Cardiology Research and Practice

Early Diagnosis and Treatment of Atherosclerosis

Lead Guest Editor: Fanbo Meng
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Cardiology Research and Practice
Volume 2023, Article ID 7630594, 11 pages
https://doi.org/10.1155/2023/7630594

Review Article

The Effect of High-Intensity Interval Training on Exercise Capacity in Patients with Coronary Artery Disease: A Systematic Review and Meta-Analysis

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Received 9 April 2022; Revised 10 November 2022; Accepted 25 November 2022; Published 3 April 2023

Academic Editor: Anwer Habib

Background. The optimal exercise prescription for coronary artery disease (CAD) remains under debate. The aim of our meta-analysis is to investigate the efficacy of high-intensity interval training (HIIT) versus moderate-intensity continuous training (MICT) of coronary artery disease patients. *Methods*. Electronic databases were searched from their inception date until October 23, 2021, and the articles include randomized controlled trials. The mean differences and 95% confidence intervals were calculated, and heterogeneity was assessed using the I^2 test. *Results*. The study standards were met by seventeen studies. The pooled studies included 902 patients. HIIT resulted in improvement in peak oxygen uptake (1.50 ml/kg/min, 95% confidence interval: 0.48 to 2.53, $n = 853$ patients, and low quality evidence) compared with MICT. There was no discernible difference between the individuals in the HIIT group and the MICT group in terms of systolic/diastolic blood pressure or peak/resting heart rate. *Conclusion*. This systematic review and meta-analysis reported the superiority of HIIT versus MICT in enhancing peak oxygen uptake in CAD patients.

1. Introduction

The main cause of death worldwide has been coronary artery disease (CAD) [1]. Cardiac rehabilitation (CR) based on exercise training is an approach to enhance cardiopulmonary capacity, metabolic parameters, and quality of life [2]. CR in patients with CAD decreases angina [3], hospitalizations [4], and mortality [5].

According to the intensity and method of training protocols, interrelated exercise rehabilitation can be divided into high-intensity interval training (HIIT) and moderate-intensity continuous exercise (MICT). MICT has shown some advantages in decreasing the cardiovascular risk and mortality [6]. Due to the exercise protocol of MICT, there remains a low level of compliance with CR. In 2007, the American Heart Association recommended HIIT, which consists of repetition of quick and intense bursts of exercise, followed by short recovery periods [7].

In recent years, a growing amount of evidence proved that HIIT has beneficial effects on exercise capacity and cardiovascular function. However, these studies were limited by the small sample size and short follow-up period. Therefore, there is not sufficient clinical evidence to prove the efficiency of HIIT in CAD patients. Previous systematic reviews [8–10] also showed the superiority of HIIT on exercise capacity in patients involved with an exercise-based cardiac rehabilitation program. However, the most updated systematic review performed their literature search in

November 2016 [11]. The study has since been followed by the publication of new studies.

The objective of this systematic review with meta-analysis was to evaluate the benefits of HIIT compared with MICT. In addition, we evaluated for the effects of HIIT on exercise capacity, blood pressure, and heart rate in CAD patients.

2. Methods

This systematic review was conducted and reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement (Supplementary Materials: PRISMA 2009 Checklist) [12] and the Cochrane Handbook for Interventional Reviews [13]. The study protocol has been published previously in INPLASY, the registration number is INPLASY202240036 (available in https://inplasy.com/inplasy-2022-4-0036/).

2.1. Search Strategy. The electronic databases PubMed, Cochrane Central Register of Controlled Trials (CENTRAL), EMBASE, and CINAHL were searched from their inception until October 23, 2021. The searches were restricted to articles written in English. The search strategy details are provided in the Supplemental Materials—search strategy.

2.2. Study Selection. The full text was reviewed of all included articles. Two reviewers (S. L. and X. C.) independently screened the titles and abstracts. Furthermore, full-text screening was conducted according to the criteria for inclusion and exclusion. Disagreements for inclusion were discussed by the two reviewers and resolved by senior authors (Y. X.). Randomized controlled trials (RCTs) were included and the selection criteria are described below. The inclusion criteria were as follows: (1) RCTs comparing the effectiveness of HIIT with MICT in participants with CAD; (2) at least one of the following outcomes were measured—VO_{2peak}, peak heart rate (HRpeak), resting heart rate (HRrest), resting systolic blood pressure (SBP), and resting diastolic blood pressure (DBP); and (3) the language was restricted to English. The exclusion criteria were as follows: (1) single-arm research and animal experiment research; (2) conference papers, letters, or abstracts where the full text was not available; and (3) incomplete data.

2.3. Data Collection. The data extraction form was predefined and included the following: population characteristics, intervention duration, training protocols, and outcome measures. One reviewer (S. L) used a standardized form to extract data from the included articles, and the extracted data were checked by a second reviewer (X. C). Attempts were made to contact the original investigators regarding any missing data. Any discrepancies were resolved by agreement after rechecking the source papers and via further discussion with a third reviewer (Y. X.).

2.4. Risk of Bias Assessment. In accordance with the recommendations in the Cochrane Handbook, the trials' methodological quality was independently evaluated by two reviewers (S. L. and X. C) using the Cochrane risk of bias assessment tool. Any discrepancies were resolved by agreement after rechecking the source papers and further discussion with a third reviewer (Y. X.). The following domains were considered: (1) random sequence generation, (2) allocation concealment, (3) blinding of the patients and personnel, (4) blinding of the outcome assessors for the primary outcomes, (5) incomplete outcome data, (6) selective reporting, and (7) other bias.

2.5. Quality of Evidence. The Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) [14] was used to assess the quality of evidence of outcomes, which criteria comprised the risk of bias, inconsistency, indirectness, inaccuracy, and publication bias. The quality of evidence was classified as high, moderate, low, or very low.

2.6. Statistical Analysis. Statistical analysis was performed with Review Manager (RevMan, Version 5.4.1 The Cochrane Collaboration, Copenhagen, Denmark) [15]. Given that all of the variables in the included studies consisted of continuous data, we used the mean difference (MD) when the same instrument was used, or the standardized mean difference (SMD) when different instruments were used, with 95% confidence intervals (CI) to analyze the outcomes. A p value < 0.05 was considered statistically significant. Heterogeneity was assessed with a chi-squared test ($p < 0.10$ was considered indicative of statistical significance) and the I^2 statistic (where $I^2 > 25\%$, 50%, and 75% indicated moderate, substantial, or considerable heterogeneity, respectively). When I^2 is less than 50%, it indicated low heterogeneity, and a fixed-effects model would be chosen; otherwise, a random-effects model was adopted. Potential publication bias was evaluated by visual examination of funnel plot asymmetry and Egger's test (a p value < 0.05 was considered statistically significant). When the number of articles included in one analysis was limited (i.e., less than 10), the risk for publication bias was not assessed.

3. Result

3.1. Study Selection. The process of study selection is shown in Figure 1. The initial search identified 570 articles (560 from the database search and 10 from the manual search), of which 381 were eligible for title and abstract scanning following the exclusion of duplicates. Based on the inclusion and exclusion criteria, 321 studies were excluded with 60 remaining. After the full texts of 60 articles were completely read, 16 articles met the eligibility criteria and were included in the meta-analysis [16–31].

3.2. Characteristics of Included Studies. Table 1 lists the general characteristics of the included studies, and the studies consisted of seven RCTs and one retrospective cohort study. One study [16] had a three-arm parallel group design. A total of sixteen studies comprising 853 patients were included for the analysis, and 406 patients underwent HIIT.

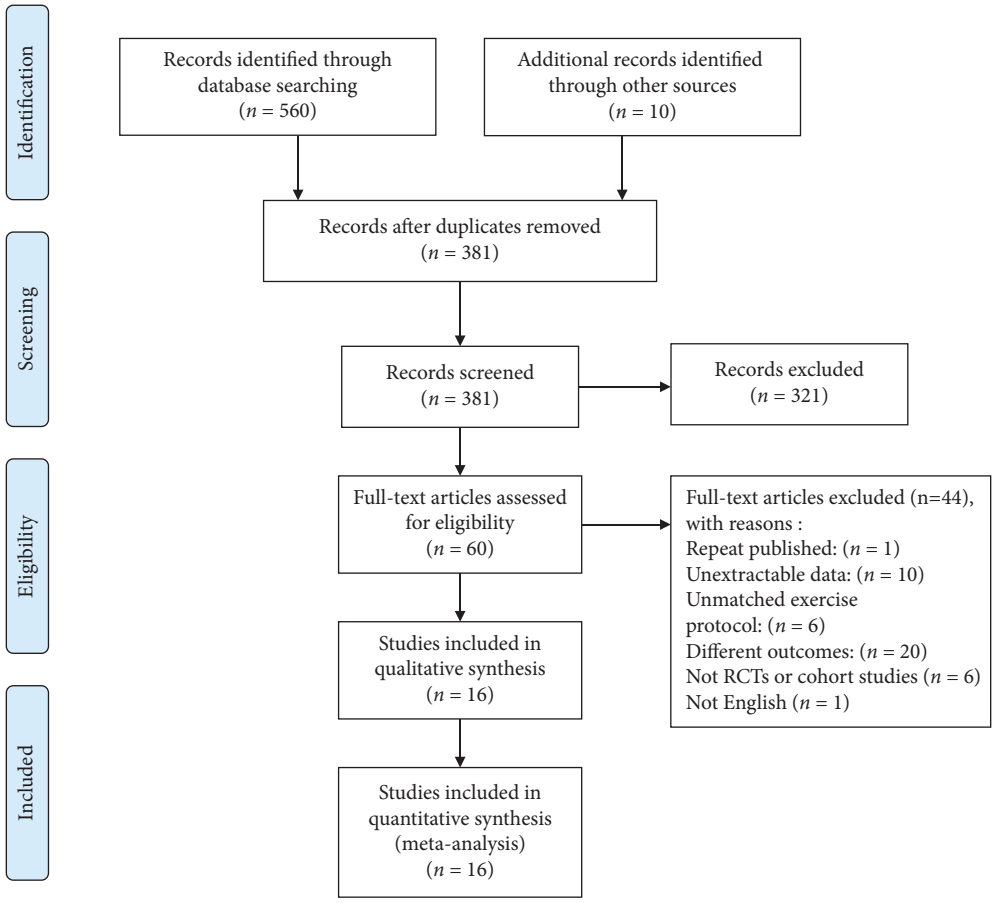


Figure 1: Flowchart of study identification and selection.

The number of participants included in each study in our meta-analysis ranged from 14 to 174, and the mean age of the included participants ranged from 55.9 to 68 years. In the included studies, MICT was applied for the intervention of the control group. The duration of the interventions ranged between 4 and 12 weeks.

3.3. Risk of Bias. The individual items on the risk of bias assessment are shown in Figure 2. Sixty percent of the included RCTs provided adequate random sequence generation but only four studies reported allocation concealment methods. As both HIIT and MICT are exercise trainings, designing an experiment with a credible placebo-control arm is challenging. Thus, all RCTs were open label. All studies claimed that the outcome assessors had been blinded to the patient treatment allocation. Four studies [21–23, 26] reported incomplete outcome data because the participants were lost to follow-up, and the reasons for loss or withdrawal were noted in the literature. Approximately 50% of the included studies were at unclear risk of selective reporting because neither their protocol nor trial registration information was available.

The risk of publication bias, as analyzed by funnel plots, showed only minor asymmetry (Supplementary Figure S1). Thus, a publication bias mechanism is not a major cause of concern.

3.4. Quality of Evidence. The GRADE system showed that the quality of evidence was low for VO_{2peak} because of unclear allocation concealment or lack of blinding. The quality of evidence was downgraded to very low for the SBP, DBP, and heart rate because of the large heterogeneity and risk of bias.

3.5. Meta-Analysis of Outcomes

3.5.1. Peak Oxygen Uptake. VO_{2peak} was measured in 16 studies [16–31] with a total of 853 patients. The pooled results showed that HIIT led to a statistically significant 1.50 mL/kg/min improvement in the patients' VO_{2peak} (95% CI, 0.48 to 2.53; $I^2 = 59\%$; Figure 3(a)). A subgroup analysis was performed on the duration of intervention (<12 and ≥ 12 weeks) for HIIT versus MICT on VO_{2peak}. The short-term group (<12 weeks) showed a significant improvement in VO_{2peak} (MD = 2.75 mL/kg/min, 95% CI, 0.98, 4.52; $I^2 = 36\%$; Figure 3(b)). The analysis long-term group (≥12 weeks) showed no significant effect on VO_{2peak} (MD = 0.58 mL/kg/min, 95% CI, −0.40, 1.57; $I^2 = 50\%$; Figure 3(b)).

3.5.2. Blood Pressure. Blood pressure included SBP and DBP, which were measured in 9 studies [16, 18–23, 27, 30] with a total of 528 patients. The results of our meta-analysis

Table 1: Characteristics of included studies.

Study	Sample size *T*/*C* (M/F)	Ages *T*/*C*	Training protocols HIIT	Training protocols MICT	Program duration
Cardozo et al., 2015	23 (14, 9)/48 (34, 14)	*T*: 56 ± 12 *C*: 62 ± 12	10 bouts ∗ 2 min (>90% HRpeak) Each interval: 2 min (<60% HRpeak)	30 min of continuous training (at 70 to 75% of HRpeak)	16 weeks
Choi et al., 2018	23 (21, 2)/21 (18,)	*T*: 60 ± 11 *C*: 62.8 ± 11.9	4 bouts ∗ 4 min (at 85–100% of the HRpeak) Each interval: 3 min (at 50–60% of the HRpeak)	28 min of continuous training (at 60 to 70% of HRpeak)	9-10 weeks
Conraads et al., 2015	85 (NA)/89 (NA)	NA	4 bouts ∗ 4 min (at 85–90% of peak VO2 90–95% of HRpeak, 15–17 Borg scale, and shortness of breath) Each interval: 3 min (at 50–70% of HRpeak)	37 min of continuous training (at least 60–70% of peak VO2, at least 65–75% of HRpeak)	12 weeks
Currie et al.,2013	11 (NA)/11 (NA)	*T*: 63 ± 8 *C*: 66 ± 8	Part 1 (week 1–4): 10 bouts ∗ 1 min (at 89% of PPO pre) Part 2 (week 5–8): 10 bouts ∗ 1 min (at 102% of PPOpre) Part 3 (week 9–12): 10 bouts ∗ 1 min (at 110% of PPOpre) Each interval: 1 min (at 10% of PPOpre)	Part1 (week 1–4): 30 min of continuous training (at 58% of PPOpre) Part 2 (week 5–8): 40 min of continuous training (at 58% of PPOpre) Part 3 (week 9–12): 50 min of continuous training (at 58% of PPOpre)	12 weeks
Currie et al., 2014	9 (9)/10 (9, 1)	*T*: 62 ± 11 *C*: 68 ± 8	Part 1 (month 1): 10 bouts ∗ 1 min (at 85% of PPOpre) Part 2 (month 2): 10 bouts ∗ 1 min (at 100% of PPOpre) Part 3 (month 3): 10 bouts ∗ 1 min (at 108% of PPOpre) Part 4 (month 4–6): 10 bouts ∗ 1 min (at 121% of PPOpre) Each interval: 1 min (at 10% of PPOpre)	Part 1 (month 1): 30 min of continuous training (at 57% PPOpre) Part 2 (month 2): 40 min of continuous training (at 57% PPOpre) Part 3 (month–3): 50 min of moderate-intensity exercise (at 57% PPOpre) Part 4 (month4–6): min of continuous training (at 78% PPOpre)	24 weeks
Dunford et al., 2021	9/11 (Total: 18/2)	Total: 61 ± 7	3 bouts ∗ 90 s stairs climbing Each interval: walking 90 s	30 min of continuous training (at 60–80% HRpeak)	12 weeks

Table 1: Continued.

Study	Sample size T/C (M/F)	Ages T/C	Training protocols HIIT	Training protocols MICT	Program duration
Jaureguizar et al., 2016	36 (33, 3)/36 (28, 8)	T: 58 ± 11 C: 58 ± 11	Part 1 (week 1): HIIT: 15 bouts ∗ 20 s (50% of the maximum load reached in the first SRT) Each interval: 40 s (10% of the maximum load reached in the first SRT) Part 2 (week 2): HIIT: 20 bouts ∗ 20 s (50% of the maximum load reached in the first SRT) Each interval: 40 s (10% of the maximum load reached in the first SRT) Part 3 (week 3): HIIT: 25 bouts ∗ 20 s (50% of the maximum load reached in the first SRT) Each interval: 40 s (10% of the maximum load reached in the first SRT) Part 4 (week 4): HIIT: 30 bouts ∗ 20 s (50% of the maximum load reached in the first SRT) Each interval: 40 s (10% of the maximum load reached in the first SRT) Part5 (week 5–8): HIIT: 30 bouts ∗ 20 s (50% of the maximum load reached in the second SRT)	Part 1: MICT: 15 mins (at (VT1) Part 2 (2 w): MICT: 20 mins (at VT1) Part 3 (3 w): MICT: 25 mins at (VT1) Part4 (4 w): MICT: 30 mins at (VT1) Part 5 (5–8 w): MICT: 30 mins at (VT1+10%)	8 weeks
Keteyian et al., 2014	36 (33, 3)/36 (28, 8)	T: 58 ± 11 C: 58 ± 11	4 bouts of 4 min (at 80–90% of the heart rate reserve) Each interval: 3 min (at 60–70% of the heart rate reserve)	30 min of continuous training (at 60% to 80% of heart rate reserve)	2 weeks
Kim 2020	23 (18, 5)/24 (16, 8)	T: 60 ± 11 C: 62.8 ± 11.9	4 bouts ∗ 4 min (at 95–100% of the HRR) Each interval: 3 min (at 60% of the HRR)	First part: 3 bouts ∗ 8 min (at 85% of the HRR) Each interval: 3 min (at 40% of the HRR)	6 weeks
Moholdt et al., 2009	28 (24, 4)/31 (24, 7)	T: 60.2 ± 6.9 C: 62.0 ± 7.6	4 bouts ∗ 4 min (at 90% of the HRpeak) Each interval: 3 min (70% of the HRpeak)	46 min of continuous training (at least 70% of HRpeak)	4 weeks
Moholdt et al., 2012	30 (25, 5)/59 (49, 10)	T: 56.7 ± 10.4 C: 57.7 ± 9.3	4 bouts ∗ 4 min (at 85–95% HRpeak) Each interval: 1 min (70% HRpeak)	Usual care exercise: 60 min of aerobic exercises	12 weeks
Pattyn et al., 2016	80 (76, 4)/83 (76, 7)	T: 57.4 ± 8.7 C: 59.9 ± 9.2	4 bouts of 4 min (at 85–95% of the HRpeak) Each interval: 3 min (50%–70% of the HRpeak)	37 min of continuous training (at least 70–75% of HRpeak)	12 weeks
Prado et al., 2016	17(14, 3)/18 (14, 4)	T: 56.5 ± 2.7 C: 61.3 ± 2.2	7 bouts ∗ 3 min (at RCP) Each interval: 3 min (at VAT)	50 min of continuous training (at VAT)	12 weeks
Rocco et al., 2012	17(14, 3)/20 (15, 5)	T: 56.5 ± 3.0 C: 62.3 ± 2.0)	7 bouts ∗ 3 min (at RCP) Each interval: 3 min (at VAT)	50 min of continuous training (at VAT)	12 weeks
Rognmo et al., 2004	8(6, 2)/9(8, 1)	T: 62.9 ± 11.2 C: 61.2 ± 7.3	4 bouts ∗ 4 min (at 80–90% oVO2peak (85–95% of HRpeak) Each interval: 3 min (at 50–60% of VO2peak)	41 min of continuous training (at 50–60% of VO2 peak)	10 weeks
Warburton et al., 2005	7(NA)/7(NA)	T: 55.9 ± 7 C: 57 ± 8	15 bouts ∗ 2 min (at 90% of heart rate/VO2reserve (range 85% to 95%)) Each interval: 2 min (at 40% of heart rate/VO2reserve (range 35% to 45%))	30 min of continuous training (at 65% of heart rate/ VO2reserve)	16 weeks

indicated a small but significant benefit from HIIT on SBP (MD = 2.59 mmHg; 95% CI, 0.09 to 5.09; $I^2 = 0\%$; Figure 4(a)). Moreover, the beneficial effect of HIIT on DBP was also small but significant (MD = 1.86 mmHg, 95% CI: 0.40 to 3.32; $I^2 = 24\%$; Figure 4(b)).

3.5.3. Heart Rate. HRpeak was available for 13 [16, 18–25, 27, 28, 30, 31] studies with a total of 713 patients. The pooled results showed that HIIT led to a statistically significant increase in Hrpeak (MD = 5.51 bpm; 95% CI, 2.13 to 8.89), but the heterogeneity was considerable ($I^2 = 40\%$; Figure 4(c)). HRrest was available for 10 [18–22, 24, 26, 27, 30] studies with a total of 588 patients. The results of the meta-analysis indicated no significantly greater effect from HIIT on HRrest (MD = 0.19 bpm; 95% CI, −0.40 to 2.23; Figure 4(d)).

4. Discussion

The overall results of this study, which includes data from 16 RCTs and 853 patients, confirm a significantly larger effect size for VO_{2peak} (+1.50 ml/min/kg) in favor of HIIT. But the results of our meta-analysis found no significant effect on SBP and DBP, or HRpeak and HRrest. Although the meta-analysis of each outcome shows a certain degree of heterogeneity (I^2<50%), we also used the random effect model, sensitivity analysis, and subgroup analysis to indicate the robustness of the results. Therefore, the results of our meta-analysis are relatively reliable.

Aerobic exercise has long been the cornerstone of cardiac rehabilitation programs for patients with CAD, and improving the aerobic exercise capacity of patients with CAD is its most significant benefit [31]. Aerobic exercise capacity is the strongest predictor of all cardiovascular morbidity and mortality and is the process of uptake, transport, and utilization of oxygen [5, 18]. In recent decades, MICT has been recommended for CAD patients according to the guidelines [32]. Several studies have already investigated the benefits of HIIT in exercise capacity [33].

VO_{2peak} is the gold standard method to assess the aerobic exercise capacity [31, 34]. In our meta-analysis of patients with CAD, HIIT showed a superiority compared with MICT in improving the VO_{2peak} of patients. Given the significant heterogeneity found in the primary analyses due to the variance in exercise protocols (variable intensities and different durations of the exercise programs), caution is warranted when interpreting our results. Our finding showed that HIIT resulted in a larger gain of 1.50 mL/kg/min on VO_{2peak} than MICT, and these results are in line with previous meta-analyses [8–10].

According to the duration of the total intervention, our research showed that <12 weeks group resulted in a greater improvement in VO_{2peak} by 2.75 mL/kg/min in MD than ≥ 12 weeks group did, which is in line with Taylor et al.'s finding [35], which reported home-based HIIT and MICT had low rates of adherence features compared with the supervised stage. Only one included trial [21] stated the protocol consisted of 6 supervised sessions (4 weeks) and 24 unsupervised sessions for an additional 8 weeks (12 weeks total). Therefore, higher patient acceptance of short-term exercise may have contributed to this outcome.

