigations of the associations between smoking and outcomes in CVD patients are warranted. The primary purpose of this study was to examine the effect of smoking status, smoking intensity, duration of smoking cessation and age of smoking initiation on the risk of all-cause mortality among CVD patients. The association between smoking status, smoking intensity, duration of smoking cessation, age of smoking initiation and cause-specific mortality was evaluated among CVD patients as a secondary objective. The broader purpose was to provide direct evidence for clinical practice and behavior changes.

METHODS

Data Source

This study was conducted using data from the National Health Interview Survey (NHIS), which has been conducted continuously since 1957. The NHIS is a nationally representative annual cross-sectional household survey administered by the National Center for Health Statistics (NCHS) and uses a stratified, multistage sampling design to collect information

from the civilian, non-institutionalized population of the United States. The content and structure of the NHIS has been revised approximately every 10–15 years. Two major revisions were made to the NHIS questionnaire in 1982 and 1997. To ensure the consistency of the self-reported information of the survey participants, we used data after 1997. Additional information about the NHIS has been described previously (21) and is provided by the Centers for Disease Control and Prevention (http://www.cdc.gov/nchs/nhis/about_nhis.htm). Adult participants ≥ 18 years of age from the annual 1997 to 2013 waves of the NHIS were included in the study (22, 23). Of these, 67,811 individuals with CVD were eligible to be linked to the National Death Index through December 31, 2015 (21). In addition, participants were excluded if they had incomplete information on smoking status and CVD types at baseline. After exclusion, 66,190 subjects were included in the final analysis. In this study, only public-use NHIS data were used. The public-use NHIS data are de-identified and do not include any protected health information. Our study was based on secondary analysis of public-use data. The publicly available data are considered as exempt under the ethical board review of the corresponding author's institution (the Third Xiangya Hospital of Central South University).

Definition of CVD

Individuals were identified as having CVD based on positive responses to the following question: Have you ever been told by a doctor or other health professional that you had coronary heart disease (CHD), a heart attack (also MI), angina (also called angina pectoris), a stroke, or any kind of heart condition or heart disease?

Assessment of Smoking

NHIS study participants were administered questionnaires relating to their smoking status, the age of smoking initiation, the number of cigarettes smoked per day, years since quitting and the number of cigarettes smoked per day before quitting. Based on self-reported responses for these questionnaires, survey participants were categorized into 3 categories for analysis: never smokers, former smokers, and current smokers. To analyse the effect of the age of smoking initiation and smoking intensity on outcomes, we classified current and former smokers into categories of age at smoking initiation: ≤ 12, 13–17, and ≥ 18+ years and categories of ≤ 9, 10–19 and ≥ 20 cigarettes smoked per day. In addition, we divided former smokers into categories of years since cessation: 0 to<10, 10 to <20 and ≥ 20 years in the smoking cessation analysis.

Outcomes

The primary outcome was all-cause mortality. The secondary outcomes included CVD mortality (codes I00-I09, I11, I13, and I20-I51, I60-I69) and cancer mortality (codes C00-C97), according to the International Statistical Classification of Diseases, Injuries, and Causes of Death (ICD-9 for deaths prior to 1999 and ICD-10 for deaths occurring from 1999 onwards).

Covariates

Several baseline variables were included as confounders in this study. Demographic data on age at enrolment, self-reported sex, self-reported race (Hispanic, non-Hispanic white, non-Hispanic black and non-Hispanic others), educational attainment (did not complete high school, completed high school, education beyond high school and missing education information) were collected. The following lifestyle behavior information was also included: alcohol consumption (lifetime abstainer, former drinker, current drinker, missing alcohol consumption information); physical activity, which was grouped into meeting recommendation, not meeting recommendation and missing data groups based on the 2008 Physical Activity Guidelines for Americans (24); and body mass index (BMI), which was calculated as weight in kilograms divided by height in meters squared and was categorized as underweight and normal weight (BMI, < 25 kg/m^2), overweight (BMI, 25 to < 30 kg/m^2) and obesity (BMI, ≥ 30 kg/m^2). Socioeconomic status included household income, which was divided into low [poverty-to-income ratio (PIR), ≤1], moderate (PIR, 1 to <4) and high (PIR, ≥ 4) groups based on the family PIR (25). We also included self-reported physician-diagnosed medical comorbidities (diabetes, hypertension, cancer). Missing values were set to a separate missing data category for that particular covariate and were included as an indicator variable in the analysis.

Statistical Analysis

Continuous variables are presented as the means with standard deviations (SEs), and categorical variables are presented as proportions. All the analyses performed were pre-specified in an analysis protocol, prior to the conduction of the study. First, for the analysis of smoking status, participants were categorized into non-smokers, former smokers, current smokers, and non-smokers, which was used as the reference group. We used Cox proportional hazards models to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for the association between smoking status and mortality. In the minimally adjusted model, age, sex, and race were adjusted. We additionally adjusted for education, income, BMI, physical activity, alcohol intake, baseline hypertension, diabetes and cancer variables in a multivariable-adjusted model. Second, we used Cox proportional hazards models to calculate HRs and 95% CIs for all-cause, CVD and cancer mortality in association with smoking intensity and the years of smoking cessation compared with the parameters in non-smokers. To further evaluate whether the age of smoking initiation was associated with all-cause mortality, we compared all-cause mortality in former and current smokers who started smoking at 12 or younger than 12 years and 13 to 17 years with those who started smoking at 18 or older than 18 years (reference group). Subsequently, subgroup analysis by five major CVDs, namely, CHD, stroke, MI, angina pectoris and other heart disease also was performed. Finally, we conducted a sensitivity analysis excluding the first 2 years of follow-up and excluding patients with cancer at baseline to test the robustness of our primary findings. The results should be interpreted as exploratory because this study did not account for multiple comparisons. For all analyses, survey weights were applied to account for the complex

sampling design. All analyses were performed with Stata 13.0 statistical software (Stata Corp LP, College Station, TX, USA). Two-sided statistical tests were used, and a *P* value of less than 0.05 was considered an indication of statistical significance.

Patient and Public Involvement

There was no patients and public involvement in the development, design or analysis of this study.

RESULTS

Characteristics of Study Population

This study population consisted of 66,190 patients with CVD, of whom 32.6, 19.6, 25.6, 18.8 and 57.9% were diagnosed with CHD, stroke, MI, angina pectoris and other heart disease, respectively. **Table 1** shows the participants' characteristics, subdivided by their self-reported smoking status. Among these participants, 50.8, 79.9 and 55.2% were women and non-Hispanic White and had a moderate household income, respectively. Overall, approximately 44.0% (29,668) did not smoke, 36.5% (23,439) were former smokers, and 19.6% were current smokers. There was a strong association between smoking status and alcohol drinking status. Namely, never smokers were more likely to be lifetime abstainers. However, current smokers were more likely to be current alcohol drinkers. Compared with current smokers, never and former smokers had higher education levels. In addition, physical activity was similar but less prevalent across all 3 categories of smoking status.

TABLE 1 | Baseline demographic characteristics of CVD patients, according to smoking status.