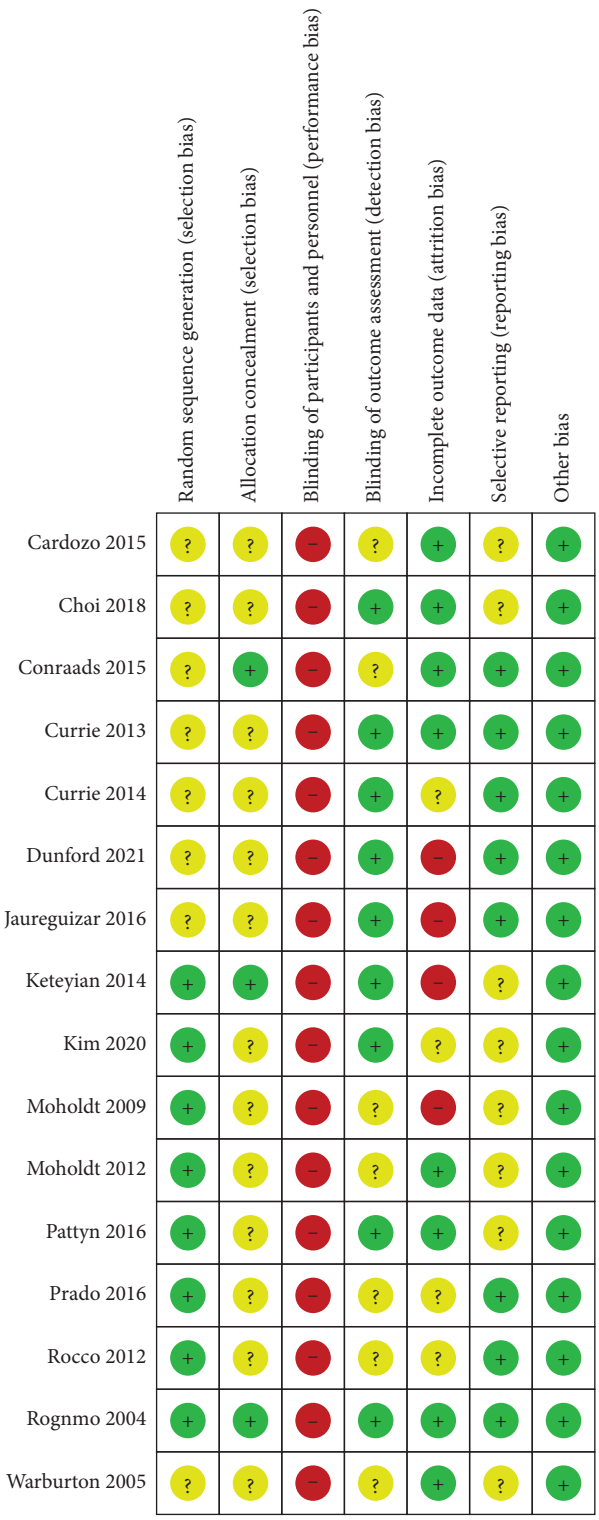
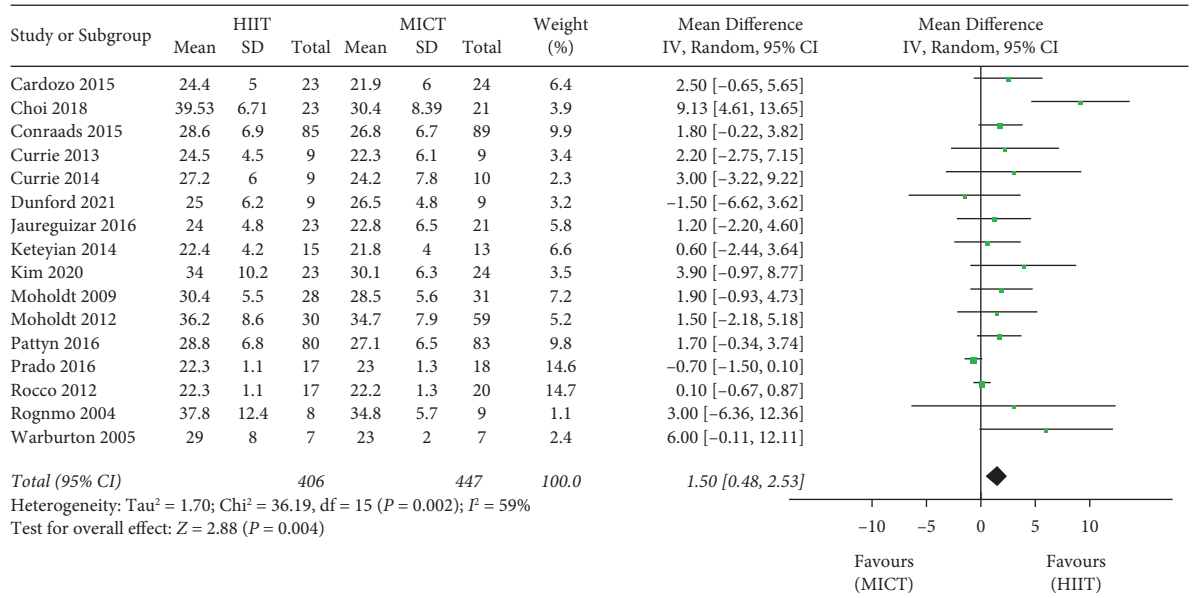


Figure 2: Risk of bias summary.

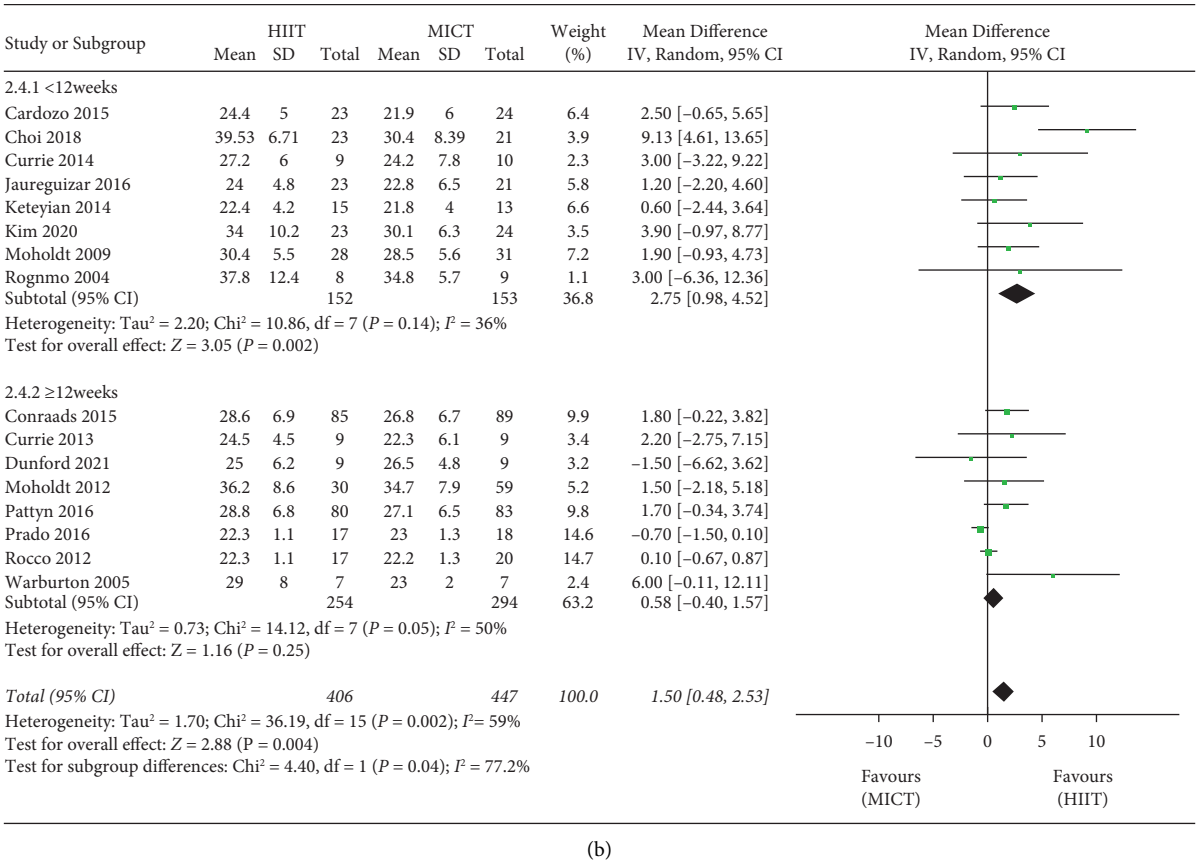
A meta-analysis involved one million adults suggested that 10 mmHg decrease of SBP and DBP could reduce the risk of premature death from stroke and ischemic heart



Study or Subgroup	HIIT Mean	HIIT SD	HIIT Total	MICT Mean	MICT SD	MICT Total	Weight (%)	Mean Difference IV, Random, 95% CI
Cardozo 2015	24.4	5	23	21.9	6	24	6.4	2.50 [−0.65, 5.65]
Choi 2018	39.53	6.71	23	30.4	8.39	21	3.9	9.13 [4.61, 13.65]
Conraads 2015	28.6	6.9	85	26.8	6.7	89	9.9	1.80 [−0.22, 3.82]
Currie 2013	24.5	4.5	9	22.3	6.1	9	3.4	2.20 [−2.75, 7.15]
Currie 2014	27.2	6	9	24.2	7.8	10	2.3	3.00 [−3.22, 9.22]
Dunford 2021	25	6.2	9	26.5	4.8	9	3.2	−1.50 [−6.62, 3.62]
Jaureguizar 2016	24	4.8	23	22.8	6.5	21	5.8	1.20 [−2.20, 4.60]
Keteyian 2014	22.4	4.2	15	21.8	4	13	6.6	0.60 [−2.44, 3.64]
Kim 2020	34	10.2	23	30.1	6.3	24	3.5	3.90 [−0.97, 8.77]
Moholdt 2009	30.4	5.5	28	28.5	5.6	31	7.2	1.90 [−0.93, 4.73]
Moholdt 2012	36.2	8.6	30	34.7	7.9	59	5.2	1.50 [−2.18, 5.18]
Pattyn 2016	28.8	6.8	80	27.1	6.5	83	9.8	1.70 [−0.34, 3.74]
Prado 2016	22.3	1.1	17	23	1.3	18	14.6	−0.70 [−1.50, 0.10]
Rocco 2012	22.3	1.1	17	22.2	1.3	20	14.7	0.10 [−0.67, 0.87]
Rognmo 2004	37.8	12.4	8	34.8	5.7	9	1.1	3.00 [−6.36, 12.36]
Warburton 2005	29	8	7	23	2	7	2.4	6.00 [−0.11, 12.11]
Total (95% CI)			*406*			*447*	*100.0*	*1.50 [0.48, 2.53]*

Heterogeneity: Tau² = 1.70; Chi² = 36.19, df = 15 (*P* = 0.002); *I*² = 59%
Test for overall effect: *Z* = 2.88 (*P* = 0.004)

(a)



Study or Subgroup	HIIT Mean	HIIT SD	HIIT Total	MICT Mean	MICT SD	MICT Total	Weight (%)	Mean Difference IV, Random, 95% CI
2.4.1 <12weeks								
Cardozo 2015	24.4	5	23	21.9	6	24	6.4	2.50 [−0.65, 5.65]
Choi 2018	39.53	6.71	23	30.4	8.39	21	3.9	9.13 [4.61, 13.65]
Currie 2014	27.2	6	9	24.2	7.8	10	2.3	3.00 [−3.22, 9.22]
Jaureguizar 2016	24	4.8	23	22.8	6.5	21	5.8	1.20 [−2.20, 4.60]
Keteyian 2014	22.4	4.2	15	21.8	4	13	6.6	0.60 [−2.44, 3.64]
Kim 2020	34	10.2	23	30.1	6.3	24	3.5	3.90 [−0.97, 8.77]
Moholdt 2009	30.4	5.5	28	28.5	5.6	31	7.2	1.90 [−0.93, 4.73]
Rognmo 2004	37.8	12.4	8	34.8	5.7	9	1.1	3.00 [−6.36, 12.36]
Subtotal (95% CI)			152			153	36.8	2.75 [0.98, 4.52]
Heterogeneity: Tau² = 2.20; Chi² = 10.86, df = 7 (*P* = 0.14); *I*² = 36%								
Test for overall effect: *Z* = 3.05 (*P* = 0.002)								
2.4.2 ≥12weeks								
Conraads 2015	28.6	6.9	85	26.8	6.7	89	9.9	1.80 [−0.22, 3.82]
Currie 2013	24.5	4.5	9	22.3	6.1	9	3.4	2.20 [−2.75, 7.15]
Dunford 2021	25	6.2	9	26.5	4.8	9	3.2	−1.50 [−6.62, 3.62]
Moholdt 2012	36.2	8.6	30	34.7	7.9	59	5.2	1.50 [−2.18, 5.18]
Pattyn 2016	28.8	6.8	80	27.1	6.5	83	9.8	1.70 [−0.34, 3.74]
Prado 2016	22.3	1.1	17	23	1.3	18	14.6	−0.70 [−1.50, 0.10]
Rocco 2012	22.3	1.1	17	22.2	1.3	20	14.7	0.10 [−0.67, 0.87]
Warburton 2005	29	8	7	23	2	7	2.4	6.00 [−0.11, 12.11]
Subtotal (95% CI)			254			294	63.2	0.58 [−0.40, 1.57]
Heterogeneity: Tau² = 0.73; Chi² = 14.12, df = 7 (*P* = 0.05); *I*² = 50%								
Test for overall effect: Z = 1.16 (*P* = 0.25)								
Total (95% CI)			*406*			*447*	*100.0*	*1.50 [0.48, 2.53]*
Heterogeneity: Tau² = 1.70; Chi² = 36.19, df = 15 (*P* = 0.002); *I*²= 59%								
Test for overall effect: *Z* = 2.88 (P = 0.004)								
Test for subgroup differences: Chi² = 4.40, df = 1 (*P* = 0.04); *I*² = 77.2%								

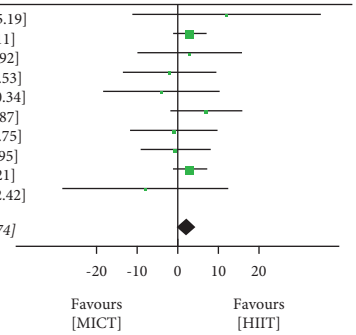
(b)

Figure 3: Meta-analysis results for VO_{2peak} (mL/kg/min).

disease by 40% and 30%, respectively [36]. In patients with hypertension, both HIIT and MICT reduced ambulatory blood pressure, increasing the percentage of patients with normal ambulatory blood pressure values [37]. However, no significant changes were found in our meta-analysis of SBP and DBP after HIIT and MICT intervention. With the reason for the significant heterogeneity among studies being unknown, whether there was a significantly greater effect on blood pressure in HIIT compared with MICT is still uncertain. This may be attributed to the inclusion of CAD patients rather than hypertensive patients in this meta-analysis. It seems that HIIT reduced SBP better than

Study or Subgroup	Experimental Mean	Experimental SD	Experimental Total	Control Mean	Control SD	Control Total	Weight (%)	Mean Difference IV, Fixed, 95% CI
Cardozo 2015	169	23	23	157	53	24	1.1	12.00 [-11.19, 35.19]
Conraads 2015	125	14.3	85	122	13.3	89	34.2	3.00 [-1.11, 7.11]
Currie 2013	121	9	11	118	19	10	3.5	3.00 [-9.92, 15.92]
Currie 2014	123	9	8	125	14	8	4.3	-2.00 [-13.53, 9.53]
Dunford 2021	116	11	9	120	19	9	2.8	-4.00 [-18.34, 10.34]
Jaureguizar 2016	123	15	23	116	15	21	7.3	7.00 [-1.87, 15.87]
Keteyian 2014	117	15	15	118	14	13	5.0	-1.00 [-11.75, 9.75]
Madssen 2014	133.7	16.4	24	134.3	14	25	7.9	-0.60 [-9.15, 7.95]
Pattyn 2016	125	13.6	80	122	13.8	83	32.6	3.00 [-1.21, 7.21]
Rognmo 2004	137	11	8	145	29	9	1.4	-8.00 [-28.42, 12.42]
Total (95% CI)			*286*			*291*	*100.0*	*2.34 [-0.06, 4.74]*

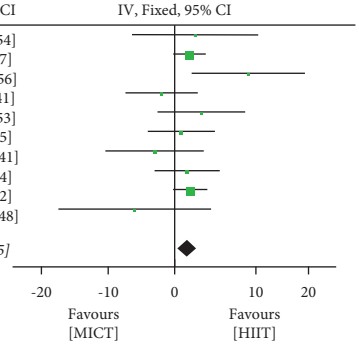
Heterogeneity: $Chi^2 = 5.03$, df = 9 ($P = 0.83$); $I^2 = 0\%$
Test for overall effect: $Z = 1.91$ ($P = 0.06$)



(a)

Study or Subgroup	HIIT Mean	HIIT SD	HIIT Total	Control Mean	Control SD	Control Total	Weight (%)	Mean Difference IV, Fixed, 95% CI
Cardozo 2015	69	9	23	66	22	24	2.2	3.00 [-6.54, 12.54]
Conraads 2015	74.7	8.4	85	72.5	8.2	89	32.2	2.20 [-0.27, 4.67]
Currie 2013	79	10	11	68	10	10	2.7	11.00 [2.44, 19.56]
Currie 2014	64	5	8	66	6	8	6.7	-2.00 [-7.41, 3.41]
Dunford 2021	76	6	9	72	8	9	4.6	4.00 [-2.53, 10.53]
Jaureguizar 2016	74	8	23	73	9	21	7.7	1.00 [-4.05, 6.05]
Keteyian 2014	71	11	15	74	9	13	3.6	-3.00 [-10.41, 4.41]
Madssen 2014	79.3	7.5	24	77.5	10	25	8.0	1.80 [-3.14, 6.74]
Pattyn 2016	74.8	8.4	80	72.5	8	83	30.9	2.30 [-0.22, 4.82]
Rognmo 2004	73	10	8	79	14	9	1.5	-6.00 [-17.48, 5.48]
Total (95% CI)			*286*			*291*	*100.0*	*1.85 [0.45, 3.25]*

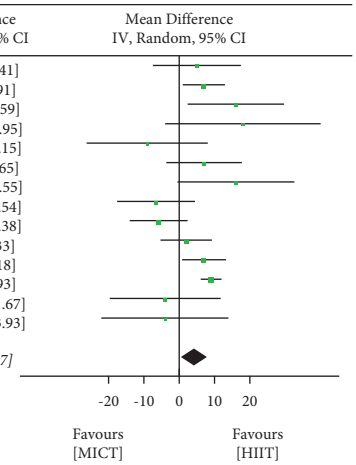
Heterogeneity: $Chi^2 = 10.55$, df = 9 ($P = 0.31$); $I^2 = 15\%$
Test for overall effect: $Z = 2.59$ ($P = 0.010$)



(b)

Study or Subgroup	HIIT Mean	HIIT SD	HIIT Total	MICT Mean	MICT SD	MICT Total	Weight (%)	Mean Difference IV, Random, 95% CI
Cardozo 2015	133	24	23	128	19	24	5.8	5.00 [-7.41, 17.41]
Conraads 2015	145	18.2	85	138	21.5	89	11.7	7.00 [1.09, 12.91]
Currie 2013	139	15	10	123	16	10	5.2	16.00 [2.41, 29.59]
Currie 2014	146	16	8	128	29	9	2.5	18.00 [-3.95, 39.95]
Dunford 2021	124	20	9	133	17	9	3.7	-9.00 [-26.15, 8.15]
Jaureguizar 2016	126	14	23	119	21	21	7.1	7.00 [-3.65, 17.65]
Keteyian 2014	151	17	15	135	26	13	3.9	16.00 [-0.55, 32.55]
Kim 2020	141	19.2	23	147.5	19.4	24	6.8	-6.50 [-17.54, 4.54]
Madssen 2014	155.8	15.9	24	161.6	13.1	25	9.2	-5.80 [-13.98, 2.38]
Moholdt 2012	156	17	30	154	16	59	10.1	2.00 [-5.33, 9.33]
Pattyn 2016	146	18.4	80	139	21.8	83	11.4	7.00 [0.82, 13.18]
Prado 2016	131	4.7	17	122	4.1	18	15.0	9.00 [6.07, 11.93]
Rognmo 2004	151	19	8	155	13	9	4.2	-4.00 [-19.67, 11.67]
Warburton 2005	155	19	7	159	15	7	3.4	-4.00 [-21.93, 13.93]
Total (95% CI)			*362*			*400*	*100.0*	*4.24 [0.51, 7.97]*

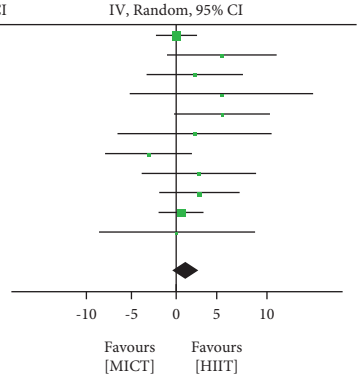
Heterogeneity: $Tau^2 = 21.88$; $Chi^2 = 28.67$, df = 13 ($P = 0.007$); $I^2 = 55\%$
Test for overall effect: $Z = 2.23$ ($P = 0.03$)



(c)

Study or Subgroup	HIIT Mean	HIIT SD	HIIT Total	MICT Mean	MICT SD	MICT Total	Weight (%)	Mean Difference IV, Random, 95% CI
Conraads 2015	55.4	7.4	85	55.4	8.1	89	31.3	0.00 [-2.30, 2.30]
Currie 2013	57	6	11	52	8	10	4.5	5.00 [-1.10, 11.10]
Currie 2014	57	4	8	55	7	9	5.8	2.00 [-3.35, 7.35]
Dunford 2021	69	10	9	64	12	9	1.6	5.00 [-5.21, 15.21]
Jaureguizar 2016	64	10	23	59	8	21	5.8	5.00 [-0.33, 10.33]
Keteyian 2014	66	13	15	64	10	13	2.3	2.00 [-6.53, 10.53]
Kim 2020	62	8.5	23	65.1	8.3	24	7.2	-3.10 [-7.91, 1.71]
Madssen 2014	65.7	11.6	24	63.2	11.1	25	4.1	2.50 [-3.86, 8.86]
Moholdt 2009	66.4	8.7	28	63.9	8.8	31	8.3	2.50 [-1.97, 6.97]
Pattyn 2016	56.3	7.7	80	55.8	8.5	83	26.8	0.50 [-1.99, 2.99]
Rognmo 2004	63	7	8	63	11	9	2.2	0.00 [-8.67, 8.67]
Total (95% CI)			*314*			*323*	*100.0*	*0.98 [-0.31, 2.27]*

Heterogeneity: $Chi^2 = 8.97$, df = 10 ($P = 0.54$); $I^2 = 0\%$
Test for overall effect: $Z = 1.49$ ($P = 0.14$)



(d)

Figure 4: Meta-analysis results for (a) SBP (mmHg), (b) DBP (mmHg), (c) HR peak (mmHg), and (d) HR rest (mmHg).

MICT in our report. Our results are inconsistent with Du et al.'s [38], who reported MICT seemed to induce a larger reduction in both SBP and DBP than HIIT. Three [20, 23, 25] included trials reported changes in medications during the invention. This would make it difficult to interpret and discuss the underlying mechanisms. Factors associated with medications should be considered when making personalized prescriptions.

In resent epidemiological studies, Aboyans and Criqui [39] indicated that elevated HRrest is independently associated with atherosclerosis and increased cardiovascular morbidity and mortality in cardiovascular diseases. Our results suggested that HRpeak and HRrest are equally influenced by HIIT and MICT. It is suggested that vigorous exercise could increase the risk of sudden cardiac events in susceptible individuals [40]. According to the results of Rognmo et al.'s study [41], the risk of cardiovascular events is low after performing high-intensity exercise or moderate-intensity exercise in cardiovascular rehabilitation.

5. Strengths and Limitations

The strength of this systematic review provided an updated analysis of data from RCTs that compared HIIT to MICT in patients with CAD. Moreover, this study was conducted in compliance with the PRISMA checklist for clear reporting, registration on INPLASY platform with protocol, and applying the GRADE tool to assess the certainty of the evidence. The study has potential limitations. First, few trials reported in detail on randomization procedures to determine whether selection bias might have affected study outcomes. Another important limitation is the small number of studies comparing HIIT and MICT with isocaloric protocols. On the other hand, the pooled studies lack large-scale clinical RCTs, which may affect the objectivity and reliability of this meta-analysis. In addition, the duration of the training program ranged from 4 to 24 weeks. The long-term safety and effects of HIIT are still unknown.

6. Conclusion

This meta-analysis and systematic review reported the superiority of HIIT in improving VO_{2peak} in CAD patients compared with MICT. These findings suggest that HIIT is a promising alternative exercise protocol for improving cardiorespiratory function in patients with CAD. The duration of the intervention and the availability of supervision are further considerations for the exercise protocols. Moreover, there was no difference between the HIIT and MICT effects on SBP and DBP or peak and resting HR. In further studies, larger and longer-term studies are needed to address inadequate evidence.

Abbreviations

CAD: Coronary artery disease
CR: Cardiac rehabilitation
HIIT: High-intensity interval training
MICT: Moderate-intensity continuous exercise
VO_{2peak}: Peak oxygen consumption
HRpeak: Heart rate
HRrest: Resting heart rate
SBP: Systolic blood pressure
DBP: Diastolic blood pressure
RCTs: Randomized controlled trials.

Data Availability

The data used to support the findings of this study are available from the authors upon request.

Disclosure

The authors have submitted in the INPLASY PROTOCOL "https://inplasy.com/wp-content/uploads/2022/04/INPLASY-Protocol-3118-1.pdf" and uploaded the updated manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors thank all the authors of the original studies included in this meta-analysis. The authors disclose that the following grant has been received for research, authorship, and publication of this article and thank the National Natural Science Foundation of China (no. 81774247) for its support.

Supplementary Materials

(1) Table S1: search strategy in English databases. (2) Figure S1: funnel plot of publication bias. (3) PRISMA 2009 Checklist. (*Supplementary Materials*)

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Hindawi
Cardiology Research and Practice
Volume 2022, Article ID 4352484, 7 pages
https://doi.org/10.1155/2022/4352484

Research Article

Serum Zinc Ion Concentration Associated with Coronary Heart Disease: A Systematic Review and Meta-Analysis

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Received 25 February 2022; Accepted 7 September 2022; Published 6 October 2022

Academic Editor: Anwer Habib

Aim. Coronary heart disease is a major cause of mortality in developed and developing countries. Changes in the trace element concentration in the human body are one of the main reasons for the transition of the human body from a healthy to a diseased state. In this meta-analysis, we have studied the relationship between the reduction in serum zinc ion concentration and coronary heart disease. *Methods*. We used PubMed and Cochrane (as of June 30, 2021) databases for the literature search. Per the requirements of this systematic review, case-control studies involving serum zinc ion concentration and coronary heart disease were searched, and the quality of the included studies was evaluated before the meta-analysis. *Results*. A total of 3,981 cases were found across seven articles. The standard mean deviation (SMD) of serum zinc ion concentration was −0.22 [−0.28, −0.15], $z = 6.52$, and $P < 0.05$ indicated that the difference was statistically significant. The forest plot results show that $I^2 = 34\% < 50\%$, and the Q test showed $P = 0.17 > 0.1$. These results suggest a lack of heterogeneity among the selected articles. Results from the funnel chart indicated that this study was free from publication bias. *Conclusion*. The results of this meta-analysis reveal that a decrease in serum zinc ion concentration is related to the occurrence of coronary heart disease. Clinically, monitoring the serum zinc ion levels is proven to be of great significance for patients with coronary heart disease.

1. Background

Coronary artery disease (CHD) is the main cause of morbidity and mortality in developed countries [1, 2]. Although in the past 20 years, the median age percentage of patients who succumbed to CHD has decreased by 22% worldwide, CHD is still the leading cause of death in the world [2]. Although the mortality rate is declining [1–3], it is still the leading cause of hospitalization and death in the UK and worldwide [1]. The mortality rate of CHD in developing countries has also been showing an increasing trend over the years [4]. CHD caused 8.1 million deaths in 2013, accounting for 14.8% of global deaths [5]. From 1990 to 2013, CHD was the leading cause of human death worldwide [5]. A German study showed that early detection and timely treatment can increase the survival rate of patients with circulatory blocks by 40% [6], suggesting that early detection and treatment are still key in treating not just CHD but also other diseases.

Changes in the trace element concentration in the body are considered to be the main factor leading to the transition of the human body from a healthy to a diseased state [7–10]. Trace elements, especially zinc ions, are likely involved in the pathogenesis of CHD [11–13]. Zinc is an important element in more than 70 enzymes, including superoxide dismutase and glutathione peroxidase. Zinc can be a cofactor of Cu-Zn superoxide dismutase (Cu, Zn-SOD) and subsequently aid

in treating CHD. Zinc ions participate in the regulation of various cellular metabolic activities, including the metabolism of different proteins, lipids, and carbohydrates in the body [14–16]. Most importantly, zinc exerts antioxidant and anti-inflammatory effects [17, 18]. An increase in the zinc ion concentration improves the antioxidant capacity of cells and ensures the secretion of a sufficient amount of NO to maintain normal endothelial function.

Based on the potential relationship between zinc ions and the occurrence of CHD and previous studies on zinc ions and CHD [16], we hypothesized that the decrease in serum zinc ion levels is related to the occurrence of CHD. To that end, we attempted to determine the relationship between serum zinc ion levels and CHD through articles on serum zinc ion concentration and CHD published in the past 10 years.

2. Methods

2.1. Search Strategy. We strictly followed the guidelines laid down for systematic reviews and meta-analysis (PRISMA) [19]. We used the PubMed and Cochrane databases for literature searches. The search keywords were used either singly or in a combination and included subject words and synonym words identified using MeSH. The subject word and synonym word for zinc ion is Zinc. The subject keyword of coronary heart disease is coronary disease, and the synonym words are coronary diseases; disease, coronary; diseases, coronary; coronary heart disease; coronary heart diseases; disease, coronary heart; diseases; coronary heart; heart disease, coronary; heart diseases, coronary. At the same time, we limited the scope of the search to reports published in English, and there was no limit to the time of publication of the literature. Before the final analysis, we once again perused and inspected the quality of the literature to ensure that only studies that met the review criteria were included. For example, in PubMed, the retrieval relationship between synonym words was "OR," and the retrieval relationship between subject words and synonym words was "AND."

2.2. Inclusion and Exclusion Criteria. The articles retrieved and selected were independently screened by two authors (HM, JR) based on the title, abstract, and full text. In addition, the points of disagreement were resolved through discussion. The inclusion criteria were research related to the topic, with data, including the average value of zinc concentration and its standard deviation. The exclusion criterion was repeated studies, review or meta-analysis, animal experiments, undetected zinc ion concentrations, and unstable studies (the unstable studies are the ones with no strict selection criteria, unreasonable statistics, and exaggerated conclusions). See Figure 1 for details.

2.3. Quality Evaluation. The quality and data extracted from each study were evaluated per the Methodological index for nonrandomized studies (MINORS) guidelines. All data were independently extracted by two reviewers (HM and JR).

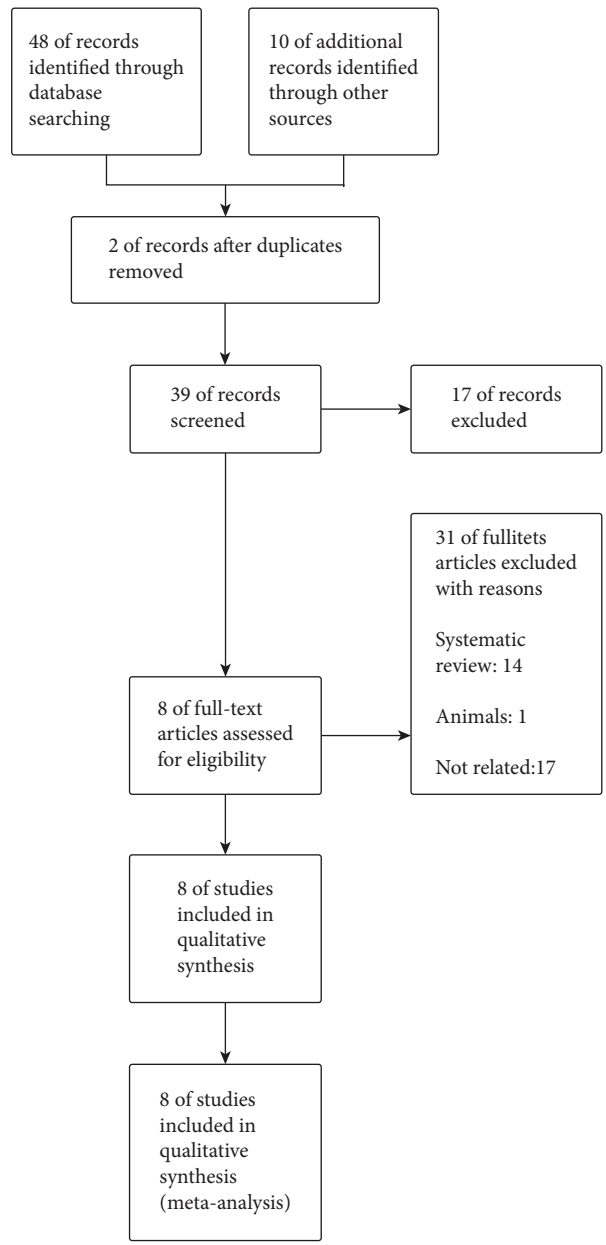

Figure 1: Flowchart for article screening.

Disagreements were resolved by involving a third unbiased reviewer. The extracted data included the name of the first author, year of publication, country/region, study design, sample size, and baseline characteristics, and whether the gold standard was applied for disease detection.

2.4. Statistical Analysis. A meta-analysis was performed to comprehensively analyze different studies. The standard mean deviation (SMD) and corresponding 95% confidence interval (CI) were used to evaluate the difference between serum zinc ion and CHD in the selected articles. For determining heterogeneity, the random-effects or fixed-effects models were used. The I^2 test was used to evaluate the statistical heterogeneity between the studies, where the values of $I^2 > 25\%$ and 50% were regarded as moderate and

high heterogeneity, respectively. In Statistics, $P < 0.05$ was considered statistically significant. In addition, we performed a sensitivity analysis to assess the robustness of the results. We also used a funnel chart to assess publication bias [20]. All analyses were performed using Review Manager 5.3 (Copenhagen, the Nordic Cochrane Centre, the Cochrane Collaboration).

3. Results

3.1. Characteristics of Each Study. In Table 1, (1) the purpose of the study is clearly given; (2) the consistency of the included patients; (3) the collection of expected data; (4) the endpoint indicators appropriately reflect the purpose of the research; (5) the objectivity of the evaluation of the endpoint indicators; (6) whether the follow-up time is sufficient; (7) the loss to follow-up rate is less than 5%; (8) whether the sample size is estimated; (9) whether the selection of the control group is appropriate; (10) whether the control group is synchronized; (11) whether the baselines between the groups are comparable; and (12) whether the statistical analysis is appropriate. Scoring method: 0 point means not reported; 1 point means reported but insufficient information; 2 points mean reported and with sufficient information. Articles with a score of 0–8 are classified as low-quality articles, 9–16 as medium-quality articles, and 17–24 as high-quality documents. The MINORS quality evaluation form denotes literature with a score of fewer than 12 points as excluded from the meta-analysis. Scoring was performed independently by two researchers. Inconsistent scoring results were resolved through discussion or consultation with an independent third party until an agreement was reached. The seven articles included in the study had scores of 15–21 points, suggesting them all to be medium- and high-quality articles.

3.2. Heterogeneity Test. The seven articles included in this study were tested for heterogeneity, if $I^2 = 34\% < 50\%$, and Q test $P = 0.17 > 0.1$. These results suggest a lack of heterogeneity between the selected articles in this study, and the fixed-effects model was chosen for the meta-analysis. To ensure the accuracy and stability of the study, we conducted a sensitivity analysis.

3.3. Sensitivity Analysis. A sensitivity analysis was carried out on the seven articles included in this study. One article was removed at a time, and none of them interfered with the results of this meta-analysis, indicating that the study had good stability. See Table 2 for details.

3.4. Meta-Analysis of Fixed Effects. The SMD value of the seven studies was −0.22, 95% confidence interval was −0.28~−0.15, $z = 6.52$, and $P < 0.05$, which was statistically significant. These results suggest that serum zinc ion concentration was related to CHD. The results are shown in the forest diagram (Figure 2).

3.5. Bias Test. A funnel chart was constructed to investigate whether there was a publication bias in this study; the symmetry in the funnel chart indicated no publication bias, as observed in Figure 3.

4. Discussion

Through this meta-analysis, we aimed to correlate the serum zinc ion concentration to the occurrence of CHD by including studies that compared the serum zinc ion concentration in patients with CHD to that in the controls. A total of seven articles met our criteria. The results showed that the serum zinc ion concentration in CHD patients was higher than that of the control group, suggesting that the serum zinc ion level has a potential impact on the occurrence of CHD.

Medical-physiological studies have shown a correlation between trace element content and CHD [27–30]. He et al. proposed that an increase in zinc ion concentration can significantly reduce high-density lipoprotein (HDL) levels and increase triglyceride (TG), cholesterol (CH), and low-density lipoprotein (LDL) levels, thereby causing atherosclerosis and cardiovascular disease [28, 31, 32]. Zinc is an important component of the antioxidant enzyme superoxide dismutase (Cu-ZnSOD) [32, 33]. The zinc ion concentration in the human body is an important parameter that helps regulate the antioxidant defense system of the body [34–36]. In the human body, the antioxidant activity of vitamin A depends on sufficient zinc ion concentration [37–39]. Vitamin E, another effective antioxidant, has many functions that overlap with zinc ions, including the maintenance of cell membrane stability, antioxidant function, and regulation of prostaglandins [38, 40]. Studies have shown that malabsorption of vitamin E is accompanied by a deficiency of zinc ions, thus indicating some interaction between the two nutrients [38, 41]. One possible explanation for the relationship between zinc ions, vitamins, and oxidation is that the body lacks zinc ions, which in turn leads to a decrease in the supply or utilization of vitamins A and E and ultimately leads to an increase in oxidation. This can create an imbalance in the ratio of oxidants to antioxidants (oxidative stress) in the body [41].

In addition, this reduction in antioxidant capacity indicates that LDL is more likely to be oxidized. The results of this study are consistent with those obtained by other researchers who observed low antioxidant levels in smokers; LDL in smokers is more likely to be oxidized. Early studies have shown the uptake of oxidized LDL cholesterol by monocytes and macrophages, forming foam cells, ultimately leading to atherosclerosis [42–44]. Therefore, the risk of atherosclerosis in diabetic patients is higher, not because of increased serum LDL levels but because of a higher likelihood of oxidized serum LDL. As the antioxidant concentration is reduced, oxidized LDL cholesterol is more likely to cause atherosclerosis [45, 46].

The serum zinc ion concentration that this study focuses on is a more accurate indicator for CHD detection and has a greater clinical application value than other indicators. In summary, this meta-analysis emphasized that low zinc ion concentration is related to the occurrence of CHD. For monitoring CHD, it is necessary to detect serum zinc ion concentration.

Table 1: Baseline information. The gold standard for diagnosing CHD in the experimental group is coronary angiography.

Id	Author	Country	Years	Is the method of diagnosing disease the gold standard	Types of test specimens for zinc ions	The mean value of zinc ion concentration in the control group	The standard deviation of zinc ion concentration in the control group	Number of the control group	Mean value of zinc ion concentration in the experimental group	The standard deviation of zinc ion concentration in the experimental group	Number of the experimental group
No. 1	Meng et al. [16]	China	2021	Yes	Serum	14.77 μmol/L	2.86	1103	14.18 μmol/L	2.87	1103
No. 2	Soinio et al. [21]	Finland	2007	Yes	Serum	15.8 μmol/L	2.5	796	15.2 μmol/L	2.5	254
No. 3	Basnet et al. [22]	Nepal	2020	Yes	Serum	13.7 mg	3.3	233	13.5 mg	2.6	233
No. 4	Cebi et al. [23]	Turkey	2011	Yes	Serum	0.91 μg/dI	0.18	20	0.85 μg/dI	0.55	30
No. 5	Hasanato [24]	Saudi Arabia	2020	Yes	Serum	15 μmol/L	3.2	50	13.5 μmol/L	1.9	50
No. 6	Islamoglu et al. [25]	Turkey	2011	Yes	Serum	0.67 ng/L	0.14	34	0.61 ng/L	0.16	33
No. 7	Li et al. [26]	China	1990	Partly	Serum	20.17 μmol/L	3.97	31	14.39 μmol/L	4.44	31

Table 2: Quality assessment form.

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9	No. 10	No. 11	No. 12	Total score
Li et al. [26]	1	1	2	2	0	0	0	1	2	2	2	2	15
Rana-2020	2	1	2	1	0	0	0	2	1	2	2	2	15
Islamoglu et al. [25]	2	2	2	2	0	0	0	2	2	2	2	2	18
Soinio et al. [21]	2	2	2	2	0	1	0	2	2	2	2	2	19
Meng et al. [16]	2	2	2	2	0	0	0	2	2	2	2	2	18
Cebi et al. [23]	2	1	2	2	0	0	0	2	2	2	2	2	17
Basnet et al. [22]	2	2	2	2	1	1	1	2	2	2	2	2	21

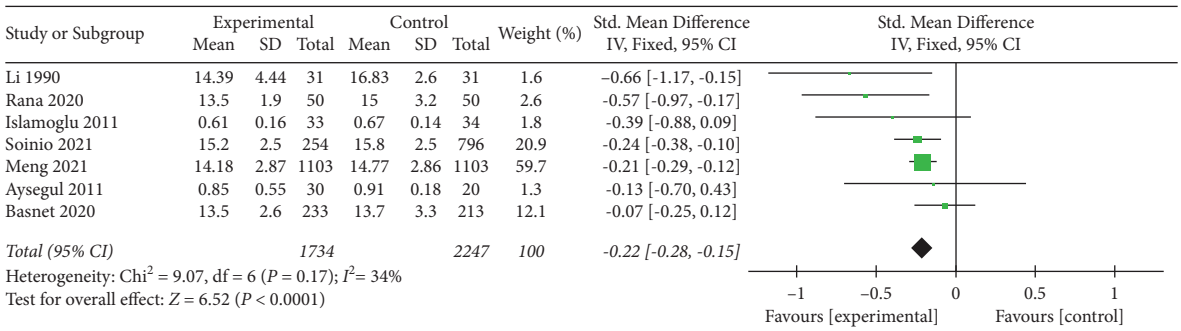

Figure 2: Forest diagram.

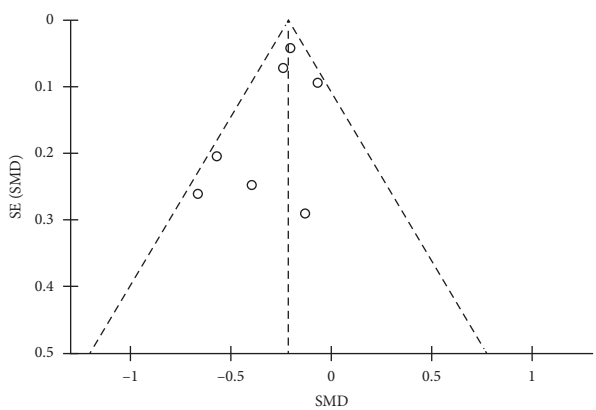

Figure 3: Funnel chart the funnel plot is symmetrical on both sides and there is no publication bias.

5. Conclusion

The results of this meta-analysis reveal that a decrease in serum zinc ion concentration is related to the occurrence of coronary heart disease. Clinically, monitoring the serum zinc ion levels is proven to be of great significance for patients with coronary heart disease.

Data Availability

The literature data supporting this meta-analysis are from previously reported studies and datasets, which have been cited. The processed data are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Heyu Meng carried out the research design and manuscript editing. Literature search and manuscript writing was performed by Jianjun Ruan. Yanqiu Chen, Zhaohan Yan, Jinsha Liu, and Xiangdong Li conducted data analysis. Cuiying Mao and Ping Yang concentrated on the study concepts. Cuiying Mao and Ping Yang contributed equally to this paper. All authors read and approved the final manuscript.

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Hindawi
Cardiology Research and Practice
Volume 2022, Article ID 9742071, 10 pages
https://doi.org/10.1155/2022/9742071

Research Article

Outcomes of Genetic Testing-Based Cardiac Rehabilitation Program in Patients with Acute Myocardial Infarction after Percutaneous Coronary Intervention

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Received 24 April 2022; Revised 20 June 2022; Accepted 21 June 2022; Published 17 August 2022

Academic Editor: Wei Jiang

Objective. There can be extreme variability between individual responses to exercise training, and the identification of genetic variants associated with individual variabilities in exercise-related traits could guide individualized exercise programs. We aimed to screen the exercise-related gene sensitivity of patients with acute myocardial infarction after PCI by establishing the gene spectrum of aerobic exercise and cardiopulmonary function sensitivity, test the effect of individualized precision exercise therapy, and provide evidence for the establishment of a precision medicine program for clinical research. *Methods*. Aerobic exercise- and cardiopulmonary function-related genes and single-nucleotide polymorphisms (SNPs) were obtained by data mining utilizing a major publicly available biomedical repository, the NCBI PubMed database. Biological samples from all participants underwent DNA testing. We performed SNP detection using Samtools. A total of 122 patients who underwent PCI were enrolled in the study. We screened the first 24 cases with a high mutation frequency for aerobic exercise- and cardiopulmonary function-related genes and the last 24 cases with a low mutation frequency and separated them into two groups for the exercise intervention experiment. *Results*. In both the low mutation frequency group and the high mutation frequency group, after 8 weeks of exercise intervention, 6 MWT distance, 6 MWT%, VO_2/kg at peak, and VO_2/kg at AT were significantly improved, and the effect in the high mutation frequency group was significantly higher than that in the low mutation frequency group (6 MWT distance: 468 vs. 439, $P = 0.003$; 6 MWT%: 85 vs. 77, $P = 0.002$, VO_2/kg at peak: 14.7 vs. 13.3, $P = 0.002$; VO_2/kg at AT: 11.9 vs. 13.3, $P = 0.003$). *Conclusions*. There is extreme variability between individual responses to exercise training. The identification of genetic variants associated with individual variabilities in exercise-related traits could guide individualized exercise programs. We found that the subjects with a high mutation frequency in aerobic exercise and cardiopulmonary function-related genes achieved more cardiorespiratory fitness benefits in the aerobic exercise rehabilitation program and provided evidence for the establishment of a precision medicine program for clinical research.

1. Introduction

Cardiovascular disease is becoming the most common cause of mortality, especially in high-income countries [1]. According to the annual report on cardiovascular health and diseases in China, the prevalence of the cardiovascular disease among Chinese residents has gradually increased, and the prevalence of coronary heart disease among people over 60 years old has reached 27.8% [2]. Since 2005, the mortality of patients with acute myocardial infarction has increased rapidly [2]. Percutaneous coronary intervention (PCI) is an effective treatment to reduce mortality, myocardial infarction, and hospitalization rate of people with the acute coronary syndrome in the treatment of acute myocardial infarction [3, 4].