Characteristics	Never smoker	Former smoker	Current smoker
No. (%)	29,668 (44.0)	23,439 (36.5)	13,083 (19.6)
Age, mean (years)	60.04 (0.2)	65.4 (0.1)	51.8 (0.2)
Sex (%)			
Female	20,011 (62.7)	9,954 (38.0)	6,756 (48.2)
Race (%)			
Hispanic	3,713 (8.8)	1,756 (5.2)	1,086 (5.8)
Non-Hispanic White	20,344 (75.8)	18,548 (85.0)	9,400 (79.6)
Non-Hispanic Black	4,523 (11.7)	2,608 (7.6)	2,252 (12.0)
Non-Hispanic Other	1, 088 (3.6)	527 (2.2)	345 (2.5)
Education level (%)			
Less than high school degree	7,453 (20.6)	6,055 (23.1)	3,758 (26.6)
High school degree	8,148 (28.2)	6,808 (29.8)	4,308 (34.9)
More than high school degree	13,830 (50.4)	10,450 (46.5)	4,938 (37.9)
Missing	237 (0.8)	126 (0.6)	79 (0.6)
Income (%)			
Low	5,494 (13.5)	3,099 (9.8)	3,684 (22.4)
Moderate	16,330 (53.6)	13,745 (56.8)	7,064 (55.8)
High	7,844 (32.9)	6,595 (33.4)	2,335 (21.8)
BMI (kg/m^2)			
<25 (%)	10,191 (34.0)	6,917 (28.5)	5,221 (39.0)
25-30 (%)	9,703 (32.9)	8,517 (37.2)	4,176 (32.4)
>30 (%)	8,868 (30.1)	7,503 (32.2)	3,432 (26.6)
Missing	906 (3.0)	502 (2.1)	254 (1.9)
Alcohol intake (%)			
Lifetime abstainer	11,560 (36.3)	2,968 (11.6)	1,612 (11.7)
Former drinker	5,895 (19.1)	8,368 (34.2)	3,553 (26.6)
Current drinker	11,946 (43.8)	11,900 (53.4)	7,752 (60.4)
Missing	267 (0.9)	203 (0.8)	166 (1.3)
Physical activity (%)			
No meeting	20,206 (65.0)	6,917 (28.5)	5,221 (39.0)
Meeting	8,847 (32.8)	8,517 (37.2)	4,176 (32.4)
Missing	615 (2.2)	572 (2.6)	326 (2.8)
Physician-diagnosed disease (%)			
Hypertension	17,750 (56.6)	15,335 (63.9)	7,245 (53.4)
Diabetes	6,149 (19.5)	5,828 (24.7)	2,151 (15.9)
Cancer	4,583 (15.2)	4,977 (21.6)	1,880 (13.8)
CVD types			
CHD	8,456 (28.0)	9,305 (40.6)	3,763 (28.1)
Stroke	5,841 (18.6)	4,957 (20.2)	2,920 (20.6)
Heart attack	5,932 (19.2)	7,419 (31.9)	3,741 (28.0)
Angina	5,020 (15.9)	5,158 (22.2)	2,468 (18.9)
Other heart disease	17,754 (61.7)	12,454 (54.1)	7,174 (56.4)

Values are n (%). CVD, cardiovascular disease; CHD, coronary heart disease; BMI, body mass index.

Association of Smoking Status With Mortality

During a mean of 8.1 years of follow-up, a total of 22,518 deaths were recorded, including 6,473 CVD deaths, 4,050 cancer deaths and 11,995 other cause deaths. In the multivariable-adjusted model, current smokers and former smokers had a higher risk of all-cause, CVD and cancer mortality than never smokers (**Figure 1**). The corresponding HRs and 95% CIs among former smokers and current smokers were 1.26 (95% CI: 1.21 to 1.31) and 1.96 (95% CI: 1.86 to 2.07) for all-cause mortality, 1.12 (95% CI: 1.05 to 1.21) and 1.80 (95% CI: 1.64 to 1.97) for CVD mortality, and 1.49 (95% CI: 1.35 to 1.64) and 2.78 (95% CI: 2.49 to 3.09) for cancer mortality, respectively. Furthermore, similar results were found when the study subjects were stratified according to sex (**Appendix Table 1**). The results were also similar in patients with specific types of CVD, except CVD mortality among former smokers with the presence of stroke (HR, 1.06; 95% CI: 0.94 to 1.21) (**Figure 1**).

Aassociation of Smoking Intensity With Mortality

In comparison to never smokers, people who smoked ≤9, 10–19, and ≥20 cigarettes per day had a substantially higher risk of dying from all causes, CVD and cancer, regardless of the specific type of CVD (**Figure 2**). It is worth noting that there was a dose-dependent association between smoking intensity and cancer-related mortality in each CVD group. However, the risks of all-cause and CVD mortality did not increase in a monotonic fashion with an increasing number of cigarettes smoked per day in each CVD group (**Figure 2**). More interestingly, we found that risks decreased as the number of cigarettes smoked daily

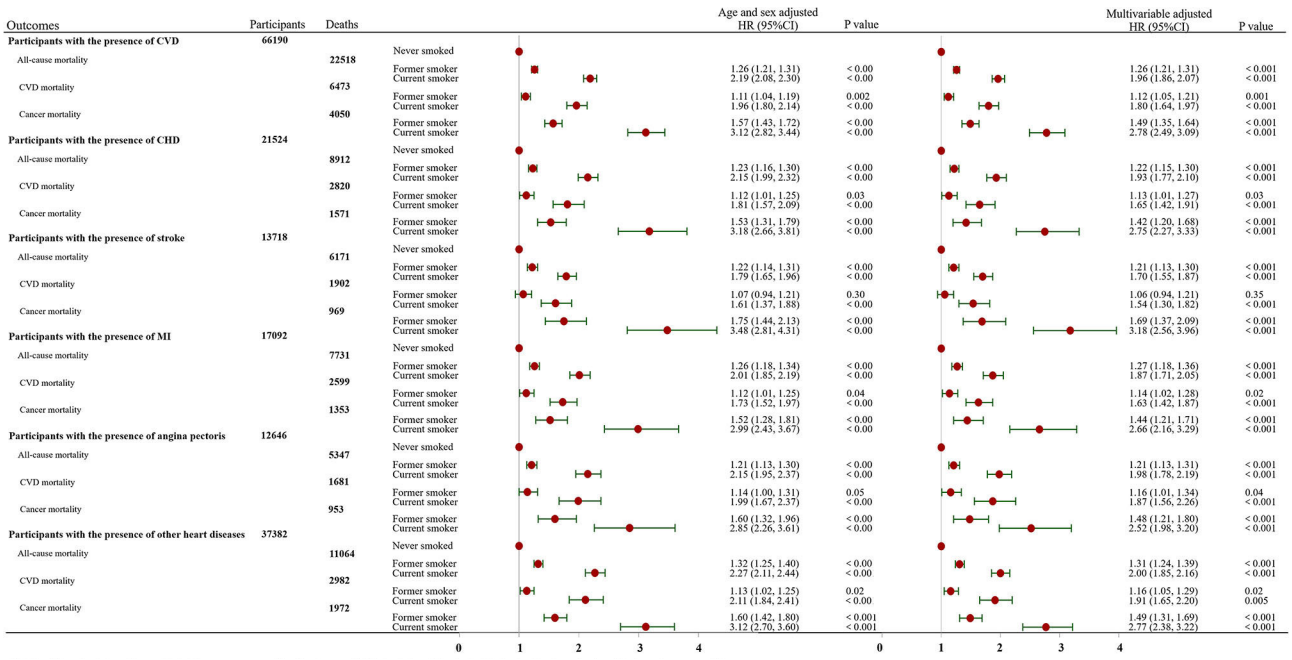


FIGURE 1 | Association of smoking status with all-cause, CVD and cancer mortality stratified by the presence of cardiovascular disease. The multivariable-adjusted model was adjusted for age, sex, race, education, income, body mass index, physical activity, alcohol intake, baseline hypertension, diabetes and cancer variables. CI, confidence interval; HR, hazard ratio; CVD, cardiovascular disease; CHD, coronary heart disease; MI, myocardial infarction.

increased in the case of deaths from CVD in participants with angina pectoris and stroke but not in participants in other CVD groups (**Figure 2**).

Association of the Number of Years Since Smoking Cessation With Mortality

As reported in **Figure 3**, the risks for all-cause, CVD-related and cancer-related mortality decreased as the number of years since quitting increased in former smokers among all CVD groups. For smokers who had stopped smoking for ≥20 years, the risk of CVD mortality gradually approached the level of never smokers (**Figure 3**). Nevertheless, former smokers who had quit smoking ≥20 years prior did not have the same risk of all-cause mortality as never smokers in any CVD groups except the angina pectoris group (**Figure 3**). The cancer mortality risk for smokers with CVD, stroke and other heart disease who had stopped smoking for ≥20 years was also not identical to that of participants who had never smoked (**Figure 3**). Additionally, former smokers who had stopped smoking for ≥30 years had significant 45, 45, and 64% reductions in the risk of all-cause, CVD and cancer mortality, respectively, compared to continuing smokers in the group of CVD patients (**Appendix Table 2**).