Although PCI has become the most important revascularization treatment for patients with coronary heart disease, PCI and drug therapy alone cannot continuously and effectively improve the prognosis of patients [5]. It is necessary to prevent the development of coronary heart disease, reduce the recurrence rate and mortality of cardiovascular events, prolong life, and improve the quality of life after discharge [5, 6]. Currently, many international clinical guidelines recommend that patients join an exercise rehabilitation program after PCI [7, 8]. Research shows that exercise rehabilitation can significantly reduce all-cause mortality, cardiovascular disease-related mortality, rehospitalization rate, and the incidence of revascularization, reduce related dysfunction and emotional abnormalities, and increase the quality of life of patients [5, 9]. While exercise is recommended by essentially every major medical organization, it is also recognized that there can be extreme variability between individual responses to exercise training [10].

The idea of personalized medicine has been gaining significant interest since the sequencing of the human genome, and the identification of specific sport- and exercise-related genes is expected to be used for precision sports medicine to provide tailor-made training as well as to select optimal sports and/or other exercise activities for each individual [10–12]. Research has found that sprinters with the RR + RX genotype of the alpha-actinin-3 (ACTN3) gene had significantly faster personal best times for the 100 m race than those with the XX genotype [13]. Thus, the identification of genetic variants associated with individual variabilities in exercise-related traits could guide individualized exercise programs, which is one of the goals of precision medicine [11, 14, 15]. Therefore, we aimed to screen the exercise-related gene sensitivity of patients with acute myocardial infarction after PCI by establishing the gene spectrum of aerobic exercise and cardiopulmonary function sensitivity, test the effect of individualized precision exercise therapy, and provide evidence for the establishment of a precision medicine program for clinical research.

2. Methods

2.1. Data Mining of the Gene Set. We utilized a major publicly available biomedical repository, the NCBI PubMed database, for data mining. Search strategies were combined as follows: ("athletic performance" OR "physical performance" OR "elite athlete" OR "athletic status" OR "endurance performance" OR "aerobic exercise" OR "strength training") AND (genes OR gene OR loci OR locus). Database searching retrieved a total of 951 studies. Information on a total of 111 exercise-related SNPs and 76 exercise-related genes was obtained after analysis by a text mining program (see Table S1). Our text mining program consisted of five steps: (1) Document searching and formatting, in which keywords were used to search documents and organize documents into XML format. (2) Gene mentions tagging using ABNER software to describe and locate genes [16]. (3) Conjunction resolution, in which the description of extracted genes, such as the "STAT3/5 gene," was resolved into the STAT3 gene and STAT5 gene. (4) Gene name normalization based on the Entrez database; because the names of genes in the free text were confusing, it was necessary to unify the gene descriptions in the article into official gene symbols to facilitate analysis and comparison. The gene symbol was based on the Entrez gene database of the NCBI. (5) Statistical analysis, in which the frequency of each gene was determined. The higher the frequency of the gene, the greater the possibility that the gene was related to the disease. The total number of documents in the PubMed database was defined as N. The frequency of independent occurrence of genes and corresponding diseases in the PubMed literature database was recorded as m and n, respectively. Supposing that the number of simultaneous occurrences of gene disease is k, we can calculate the probability of more than k power co-citations under completely random conditions through hypergeometric distribution, as follows:

$$p = 1 - \sum_{i=0}^{k-1} p(i|n, m, N), \tag{1}$$

and

$$p(i|n, m, N) = \frac{n!(N-n)!m!(N-m)!}{(n-i)!i!(n-m)!(N-n-m+i)!N!}. \tag{2}$$

Through the classification of aerobic exercise- and cardiopulmonary function-related genes involved in this exercise program, 36 aerobic exercise- and cardiopulmonary function-related genes and 45 related SNPs were obtained (see Figure 1).

The red color indicates that the SNP appears, and grey indicates that it does not appear.

2.2. Subjects and Groups. Patients were recruited from DAQING Oilfield General Hospital. In the inclusion criteria, patients were recruited from Daqing Oilfield General Hospital. The inclusion criteria were as follows: (1) all patients were treated with PCI for the first time and whose Killip class was I-II; (2) all patients were treated within 6 hours after the onset of disease, and (3) clinical data and imaging data during the treatment period were complete, and there were no missing data. The exclusion criteria were as follows: (1) exercise-induced syncope or ventricular arrhythmias; (2) inability to exercise and walk owing to comorbidities; (3) suffering from end-stage diseases such as malignancies; (4) severe complications such as pulmonary edema, severe arrhythmia, or cardiogenic shock; and (5) suffering from mental illness or family history of mental illness. All percutaneous coronary interventions were performed by the same team [17]. A total of 122 patients who underwent PCI after acute myocardial infarction were enrolled in the study. Informed written consent was obtained from all participants. Biological samples from all participants underwent DNA testing. The DNA of the 122 subjects was extracted using a Universal Cylindrical Genome Extraction Kit (KangWei Century, CW2298 M). Agarose gel electrophoresis was used to analyze the degree of DNA degradation and the presence of impurity bands and RNA

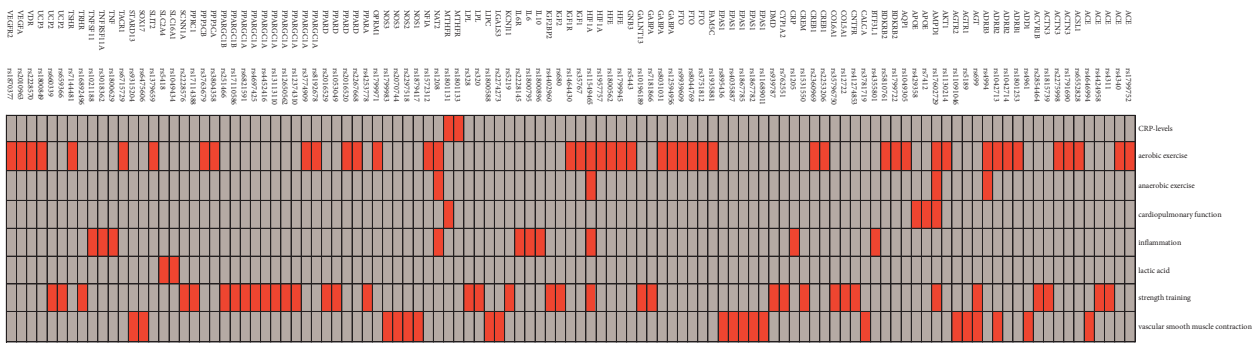

Figure 1: Heatmap of 111 SNP.

and protein contamination in the 122 subjects. The quality of the DNA was determined by a NanoDrop ND-2000 ultra-micro spectrophotometer. Biological samples were gene sequenced using the Illumina HiSeq PE150. Quality control (QC) analyses were performed on sequenced Reads. Mapping reads to a reference after QC. The sequencing results were compared with the reference genome using BWA software which mainly uses the location comparison between a large number of short fragments after second-generation sequencing and the reference genome. We performed SNP genotyping through SAMtools software [18, 19]. The regional distribution statistics of 45 aerobic exercise- and cardiopulmonary function-related SNP loci of the 122 samples are shown in Table 1.

We drew a panorama of 45 SNP genotypes from 122 samples, screened the first 24 cases with high mutation frequency and the last 24 cases with low mutation frequency, and divided them into two groups for the exercise intervention experiment, as shown in Figure 2. There were no statistically significant differences between the 2 groups of patients regarding general data such as age, sex, body mass index (BMI), Killip classification, coronary lesions, and comorbidities such as hypertension or diabetes ($P > 0.05$); the groups were comparable.

2.3. Procedures. A total of 122 patients who underwent PCI after acute myocardial infarction were enrolled in the study, and the first 24 cases with a high mutation frequency in aerobic exercise and cardiopulmonary function-related genes and the last 24 cases with a low mutation frequency were screened into two groups for the exercise intervention experiment. The flow chart is shown in Figure 3. After PCI treatment, all patients underwent blood pressure regulation, sedation, and other treatments, and symptomatic care, such as oxygen and medication administration, was also delivered. These two groups were given an exercise prescription and guidance from the same team. Each exercise period consisted of warm-up exercises for 5 min (20–40% of VO_2 max), followed by moderate-intensity continuous aerobic exercise (50–60% of VO_2 max), light exercise for 40 min, and a 5-min cooldown; the regimen had a Borg rating of 11–13. The exercise was performed for 50 minutes each time, 4 times/week, for a total of 8 weeks. The exercise was advised to be halted if any of the following occurred: (1) chest pain, dyspnoea, or dizziness during or after exercise; (2) heart rate fluctuation >30 beats/min; (3) blood pressure >200/100 mmHg or systolic blood pressure increase >30 mmHg or decrease >10 mmHg; (4) electrocardiogram monitoring during exercise showed ST-segment depression ≥0.1 mV or elevation ≥0.2 mV; or (5) severe arrhythmia during or after exercise. This study was approved by the DAQING Oilfield General Hospital ethics committee.

Table 1: Regional distribution of 45 SNP loci.

Func.refGene[1]	Number
Exonic[2]	16
ncRNA_intronic[3]	2
Intronic[4]	15
UTR3[5]	3
UTR5[6]	5
Downstream[7]	1
Intergenic[8]	3
Total[9]	45

[1]The functional region where the mutation site is located; [2]exonic region; [3]noncoding RNA intron region; [4]inner subregion; [5]3′ UTR area; [6]5′ UTR area;[7]1 KB region downstream of transcription termination site; [8]gene spacer region;[9] Total SNP.

2.4. Outcome Measures. Cardiopulmonary exercise testing with respiratory gas analysis was performed using the individualized ramp protocol recommended by the American Heart Association [20]. The specific protocol was as follows: 0 W: rest for 1 min; 0 W: warm-up for 2 min; treadmill intensity started at 5 W. Thereafter, according to the exercise ability of the subjects, the intensity was increased by 15–25 W per minute until the subjects reached the outcome measures at 8–12 minutes. Calibration was performed before each testing period. Peak O_2 utilization (VO_2 peak) was defined as the highest VO_2 value (without reaching an oxygen uptake steady-state plateau), achieved at individual maximum load during incremental exercise testing [20]. During CPET, data on the subjects' static electrocardiogram and static lung function (vital capacity/maximum ventilation) were individually collected. The indications for termination of CPET in our study were following the scientific statement from the American Heart Association [21]. The 6-minute walk test (6 MWT) was used in this study. The procedures and indications for termination of the 6 MWT were performed according to the recommendations of the

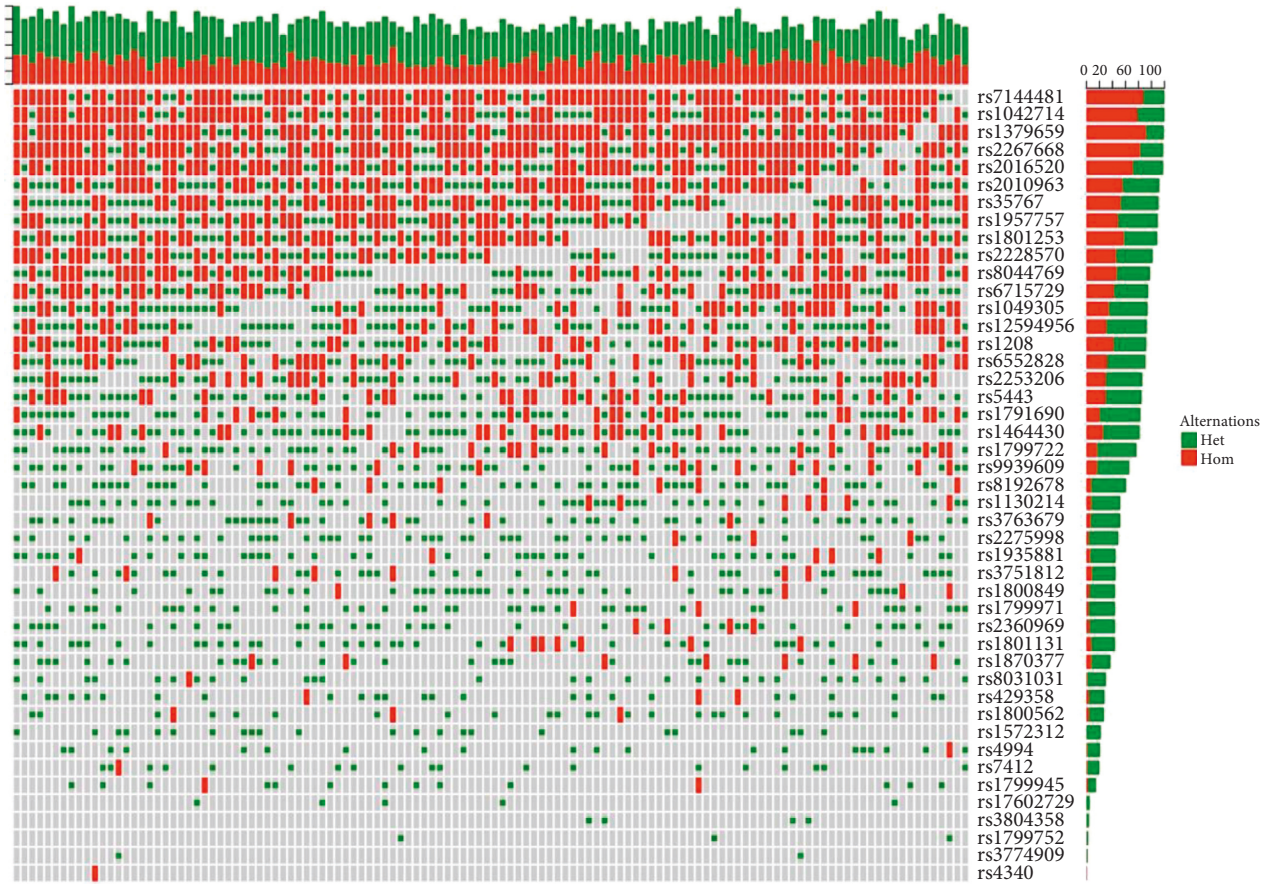

Figure 2: Panorama of 45 SNP gene mutations in subjects.

American Thoracic Society [22]. The participants walked for 6 minutes along an indoor 30-m corridor, and the distance walked was recorded for analysis.

2.5. Statistical Analyses. Continuous variables are expressed as the mean ± SD or median and were compared using a two-sided independent samples *t* test. Frequency data were compared between the groups using the chi-square test. A value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using *R* version 4.0.5.

3. Results

The average age of the high mutation frequency group was 52.76 ± 7.74 years, and that of the low mutation frequency group was 50.76 ± 9.34 years. There was no significant difference in sex, age, or exercise ability indices, including the 6 MWT, VO_2/kg at peak, and VO_2/kg at AT, between the low mutation frequency group and the high mutation frequency group, as shown in Table 2.

After 8 weeks of exercise training, the 6-minute walk test distance (468 vs. 439, $P = 0.003$), 6 MWT% (85 vs. 77, $P = 0.002$), VO_2/kg at peak (14.7 vs. 13.3, $P = 0.002$), and VO_2/kg at AT (11.9 vs. 13.3, $P = 0.003$) in the high mutation frequency group were significantly higher than those in the low mutation frequency group, as shown in Table 3.

Through the independent samples *t*-test analysis of the results in the group, it was found that the 6-minute walk test distance (388 vs. 468, $P < 0.01$), 6 MWT% (68 vs. 85, $P < 0.01$), VO_2/kg at peak (12.1 vs. 14.7, $P < 0.01$) and VO_2/kg at AT (9.1 vs. 11.9, $P < 0.01$) in the high mutation frequency group after 8 weeks of training were significantly higher than those before training, as shown in Table 4.

Through the independent samples *t*-test analysis of the results in the group, it was found that the 6-minute walk test distance (385 vs. 439, $P < 0.01$), 6MWT% (67 vs. 77, $P < 0.01$), VO_2/kg at peak (12.4 vs. 13.3, $P < 0.01$), and VO_2/kg at AT (9.3 vs. 10.1, $P < 0.01$) in the low mutation frequency group after 8 weeks of training were significantly higher than those before training, as shown in Table 5.

In both the low mutation frequency group and the high mutation frequency group, after 8 weeks of exercise intervention, 6MWT distance (low mutation frequency group: 385 vs. 439, $P < 0.01$; high mutation frequency group: 388 vs. 468, $P < 0.01$) and 6MWT% (low mutation frequency group: 67 vs. 77, $P < 0.01$; high mutation frequency group: 68 vs. 85, $P < 0.01$) were significantly improved, and the effect in the high mutation frequency group was significantly higher than that in the low mutation frequency group (6 MWT distance: 468 vs. 439, $P = 0.003$; 6 MWT%: 85 vs. 77, $P = 0.002$), as shown in Figure 4.

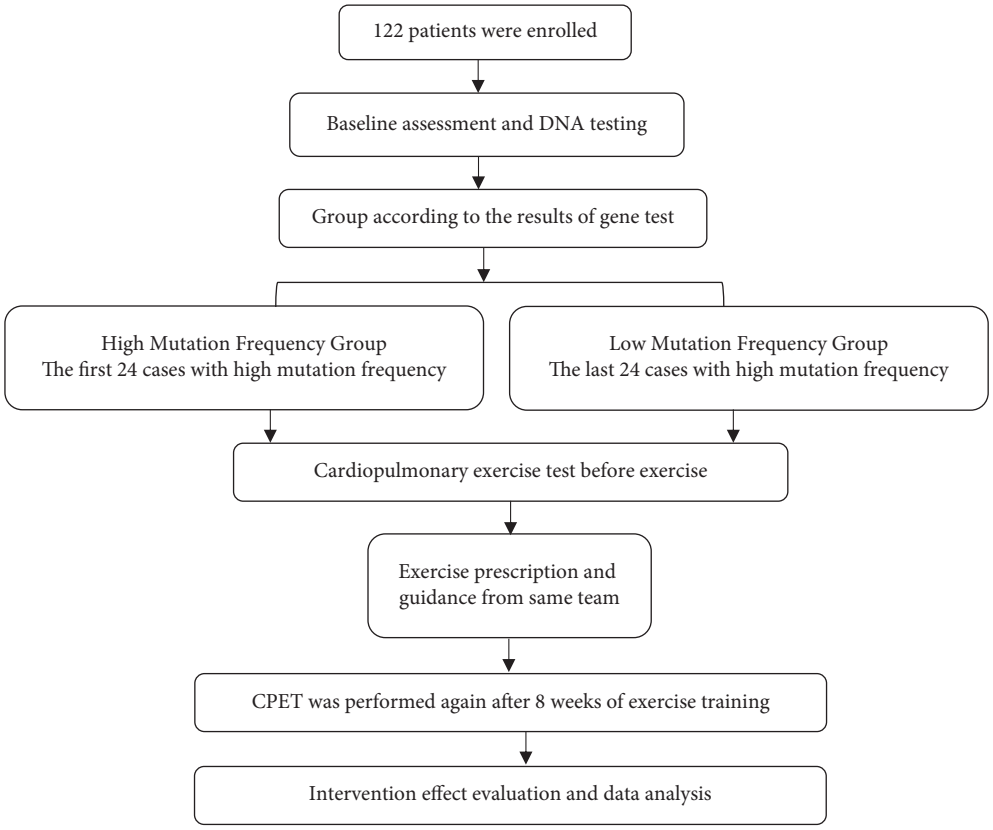

Figure 3: Intervention flow chart.

Table 2: Comparison of baseline indexes between the high mutation frequency group and the low mutation frequency group.

	High mutation frequency group ($n = 24$)	Low mutation frequency group ($n = 24$)	P value
Age (Y)	52.76 ± 7.74	50.76 ± 9.34	0.41
Gender			0.63
Male %	88	92	
Female %	12	8	
6 MWT (m)	388	385	0.16
6 MWT%	68	67	0.77
VO_2/kg PEAK (ml/kg/min)	12.1	12.4	0.68
VO_2/kg AT (ml/kg/min)	9.1	9.3	0.91

6 MWT: 6-minute walk test. AT: anaerobic threshold.

Table 3: Comparison of exercise ability indices after 8 weeks of exercise intervention.

	High mutation frequency group ($n = 24$)	Low mutation frequency group ($n = 24$)	P value
6 MWT (m)	468	439	0.003
6 MWT%	85	77	0.002
VO_2/kg PEAK (ml/kg/min)	14.7	13.3	0.002
VO_2/kg AT (ml/kg/min)	11.9	10.1	0.003

Table 4: Comparison of indices in the high mutation frequency group before and after the 8-week exercise intervention.

	High mutation Frequency group before ($n = 24$)	High mutation Frequency group after ($n = 24$)	P value
6MWT (m)	388	468	<0.01
6MWT%	68	85	<0.01
VO_2/kg PEAK (ml/kg/min)	12.1	14.7	<0.01
VO_2/kg AT (ml/kg/min)	9.1	11.9	<0.01

Table 5: Comparison of indices in the low mutation frequency group before and after the 8-week exercise intervention.

	Low mutation frequency group before ($n = 24$)	Low mutation frequency group after ($n = 24$)	P value
6 MWT (m)	385	439	<0.01
6 MWT%	67	77	<0.01
VO_2/kg PEAK (ml/kg/min)	12.4	13.3	<0.01
VO_2/kg AT (ml/kg/min)	9.3	10.1	<0.01

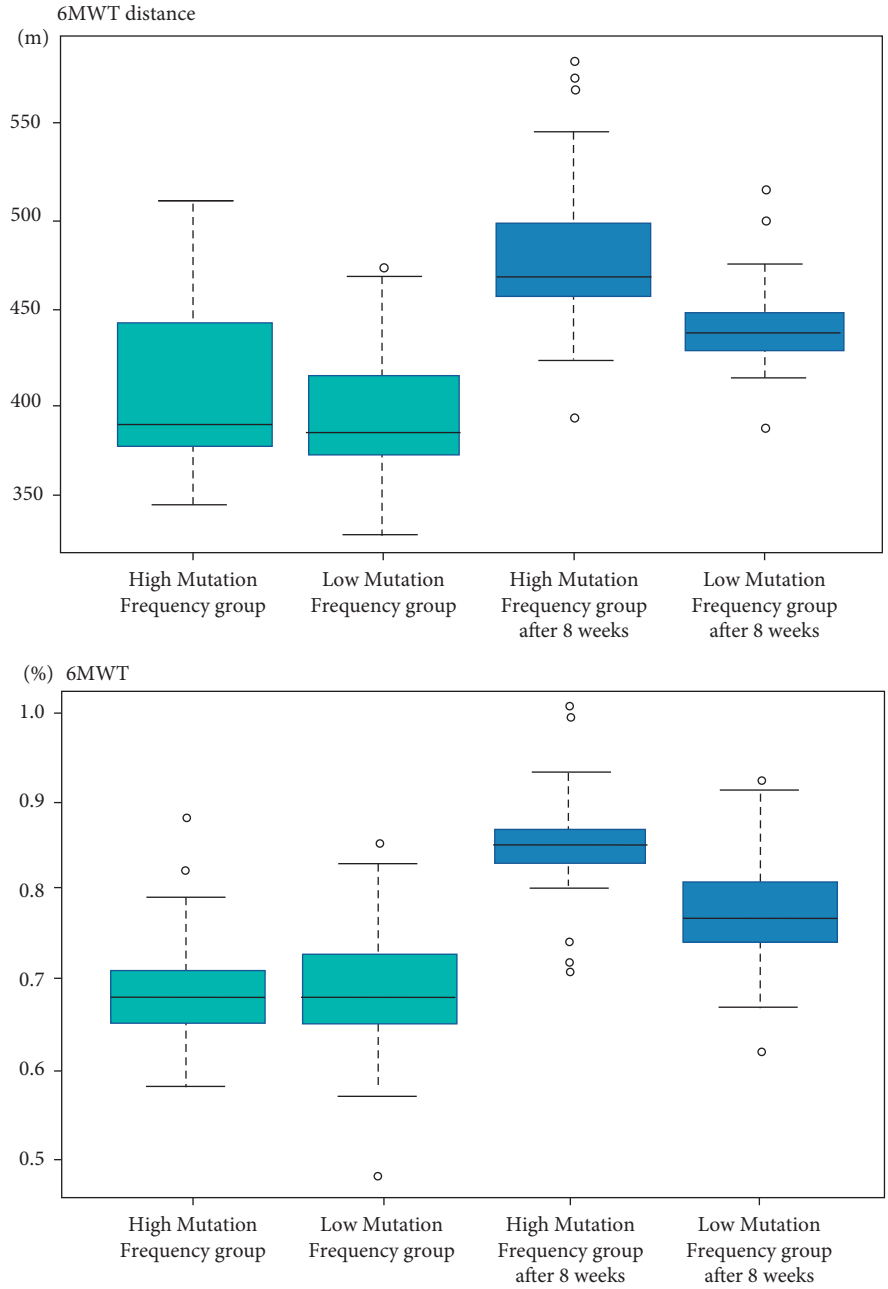

Figure 4: 6MWT distance and 6MWT% before and after the 8-week exercise intervention.