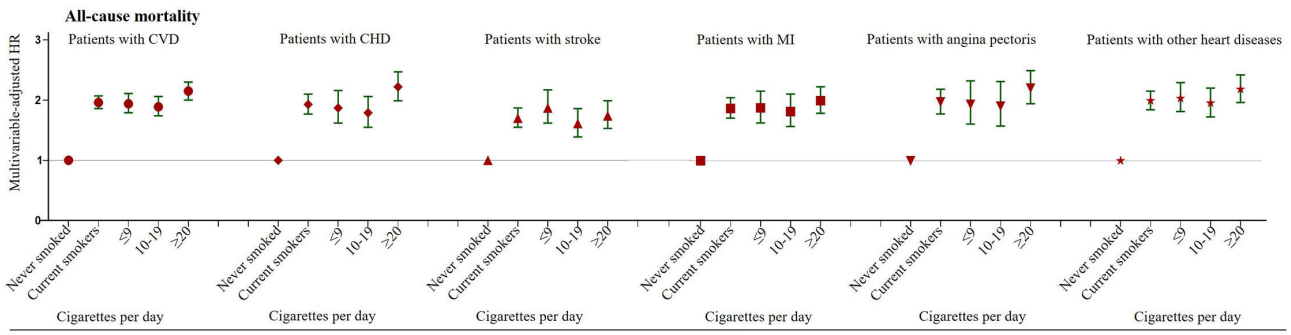
Nevertheless, when never smokers were used as the reference group, we further observed that former smokers who quitted for ≥30 years still had higher all-cause mortality than never-smokers (**Appendix Table 2**). However, the differences in CVD mortality among never smokers and former smokers who quitted for ≥20 years, were not statistically significant (**Appendix Table 2**). Moreover, after 30 years since quitting, the risk of dying from cancer causes among former smokers were also at the same level of that observed among never smokers (**Appendix Table 2**). Therefore, the risk of dying from CVD and cancer causes returned similar to that of never smoker after 20 and 30 years since quitting, respectively. Yet, the risk of dying from all causes did not return similar to that of never smoker after 30 years since quitting.

Association of the age of Smoking Initiation With Mortality

For current smokers, all-cause mortality hazards were 19% higher and 27% higher for those who started smoking before the age of 12 years in the CVD group (HR, 1.19; 95% CI, 1.01 to 1.41) and in the other heart disease group (HR, 1.27; 95% CI, 1.01 to 1.61), respectively, but not among the remaining 4 CVD groups (**Figure 4**). For former smokers, in comparison to the age of smoking initiation in the 18+ years category, participants who started smoking at age 12 or younger than 12 years had an increased risk of all-cause mortality in the CHD, MI and angina pectoris groups (**Figure 4**). Compared with current and former smokers who started smoking at age 18 or older, current and former smokers who started smoking at age 13 to 17 years did not have different all-cause mortality regardless of the CVD group (**Figure 4**).

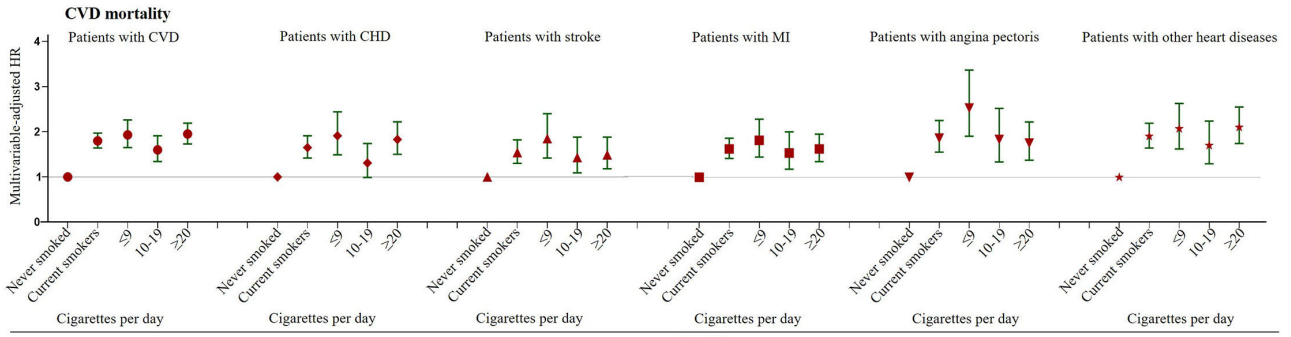
Sensitivity Analysis

Excluding deaths that occurred during the first 2 years of follow-up or participants who had cancer at baseline did not substantially alter the significant



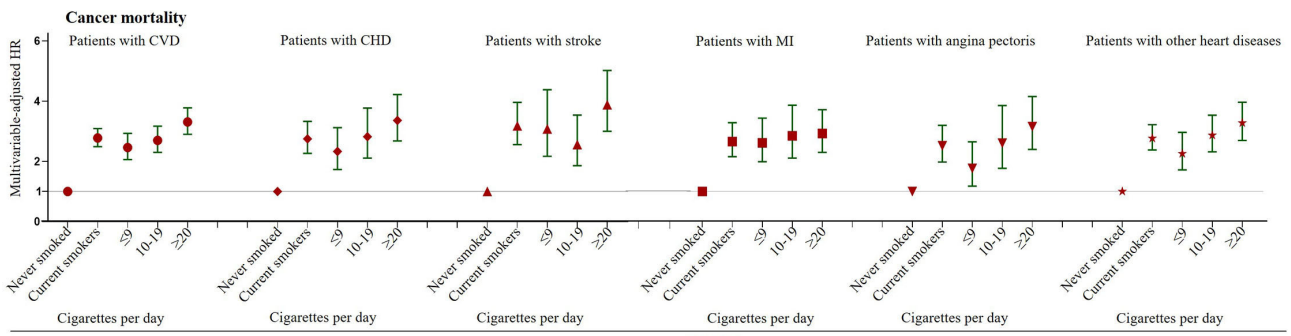
HR(95%CI)

Smoking status	Patients with CVD	Patients with CHD	Patients with stroke	Patients with MI	Patients with angina pectoris	Patients with other heart diseases
Never smoked	Reference	Reference	Reference	Reference	Reference	Reference
Current smokers	1.96 (1.86-2.07)	1.93 (1.77-2.10)	1.70 (1.55-1.87)	1.87 (1.71-2.05)	1.98 (1.78-2.19)	2.00 (1.85-2.16)
≤9 Cigarettes per day	1.94 (1.79-2.11)	1.87 (1.62-2.16)	1.87 (1.62-2.17)	1.88 (1.63-2.16)	1.94 (1.61-2.33)	2.04 (1.82-2.30)
10~19 Cigarettes per day	1.89 (1.74-2.06)	1.79 (1.55-2.06)	1.61 (1.39-1.86)	1.82 (1.57-2.11)	1.91 (1.58-2.32)	1.96 (1.73-2.21)
≥20 Cigarettes per day	2.15 (2.00-2.30)	2.22 (1.99-2.47)	1.74 (1.53-1.99)	2.00 (1.79-2.23)	2.21 (1.95-2.50)	2.19 (1.97-2.43)



HR(95%CI)

Smoking status	Patients with CVD	Patients with CHD	Patients with stroke	Patients with MI	Patients with angina pectoris	Patients with other heart diseases
Never smoked	Reference	Reference	Reference	Reference	Reference	Reference
Current smokers	1.80 (1.64-1.97)	1.65 (1.42-1.91)	1.54 (1.30-1.82)	1.63 (1.42-1.87)	1.87 (1.56-2.26)	1.91 (1.65-2.20)
≤9 Cigarettes per day	1.93 (1.65-2.26)	1.91 (1.49-2.44)	1.85 (1.42-2.40)	1.82 (1.45-2.29)	2.54 (1.91-3.38)	2.08 (1.63-2.64)
10~19 Cigarettes per day	1.60 (1.34-1.91)	1.31 (0.99-1.74)	1.43 (1.09-1.88)	1.54 (1.18-2.01)	1.84 (1.34-2.53)	1.71 (1.30-2.25)
≥20 Cigarettes per day	1.95 (1.73-2.19)	1.83 (1.50-2.22)	1.49 (1.18-1.88)	1.63 (1.35-1.96)	1.76 (1.38-2.23)	2.11 (1.75 -2.56)



HR(95%CI)

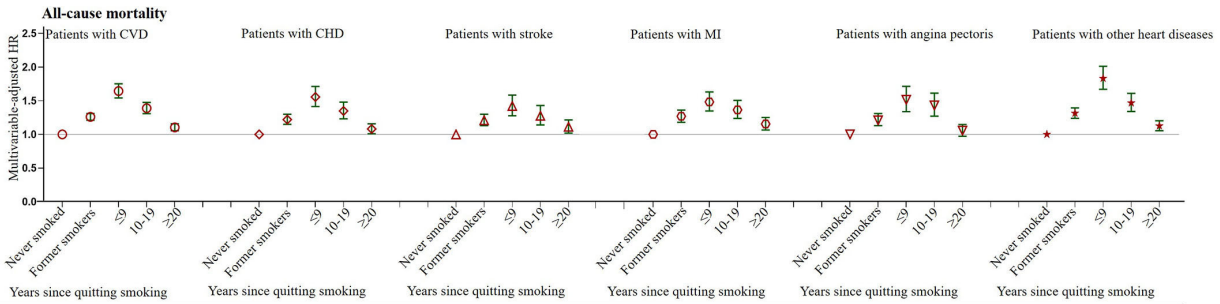
Smoking status	Patients with CVD	Patients with CHD	Patients with stroke	Patients with MI	Patients with angina pectoris	Patients with other heart diseases
Never smoked	Reference	Reference	Reference	Reference	Reference	Reference
Current smokers	2.78 (2.49-3.09)	2.75 (2.27-3.33)	3.18 (2.56-3.96)	2.66 (2.16-3.29)	2.52 (1.98-3.20)	2.77 (2.38-3.22)
≤9 Cigarettes per day	2.46 (2.06-2.93)	2.33 (1.73-3.12)	3.08 (2.17-4.38)	2.62 (1.99-3.44)	1.77 (1.18-2.65)	2.26 (1.72 -2.97)
10~19 Cigarettes per day	2.70 (2.30-3.17)	2.82 (2.11-3.77)	2.56 (1.86-3.54)	2.85 (2.11-3.87)	2.61 (1.77-3.86)	2.87 (2.32-3.54)
≥20 Cigarettes per day	3.31 (2.90-3.78)	3.36 (2.68-4.22)	3.88 (3.00-5.02)	2.93 (2.30-3.72)	3.16 (2.40-4.16)	3.28 (2.70 -3.97)

FIGURE 2 | Association of Smoking Intensity with All-cause, CVD and Cancer Mortality Stratified by the Presence of Cardiovascular Disease. The multivariable-adjusted model was adjusted for age, sex, race, education, income, body mass index, physical activity, alcohol intake, baseline hypertension, diabetes and cancer variables. CI, confidence interval; HR, hazard ratio; CVD, cardiovascular disease; CHD, coronary heart disease; MI, myocardial infarction.

associations of smoking status (never smokers, former smokers and current smokers) with all-cause or CVD mortality among patients with CVD **(Appendix Table 3).**

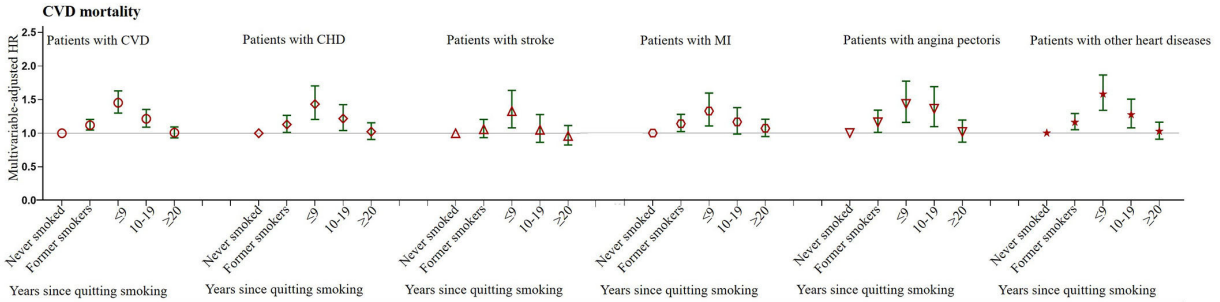
DISCUSSION

The present study showed that current and former smokers had a higher risk of all-cause, CVD and cancer mortality



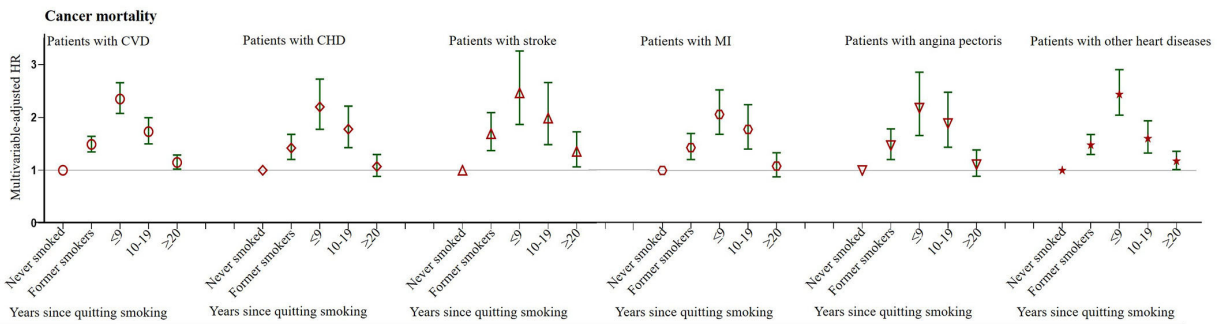
HR(95%CI)

Smoking status	Patients with CVD	Patients with CHD	Patients with stroke	Patients with MI	Patients with angina pectoris	Patients with other heart diseases
Never smoked	Reference	Reference	Reference	Reference	Reference	Reference
Former smokers	1.26 (1.21-1.31)	1.22 (1.15-1.30)	1.21 (1.13-1.30)	1.27 (1.18-1.36)	1.21 (1.13-1.31)	1.31 (1.24-1.39)
≤9 years since quitting smoking	1.64 (1.54-1.75)	1.56 (1.41-1.71)	1.42 (1.28-1.58)	1.48 (1.35-1.63)	1.51 (1.34-1.71)	1.83 (1.67-2.01)
10~19 years since quitting smoking	1.39 (1.31-1.48)	1.35 (1.23-1.48)	1.28 (1.14-1.43)	1.36 (1.24-1.50)	1.43 (1.27-1.61)	1.47 (1.34-1.61)
≥20 years since quitting smoking	1.10 (1.05-1.16)	1.08 (1.01-1.16)	1.11 (1.02-1.21)	1.16 (1.07-1.25)	1.06 (0.97-1.15)	1.13 (1.06-1.20)



HR(95%CI)

Smoking status	Patients with CVD	Patients with CHD	Patients with stroke	Patients with MI	Patients with angina pectoris	Patients with other heart diseases
Never smoked	Reference	Reference	Reference	Reference	Reference	Reference
Former smokers	1.12 (1.05-1.21)	1.13 (1.01-1.27)	1.06 (0.94-1.21)	1.14 (1.02-1.28)	1.16 (1.01-1.34)	1.16 (1.05-1.29)
≤9 years since quitting smoking	1.45 (1.30-1.63)	1.43 (1.21-1.70)	1.33 (1.08-1.64)	1.33 (1.11-1.59)	1.43 (1.16-1.77)	1.58 (1.34-1.86)
10~19 years since quitting smoking	1.22 (1.09-1.35)	1.22 (1.04-1.43)	1.05 (0.86-1.28)	1.17 (0.98-1.38)	1.36 (1.10-1.69)	1.27 (1.08-1.50)
≥20 years since quitting smoking	1.01 (0.93-1.09)	1.02 (0.91-1.15)	0.96 (0.83-1.11)	1.07 (0.95-1.21)	1.02 (0.86-1.19)	1.03 (0.91-1.16)



HR(95%CI)

Smoking status	Patients with CVD	Patients with CHD	Patients with stroke	Patients with MI	Patients with angina pectoris	Patients with other heart diseases
Never smoked	Reference	Reference	Reference	Reference	Reference	Reference
Former smokers	1.49 (1.35-1.64)	1.42 (1.20-1.68)	1.69 (1.37-2.09)	1.44 (1.21-1.71)	1.48 (1.21-1.80)	1.49 (1.31-1.69)
≤9 years since quitting smoking	2.35 (2.07-2.66)	2.20 (1.77-2.72)	2.46 (1.87-3.25)	2.08 (1.69-2.55)	2.20 (1.67-2.89)	2.46 (2.06-2.94)
10~19 years since quitting smoking	1.73 (1.50-1.99)	1.78 (1.43-2.21)	1.99 (1.48-2.66)	1.79 (1.41-2.27)	1.90 (1.45-2.50)	1.62 (1.34-1.96)
≥20 years since quitting smoking	1.15 (1.02-1.29)	1.07 (0.88-1.30)	1.35 (1.06-1.73)	1.09 (0.88-1.34)	1.11 (0.89-1.40)	1.18 (1.02-1.37)

FIGURE 3 | Association of the Number of Years Since Smoking Cessation with All-cause, CVD and Cancer Mortality Stratified by the Presence of Cardiovascular Disease. The multivariable-adjusted model was adjusted for age, sex, race, education, income, body mass index, physical activity, alcohol intake, baseline hypertension, diabetes and cancer variables. CI, confidence interval; HR, hazard ratio; CVD, cardiovascular disease; CHD, coronary heart disease; MI, myocardial infarction.