After 8 weeks of exercise training, VO_2/kg at peak (low mutation frequency group: 12.4 vs. 13.3, $P < 0.01$; high mutation frequency group: 12.1 vs. 14.7, $P < 0.01$) and VO_2/kg at AT (low mutation frequency group: 9.3 vs. 10.1, $P < 0.01$; high mutation frequency group: 9.1 vs. 11.9, $P < 0.01$) were significantly improved in both the low mutation frequency group and the high mutation frequency group, and the effect in the high mutation frequency group

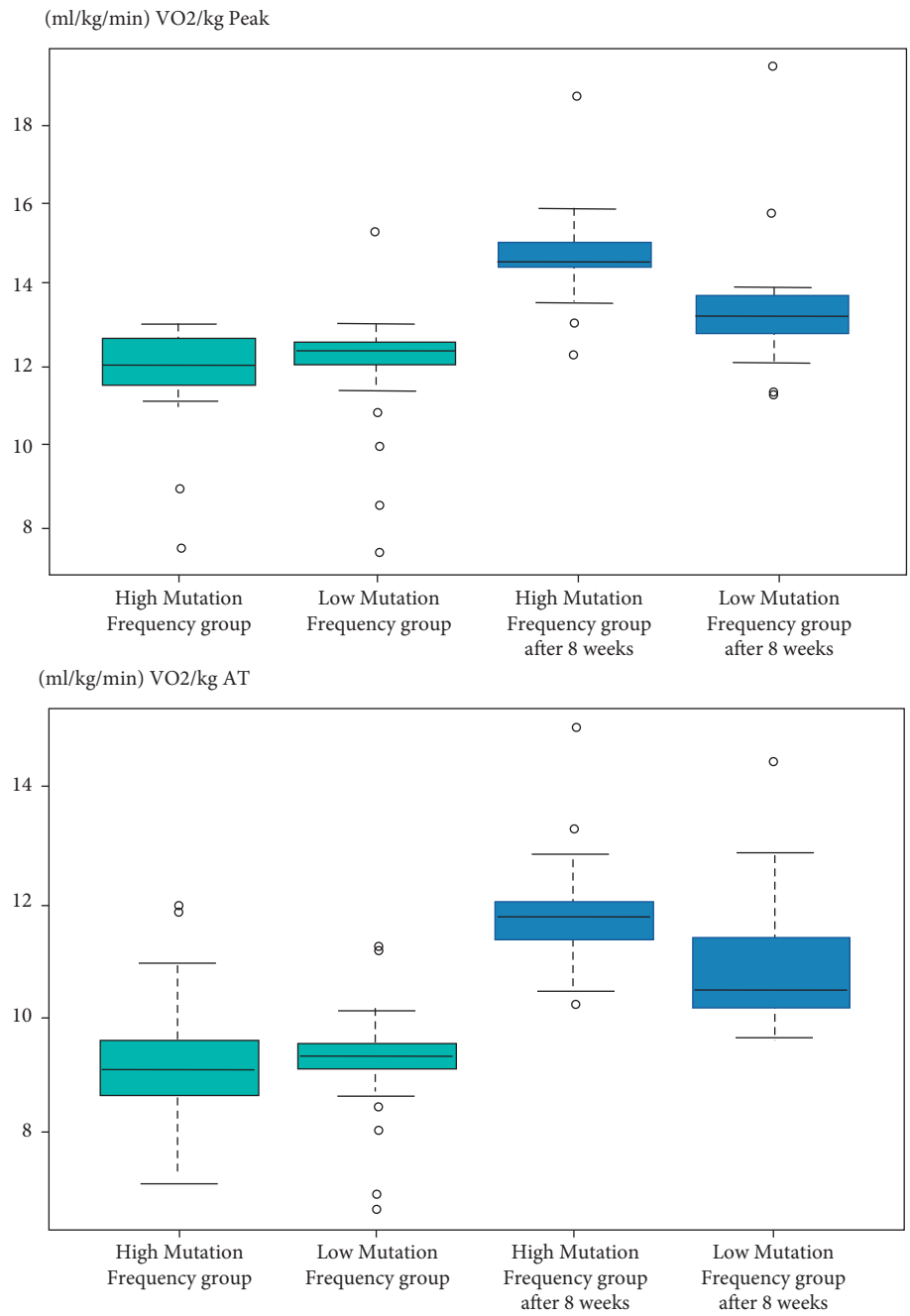

Figure 5: VO_2/kg at peak and VO_2/kg at AT before and after the 8-week exercise intervention.

was significantly higher than that in the low mutation frequency group (VO_2/kg at peak: 14.7 vs. 13.3, $P = 0.002$; VO_2/kg at AT: 11.9 vs. 13.3, $P = 0.003$), as shown in Figure 5.

4. Discussion

As previous studies have suggested, genetic modifiers have been identified from the study of affected patient populations to identify common genomic variations. In precision medicine, these findings could provide the most useful results in terms of applicability in the clinic [10]. Findings from numerous investigations demonstrate extraordinary interindividual variability in response to a standard dose of exercise [23], and the issue of individual response to treatment is one of the most important in exercise medicine. In our study, we tried to select patients with more mutations in aerobic exercise and cardiopulmonary function sensitivity genes through gene mutation detection to carry out an aerobic exercise intervention to detect whether they would obtain more benefits compared with a low mutation frequency group, which is consistent with some previous studies [11, 14]. Previous twin and familial studies suggest that there is moderate heritability of "sport and exercise-related traits" [22], thus, the identification of genetic variants

determining variabilities in sport and exercise-related traits may offer significant benefits to athletes and the general population [10,11]. We found that the group with a high mutation frequency for aerobic exercise- and cardiopulmonary function-related genes gleaned more benefits from the 8-week aerobic exercise rehabilitation program (MWT distance: 468 vs. 439, $P = 0.003$; 6MWT%: 85 vs. 77, $P = 0.002$, VO_2/kg at peak: 14.7 vs. 13.3, $P = 0.002$; VO_2/kg at AT: 11.9 vs. 13.3, $P = 0.003$).

Research has found considerable interindividual responses to a single-dose exercise program for maximal oxygen uptake (VO_2 max), which is achieved at the individual maximum load during incremental exercise testing [23]. The concept of genetic variation being associated with trainability has been extensively studied in relation to peak VO_2, potentially explaining up to 50% of the variability in the change in peak VO_2 after endurance training [24, 25]. Many previous large-scale trials and meta-analyses used the 6 MWT and peak VO_2 to demonstrate the physical and physiological benefits of routine cardiac rehabilitation [5, 26, 27]. VO_2 max describes the maximum ability of a whole organism to transport oxygen from the air to the tissues and especially the exercising skeletal muscles [24, 28]. The maximal amount of O_2 per unit of time that can be delivered to peripheral organs, including skeletal muscle, where it is used to sustain muscular contraction at peak exercise, is considered the gold standard measure of cardiorespiratory fitness [29, 30]. Peak or maximum cardiac output and total body hemoglobin mass seem to predominate as determinants of max VO_2. Cardiorespiratory fitness is closely associated with all-cause mortality and cardiovascular mortality. Thus, we will further carry out exercise intervention projects for cardiovascular disease patients to reduce the incidence and mortality of heart failure.

In addition, human athletic performance has long been assumed to be polygenic. In addition to single-nucleotide variants in the gene regions, other types of genomic variation, such as structural variation and variants in noncoding RNA, may also contribute to the complexity of the athletic phenotype [31, 32]. Given that exercise is polygenic within a given organ and affects multiple organ systems, there are likely other undetermined adaptations that do respond to exercise [33]. Studies have suggested that subjects show improvements in oxidative enzyme activities in muscles even in the group that did not show an increase in VO_2 max in response to aerobic exercise [34]. The evaluation indicators included in our study are limited; thus, failure to improve one specific phenotype is not reason enough to cease or fail to recommend or prescribe exercise because VO_2 max does not increase. We will screen to carry out exercise therapy and interventions efficiently and accurately for cardiovascular disease patients. In addition to clinical efficacy and safety, the costs and cost-effectiveness of cardiac rehabilitation need to be considered with the growing cost pressures on healthcare systems across the world [35]. Previous studies concluded that cardiac rehabilitation was cost-effective compared with no cardiac rehabilitation (incremental cost-effectiveness ratios (ICERs) ranged from US$1,065 to US$71,755 per quality-adjusted life-year (QALY)), and exercise intervention in cardiac rehabilitation appears to cost-effective, though uncertainty was high [36]. Thus, optimal tailored medical therapies for the individual based on the individual's complete clinical and risk profiles which include their genomic information may revolutionize healthcare by substantially enhancing the efficacy of treatment with a promise to significantly reduce the costs associated with healthcare provision [37].

On the other hand, although a large number of studies have been conducted to identify sport- and exercise-related genes, the findings are mostly inconclusive because of a lack of replication, which is caused by the small sample sizes [11, 38]. Similarly, the sample size in our study is limited. Common SNPs associated with polygenic traits (including sport- and exercise-related traits) generally show a modest OR of 1.1–1.5 [39], and each physiological marker of performance is a complex trait regulated by a network of genes and pathways [11, 40]. A study suggests that a sample size of less than 1000 is still insufficient despite a well-standardized intervention protocol and precise phenotyping [11], therefore, both a large sample size and precise phenotyping are necessary to reduce the SE and increase statistical power to detect a significant SNP-trait association [11]. In addition, although the variability in individual training responses to improved maximal aerobic capacity after exercise-based cardiovascular rehabilitation exists in both healthy subjects and patients with established cardiovascular disease [41], but the interaction between gene variants and disease-modifying factors adds to the complexity, it is unclear whether genomic predictors of training response are the same in healthy and at-risk or diseased populations, and this study lacks the comparison of cardiac rehabilitation effect between normal people and patients with acute myocardial infarction after PCI. In the future, we will plan further exercise intervention projects combined with large-scale gene testing and screening to carry out exercise therapy and interventions efficiently and accurately for cardiovascular disease patients, reduce the incidence and mortality of heart failure, and provide evidence for clinical research.

5. Conclusions

Cardiovascular disease is a major public health problem worldwide. PCI is an effective treatment to reduce mortality, myocardial infarction, and hospitalization rate of the acute coronary syndrome in the treatment of acute myocardial infarction. While exercise is recommended by essentially every major medical organization, it is also recognized that there can be extreme variability between individual responses to exercise training. We found that the intervention group with a high mutation frequency in aerobic exercise- and cardiopulmonary function-related genes achieved more benefits in the 8-week aerobic exercise rehabilitation program. Thus, we will plan further exercise intervention projects combined with large-scale gene testing and screening to carry out exercise therapy and interventions efficiently and accurately for cardiovascular disease patients, reduce the incidence and mortality of heart failure, and provide evidence for clinical research.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors report no conflicts of interest in this work.

Authors' Contributions

Xing Yu and Yuxuan Fan contributed equally to this article.

Acknowledgments

The authors would like to thank the funding of the National Natural Science Foundation of China (grant number: 82172552), sponsored by the Scientific Research Foundation of SUMHS (SSF-21-03-005) and by the Scientific Research Foundation of SUMHS (SSF-22-03-001).

Supplementary Materials

Table S1. A total of 111 exercise-related single nucleotide polymorphisms (SNP) and 76 exercise-related genes were obtained after analysis by a text mining program. (*Supplementary Materials*)

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Hindawi
Cardiology Research and Practice
Volume 2022, Article ID 6532003, 11 pages
https://doi.org/10.1155/2022/6532003

Research Article

Nontargeted Metabolomic Profiling of Huo-Tan-Chu-Shi Decoction in the Treatment of Coronary Heart Disease with Phlegm-damp Syndrome

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Received 7 April 2022; Revised 1 June 2022; Accepted 27 June 2022; Published 12 August 2022

Academic Editor: Fanbo Meng

Background. Considered an effective supplementary therapy, traditional Chinese medicine (TCM) has been widely applied in the treatment of coronary heart disease (CHD). In this study, we aim to investigate the effects and mechanisms of Huo-Tan-Chu-Shi decoction (HTCSD, an in-hospital TCM prescription) in the treatment of CHD with the phlegm-damp syndrome in mice by nontargeted metabolomics with liquid chromatography-mass spectrometry (LC-MS)/MS. *Methods*. A CHD with phlegm-damp syndrome model was established with ApoE$^{-/-}$ mice by subcutaneous injection with isoproterenol combined with high temperature, high humidity, and a high-fat diet, and divided into the HTCSD and Tanshi groups. C57BL/6 mice were set as the control group with an ordinary environment and diet. After administration, electrocardiogram (ECG), interventricular septum thickness (IVS) and left ventricular posterior wall thickness (LVPW), serum levels of creatine phosphokinase-Mb (CK-MB), cardiac troponin T (cTnT), lactic dehydrogenase (LDH) and oxidized low-density lipoprotein (oxLDL), and myocardial histopathological changes were recorded to assess myocardial damage. LC-MS/MS was applied to demonstrate the serum metabolic profile and explore potential mechanisms. *Results*. The obvious depressions of the ST segment and T wave presented in the ECG of Tanshi mice, while the depressions in ECG of HTCSD mice were significantly reduced. Compared with the control group, IVS, LVPW, and serum levels of CK-MB, cTnT, LDH, and oxLDL increased greatly in the Tanshi group, while these indicators decreased remarkably in the HTCSD group compared with those of the Tanshi group. Histopathology showed severe structural disorder, necrosis, and fibrosis of myocardial cells in Tanshi mice, which were alleviated in HTCSD mice. Metabonomics analysis showed obvious metabolic alterations among the experimental mice and revealed that the relevant metabolic pathways mainly included phospholipid metabolism, necroptosis, and autophagy. *Conclusions*. HTCSD has a certain therapeutic effect in mice with CHD with phlegm-damp syndrome via reducing myocardial ischemia, hypertrophy, and fibrosis. The underlying mechanisms involve the regulation of phospholipid metabolism, necroptosis, and autophagy.

1. Introduction

Coronary heart disease (CHD) also known as ischemic heart disease, is one of the leading causes of death worldwide, which seriously threatens human health. Traditional Chinese medicine (TCM), characterized as high safety and having multitargeted effects, has been considered a reliable alternative therapy and is extensively used in CHD treatment. In

South China with a hot and humid climate, phlegm-damp syndrome, known as "Tanshi" in Chinese, is one of the major pathogenic factors of CHD [1]. Hence, Prof. Keji Chen (a famous TCM doctor) invented the Huo-Tan-Chu-Shi Decoction (HTCSD) based on Gualou-Xiebai series decoctions (the classical TCM formula used to treat CHD for thousands of years). HTCSD is made up of six Chinese herbal medicines, including Fructus Trichosanthis, Bulbus Allii Macrostemi, Rhizoma Pinelliae Praeparatum, Rhizoma Coptidis, Rhizoma Curcumae, and Radix Codonopsis. As an effective in-hospital prescription in the Guangdong Provincial Hospital of Chinese Medicine, previous research has found that several chemicals and CHD-related targets were regarded as the pivotal components and targets of HTCSD in the treatment of CHD [2]. On account of syndrome differentiation and overall adjustment of TCM treatment, further studies need to explore the underlying mechanism of HTCSD in treating CHD with phlegm-damp syndrome.

Metabolomics is a mature systematic biological technology in detecting and analyzing the overall and dynamic changes of endogenous metabolites, reflecting the biochemical process, and the physiological and pathological stages of the body [3]. It is analogous to the characteristics of multicomponent, multitarget, and holistic regulation of TCD treatment, which benefit the revelation of pharmacological mechanisms and pathways of TCM prescriptions [4]. Based on nontargeted metabolomics with liquid chromatography-mass spectrometry (LC-MS)/MS, this study aims to investigate the effect of HTCSD on serum metabolic profiling and the involved mechanisms in treating mice with CHD with phlegm-damp syndrome.

2. Methods

2.1. Materials and reagents. HTCSD contains the following Chinese herbal medicines: Fructus Trichosanthis 30 g, Bulbus Allii Macrostemi 30 g, Rhizoma Pinelliae Praeparatum 15 g, Radix Codonopsis 20 g, Rhizoma Curcumae 15 g, and Rhizoma Coptidis 6 g, which were purchased from Kangmei Pharmaceutical Co., Ltd (Guangdong, China). These herbs were soaked in 8-fold of water for 30 min, and then decocted twice for 30 min for each time, evaporated to a concentration of 1.16 g/mL, and stored at 4°C.

Isoproterenol (ISO) was obtained from Sigma-Aldrich (St. Louis, USA). Creatine phosphokinase-Mb (CK-MB), cardiac troponin T (cTnT), lactic dehydrogenase (LDH), and oxidized low-density lipoprotein (oxLDL) ELISA kits were purchased from Cusabio (Wuhan, China). Hematoxylin staining and eosin staining solutions were purchased from Yingjin Biotechnology Co., Ltd (Guangdong, China). Water, ethanol, acetonitrile, and formic acid were purchased from CNW Technologies GmbH (Düsseldorf, Germany). L-2-chlorophenylalanine was purchased from Shanghai Hengchuang Biotechnology Co., Ltd. (Shanghai, China).

All the chemicals and solvents were analytical or high-performance liquid chromatography grade.

2.2. Animal Model, Grouping, and Administration. Specific pathogen-free (SPF) male C57BL/6 and ApoE$^{-/-}$ mice were provided by Beijing HFK Bioscience CO., LTD, and the animal experiment was approved by the Animal Care and Use Committee of the Guangdong Provincial Hospital of Chinese medicine. After 1 week of accommodation to the environment, ApoE$^{-/-}$ mice were fed a western diet (21% fat, 1.5% cholesterol). After 4 weeks on a high-fat diet, ApoE$^{-/-}$ mice were housed in a room at a temperature of 35 ± 0.5°C and a humidity of $90 \pm 5\%$ for 7 hours a day (the rest of the day in a room temperature of 20–25°C and a humidity of 50–60%). The mice were randomized into the Tanshi group and HTCSD group after 12 weeks of western diet and 8 weeks of high-temperature and high-humidity environment. C57BL/6 mice were housed in the laboratory environment (a temperature of 20–25°C and a humidity of 50–60%) and fed with a standard laboratory diet for 12 weeks as the control group. HTCSD mice were administered HTCSD intragastrically (23.2 g/kg/day) for 4 weeks, while the same volume of saline was given in the Tanshi and control group. ISO-induced myocardial ischemia is a classical model to explore the cardioprotective effects of various pharmacological interventions [5]. Subcutaneous injections with ISO (10 mg/kg/day) were executed in Tanshi and HTCSD mice for 7 days to simulate myocardial ischemia of CHD before euthanasia, while the control mice were given the same volume of saline.

2.3. Electrocardiography & Echocardiography. At 2 hours after the last ISO injection, electrocardiography was performed to record the limb lead II electrocardiogram (ECG) of the mice and analyze respectively the changes of the ST segment and T wave after 0 s, 1 min, 5 min, 10 min, and 20 min. Meanwhile, the echocardiogram equipped with the VisualSonics Vevo 2100 system and 21-MHz linear array transducer was applied to measure the left ventricular posterior wall thickness (LVPW) and interventricular septal thickness (IVS) via the parasternal short-axis view at the level of the papillary muscle by the M-mode tracing method.

2.4. Serum Biochemical Analysis & Histomorphology. After fasting for 12 hours, the mice were anesthetized with 20% urethane, and the eyeballs were removed for blood collection. The blood samples were placed in 1.5 mL EP tubes, centrifuged at 3500 rpm, and placed at 4°C for 10 minutes to collect serum samples. Enzymatic biochemical kits were used to separately determine the serum levels of CK-MB, cTnT, LDH, and oxLDL.

Mice hearts were separated and myocardial tissues with 0.5 cm in thickness were cut along the transverse axis in the middle of the left ventricle, fixing with 4% paraformaldehyde solution. The fixed tissues were embedded in paraffin wax and made into sections of 3.5 μm thickness and subjected to hematoxylin & eosin (H&E) staining to observe the pathological changes.

All the protocols were followed in accordance with the manufacturer's recommendations.

2.5. Nontargeted LC-MS-Based Analysis. Serum samples of each group ($n = 6$) stored at −80°C were thawed at room temperature. 100 μL of the sample was added to a 1.5 mL Eppendorf tube with 10 μL of 2-chlorol-phenylalanine (0.3 mg/mL) and Lyso PC17 : 0 (0.01 mg/ml), respectively, dissolved in methanol as the internal standard, and the tube was vortexed for 10 s. Subsequently, 300 μL of methanol and acetonitrile (2/1, v/v) was added, and the mixtures were vortexed for 1 min, ultrasonicated in an ice-water bath for 10 min, and stored at −20°C for 30 min. The extract was centrifuged at 13000 rpm, and placed at 4°C for 10 min. 300 μL of the supernatant in a brown and glass vial was dried in a freeze concentration centrifugal dryer. 400 μL mixture of methanol and water (1/4, v/v) were added to each sample, the samples were vortexed for 30 s, ultrasonicated for 3 min, and then placed at 4°C for 2 hours. Samples were centrifuged at 13000 rpm and placed at 4°C for 10 min. The supernatants (150 μL) from each tube were collected using crystal syringes, filtered through 0.22 μm microfilters, transferred to LC vials, and stored at −80°C.

A Dionex UltiMate 3000 RS UHPLC system fitted with a Q-Exactive Quadrupole-Orbitrap mass spectrometer and equipped with a heated electrospray ionization (ESI) source (Thermo Fisher Scientific, Waltham, MA, USA) was used to analyze the metabolic profiling in both ESI-positive and ESI-negative ion modes. An ACQUITY UPLC HSS T3 column (1.8 μm, 2.1 × 100 mm) was employed in both the positive and negative modes. The binary gradient elution system consisted of (a) water (containing 0.1% formic acid, v/v) and (b) acetonitrile (containing 0.1% formic acid, v/v), and the separation was achieved using the gradient as shown in Supplementary Table S1. The flow rate was 0.35 mL/min and the column temperature was 50°C. All the samples were kept at 4°C during the analysis. The injection volume was 2 μL. The mass range was from m/z 66.7 to 1,000.5. The resolution was set at 70,000 for the full MS scans and 35,000 for HCD MS/MS scans. The collision energy was set at 10, 20, and 40 eV. The mass spectrometer was operated as shown in Supplementary Table S2.

2.6. Data Processing and Statistical Analysis. The acquired LC-MS raw data were preanalyzed by the Progenesis QI software with ver2.3 (Nonlinear Dynamics, Newcastle, UK). Principle component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were carried out to assess the general metabolic alterations among the three groups. A specific plot was used to present the number of differential metabolites among the groups. A heatmap and cluster plots were made to apparently exhibit the expression change of differential metabolites among the experimental groups. Metabolic pathways enrichment analysis was performed on differential metabolites based on the KEGG database. The selection of differential metabolites was based on a variable influence on the projection (VIP) values obtained from the OPLS- DA model and the p values from a two-tailed Student's t-test. Metabolites with VIP values larger than 1.0 and p values less than 0.05 were regarded as differential metabolites.