among patients with CVD, irrespective of the specific type of CVD. Among CVD patients, smokers had a higher risk for cancer mortality than for CVD mortality. Additionally, we found a dose-dependent positive association between smoking and cancer mortality, but the association for all-cause mortality was presented in a non-dose-response manner. Moreover, we observed that the risk of all-cause, CVD and cancer mortality declined as the number of years since quitting increased among

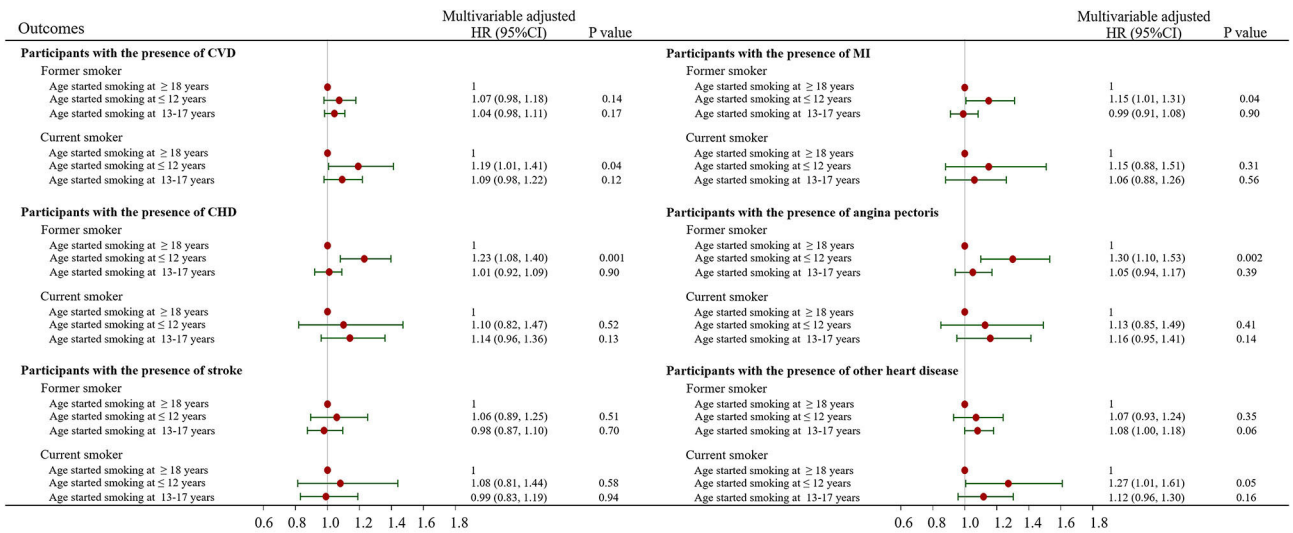


FIGURE 4 | Association of the age of smoking initiation with all-cause, CVD and cancer mortality stratified by the presence of cardiovascular disease. The multivariable-adjusted model was adjusted for age, sex, race, education, income, body mass index, physical activity, alcohol intake, baseline hypertension, diabetes and cancer variables. CI, confidence interval; HR, hazard ratio; CVD, cardiovascular disease; CHD, coronary heart disease; MI, myocardial infarction.

patients with a previous CVD, and 20 or more than 20 years of smoking cessation ceased to be significantly related to increased mortality risk. Finally, early smoking initiation before 12 years of age was associated with increased risk for all-cause mortality among current smokers who had a history of CVD.

Numerous previous studies showed that smoking increased the risk of long-term mortality in patients with acute myocardial infarction by more than 19 to 43% (20, 26, 27). Similar results were also observed in patients who had been diagnosed with stable ischaemic heart disease (49%) (28), arterial disease (66%) (29), atrial fibrillation (82%) (30), and stroke (36%) (31). Moreover, our findings have also revealed that smokers had a 96% higher risk of long-term all-cause mortality than never smokers among patients with CVD. Obviously, these findings are much lower than those related to the contemporary risks of smoking from the general UK, US and Japan populations, where current smokers had a 180 to 200% excess risk of all-cause mortality (HR, 2.8 to 3.0) compared with the mortality of never smokers (21, 32, 33). The reasons for these inconsistencies between the CVD population and the general population are unclear, and further study is required.

Numerous prior studies tended to focus on the all-cause and CVD mortality effects of smoking on CVD patients. Our study improves upon prior long-term studies by exploring the differences in this association with smoking between various causes of death, especially cancer. Surprisingly, in the present study, former and current smokers had a higher HR for mortality due to cancer than for mortality due to CVD. Our findings are consistent with emerging evidence from the stroke population that showed that smoking increased the risk of CVD and cancer mortality, with HRs of 1.15 (95% CI, 0.88 to 1.50) and 3.83 (95% CI, 2.48 to 5.91) for CVD and cancer mortality, respectively (31). Several explanations may account for the findings that smoking in CVD patients has a greater impact on cancer mortality than on CVD mortality. First, some socioeconomic and clinical factors might attenuate the association between smoking and death from CVD (31). Another explanation is that smokers with CVD might undergo more aggressive CVD treatments and secondary prevention, which reduce their risk of CVD mortality. In addition, smoking potentiates responsiveness to clinical treatments, particularly antiplatelet therapies, in the CVD population (34–37).

Our analysis showed a significant positive dose-response association between smoking intensity and cancer mortality but not all-cause or CVD mortality. Inconsistent with our findings, previous studies showed that the risk of all-cause CVD and cancer mortality increased significantly with an increasing number of cigarettes smoked per day (13, 14, 32). Furthermore, we found a more interesting result that the risk of death from CVD decreased significantly with smoking intensity in stroke and angina patients. A possible biological mechanism to explain this phenomenon might be an ingestion of increased amounts of nicotine with smoking intensity that might induce P2Y12 receptor expression in human platelet lysates (within the dose range) (37), which may increase the relative benefit of antiplatelet therapy in smokers compared to that of non-smokers. However, the exact underlying mechanisms remain to be determined.

It turns out that smoking cessation has multiple benefits for different populations, and the CVD population is no exception (5, 13, 15, 21, 29, 31, 32, 38, 39). Previous studies showed that smokers who ceased smoking at 25 to 34, 35 to 44, or 45 to 54 years of age gained approximately 10, 9, and 6 years of life, respectively, relative to persistent smokers (21). This result revealed the beneficial effect of smoking cessation on mortality. In agreement with previous studies, our results also confirm that the risk of death from all causes, CVD and cancer substantially declined with an increasing number of years since quitting, with risks equivalent to those of never smokers after 30 years of cessation. These findings, the absolute benefits of quitting smoking, suggest that further work is

needed to encourage smoking cessation in patients with CVD. Contemporary guidelines also emphasize that cigarette/tobacco cessation is an important treatment for primary and secondary CVD prevention and is the most cost-effective strategy for CVD prevention (40, 41). Although the benefits of smoking cessation among patients with CVD are well established in this study and other previous studies, the true gain of life-years from the time of cessation in the CVD population remains unclear. Therefore, future studies should focus on the quantitative relationship between the time of smoking cessation and the true gain of life-years in the CVD population.

Numerous prior studies have assessed the relationship between the age of smoking initiation and smoking-related morbidities and mortality in the general population (32, 42–45), and most studies have shown a strong inverse association of mortality with age at initiation (32, 42–45). However, data on the impact of the age at smoking initiation on adverse outcomes in CVD patients are weak and unclear. Although our study showed that a young age at smoking initiation was associated with increased risk, the differences were not statistically significant. It is likely that the intensity and duration of smoking may have disturbed the relationship between the age of smoking initiation and mortality.

LIMITATIONS

The study has several potential limitations. First, although our results remained robust after a series of sensitivity analyses, we cannot establish a cause-effect relationship between smoking and mortality in CVD patients due to the observational nature of our study. Second, smoking status was collected with the use of self-reported responses only at baseline, and it is possible that smoking status is subject to measurement error and might have changed throughout follow-up. Third, this study did not collect information on what types of tobacco products were smoked, and the potential influence of types of tobacco products could not be tested, which should be evaluated in future studies. Fourth, although we were able to adjust for many confounding factors in our study, residual and unmeasured confounding factors cannot be fully ruled out. For example, blood pressure, blood glucose, lipid profiles (Low Density Lipoprotein-Cholesterol, High Density Lipoprotein-Cholesterol, Tryglicerides) and treatments are some of the factors that can clearly bias the relationship between smoke and mortality outcomes. Therefore, the true strength of association remains uncertain. Fifth, this study did not adjust for multiple comparison. In addition, there were overlaps in the subgroups of CVD, which might make it harder to draw conclusions about the impact of smoking status on the risk of all-cause, CVD and cancer mortality in the specific CVD subgroup. Therefore, future studies should focus on examining the effect of smoking status on the risk of death from all-cause, CVD and cancer in patients with a specific CVD disease. Finally, since the NHIS is a passive follow-up study that relies on probabilistic matching to the NDI to assess the death of participants, the cause of death may be misclassified and the estimated hazards of cause-specific mortality, but not the estimated hazards of all-cause mortality, may have been affected.