In vivo data were presented as mean ± standard deviation and statistical analysis between groups was performed by one-way analysis of variance with the LSD test via the GraphPad Prism software v7.0. P values less than 0.05 were considered statistically significant.

3. Results

3.1. HTCSD Reduced Myocardial Injury in Mice Induced by the Administration of ISO Combined with High Temperature and High Humidity, and a High-Fat Diet. From the results of ECG and echocardiography, as shown in Figure 1, 1(a) and Table 1&2, the significant depressions of the ST segment and T wave were presented in the ECG of the Tanshi group compared with that of the control group, while the depressions in ECG of the HTCSD group mitigated remarkably. As presented in Figure 1, 1(b) and Table 3, compared with the control group, IVS and LVPW at systole and diastole increased greatly in the Tanshi group, while these values obviously improved in the HTCSD group. These data indicated that critical myocardial ischemia and hypertrophy arose in Tanshi mice, and HTCSD can effectively ameliorate the ischemic damage and pathological hypertrophy of the myocardium.

Besides, as shown in Figure 2, 2(a), the serum levels of CK-MB, cTnT, LDH, and oxLDL elevated significantly in Tanshi mice compared with those of control mice, while decreases in various degrees were found in HTCSD mice compared with Tanshi mice. Furthermore, as presented in (Figure 2, 2(b)) by H&E staining, the myocardial cells in the control mice had normal morphology and a structure with a distinct texture. However, notably, the structure disordered of myocardial cells with inflammatory cells infiltration, extensive swelling, and rupture of myocardial fibers, and dramatic edema of the intercellular spaces were found in Tanshi mice. In HTCSD mice, the myocardial pathological changes improved with the mitigation of myocardial fiber rupture, inflammatory infiltration, and tissue edema. These results suggested that Tanshi mice suffered from severe myocardial injury and fibrosis, while the damage was improved with the treatment of HTCSD.

Taken together, our study demonstrated that the application of ISO combined with high temperature and high humidity, and a high-fat diet induced severe myocardial injury in mice. In addition, HTCSD showed a certain therapeutic effect in mice with CHD with phlegm-damp syndrome by reducing myocardial ischemia, hypertrophy, and fibrosis.

3.2. Analysis of Serum Metabolomic Profiling. In this study, score plots of PCA and PLS-DA were used to generally evaluate the metabolic alterations. As presented in Figures 3, 3(a)& 3(b), the score plots of PCA manifested a complete distinction between the control and Tanshi groups,

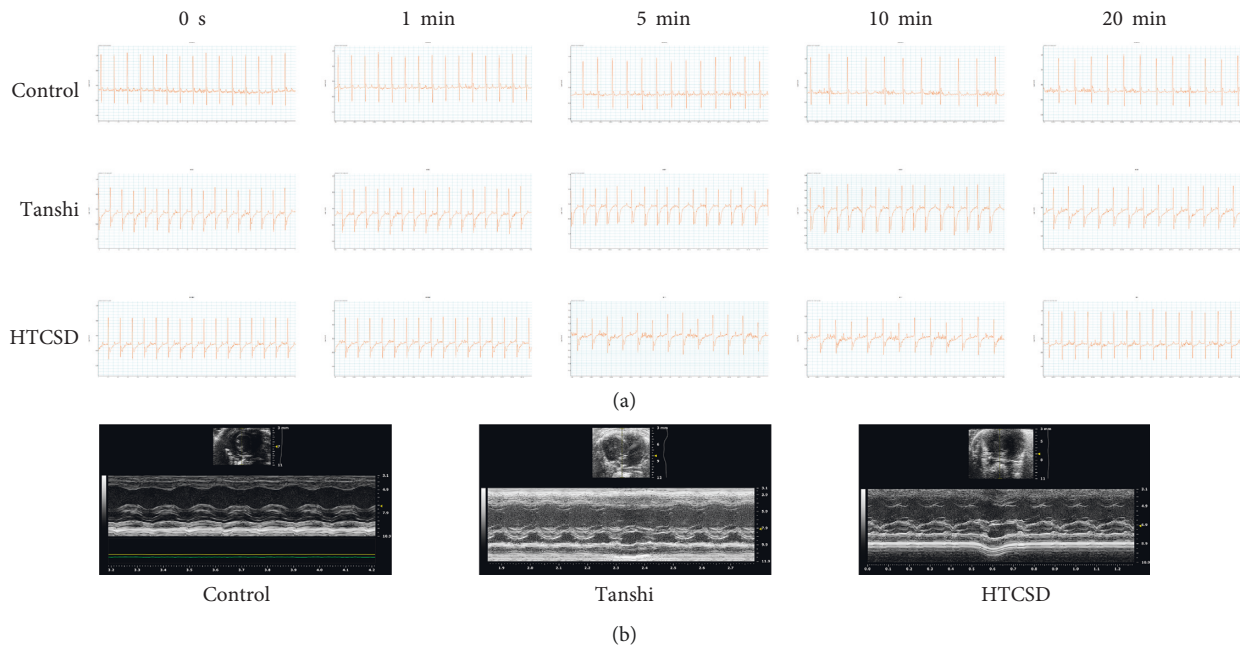

Figure 1: Electrocardiography and echocardiography. (a) ECGs of limb lead II of mice were recorded to evaluate the changes of the ST segment and T wave. (b) LVPW and IVS of mice were measured by echocardiography. Abbreviations: ECG: electrocardiogram, LVPW: left ventricular posterior wall thickness, IVS: interventricular septal thickness, HTCSD : Huo-Tan-Chu-Shi decoction.

Table 1: Changes of the ST segment in the ECG of mice (mean ± S.D, $n = 5$–6).

	ST height (mV)				
	0s	1 min	5 min	10 min	20 min
Control	0.1118 ± 0.0383	0.0991 ± 0.0394	0.1145 ± 0.0357	0.1531 ± 0.0399	0.1456 ± 0.0453
Tanshi	−0.2387 ± 0.0450**	−0.2557 ± 0.0614**	−0.2321 ± 0.0958**	−0.2291 ± 0.0977**	−0.2062 ± 0.0694**
HTCSD	−0.1608 ± 0.0393#	−0.1148 ± 0.0781##	−0.1305 ± 0.0551#	−0.1169 ± 0.0438#	−0.0800 ± 0.0413##

Note:** P-value<0.01 (Tanshi vs Control), # P-value<0.05 (HTCSD vs Tanshi), and ## P-value<0.01 (HTCSD vs Tanshi). ECG: electrocardiogram, HTCSD: Huo-Tan-Chu-Shi decoction.

Table 2: Changes of T wave in the ECG of mice (mean ± S.D, $n = 5$–6).

	T Amplitude (mV)				
	0s	1 min	5 min	10 min	20 min
Control	0.1289 ± 0.0291	0.1133 ± 0.0313	0.1270 ± 0.0357	0.1415 ± 0.0355	0.1638 ± 0.0413
Tanshi	−0.1980 ± 0.0265**	−0.2169 ± 0.0460**	−0.2276 ± 0.0722**	−0.2256 ± 0.0538**	−0.1838 ± 0.0578**
HTCSD	−0.1494 ± 0.0419	−0.1297 ± 0.0738#	−0.1250 ± 0.0489##	−0.1218 ± 0.0558##	−0.1161 ± 0.0748

Note:** P-value<0.01 (Tanshi vs Control), # P-value<0.05 (HTCSD vs Tanshi), and ## P-value<0.01 (HTCSD vs Tanshi). ECG: electrocardiogram, HTCSD: Huo-Tan-Chu-Shi decoction.

and an overlap was observed between the Tanshi and HTCSD groups. PLS-DA showed apparent separations among the control, Tanshi, and HTCSD groups. The results showed a significant difference of the metabolic profiling among the three groups.

Considering the screening criterion of VIP >1 and $P < 0.05$, 456 differential metabolites were found between the control and Tanshi groups, 112 differential metabolites between the Tanshi and HTCSD groups, and 53 differential metabolites in common as shown in Figure 4, 4(a). According to VIP, the top 50 differential metabolites between the HTCSD and Tanshi groups were shown in heatmaps (Figure 4, 4(b)). The color from blue to red indicates the expression of metabolites from low to high, and there was a remarked differentiation between HTCSD and Tanshi groups. Furthermore, on the basis of the theory of the R package Mfuzz fuzzy clustering, differential metabolites in common among the three groups were collected into several clusters, and then, time sequence analysis was performed for observing the variation trend. Figures 4, 4(c), and Table 4 show differential metabolites of clusters 2, 5, and 8. In Table 4, the value of membership indicatesthe degree of differential metabolites conform with the relevant cluster. The metabolites

Table 3: Comparison of LVPW and IVS measured by echocardiography (mean ± S.D, $n = 5–6$).

	IVSD (mm)	IVSS (mm)	LVPWD (mm)	LVPWS (mm)
Control	0.88 ± 0.24	1.35 ± 0.34	1.14 ± 0.30	1.50 ± 0.29
Tanshi	1.20 ± 0.10**	2.08 ± 0.35**	1.68 ± 0.24**	2.03 ± 0.44*
HTCSD	0.91 ± 0.26#	1.83 ± 0.17	1.28 ± 0.27#	1.51 ± 0.33#

Note: *p-value<0.05 (Tanshi vs Control), **P-value<0.01 (Tanshi vs Control), and #P-value<0.05 (HTCSD vs Tanshi). IVSD: interventricular septal thickness at diastole, IVSS: interventricular septal thickness at systole, LVPWD: left ventricular posterior wall thickness at diastole, LVPWS: left ventricular posterior wall thickness at systole, ECG: electrocardiogram, HTCSD: Huo-Tan-Chu-Shi decoction.

with membership> 0.4 were select for further analysis, the superclass of which was mainly lipids and lipid-like molecules.

To comprehensively investigate metabolic disturbances among experimental groups, metabolic pathway analyses were performed by the KEGG database. The column charts of the top 20 KEGG pathways in the Tanshi vs control group and the HTCSD vs Tanshi group are presented in Figures 4, 4(d) and 4, 4(e). Several same pathways were found among three groups, mainly including glycerophospholipid metabolism, sphingolipid metabolism, necroptosis, and autophagy. Combing the selected differential metabolites in Table 4 with the metabolic pathways in common, the molecule mechanism of HTCSD in the treatment of CHD with phlegm-damp syndrome may involve the regulation of lipid metabolism, necroptosis, and autophagy.

4. Discussion

TCM combined with modern medicine has become an effective therapeutic strategy and is widely applied in China [6]. In addition to the therapeutic concept of holism and syndrome differentiation, TCM emphasizes the treatment in accordance with local conditions. With the hot and humid climate in South China, spleen qi deficiency and phlegm-damp syndrome are considered the main types of TCM constitutions in the local people [7]. With the effect of improving qi, promoting blood circulation, resolving phlegm, and eliminating dampness, HTCSD was used to treat patients with CHD with phlegm-damp syndrome.

In our study, the administration of ISO was applied to stimulate myocardial ischemia. The changes of the ST segment and T wave of ECG, biochemical indexes such as cTnT, CK-MB, and LDH, and histopathology are crucial evaluation indicators for myocardial ischemia and cell injury. Meanwhile, high temperature, high humidity, and a high-fat diet were adopted to induce phlegm-damp syndrome. As shown in the experimental results, we built a CHD with phlegm-damp syndrome model in mice. And, we demonstrated that the Chinese herbal formula HTCSD has a therapeutic effect in reducing myocardial ischemia, hypertrophy, and fibrosis in mice. Based on metabolomic analysis, the associated mechanisms primarily included phospholipid metabolism, cell necroptosis, and autophagy.

4.1. Regulation of Phospholipid Metabolism. As shown in this study, phospholipids are the main type of lipids expressing abnormally among the experimental mice. Phospholipids mainly include glycerophospholipids and sphingolipids. Among them, glycerophospholipids with high content in the body include phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositide (PI) [8], of which PC and PE were considered as the potential biomarkers of cardiovascular diseases [9, 10]. With the effect of phospholipase A2, PC transforms into lysophosphatidylcholine (LysoPC), which is the main component of oxLDL [11]. In our study, it was found that the serum levels of PC (14 : 0) and PC (O-16 : 0) increased while PC (16 : 1), PC (18 : 0), PC (18 : 1), PC (20 : 2), LysoPC (18 : (2), and LysoPC (20 : (4) decreased in Tanshi mice compared with the control group. And, the treatment of HTCSD downregulated the PC (14 : 0) and PC (O-16 : 0) levels while upregulating the serum levels of PC (16 : 1), PC (18 : 0), PC (18 : 1), PC (20 : 2), LysoPC (18 : (2), and LysoPC (20 : 4), which is in accordance with the result of a previous study [12]. Based on the type of cell and the stage of inflammatory response and oxidizing reaction, LysoPC plays various roles in the progression of AS [13–15]. Research has shown that LysoPC induces the migration of lymphocyte and macrophage and the activation of oxidative stress, and increases proinflammatory cytokines levels, accelerating the development of CHD [16, 17]. However, some types of LysoPC show a negative association with CHD [10], which can inhibit the synthesis and foaming of macrophages and reduce cholesterol accumulation [18]. In addition, PE is associated with oxidative phosphorylation, the stability of mitochondrial, and autophagy [19]. A study found that PE containing unsaturated fatty acids increases the risk of CHD [20]. Our present study showed that the concentration of PE(0 : 0/22 : 6(4Z,7Z,10Z,13Z,16Z,19Z)) and PE(O-18 : 1(9Z)/0 : 0) elevated in mice with CHD with phlegm-damp syndrome the administration of HTCSD was reduced. Furthermore, PS was proved to have an atheroprotective effect by regulating cholesterol metabolism, inhibiting inflammatory response, and enhancing the function of high-density lipoprotein (HDL) [21]. This study presented that PS(P-18 : 0/0 : 0) and PS(20 : 4(5Z,8Z,11Z,14Z)/19 : 0) levels decreased in Tanshi mice, while increased in HTCSD mice. As mentioned previously, with the regulation of glycerophospholipids, HTCSD may improve coronary AS in mice by modulating the function of immune cells and cholesterol metabolism, inhibiting oxidative stress and inflammatory response. Sphingolipid metabolism is another phospholipid-associated pathway found in our study. As the key of sphingolipid metabolism, ceramide can form sphingomyelin (SM) with the incorporation of phosphocholine. And, ceramide can be metabolized to generate sphingosine (sph), which can further phosphorylate to form sph 1-phosphate (S1P) [22]. Studies have shown that both SM and S1P can induce inflammatory responses of smooth muscle cells in the coronary artery, and SM is associated with atherosclerotic plaque instability [23]. In addition, it has been reported that the S1P bond to HDL has antiatherosclerotic effects, while the S1P bond to non-HDL is negatively associated with CHD

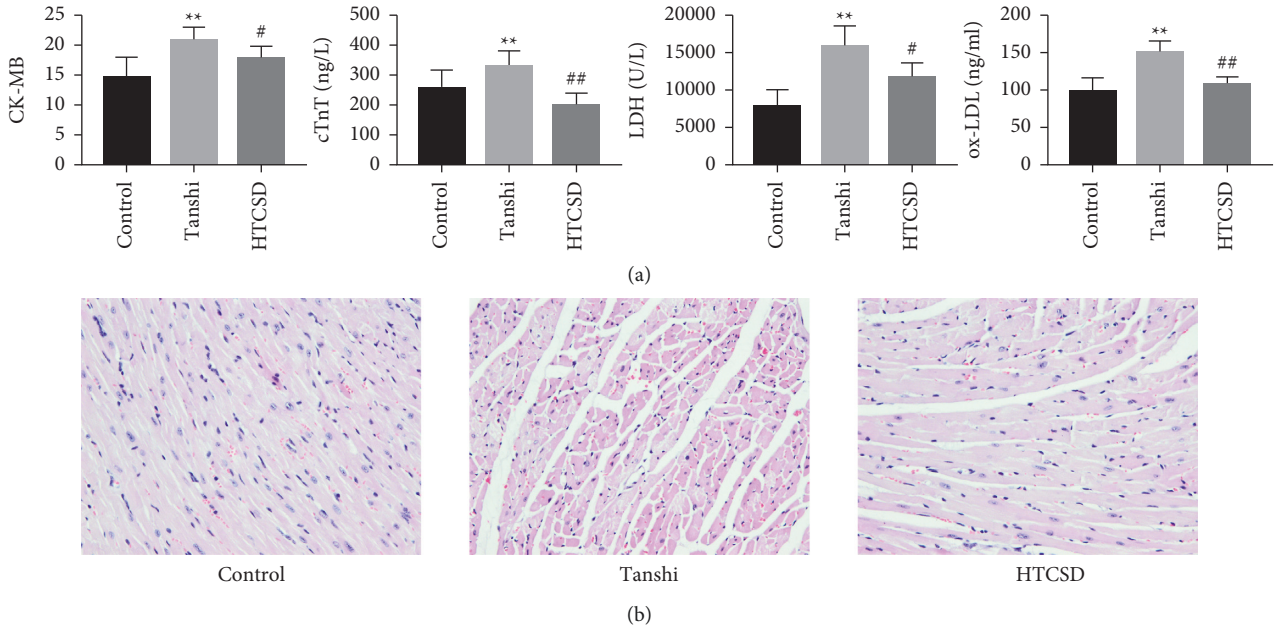

Figure 2: Serum biochemical analysis and histomorphology. (a) Serums were collected to evaluate the levels of CK-MB, cTnT, LDH, and oxLDL and are shown as mean ± S.D ($n = 5$–6). $^{**}P$-value<0.01 (Tanshi vs Control), $^{\#\#}P$-value<0.01 (HTCSD vs Tanshi), and $^{\#}P$-value<0.05 (HTCSD vs Tanshi). (b) H&E staining (scale bar = 50 μm) was used to observe the pathological changes of the myocardium of the left ventricle. Abbreviations: CK-MB: creatine phosphokinase-Mb, cTnT: cardiac troponin T LDH: lactic dehydrogenase, oxLDL: oxidized low-density lipoprotein, H&E: hematoxylin and eosin. HTCSD: Huo-Tan-Chu-Shi decoction.

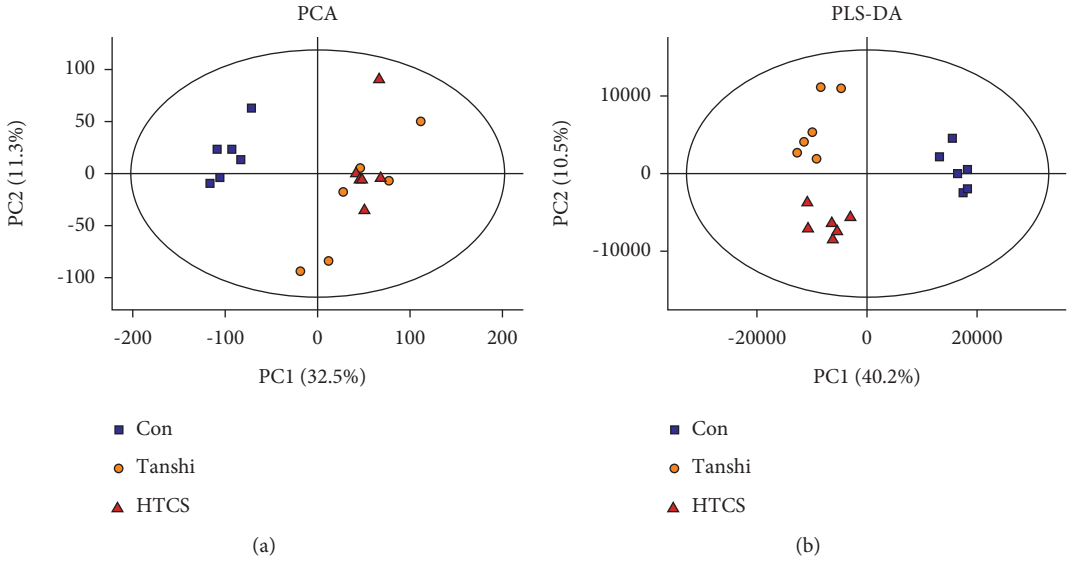

Figure 3: Score plots of PCA and PLS-DA. (a) Score plot of principle component analysis (PCA). (b) Score plot of partial least squares-discriminant analysis (PLS-DA). Abbreviations: HTCSD : Huo-Tan-Chu-Shi decoction.

[24]. Our present study shows that the levels of SM (d18 : 0/16 : 0) and sph elevated in Tanshi mice, while decreased after medication. This study suggested that HTCSD might reduce smooth muscle cell inflammation and enhance plaque stability via downregulating the levels of SM and sph.

Collectively, with the regulation of glycerophospholipids and sphingolipids, HTCSD can improve the disorder of lipid profile and finally mitigate myocardial injury in mice. Previous research proved that lipids are associated with the occurrence and progression of CHD [25, 26]. In addition, a study indicated that patients with CHD with phlegm syndrome were prone to have inordinate lipid metabolism, and the medication of Gualou-Xiebai-Banxia decoction (one of the Gualou-Xiebai series decoctions) promoted the improvement of lipid metabolism and the stability of the cell membrane [27]. As the herbs reserved from the classical prescription, Fructus Trichosanthis,

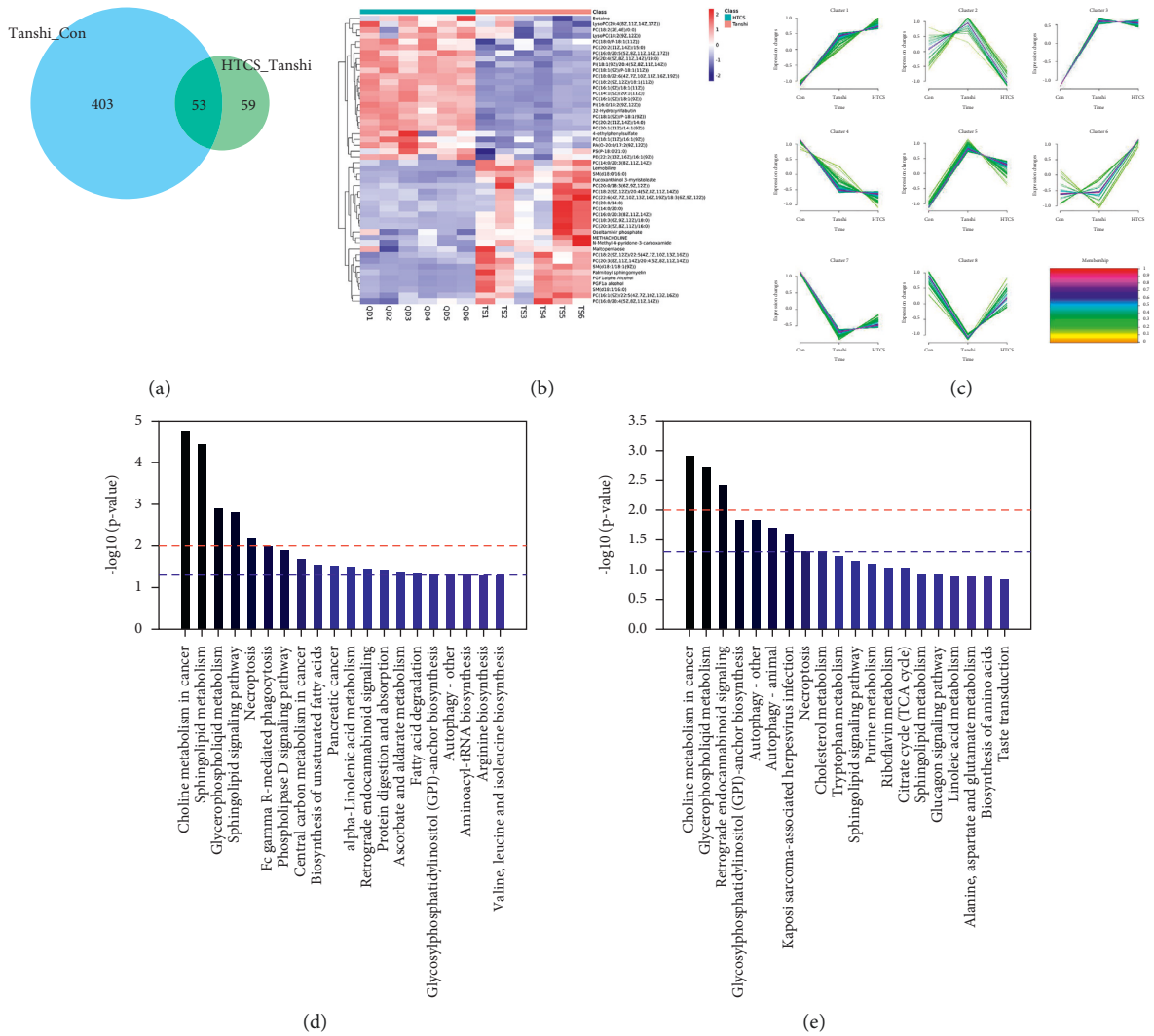

Figure 4: Serum metabolism profile alterations and metabolite-associated pathways. (a) The amount of differential metabolites among the experimental groups. (b) Heatmap of top 50 differential metabolites in the HTCSD vs Tanshi group. (c) Clusters of time sequence analysis of differential metabolites among the experimental groups. (d) The column chart of the top 20 KEGG pathways in the Tanshi vs control group. (e) The column chart of the top 20 KEGG pathways in the HTCSD vs Tanshi group. Abbreviations: HTCSD : Huo-Tan-Chu-Shi decoction.

Bulbus Allii Macrostemi, and Rhizoma Pinelliae Praeparatum in HTCSD have a dramatic antihyperlipidemia effect by regulating the lipid level and inhibiting lipid accumulation [28]. With the improvement of lipid metabolism, these herbs protect the damaged myocardium.

4.2. Regulation of Cell Necroptosis. In this study, we found that necroptosis is another important metabolic pathway involved in the treatment of CHD with phlegm-damp syndrome by HTCSD. With elaborate regulation by the intracellular signaling molecular pathways, necroptosis is induced by a series of death receptors (including tumor necrosis factor receptors (TNFR) 1, 2 and fatty acid synthase) and activated by the formation of a receptor-interacting protein (RIPK) 1/RIPK3 necrosome. The further combination with the RIPK 1/RIPK3 necrosome and mixed lineage kinase domain-like (MLKL) protein forms a necrotic complex, which mediates the process of necroptosis [29]. Necroptosis conducts cytolysis and contributes to severe inflammatory responses on account of the rapid loss of the plasma membrane integrity and the release of intracellular proinflammatory contents, which is considered an important pathological and physiological procedure of ischemia-reperfusion injury, myocardial infarction, and cardiac remodeling [30]. In early AS, vascular monocytes phagocytose modified lipoproteins (such as oxLDL) to form macrophage foam cells, which further increases the expression of RIP3 and MLKL in macrophage foam cells, inducing inflammatory responses and aggravating AS progression [31]. The expression of RIP3 in myocardial cells mediates the production of inflammation and reactive oxygen species and the pathological myocardial remodeling [32]. In addition, the inhibition of RIP1 can reduce the area of myocardial infarction after induction of ischemia [33]. As shown in our study, excessive necroptosis might generate in mice of the Tanshi group, inducing wide inflammation and myocardial damage. With the regulation of

Table 4: Differential metabolites of cluster 2, cluster 5, and cluster 8 (membership>0.4).

Cluster number	Metabolites	Membership
Cluster 2	FAD	0.710145334
Cluster 2	Fucoxanthinol 3-myristoleate	0.693372847
Cluster 2	PE(0 : 0/22 : 6(4Z,7Z,10Z,13Z,16Z,19Z))	0.664223572
Cluster 2	SM(d18 : 0/16 : 0)	0.450356799
Cluster 2	Cadusafos	0.416098027
Cluster 2	(3Z)-2-propylpent-3-enoic acid	0.401266799
Cluster 5	5-Deoxydiplosporin	0.859002946
Cluster 5	Inosine triphosphate	0.853761932
Cluster 5	SLF	0.839544464
Cluster 5	Garcinia lactone dibutyl ester	0.835935897
Cluster 5	9-Hydroperoxy-12,13-dihydroxy-10-octadecenoic acid	0.832606958
Cluster 5	PC(14 : 0/20 : 3(8Z,11Z,14Z))	0.806742973
Cluster 5	2,5-Octadien-1-ol	0.784832227
Cluster 5	3-Methyl-3-butenyl apiosyl-(1->6)-glucoside	0.68681373
Cluster 5	Histidine-Phenylalanine	0.665285993
Cluster 5	5,10-Methenyltetrahydrofolate	0.658926736
Cluster 5	3a,21-Dihydroxy-5b-pregnane-11,20-dione	0.633962294
Cluster 5	(2-Methyl-3-phenylpropoxy)sulfonic acid	0.632564399
Cluster 5	Zizyphine A	0.616615337
Cluster 5	DG(22 : 4(7Z,10Z,13Z,16Z)/24 : 0/0 : 0)	0.615378609
Cluster 5	Militarinone B	0.610631061
Cluster 5	PE(O-18 : 1(9Z)/0 : 0)	0.596672725
Cluster 5	Lipoxin D4	0.580309909
Cluster 5	Sphingosine	0.57731702
Cluster 5	PC(O-16 : 0/0 : 0)	0.564222357
Cluster 5	Uralenneoside	0.56046916
Cluster 5	Hoffmanniolide	0.552641588
Cluster 5	4-(2,6,6-Trimethyl-1-cyclohexenyl)-2-butanol	0.552081026
Cluster 5	Tetraneurin A	0.531319613
Cluster 5	2-O-(beta-D-galactopyranosyl-(1->6)-beta-D-galactopyranosyl) 2S,3R-dihydroxynonanoic acid	0.511911474
Cluster 5	Terbutaline-1-sulfate	0.500476178
Cluster 5	OKDdiA-PE	0.498764561
Cluster 5	Valorphin	0.480345113
Cluster 5	PHOHA-PS	0.466379377
Cluster 5	Galabiosylceramide (d18 : 1/24 : 1(15Z))	0.465226025
Cluster 5	((3,4,5-Trihydroxy-6-(1,2,6-trihydroxy-3-(hydroxy(3,4,5-trihydroxyoxan-2-yl) methyl)-4-oxocyclohexa-2,5-dien-1-yl)oxan-2-yl) methoxy)sulfonic acid	0.460888285
Cluster 5	15-Cyclohexyl pentanor PGF2alpha	0.45349852
Cluster 5	Tryptophyl-asparagine	0.434188447
Cluster 5	Beauvericin	0.418981588
Cluster 5	9,10-Dihydroxy-13-hydroperoxy-11-octadecenoic acid	0.408170734
Cluster 5	11-Deoxy-11-methylene-PGD2	0.402497413
Cluster 5	OKOOA-PC	0.402386826
Cluster 8	PC(18 : 0/P-18 : 1(11z))	0.762565962
Cluster 8	PS(P-18 : 0/0 : 0)	0.751504134
Cluster 8	N-Methyl-a-aminoisobutyric acid	0.718517348
Cluster 8	Rutagravine	0.701523565
Cluster 8	PS(20 : 4(5Z,8Z,11Z,14Z)/19 : 0)	0.690096004
Cluster 8	Streptidine	0.574309645
Cluster 8	LysoPC(20 : 4(8Z,11Z,14Z,17Z))	0.567252783
Cluster 8	PC(18 : 1(9Z)/P-18 : 1(9z))	0.508347134
Cluster 8	LysoPC(18 : 2(9Z,12Z))	0.477289213
Cluster 8	PC(16 : 1(9Z)/18 : 1(9z))	0.437316248
Cluster 8	PC(20 : 2(11Z,14Z)/14 : 0)	0.428489908
Cluster 8	31-Hydroxy rifabutin	0.423115022

Note: The value of membership indicates the degree of differential metabolites conform with the relevant cluster.

necroptosis, HTCSD may inhibit inflammatory responses and AS progression in mice by downregulating the serum level of oxLDL and the expressions of RIP3 and MLKL.

4.3. Regulation of Autophagy. Autophagy is also one of the underlying mechanisms involved in the pathological and medication process. The molecular pathway of autophagy mainly includes a mammalian target of rapamycin (mTOR), adenosine monophosphate-activated protein kinase (AMPK), endoplasmic reticulum stress, and p53 [34]. Activated as a cytoprotective procedure, autophagy promotes the release of nutrients from amino acids, fatty acids, and monosaccharides into the cytoplasm, resulting in the recycling of cytoplasmic components for protein synthesis and adenosine triphosphate (ATP) production [35, 36]. Research has demonstrated that autophagy has an atheroprotective effect via inhibiting inflammation and apoptosis, promoting cholesterol efflux, and reducing lipid deposition, while the dysfunction of autophagy exacerbates AS progression [37]. With the lack of sufficient glucose, amino acids, and energy, the ischemia-induced autophagy response is initiated by activating AMPK and inhibiting mTOR signaling, showing a cardioprotection effect [38–40]. In contrast, under the circumstance of a high-fat diet, the maturation of autophagy is disrupted in myocardial cells and the cardioprotection effect is diminished [41]. Moreover, a study has proven that damp-heat syndrome can lead to the dysfunction of autophagy in atherosclerotic rats. Berberine, the main active ingredient in Rhizoma Coptidis (one of the herbs of HTCSD), was found to have an atheroprotective effect by enhancing plaque stability, the mechanism which may be involved with the regulation of autophagy [42, 43]. In our study, it is found that the autophagy activity may decrease in mice with CHD with phlegm-damp syndrome. The administration of HTCSD may improve the autophagy activity via the AMPK/mTOR signaling pathway, resulting in inhibiting inflammatory reactions and enhancing plaque stability.

5. Limitation

Our study has some limitations. First, metabolomics data are massive and complex. The number and variety of metabolites identified in serum may differ from cells or tissues. Second, a small sample was adopted in our study and there are only few metabolomics studies of TCM treatment in CHD. Some experiment results were inconsistent and difficult to determine. In summary, this study was a preliminary exploration of the mechanism of HTCSD via nontargeted metabolomics, extensive research is needed to investigate the deeper molecular mechanisms.

6. Conclusion

In conclusion, this study demonstrated that HTCSD alleviated myocardial ischemia, hypertrophy, and fibrosis in mice. The mechanism involved may be as followed: regulation of phospholipid metabolism, necroptosis, and autophagy. It provides directions for further investigations to excavate the more precise molecular mechanisms. [37–42]

Data Availability

All relevant data are accessible from the corresponding author (e-mail: weihui.lu@gzucm.edu.cn).

Ethical Approval

Animal experiments were conducted with the consent of the Animal Care and Use Committee of the Guangdong Provincial Hospital of Chinese medicine.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Weihui Lu and Taohua Lan contributed to the design and supervision of the study, Zhaoying Liang performed the data analyses and wrote the manuscript, Qiaohuang Zeng conducted the experiment and data collection, and Xiaomin Ou and Jing Cai were responsible for the data collection and analyses.

Acknowledgments

The authors thank the Shanghai LuMing Biological Technology co., Ltd (Shanghai, China) for providing metabolomics services. The present work was supported by the Specific Fund of the State Key Laboratory of Dampness Syndrome of Chinese Medicine (Grant nos. SZ2021ZZ10, SZ2021ZZ1002, SZ2021ZZ12, SZ2021ZZ26, SZ2021ZZ46), National Natural Science Foundation of China (Grant nos. 81874432 and 82074369), Science and Technology Foundation of Guangzhou City (No. 202201020348), Science and Technology Planning Project of Guangdong Province (Grant no. 2018B030322012), and the Key Project of the First-Class University Construction of the Guangzhou University of Chinese Medicine (Grant no. XK2019024)..

Supplementary Materials

Supplementary Table S1. The elution gradient. Supplementary Table S2. The mass spectrometry parameter. (*Supplementary Materials*)

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Hindawi
Cardiology Research and Practice
Volume 2022, Article ID 1647809, 13 pages
https://doi.org/10.1155/2022/1647809

Research Article

Effectiveness and Safety of Four Aerobic Exercise Intensity Prescription Techniques in Rehabilitation Training for Patients with Coronary Heart Disease

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Received 27 March 2022; Revised 6 May 2022; Accepted 13 June 2022; Published 15 July 2022

Academic Editor: Binhua Liang

Background and Objectives. Exercise intensity is a key indicator for the safety and effectiveness of aerobic exercise program in cardiac rehabilitation (CR) in patients with coronary heart disease (CHD). The majority of CR guidelines recommend aerobic exercise prescription based on moderate intensity and suggest many techniques for setting the heart rate target of exercise to match the intensity. But even high-risk CHD patients rarely adhere to exercise training under medical monitoring. The effectiveness and safety of exercise under these high-intensity techniques is still a paucity of evidence. The purpose of this study was to determine if these techniques can safely and effectively inform exercise prescription for individuals with CHD. *Methods*. A retrospective study was conducted on all patients with CHD who were admitted to CR and completed cardiopulmonary exercise tests (CPET) in Guangdong Hospital of traditional Chinese medicine. According to the risk stratification method of CHD, all participants were divided into three groups: low, moderate, and high risk. The training target heart rates (HRt) of each participant were calculated according to the formula of heart-rate-reserve (HRR), maximum-heart-rate (MHR), target-heart-rate (THR), and anaerobic threshold (AT) method provided in the guideline. Among them, the HRR method using the maximum-heart-rate obtained by the age formula was named "HRR method A," and that using the actual measured peak heart rate was named "HRR method B." For the three groups, the effectiveness and safety indexes at the target-heart-rate zone set by the different formulas above are counted and compared using CPET data. *Results*. A total of 324 patients were included in the analysis. There was no significant difference between the target-heart-rate set by the HRR method A and AT method among the three groups ($P > 0.05$). The mean value of HRt set by other methods was lower than the AT heart rate ($P < 0.05$). The HRt set by the THR method was close to the AT, while that set by the MHR method was the lowest. The frequency of patients whose HRt was set by the MHR method was lower than the AT one, which was the highest. None of the participants had serious adverse events. There were no risks of ECG abnormalities in the low- and moderate-risk groups. The HRR method A had the highest incidence of various risks of ECG abnormalities, while the MHR method had the lowest one, and the safety of the THR method is close to that of the AT method ($P < 0.05$). *Conclusion*. The heart rate calculated by HRR method A is more consistent with the actual AT. All four techniques are safe in low- and moderate-risk patients. In high-risk patients, using HRR method A has certain risks. It is recommended to use the MHR method for safety reasons, but its effectiveness is low. If considering both effectiveness and safety, the THR method can be conservatively selected at the beginning of the CR program.

1. Introduction

Coronary heart disease (CHD) is the single most common cause of death globally. The prevalence of coronary heart disease in China is estimated to be 11.0 million cases, and nearly 40% of all CVD-related deaths are due to CHD [1]. For CHD patients, cardiac rehabilitation (CR) is a comprehensive treatment process for which there is strong evidence for the benefits such as significantly reducing all-cause and cardiovascular mortality [2, 3]. The cornerstone of

a CR program is exercise training [4, 5], as aerobic exercise is a core component of it. Prescribing a safe and effective aerobic exercise program in CR is critical to improving functional capacity [6].

Exercise intensity is a key indicator that determines the safety and effectiveness of aerobic exercise program [7]. For safety reasons, patients are usually recommended to exercise without excessive intensity to avoid cardiovascular events. However, if the intensity is not appropriate, the effectiveness would not reach the target of rehabilitation. The majority of guidelines on exercise training in CR recommend aerobic exercise prescription based on moderate intensity. Guidelines recommend that stratification of risk (low, moderate, or high) for exercise complications [8] should be carried out before training in CHD patients. For patients with different risk stratification, the monitoring recommendation level during exercise is different [9]. Individuals in moderate and high-risk groups are required to exercise with electrocardiography (ECG) monitoring, especially in the early CR exercise program. Patients in the low-risk group can exercise at home in the absence of monitoring. During exercise, ECG monitoring is not only for safety but also to ensure that the heart rate of patients can reach the target intensity preset by the exercise prescription.

However, recent data suggests that the distribution density of clinics with a CR unit in China is only 1.32 per 100 million population [10]. Most of these clinics are concentrated in the economically developed areas along the southeast coast. Even in these CR clinics, CHD patients rarely exercise with ECG monitoring.

Moreover, at the beginning of the CR program, leading guidelines recommend that the exercise prescriptions should be based on a graded exercise test (GXT) or cardiopulmonary exercise test (CPET) [11]. The exercise test provides accurate information such as anaerobic threshold (AT) that we use to build patient-specific exercise prescriptions. The oxygen uptake (VO2) at anaerobic threshold match 60% peak oxygen uptake (VO2peak). It is the best intensity to exercise at with an AT heart rate for CHD patients [12]. But recent evidence shows that only 30% of CR clinics perform these baseline exercise tests [13].

In the absence of a baseline exercise test, guidelines suggest some techniques for setting heart rate targets for exercise to match moderate intensity as feasible alternatives for exercise prescription. Commonly used indicators of intensity in these techniques are percentages of heart-rate-reserve (%HRR), percentages of max heart rate (%HRmax), and resting heart rate plus 20–30°bpm (RHR + 20–30) [14, 15]. In addition, Borg's rating of perceived exertion (RPE) 6–20 scale is suggested for use to set a target of 12–16 (moderate to hard) for subjective intensity monitoring of exercise. A target of 11–13 is usually suggested at the beginning seen as moderate intensity [16].

It is plausible that these techniques are safe alternatives, but there is a paucity of evidence. Clinicians often have concerns when using these methods to prescribe exercise, especially when the patient is likely to exercise without monitoring. It is important to compare the effectiveness and safety of these tests. Therefore, our aim was to determine if these techniques can effectively and safely inform exercise prescription for individuals with CHD. The first primary objective was to determine if the corresponding exercise intensities (expressed in heart rate) set by these methods match the anaerobic threshold (representing moderate intensity). The second primary objective was to determine the frequency of exercise-induced abnormalities (both ECG abnormalities and serious adverse events) at the intensities that were set by the above methods during aerobic exercise. The third objective was to determine if the effectiveness and safety differed between them.

2. Materials and Methods

This trial was a retrospective chart study and was approved by the Ethics Committee of Guangdong Provincial Hospital of Traditional Chinese Medicine (ethics number: ZE2022-090-01). The requirement for informed consent was waived because of the retrospective nature of this study.

2.1. Setting and Participants. Both the heart rate as a certain indicator of intensity and the exercise-induced ECG abnormalities require patients to exercise under ECG monitoring. But in reality, very few patients can meet the requirements. Therefore, as an alternative, we collected the data retrospectively from the results of CPET in CHD patients under different heart rates set by the intensity techniques (detailed below) to study.

2.1.1. Case Inclusion Criteria. A. CHD is defined as any medical history (ECG is stable for more than 72 hours) of myocardial infarction (MI), previous revascularization procedure with percutaneous coronary intervention (PCI), coronary artery bypass graft (CABG), coronary angiography (CAG), or coronary computed tomographic angiography (CCTA) which shows that there was ≥1 coronary artery stenosis ≥50%, within 6 months [8].

B. post-CPET: individuals who had completed a cardiopulmonary exercise test at the Second affiliated hospital of Guangzhou University of Chinese medicine.

2.1.2. Exclusion Criteria. Patients with missing CHD related medical records or CPET examination data were excluded.

According to the inclusion and exclusion criteria, a total of 324 patients who were inpatients or outpatients in this hospital between June 2018 and February 2022 were included in this retrospective study ($n = 324$).

2.2. Calculation of Heart Rate by Exercise Intensity Techniques. When building an aerobic exercise prescription in CR for CHD patients, it is ordinarily recommended to set it at a sustained moderate intensity by the guidelines. This intensity of control, in practice, is achieved by controlling the heart rate (HR) of patients during exercise. The HR corresponding to moderate intensity exercise is usually calculated at 60%–80% of HR reserve (HRR), or 50%–70% of VO2 reserve, or HR at the AT.

The guideline provides four techniques for the actual calculation of exercise prescription: the heart-rate-reserve (HRR) method, max-heart-rate (MHR) method, target-heart-rate (THR) method, and the anaerobic threshold (AT) method to set the target-heart-rate at moderate intensity [17]. The AT method needs to be measured by CPET. The detailed calculation methods of these techniques are as follows.

2.2.1. HRR Method. Target heart rate = (HRmax—RHR) × exercise intensity% + RHR.

The intensity range is usually 60%–80%. According to the guidelines and expert consensus, 60% was used in this trial as the initial exercise intensity level. "RHR" means resting heart rate, and "HRmax" means maximum-heart-rate during exercise. There are two ways to set the HRmax. We use the formula (220—age) to calculate the first HRmax inferred from the patient's age. The second one is the peak heart rate actually measured in the exercise test (such as CPET). Therefore, we get two methods to set the target-heart-rate by the HRR method. We named the first method with calculated HRmax "HRR method A," and the other one "HRR method B" for distinguishing.

2.2.2. THR Method. Target heart rate = RHR + 20–30 bpm. We take the lower value 20 to calculate in this study as the initial exercise intensity level.

2.2.3. MHR Method. Target heart rate = age inferred HRmax × exercise intensity = (220—age) × exercise intensity. The intensity range here is 50%–85%. According to the expert consensus [8], the initial exercise intensity level is set as the lower limit of the intensity value of the formula, and then the intensity is gradually increased as the physical fitness improves. Therefore, we take it as 50% in this trial.

2.2.4. AT Method. Target heart rate = heart rate at the anaerobic threshold (HRAT).

In this study, four training heart rates were calculated using the above methods (HRR method A and B, THR method, and MHR method). Then, from the data of each patient's CPET, the indicators of safety and intensity for each patient under these four training HR were collected and compared with the HRAT.

2.3. Cardiopulmonary Exercise Test. CPET was performed on a cycle-ergometer (CS-200 Ergo-Spiro, SCHILLER, Baar, Switzerland) fitted with a facemask for all subjects. Testing was done with expired gas analysis under continuous ECG monitoring. In order to terminate the test after 10 minutes, the load incremental phase of the test protocol followed an increasing work rate of 15–30 W/min using a ramp-pattern.

Standard 12-lead ECGs were obtained after adequate skin preparation, at rest, each minute during exercise, and 5–6 minutes during the recovery phase. RHR was the heart rate at rest, and HRpeak was the heart rate at peak exercise. The heart rate at anaerobic threshold was termed HRAT. HRmax was the age-predicted maximum-heart-rate, estimated by 220-age. All the heart rate and ECG abnormalities during the test were recorded.

Oxygen uptake (VO2) and carbon dioxide output (VCO2) were measured breath by breath. Respiratory gas analysis will be performed using a metabolic cart (Cardiovit CS-200 Touch, SCHILLER, Baar, Switzerland). The highest value or the plateau of oxygen uptake was termed VO2peak. The value of oxygen uptake at an anaerobic threshold was termed VO2AT. These indicators were standardized by bodyweight (ml/kg/min). Metabolic equivalents (METs) were expressed each minute at rest, during exercise, and in the recovery phase.

Test termination criteria include symptoms (i.e., leg discomfort/fatigue, dyspnea, chest pain, or other), >2 mm of horizontal or downsloping ST segment depression, or a drop of systolic blood pressure >20 mmHg during progressive exercise, or sustained ventricular tachycardia (VT) and NSVT that interfered with hemodynamic stability.