In conclusion, the findings of this prospective cohort study using a large sample of U.S. adults with CVD suggest that there is a higher all-cause, CVD and cancer mortality among former and current smokers than never smokers but did not reveal a dose-response pattern with respect to smoking intensity in current smokers. In addition, an inverse association between the age of smoking initiation and mortality was not observed. This by no means indicates that it should not be encouraged to stop or reduce smoking among patients with CVD or to control tobacco use among youth. We further found that the risk of all-cause, CVD and cancer mortality declined significantly with increasing time since cessation of smoking, which suggests that smoking is an important modifiable risk factor for mortality in the CVD population.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.cdc.gov/nchs/nhis/index.htm.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by in this study, only public-use NHIS data were used. The public-use NHIS data are de-identified and do not include any protected health information. Our study was based on secondary analysis of public-use data. The publicly available data are considered as exempt under the ethical board review of the corresponding author's institution (The Third Xiangya Hospital of Central South University). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-lW and X-cZ: conception and design of the study and administrative support. J-lW, Y-fW, and X-cZ: data analysis and interpretation. J-lW, W-jY, L-yZ, and X-cZ: original draft preparation. All authors final approval of manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.728217/full#supplementary-material

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Impact of Conventional Cardiovascular Risk Factors on Left Internal Mammary Artery Graft Disease

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Background: The development of atherosclerosis was considered as the common cause of the stenosis of coronary artery grafts. Left internal mammary artery (LIMA) was the best artery graft for further effectiveness of coronary artery bypass grafting (CABG). We sought to assess the impact of known conventional cardiovascular risk factors (RFs) on LIMA graft stenosis.

Methods: A retrospective study including 618 participants, who had recurrence of chest pain after CABG, aged ≥18 years, hospitalized for coronary angiography in Beijing Anzhen hospital between 2010 and 2017 was performed. All the participants were confirmed to have LIMA graft. Multivariate analysis was conducted to determine the relationship between conventional RFs and LIMA graft stenosis.

Results: Of the study, 220 (35.6%) participants continued to smoke, 504 (81.6%) were overweight or obese, and 411 (66.5%) and 242 (39.2%) reported concomitant hypertension and diabetes, respectively. LIMA graft stenosis occurred in 161 participants (26.1%). Postoperative smoking, a CABG duration of ≥10 years and hyperglycemia without diabetes had an increased risk of LIMA graft stenosis, the odds ratio (OR) was 1.86 [95% confidence interval (CI): 1.26–2.78], 2.24 (95%CI:1.33–3.478), and 2.44(95% CI:1.39–4.32), respectively. Statin use (OR, 0.28; 95% CI: 0.25–0.5) and low-density lipoprotein cholesterol (LDL-C) < 1.8 mmol/L (OR, 0.27; 95% CI: 0.14–0.53) had a significantly decreased risk of LIMA graft stenosis. While, only 15.4% (95/618) achieved the target LDL-C level.

Conclusions: Postoperative smoking and hyperglycemia without diabetes had an increased risk of LIMA graft stenosis. Statin use and LDL-C <1.8 mmol/L decreased the risk.

Keywords: coronary heart disease, coronary artery bypass grafting, left internal mammary artery, atherosclerosis, risk factor

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INTRODUCTION

Coronary artery bypass graft (CABG) surgery is the most complete and durable treatment of left main coronary artery and three-vessel coronary severe stenosis or occlusion disease and has been an established therapy for nearly 50 years. Following CABG, effects should be done to reduce the recurrence of cardiovascular event. Studies have shown that the recurrence rate of angina is 17% in

1 year after CABG and 63% in 10 years after CABG (1), which was associated with native coronary artery disease progression and the development of graft atherosclerosis, and the stenosis of grafts was considered as the most common cause (2).

The grafts were saphenous vein (SV), left internal mammary artery (LIMA), right internal mammary artery (RIMA) and radial artery (RA). Using graft from LIMA to the left anterior descending artery (LAD) improves the effectiveness of CABG with high long-term patency rate (3). The great SV as a second conduit are commonly used for other target vessels of multi-vessel disease, however, they are associated high failure rate; the stenosis rate was more than 20% in 1 year, 40% in 2 years and 60% in 10 years after CABG (4). RA seems to be better graft than SV because prognosis improvement in increasing the long-term survival and decreasing the incidence of cardiovascular events were found in more and more studies (5–7). So, full arterial grafting for further effectiveness of CABG may be the direction (8, 9). Even though RA graft are strongly suggested in CABG surgery as the second artery graft (5–9), it appears to be underutilized in China with the percentage <1%. Hence, LIMA graft stenosis will be highlighted in this study.

The patency rate of grafts rapidly increases within the first year after CABG and then slowly,which was attributed to anastomotic site stenosis between 1 month and 1 year and atherosclerosis development of the bypass graft 1 year later (10). Dyslipidemia, hypertension, diabetes mellitus, obesity and smoking were considered as conventional risk factors (RFs) for atherosclerosis, which largely contribute to cardiovascular disease (CVD), approximately 70% of native coronary artery case and deaths were attributed to them (11). Are those conventional RFs associated with the development of atherosclerosis in artery grafts? Conflicting evidence was reported in previous studies (12–17). Therefore, we performed this study, enrolled patients who had recurrence of chest pain after performing CABG surgery at least 1 year, analyzed the impact of conventional cardiovascular RFs on LIMA graft stenosis.

METHODS

Participants

This study was based on a retrospective, single-center analysis of patients who had recurrence of chest pain after CABG and had subsequent coronary angiographic findings in the Department of Cardiology of Beijing Anzhen Hospital from January 2010 to December 2017. All the participants were enrolled through the hospital's electronic record system. The inclusion criteria were an age of ≥18 years; recurrence of chest pain after CABG; completed electronic medical records and coronary angiographic findings; underwent LIMA-LAD graft. The exclusion criteria were CABG within 1 year before the start of the study; second CABG; renal insufficiency requiring dialysis treatment; and/or cancer.

Abbreviations: RFs, risk factors; LIMA, left internal mammary artery; CVD, cardiovascular disease; CABG, coronary artery bypass grafting; SV, The grafts were saphenous vein; RIMA, right internal mammary artery; RA, radial artery; LAD, left anterior descending artery; OR, odds ratio; CI, confidence interval; LDL-C, low-density lipoprotein cholesterol; ARB, angiotensin receptor blockers; ACEI, angiotensin-converting enzyme inhibitors; BMI, body mass index.

Data Collection

Data regarding the patients' demographics, conventional RFs for CVD, coronary angiographic findings and medication intake were collected from the hospital records by trained abstractors, using physician notes, laboratory reports, patient histories, and discharge summaries. A structured questionnaire was developed. Demographic information consisted of gender, age; conventional RFs included Overweight/obese, medical history of hypertension and diabetes, LDL-C level and smoking details. Basic medication therapy for secondary prevention to cardiovascular events included the usage of aspirin, β-blocker, angiotensin receptor blockers (ARB)/angiotensin-converting enzyme inhibitors (ACEI) and statins (dosage and type of those drugs were not obtained in the study). Height, weight, and blood pressure were measured in ward and recorded. The fasting plasma glucose (FPG) and LDL-C concentrations were examined while the patients were hospitalized. Coronary angiographic findings was reviewed, occlusion or stenosis of the native coronary arteries (including the left main, left anterior descending, circumflex, and right coronary arteries) and artery grafts were recorded. Number and stenosis of SV graft was not collected in this study.

Measurements and Diagnostic Criteria

Chest pain was identified based on induction factor, position pain, feature of pain, pain duration, radiating pain and remission. We defined severe stenosis for native coronary artery as stenosis of ≥50% for left main, ≥70% for left anterior descending, circumflex, and right coronary arteries; defined artery graft disease as stenosis of ≥50%, and at least 1 stenosis was measure in LIMA based on angiography. Uncontrolled blood pressure was defined as SBP of ≥140 mmHg and / or DBP of ≥90 mmHg, and controlled blood pressure was defined as SBP of <140 mmHg and DBP of <90 mmHg. The body mass index (BMI) was calculated as body mass in kilograms divided by height in meters squared. Normal weight was defined as a BMI of <24.0 kg/m^2, an overweight status was defined as a BMI of 24.0 to 27.9 kg/m^2, and obese was defined as a BMI of ≥ 28.0 kg/m^2. Ideal target of LDL-C was defined as an LDL-C concentration of <1.8 mmol/L (70 mg/dl). Ideal target of blood glucose was defined as an FPG of <7 mmol/L (126 mg/dl). The presence of hypertension or diabetes was defined by a previous diagnosis of hypertension or diabetes in the document of diagnosis on admission or summary of history. Nonsmokers were defined as patients who had never smoked, ever-smokers who stopped smoking at least 6 months, and current smokers were defined as those who were smoking every day or some days after CABG or stopped smoking <6 months at the time of interview.

Statistical Analysis

The statistical analysis was performed with SPSS software for Windows, version 18.0 (SPSS, Inc., Chicago, IL, USA). Normally distributed continuous variables are presented as means ± standard deviations. A student's *t*-test was used to compare two independent samples. Categorical variables are expressed as proportions. The chi-square test was used to compare the differences in proportions between different groups. Multiple

TABLE 1 | General characteristics of the study population.

General characteristics	Total subjects
Sex, male	500 (80.9)
Age (y)	
Mean age	63.6 ± 8.8
<65	313 (50.6)
≥65	305 (49.4)
Hospital in which CABG surgery underwent	
Anzhen hospital	403 (65.2)
Other hospital	215 (34.8)
Duration of CABG (y)	
<5	236 (38.2)
5–9	266 (43.0)
≥10	116 (18.8)
Conventional risk factors	
Postoperative smoking	220 (35.6)
Overweight/obesity (BMI ≥ 24)	504 (81.5)
LDL-C ≥1.8 mmol/L	523 (84.6)
History of hypertension	411 (66.5)
History of diabetes	242 (39.2)
Prior MI	186 (30.1)
Current presentation	
Angina	577 (93.4)
Acute MI	41 (6.6)
Native coronary artery severe stenosis	
Left main stenosis	298 (48.2)
Left anterior descending stenosis	473 (76.5)
Circumflex coronary artery stenosis	383 (62.0)
Right coronary artery stenosis	269 (43.5)
LIMA graft stenosis	161 (26.1)

Data are presented as mean ± standard deviation or n (%).
CABG, coronary artery bypass grafting; BMI, body mass index; LDL-c, low-density lipoprotein cholesterol, MI, myocardial infarction; LIMA, internal mammary artery.

backward stepwise logistic regressions were performed to identify the association between risk factors and artery graft disease. All significant variables based on the chi-square test were entered into a multivariate model. All reported *P* values were two-tailed, and $P < 0.05$ were considered statistically significant.

RESULTS

General Information

A total of 618 participants (male: $n = 500$, female: $n = 118$), 13 (2.1%) together with RIMA graft and 4 (0.65%) together with RA graft. The age ranged from 37 to 75 years (mean, 63.6 ± 8.8 years). Mean interval after CABG was 6.4 ± 3.5 years (range from 1 to 18 years), and 83.8% of them had undergone CABG <10 years prior to the study. The prevalence of conventional RFs was postoperative smoking 220 (35.6%), overweight or obese 504 (81.6%), hypertension 411 (66.5%), diabetes 242 (39.2%), and 15.4% (95 of 618) achieved LDL-C target level of <1.8 mmol/L. Recurrent angina was the most common presentation (93.4%, 577/618). According to the cut-off point of coronary artery stenosis, 298 (48.2%) patients had a left main disease, 473 (76.5%), 383 (62.0%), and 269 (43.5%) had a severe stenosis in LAD, circumflex coronary artery, and right coronary artery, respectively. LIMA graft stenosis occurred in 161 participants (26.1%). Main characteristics of the overall sample are presented in **Table 1**.

TABLE 2 | Mean level and the proportion of optimal control for conventional RFs according to LIMA graft stenosis.

Conventional RFs	LIMA graft stenosis		
	No ($n = 457$)	Yes ($n = 161$)	*P* value
SBP (mmHg)	129 ± 18	129 ± 17	0.667
DBP (mmHg)	76 ± 11	76 ± 10	0.596
FPG (mmol/L)	5.87 ± 2.09	6.02 ± 2.41	0.453
LDL-C (mmol/L)	2.69 ± 0.91	2.94 ± 0.83	0.033
BMI (kg/m^2)	27.1 ± 3.6	27.5 ± 4.3	0.250
BMI<24 (kg/m^2)	86 (18.8)	28 (17.4)	0.688
LDL-C<1.8 (mmol/L)	83 (18.2)	12 (7.5)	0.001
BP < 140/90 mmHg (in patients with hypertension)	172 (55.8)	55 (53.4)	0.666
FPG < 7.0 mmol/L (in patients with diabetes)	93 (51.7)	23 (37.1)	0.048
Smoking cessation (in smokers at baseline)	88 (37.6)	12 (14.0)	<0.001

Data are presented as mean ± standard deviation or n (%).
RFs, risk factors; LIMA, left internal mammary artery; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-C, low-density lipoprotein; BMI, body mass index; BP, blood pressure; FPG, fasting plasma glucose.

Mean Level and Optimal Control for Conventional RFs According to LIMA Graft Stenosis

Mean LDL-C level was 2.94 mmol/L among patients with LIMA graft stenosis, which was higher than that in those without LIMA graft stenosis (2.69 mmol/L, P=0.033). For mean level of SBP, DBP, FPG and BMI, no correlation with LIMA graft stenosis were observed (all p >0.05). Higher proportion of patients with LDL-C <1.8 mmol/L was observed in patients without LIMA graft stenosis than that in those with LIMA graft stenosis (18.2 vs. 7.5%, $P = 0.001$). There were significant differences in the proportion of FPG <7.0 mmol/L and smoking cessation across LIMA graft stenosis groups (P<0.05). There were no significant differences of in the proportion of BMI<24 kg/m^2 and BP<140/90 mmHg between the two groups (**Table 2**).

Correlation of Conventional RFs With LIMA Graft Stenosis

Of 618 patients, 161 (26.1%) developed LIMA graft stenosis, which did not vary by sex, age group, BP group, BMI group, and use of aspirin, ACEI/ARB and β-blocker (all $P > 0.05$). It was more frequent among patients without history of diabetes who had an FPG≥7.0 mmol/L, with postoperative smoking, or with a duration of CABG≥10 years (all $P < 0.05$); lower prevalence was observed among patients with LDL-C<1.8 mmol/L and taking statin ($P = 0.001$). Multivariate logistic regression analysis was

TABLE 3 | Prevalence of LIMA graft stenosis and the relationship between LIMA graft stenosis and conventional RFs.

Characteristics		Number	Prevalence of LIMA graft stenosis		Multivariate analysis	
			n (%)	*P* value	OR (95% CI)	*P* value
Gender						
Male		500	127 (25.4)	0.447		
Female		118	34 (28.8)			
Age group (y)						
<65		313	77 (24.6)	0.405		
≥65		305	84 (27.5)			
Duration of CABG (y)						
<5		236	55 (23.3)	0.021	1	
5–9		266	64 (24.1)		1.11 (0.72–1.72)	0.641
≥10		116	42 (36.2)		2.24 (1.33–3.78)	0.003
Blood pressure (mmHg)				0.196		
HP^- BP < 140/90		185	48 (25.9)			
BP≥140/90		22	10 (45.5)			
HP^+ BP < 140/90		227	55 (24.2)			
BP≥140/90		136	48 (26.1)			
Blood glucose (mmol/L)				0.006		
DM^- FPG<7.0		303	70 (23.1)		1	
FPG≥7.0		73	29 (39.7)		2.44 (1.38–4.32)	0.002
DM^+ FPG<7.0		116	23 (19.8)		0.84 (0.48–1.48)	0.539
FPG≥7.0		126	39 (31.0)		1.51 (0.92–2.47)	0.104
LDL-C (mmol/L)				0.001		
≥2.6		331	103 (31.1)		1	
1.8–2.59		192	46 (24.0)		0.63 (0.41–0.98)	0.041
<1.8		95	12 (12.6)		0.27 (0.14–0.53)	<0.001
BMI group				0.595		
<24		114	28 (24.6)			
24–27.9		199	48 (24.1)			
≥28		305	85 (27.9)			
Postoperative smoking						
No		398	87 (21.9)	0.001	1	
Yes		220	74 (33.6)		1.86 (1.26–2.77)	0.002
Aspirin use	No	117	36 (30.8)	0.197		
	Yes	501	125 (25.0)			
ACEI/ARB use	No	413	111 (26.9)	0.476		
	Yes	205	50 (24.4)			
Statin use	No	205	74 (36.1)	<0.001	1	
	Yes	413	87 (21.1)		0.28 (0.25–0.56)	<0.001
β-blocker use	No	185	41 (22.2)	0.150		
	Yes	433	120 (27.7)			

OR, odds ratio; CI, confidence interval; CABG, coronary artery bypass grafting; HP^-, patients without hypertension; HP^+, patients with hypertension; BP, blood pressure; DM^-, patients without diabetes; DM^+, patients with diabetes; FPG, fasting plasma glucose; LDL-c, low-density lipoprotein cholesterol; BMI, body mass index; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.

performed to identify the association between conventional RFs and LIMA graft disease. LIMA graft disease correlated with high blood glucose without diabetes (OR, 2.44; 95% CI: 1.39–4.32; $P = 0.002$), postoperative smoking (OR, 1.86; 95%CI: 1.26–2.78; $P = 0.002$) and duration of CABG≥10 years (OR, 2.24; 95%CI: 1.33–3.78; $P = 0.003$), LDL-C achieved target level (OR, 0.27; 95% CI: 0.14–0.53; $P < 0.001$) and statin use (OR, 0.38; 95% CI, 0.25–0.56; $P < 0.001$). Details are given in **Table 3.**

Statin Use and LDL-C Control

Of the study, 413 patients (66.8%) were taking statins, there was no significant difference in statin use among different duration of CABG, but it appeared to be lower in women, patients aged ≥65 years, and patients with LIMA graft stenosis ($p < 0.05$). In total, 95 patients (15.4%) achieved target LDL-C level, and the figure was 17.4% (72/413) in treated patients. Regardless of the treated patients or the overall sample, the frequency of achieving target

TABLE 4 | Statin use and LDL-C control of the study population.

Characteristics		Statin use	LDL-C<1.8 mmol/L	
			Treated patients	All patients
Sex[#]	Male	345 (69.0)	60 (17.4)	75 (15.0)
	Female	68 (57.6)	12 (17.6)	20 (16.9)
Age group (y)[#]	<65	227 (72.5)	42 (18.5)	46 (14.7)
	≥65	186 (61.0)	30 (16.1)	49 (16.1)
Duration of CABG (y)	<5	160 (67.8)	26 (16.3)	33 (14.0)
	5–9	173 (65.0)	34 (19.6)	46 (17.3)
	≥10	80 (69.0)	12 (15.0)	16 (13.8)
Artery graft disease[#§]	Yes	87 (54.0)	10 (11.5)	12 (7.5)
	No	326 (71.3)	62 (19.0)	83 (18.2)

[#]*Significant difference in statin use between the two groups ($p < 0.05$);* [§]*Significant difference in LDL-C control between patients with or without artery graft disease.*
LDL-C, low-density lipoprotein cholesterol; CABG, coronary artery bypass grafting.

LDL-C level of patients with LIMA graft stenosis was lower than that of patients without LIMA graft stenosis (**Table 4**).

DISCUSSION

Our study indicated 26.1% of patients who had recurrence of chest pain after CABG developed stenosis in LIMA grafts. Postoperative smoking and hyperglycemia without diabetes had an increased occurrence of LIMA stenosis. Statin use and LDL-C <1.8 mmol/L were more common in patients without LIMA stenosis.

Strong evidence supports that conventional RFs including age, sex, hypertension, diabetes, total cholesterol, obesity, and smoking, which were widely used to estimate the risk of CVD based on the risk score system (18–20). Little was known whether all of them attributed to coronary artery grafts atherosclerosis development. In the present study, we identified long duration after CABG and postoperative smoking as a potential RF; we also demonstrated a novel association between hyperglycemia without diabetes and artery graft disease. Statin use and LDL-C <1.8 mmol/L were protective. Age, sex, hypertension, and obesity were not associated, which are quite difference from other studies. For example, Shah et al. found no patients variables (age, gender diabetes, hypertension) were significantly associated with graft patency in symptomatic patients after CABG (21). Conflicting evidence was found for each conventional RFs.

Hypertension is the largest risk factor with population attributable fraction (PAFs) of 22.3% for CVD (11). But it was not associated with artery graft stenosis in the present study, the prevalence of hypertension and mean level of SBP and DBP was similar between patients with or without artery graft stenosis, which was consistent with previous study (12, 13, 21). Individuals who had no history of hypertension with BP≥140/90 mmHg, it should be noted, had a high prevalence of artery graft stenosis, but the real associations might have been concealed because of the small sample size.

Diabetes is an important predictor of CVD (22), its influence on long-term patency of grafts is also important because nearly 30–40% of subjects undergoing CABG have diabetes (13–16). Conflicting evidence were reported in previous studies. Some identified diabetes as a potential predictor for graft failure (13), some showed negative association (21), and the rest showed that diabetes reduced the risk of LIMA graft stenosis (14–16). In the current study, four groups were divided according to the history of diabetes and the FPG level, hyperglycemia without diabetes had a higher prevalence of artery graft senosis than other groups, we than identified the association with OR = 2.44 (95%CI: 1.38–4.32). These individuals were suspected to be undiagnosed diabetes; they are more dangerous because most of them did not receive any hypoglycemic treatment and lifestyle intervention than previous diagnosis of diabetes. We now have a large population of undiagnosed diabetes with high percentage of 63% among patients with diabetes based on the latest national survey (23).

LDL-C lowering reduced the risk of recurrent ischemic cardiovascular events, guidelines on the management of blood cholesterol recommended a target LDL-C concentration of <1.8 mmol/L for patients with CVD to reduce recurrent risk (24) even <1.4 mmol/L for more benefit (25). Quin JA, et al. reported subjects who achieved a LDL-C target of <2.6 mmol/L at 1-year did not experience improved clinical outcomes or graft patency (26). A systematic review concluded that LDL-C lowering to 1.8 mmol/L may benefit post-CABG (27). The present study indicates that LDL-C<1.8 mmol/L had a low artery graft disease. However, the optimal target (LDL-C < 1.8 mmol/L) is seldom achieved in patients with coronary heart disease (27). Strong evidence supports the benefit of statins use with respect to the risk of recurrent cardiovascular events and improve survival have largely been attributed to reduction of LDL-C (28). Statin therapy also reduces the inflammatory cascade and promotes stability of coronary lesions vulnerable to rupture. The present study indicates that statin use could reduce the longer-term risk of artery graft stenosis, even in subjects who didn't achieved the LDL-C target. Systematic review about statin therapy before and after CABG concluded, postoperative statins reduce the recurrence of cardiovascular events (27).

Although some cross-sectional studies did not show the association between postoperative smoking and coronary artery graft lesions (13), follow-up studies showed smoking cessation turned out to have a great effect on reducing angina pectoris or long-term mortality, the effect even greater than that of any other intervention or treatment (16, 17, 29, 30). Positive association was also found in this current study. Therefore, patients with CHD, especially those undergoing CABG should be encouraged to stop smoking. Previous studies showed 43–68% of patients undergoing CABG stopped smoking (29, 30). Continuous smoking cessation intervention is needed for patients care after CABG.

The major limitation of this study is a single center and retrospective analysis, all the information was absorbed from the hospital electronic medical records, detail information about diet, physical inactivity and dosage of medication was not collected, therefore it is likely that there is a selection and recall bias

related to the inquiry of medical history and the structured questionnaire. The sample size of the present study was also limited. In addition, we aimed to evaluate the effect of traditional RFs for CVD on artery graft stenosis, other RFs (13) that might be associated with graft stenosis were not collected and unadjusted, such as plasma fibrinogen, creatinine, and lifestyles. Glycosylated hemoglobin level was not collected because of high percentage missing value in the electronic medical record, or undiagnosed diabetes could be identified.

CONCLUSIONS

This study provides more evidence for the RFs of coronary artery grafts artery, not all conventional risk factors were positively associated with artery graft stenosis. Long duration after CABG and postoperative smoking were identified as a potential risk factor, hyperglycemia without diabetes, it should be noted, had a high prevalence of artery graft stenosis. Statin use and LDL-C $<$1.8 mmol/L might be protective. The results will help clinicians to take both lifestyle and pharmacological interventions to reduce artery graft lesions risk.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Boards of Beijing Anzhen Hospital, Capital Medical University, Beijing, China. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

H-JZ and X-TS conceived and designed the study. H-JZ and H-XY prepared the database, performed the analysis, interpreted results, and drafted the manuscript. NN and J-WW prepared the database. X-TS interpreted results and performed critical revision of the manuscript. All authors read and approved the final version of the manuscript.

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