2.4. Measures. The following measures were extracted from the patient health record and data from the results of CPET.

2.4.1. Patient Profile. Participant characteristics were recorded, including age, gender, body mass index (BMI), history of MI/heart failure (HF), history of PCI/CABG, complete revascularization, medical history (hypertension, diabetes, dyslipidemia, carotid/cerebral arteriosclerosis, etc.), arrhythmia history such as complex ventricular arrhythmias (VA), history of sudden death, left ventricular ejection fraction (LVEF), functional reserve (i.e., maximum metabolic equivalents, max METs), and use of β-blocker medications.

2.4.2. Risk Stratification for Exercise Complications. According to the risk stratification method for CHD patient exercise complications, all participants were divided into low-risk, moderate-risk, and high-risk groups [18] (see Table 1).

2.4.3. Cardiopulmonary Exercise Test Results. The results of CPET reflect the patient's response to exercise. These indicators include RHR, HRAT, HRmax, HRpeak, %HRmax, HRR, VO2peak (and the percentage of the predicted value), peak Mets, VO2AT (and the percentage of the predicted value), exercise ECG results (i.e., positive, suspicious positive, negative, complex VA or other), exercise angina pectoris, and other symptoms. All these data were continuously monitored and qualitatively interpreted by an experienced cardiologist.

2.5. Comparison of Target-Heart-Rate. The intensity at AT is usually considered as a typical moderate exercise intensity and is recommended by the guidelines [16]. We calculated

Table 1: Risk stratification of cardiovascular events during exercise.

	Item	Risk stratification		
		Low-risk	Moderate-risk	High-risk
Exercise test index	Angina	No	Maybe yes	Yes
	Asymptomatic, with myocardial ischemia and ECG changes	No	Maybe yes, ST segment down shift <2 mm	Yes, ST segment down shift ≥2 mm
	Other obvious discomfort symptoms (e.g., dyspnea, dizziness)	No	Maybe yes	Yes
	Complex ventricular arrhythmias	No	No	Yes
	Hemodynamic response	Normal	Normal	Abnormal
	Functional reserve	≥7METs	5.0–7.0 METs	≤5 METs
Nonexercise test index	LVEF	≥50%	40%–50%	<40%
	History of sudden death/sudden death	No	No	Yes
	Resting complex ventricular arrhythmias	No	No	Yes
	Complications of MI or revascularization	No	No	Yes
	Myocardial ischemia after MI or revascularization	No	No	Yes
	Congestive heart failure	No	No	Yes
	Clinical depression	No	No	Yes

All low-risk items match belong to low-risk groups; any high-risk item matches belong to high-risk group. ECG, electrocardiography. LVEF, left ventricular ejection fraction. MI, myocardial infarction.

the target HR for training of each participant according to the above four calculation formulas (HRR method A and B, THR method, and MHR method) and then compared these HR with their own HRAT (less than, reach, or exceed) to evaluate whether exercise at these target HR can reach the AT intensity. For each method, we calculated the average target HR in the three risk groups, counted the frequency of patients who reached, less than, or exceeded the HRAT each group and compared the above indicators.

2.5.1. Comparison of Safety Indicators. In this study, safety indicators include exercise-induced clinically relevant ECG abnormalities and serious adverse events. For each intensity technique, we counted the frequency of these two safety indicators in CPET data at each calculated target HR zone, including the total frequency in all participants as well as the frequency in the three risk groups. Then we compared the characteristics of the above safety indicators in different intensity calculation methods. These two safety indicators were defined as follows:

(i) Exercise-Induced Clinically Relevant ECG Abnormalities. Clinically relevant abnormalities were defined as exercise-induced changes on ECG during exercise at the target HR zone that would prohibit exercise beyond the intensity at which it occurred. It included exercise-induced horizontal or downsloping ST segment depression or elevation of ≥1 mm from baseline; complex VA (multiform ventricular premature beats of ≥3 in 10 beats); nonsustained ventricular tachycardia (VT); second- or third-degree atrioventricular block; ventricular fibrillation (VF)/VT; or bundle branch block. All ECGs in the CPET data were reviewed and interpreted by an experienced cardiologist. A second cardiologist reviewed all ECGs to verify the findings. Discrepancies in interpretation were resolved through discussion.

(ii) Exercise-Induced Serious Adverse Events. Serious adverse events are defined as events that lead to permanent or lasting change in function, disability, death, or potentially life-threatening events (e.g., MI, sustained VT, cardiac arrest, or a condition that requires cardiopulmonary resuscitation during exercise).

2.6. Data Analysis. All statistical analyses were performed with SPSS Statistics 23 (SPSS Inc., Chicago, IL, USA). Descriptive statistics were used to characterize the patients. Continuous variables were presented as means with standard deviation (SD). Categorical variables were expressed as counts (percentages). Frequency values were used to describe the prevalence of patients who exhibited exercise-induced ECG abnormalities or serious adverse events during exercise at each calculated target HR zone. Paired *t*-tests were used to compare the test results in each intensity calculation method with those of the AT method. Analysis of variance or chi-square tests were used to compare the participant characteristics and exercise test results, as appropriate, for those in the three risk groups. A P value of <0.05 was considered statistically significant for all analyses.

3. Results

3.1. Patient Profile. According to the above-mentioned risk stratification method of CHD, all 324 patients were divided into three groups. 232 cases matched any high-risk items and were classified into the high-risk group. 15 patients matched all low-risk items and were classified into the low-risk group. The remaining 77 cases were classified into the moderate-risk group.

In order to minimize the impact of sample size difference on the results, the measurement data between the three groups are analyzed by one-way ANOVA and the post hoc comparison (Scheffe test is used when the variance is

homogeneous and Tamhane T2 test is used when the variance is uneven). The counting data among the groups are analyzed by the chi-square test.

The basic clinical data and medical history data of patients ($n = 324$) are shown in Tables 2 and 3. The numbers of patients with other diseases such as COPD, nephrosis, and atrial fibrillation are too small to be statistically significant and are not shown in table.

In a comparison among three groups, the mean age of patients in the high-risk group (64.07 ± 9.71 years) was higher than that in the moderate-risk group and low-risk group ($P < 0.05$).The sex ratio of the high-risk group was significantly different from that of the low-risk group and moderate-risk group ($P < 0.05$).

Other indicators with significant differences between groups are functional reserve, HF, incomplete revascularization, and complex VA ($P < 0.05$). The differences are due to the fact that these indicators are items for grouping themselves.

3.2. Outcome Indicators of CPET. The main outcome indicators of CPET are shown in Table 4.

13 participants failed to measure the anaerobic threshold in the CPET, which reflects the poor aerobic capacity of these 13 patients. Most of the other participants had normal VO2AT oxygen uptake (228, 70.4%). The measured mean HRAT, metabolic equivalent at (ATMETs), measured VO2AT, and the percentage of measured VO2AT to predicted value were all in line with the normal level of adults.

The indicators as VO2AT, ATMETs, and the percentage of VO2AT to the predicted value were the highest in the low-risk group and the lowest in the high-risk group ($P < 0.05$), while there was no difference among the three groups in the mean value of measured HRAT ($P > 0.05$).

It is suggested that although the HRAT level is similar, the VO2 and METS of patients in the high-risk group were significantly lower than those in the low-risk group and moderate-risk group.

At the peak exercise level, there were significant differences in VO2peak, peak METs, and the percentage of VO2peak in the predicted value among the three groups ($P < 0.05$). The average VO2peak in the low-risk group was 28.3 ± 2.6 ml/kg/min, reaching 94.3% of the predicted value, which was the level of normal adults. The VO2peak decreased in moderate- and high-risk group. The mean value of VO2peak in the high-risk group was lower than that in the moderate-risk group ($P < 0.05$). The HRpeak of the high-risk group was 121 ± 21.1 bpm, which was the lowest in three groups ($P < 0.05$).

There was a difference in the HRmax calculated according to age ($P < 0.05$). This result should be related to the significant difference in age among the groups. There was no significant difference in RHR among the three groups.

The significant differences between groups in other indicators like Complex VA, ECG positive reaction, and the number of patients with normal VO2peak are due to the fact that these indicators are items for grouping themselves.

3.3. Effectiveness Indicators. The comparison of effectiveness indicators is shown in Table 5.

A paired t-test was performed between every HR calculated by the other 4 methods and the measured HRAT. Except for HRR method A, there was a significant difference ($P < 0.05$). This shows that the HR calculated by the HRR method A is consistent with HRAT.

In comparison among the three groups, the HR calculated by the HRR method B and the MHR method were significantly different ($P < 0.05$). And post hoc comparison showed a difference between moderate- and high-risk group in the MHR method ($P < 0.05$). According to Spearman's rank correlation coefficient, there is a significant correlation between this difference and both age and gender ($P = 0.00$).

The frequencies of the participants whose target HRs were calculated lower than the HRAT were more than 50% in all four methods. Among them, the number (307, 94.8%) in the MHR method is the highest, followed by 268 (82.7%) in the HRR method B, and 208 (64.2%) in the THR method. The frequency of this type of patients in the HRR method A was the least at 164 (50.6%). There was no significant difference among the groups ($P > 0.05$).

From the above results, it can be seen that the HRR method A is most consistent with the measured HRAT. The average HR of the other three methods is lower than the measured one at. The order of the average HR from high to low is the THR method, HRR method B, and MHR method. The target HR of the THR method is closer to the measured HRAT except for HRR method A.

3.4. Safety Indicators. All participants had no serious adverse events occur through the motion test terminal. The indicator of exercise-induced horizontal or downsloping ST segment depression or elevation of ≥ 1 mm from baseline actually refers to the ECG positive reaction of an exercise load test. We found that the risk relevant ECG abnormalities in this study were mainly ECG positive reactions and complex VA, including 28 (8.6%) positive reactions and 27 (8.3%) complex VA. In addition, 1 participant developed nonsustained ventricular tachycardia and 2 participants developed paroxysmal bundle branch block. All the above participants belong to the high-risk group, of which ECG positive reactions accounted for 12.1% and complex VA accounted for 11.6%. There were no risk-relevant ECG abnormalities that occured in the low-risk and moderate-risk groups.

For each participant with risk-relevant ECG abnormalities, we counted the frequency of these indicators in CPET data at each target HR zone calculated by the above intensity technique. The result is shown in Table 6.

The distribution of ECG abnormalities in the HR interval of the above four methods and the AT method is different ($P < 0.05$). The highest frequency of all kinds of risk of ECG abnormalities occurred in the HRR method A, followed by measured HRAT and THR methods, and the MHR method has the lowest risk of ECG abnormalities ($P < 0.05$). The safety of the THR method is close to that of the AT method. From the above results, we can see that the frequency of risk

Table 2: Basic clinical data of patients.

	All Patients ($n = 324$)		Low-risk ($n = 15$)		Moderate-risk ($n = 77$)		High-risk ($n = 232$)		$P^{\#}$	P^{a}	P^{b}	P^{c}
	No. of patients (%)	Mean (SD)	No. of patients (%)	Mean (SD)	No. of patients (%)	Mean (SD)	No. of patients (%)	Mean (SD)				
Age, y	324	62.8 (9.9)	15	57.3 (6.7)	77	60.0 (10.3)	232	64.1 (9.7)	0.00	0.63	0.04	0.01
Men	210 (64.8)		14 (93.3)		62 (80.5)		134 (57.8)		0.00	0.41	0.01	0.00
BMI,												
kg/m^2	324	24.2 (3.4)	15	22.9 (2.0)	77	23.8 (2.6)	232	24.4 (3.6)	0.14	0.39	0.05	0.31
LVEF, %	324	65.9 (8.5)	15	66.9 (6.7)	77	65.9 (7.1)	232	65.8 (9.0)	0.89	0.91	0.89	1.00
Functional reserve, METs	324	5.1 (1.5)	15	8.1 (0.7)	77	6.1 (0.7)	232	4.6 (1.3)	0.00	0.00	0.00	0.00
β-blocker												
Medications	185 (57.1)		9 (60)		37 (48.1)		139 (59.9)		0.21	0.39	1.00	0.07

$P^{\#}$, P value of comparison among three groups; P^{a}, P value of comparison between low-risk and moderate-risk group; P^{b}, P value of comparison between low-risk and high-risk group; P^{c}, P value of comparison between moderate-risk and high-risk group. BMI, body mass index; LVEF, left ventricular ejection fraction.

Table 3: Medical history data of patients.

	All Patients ($n = 324$) No. of patients (%)	Low-risk ($n = 15$) No. of patients (%)	Moderate-risk ($n = 77$) No. of patients (%)	High-risk ($n = 232$) No. of patients (%)	$P^{\#}$	P^{a}	P^{b}	P^{c}
Type of CHD								
MI	91 (28.1)	5 (33.3)	18 (23.4)	68 (29.3)	0.76	0.63	0.80	0.70
HF	26 (8.0)	0	0	26 (11.2)	0.00	—	0.38	0.00
post-PCI	213 (65.7)	12(80)	48 (62.3)	153 (65.9)	0.42	0.19	0.40	0.57
post-CABG	4 (1.2)	0	0	4 (1.7)	0.45	—	1.00	0.56
Incomplete revascularization	56 (17.3)	0	0	56 (24.1)	0.00	—	0.03	0.00
Complex VA	23 (7.1)	0	0	23 (9.9)	0.01	—	0.37	0.00
History of other diseases								
Hypertension	207 (63.9)	7 (46.7)	44 (57.1)	156 (67.2)	0.10	0.46	0.10	0.11
Diabetes	128 (39.5)	4 (26.7)	32 (41.6)	92 (39.7)	0.56	0.28	0.32	0.77
Dyslipidemia	107 (33)	3 (20)	25 (32.5)	79 (34.1)	0.53	0.51	0.40	0.80
Carotid atherosclerosis	153 (47.2)	6 (40)	40 (51.9)	107 (46.1)	0.57	0.40	0.65	0.38

$P^{\#}$, P value of comparison among three groups; P^{a}, P value of comparison between low-risk and moderate-risk group; P^{b}, P value of comparison between low-risk and high-risk group; P^{c}, P value of comparison between moderate-risk and high-risk group. CHD, coronary heart disease; MI, myocardial infarction; HF, heart failure; PCI, percutaneous coronary intervention; CABG, coronary artery bypass graft; VA, complex ventricular arrhythmia.

Table 4: The main outcome indicators of CPET.

	All Patients ($n = 324$)		Low-risk ($n = 15$)		Moderate-risk ($n = 77$)		High-risk ($n = 232$)					
	No. of patients (%)	Mean (SD)	No. of patients (%)	Mean (SD)	No. of patients (%)	Mean (SD)	No. of patients (%)	Mean (SD)	$P^{\#}$	P^{a}	P^{b}	P^{c}
HRAT, bpm	311	98 (13.0)	15	103 (12.7)	77	99 (11.8)	219	97 (13.3)	0.19	0.59	0.28	0.58
ATMETs, METs	311	3.2 (0.7)	15	4.3 (0.7)	77	3.6 (0.6)	219	3.0 (0.7)	0.00	0.00	0.00	0.00
VO2AT, ml/kg/min	311	11.3 (2.5)	15	15.2 (2.3)	77	12.6 (2.0)	219	10.6 (2.3)	0.00	0.00	0.00	0.00
VO2AT/pred%, %	311	43.1 (13.6)	15	50.9 (13.3)	77	46.6 (10.2)	219	41.4 (14.3)	0.00	0.53	0.03	0.01
VO2peak, ml/kg/min	324	17.8 (5.3)	15	28.3 (2.6)	77	21.2 (2.8)	232	16.0 (4.7)	0.00	0.00	0.00	0.00
VO2peak/pred%, %	324	69.9 (17.4)	15	94.3 (18.8)	77	78.4 (13.3)	232	65.6 (16.2)	0.00	0.00	0.00	0.00
HRpeak, bpm	324	125 (20.9)	15	147 (14.8)	77	132 (15.9)	232	121 (21.1)	0.00	0.01	0.00	0.00
HRmax, bpm	324	157 (10.4)	15	163 (6.7)	77	160 (10.3)	232	156 (10.4)	0.00	0.65	0.06	0.02
RHR, bpm	324	73 (10.5)	15	71 (13.0)	77	73 (9.7)	232	73 (10.6)	0.62	0.59	0.28	0.58
Complex VA in test	27 (8.3)		0		0		27 (11.6)		0.00	-	0.38	0.00
ECG positive reaction	28 (8.6)		0		0		28 (12.1)		0.00	-	0.23	0.00
ECG suspicious positive reaction	42 (13.0)		0		13 (16.9)		29 (12.5)		0.19	0.12	0.23	0.33
With Normal VO2AT	228 (70.4)		12 (80)		59 (76.6)		157 (67.7)		0.23	1.00	0.48	0.14
With Normal VO2peak	69 (21.3)		12 (80)		26 (33.8)		31 (13.4)		0.00	0.00	0.00	0.00
With Roughly normal VO2peak	27 (8.3)		1 (6.7)		7 (9.1)		19 (8.2)		0.94	1.00	1.00	0.81

$P^{\#}$, P value of comparison among three groups; P^{a}, P value of comparison between low-risk and moderate-risk group; P^{b}, P value of comparison between low-risk and high-risk group; P^{c}, P value of comparison between moderate-risk and high-risk group. HRAT, heart rate at anaerobic threshold; ATMETS, metabolic equivalent at anaerobic threshold; VO2AT, oxygen uptake at anaerobic threshold; Pred, predicted value; VO2peak, oxygen uptake at peak; HR peak, heart rate at peak; HRmax, age-predicted maximum-heart-rate; RHR, resting heart rate; VA, ventricular arrhythmia; ECG, electrocardiography.

Table 5: The comparison of effectiveness indicators.

Intensity technique	All patients ($n = 324$)			Low-risk ($n = 15$)		Moderate-risk ($n = 77$)		High-risk ($n = 232$)		$P^{\#}$	P^{a}	P^{b}	P^{c}
	No. of patients (%)	Mean (SD)	P,*	No. of patients (%)	Mean (SD)	No. of patients (%)	Mean (SD)	No. of patients (%)	Mean (SD)				
Mean value of target-heart-rate set by exercise intensity techniques, (bpm)													
HRR-m A	324	98 (8.5)	0.45	15	99 (9.7)	77	99 (7.2)	232	98 (8.8)	0.77	1.00	0.96	0.79
HRR-m B	324	88 (11.0)	0.00	15	94 (10.5)	77	91 (9.5)	232	87 (11.3)	0.01	0.54	0.08	0.09
MHR-m	324	79 (5.2)	0.00	15	81 (3.4)	77	80 (5.1)	232	78 (5.2)	0.00	0.65	0.06	0.02
THR-m	324	93 (10.5)	0.00	15	91 (13.0)	77	93 (9.7)	232	93 (10.6)	0.62	0.90	0.73	0.81
AT-m	311	98 (13.0)		15	103 (12.7)	77	99 (11.8)	219	97 (13.3)	0.19	0.59	0.28	0.58
Frequencies of whose target HR lower than HRAT.													
HRR-m A	164 (50.6)			9 (60.0)		37 (48.1)		118 (50.9)		0.69	0.40	0.49	0.67
HRR-m B	268 (82.7)			11 (73.3)		66 (85.7)		191 (82.3)		0.49	0.42	0.60	0.49
MHR-m	307 (94.8)			14 (93.3)		72 (93.5)		221 (94.8)		0.81	1.00	1.00	0.76
THR-m	208 (64.2)			12 (80.0)		56 (72.7)		140 (60.3)		0.06	0.79	0.13	0.051
Frequencies of whose target HR higher than HRAT.													
HRR-m A	160 (49.4)			6 (40.0)		40 (51.9)		114 (49.1)		0.69	0.40	0.49	0.67
HRR-m B	53 (16.4)			4 (26.7)		11 (14.3)		38 (16.4)		0.50	0.42	0.50	0.66
MHR-m	16 (4.9)			1 (6.7)		5 (6.5)		10 (4.3)		0.71	1.00	0.51	0.64
THR-m	106 (32.7)			3 (20.0)		20 (26.0)		83 (35.8)		0.16	0.87	0.21	0.11

P,* , P value of comparison between each intensity technique and AT-m by paired t-test; $P^{\#}$, P value of comparison among three groups; P^{a}, P value of comparison between low-risk and moderate-risk group; P^{b}, P value of comparison between low-risk and high-risk group; P^{c}, P value of comparison between moderate-risk and high-risk group. HRR-m A, heart-rate-reserve method A; HRR-m B, heart-rate-reserve method B; MHR-m, max-heart-rate method; THR-m: target-heart-rate method; AT-m: anaerobic threshold method.

Table 6: Comparison of safety indicators in intensity techniques.

	a. No. and Frequencies with ECG positive reaction			*b*. No. and Frequencies with Complex VA			*c*.	*f*.
	No. of patients	(%) of all high-risk patients ($n = 232$)	(%) in all ECG positive reaction patients	No. of patients	(%) of all high-risk patients ($n = 232$)	(%) in all Complex VA patients ($n = 27$)	No. of patients	No. of patients
HRR-m A	7	3.0	25	18	7.8	66.7	1	2
HRR-m B	1	0.4	3.6	9	3.9	33.3	0	1
MHR-m	0	0	0	5	2.2	18.5	1	1
THR-m	4	1.7	14.3	13	5.6	48.1	1	1
AT-m	2	0.9	7.1	14	6.0	51.9	1	2
P△		0.00	0.00		0.00	0.00		

$P^{\triangle}$, P value of the consistency comparison among the five methods in the distribution of the safety indicators (using the nonparametric test of Related Samples—Cochran's Q test,) in the high-risk group or all the people with this safety indicators. c, no. of patients with nonsustained ventricular tachycardia; f, no. of patients with bundle branch block; HRR-m A, heart-rate-reserve method A; HRR-m B, heart-rate-reserve method B; MHR-m, max-heart-rate method; THR-m, target-heart-rate method; AT-m, anaerobic threshold method. Among the above-mentioned patients with ECG abnormalities, most of them with positive reaction have a medical history of hypertension, normal LVEF, normal anaerobic threshold, decreased functional reserve, and most of them use β-blocker medications, but there was no significant difference compared with negative patients ($P > 0.05$).

of ECG abnormalities is low overall, and the MHR method is safer than other techniques.

Most of the patients with complex VA during exercise had a history of hypertension, carotid atherosclerosis, and PCI. Most of them also had normal LVEF and anaerobic threshold and decreased functional reserve. One third of these patients had ventricular arrhythmia at rest ($P < 0.05$).

4. Discussion

The results of the first part of our study show that according to the CHD risk stratification method, most CHD patients will be classified into the moderate- and high-risk groups, and the average age and female composition ratio will increase in the high-risk group. The reason for this grouping result may only be that CHD patients with moderate- and high-risk account for a high proportion in our hospital. In the long term, it is necessary to further multicenter research and statistics on the composition ratio of each risk group in the actual clinical work.

The second part of statistical results on CPET showed that, although the VO2, METs, and the percentage of VO2 in the predicted value in the AT period of CHD patients decreased with the increase of risk grouping level ($P < 0.05$), the mean values of them were still at a normal level. This result shows that regardless of the risk group, AT level in most CHD patients is normal. The decrease of aerobic capacity in the high-risk group is mainly reflected in the peak period. HRAT of each group is at a similar level. This result proves that each risk groups can use the measured HRAT as the standard of HR for moderate intensity exercise.

The third part of the results is about the effectiveness. It is generally believed that exercise rehabilitation can achieve a better curative effect in the HR range consistent with HRAT, which represents the HR of moderate intensity exercise.

When comparing the effectiveness of the four methods, our results found that the HR calculated by the HRR method A had the highest coincidence with the measured HRAT ($P < 0.05$). The target HR calculated by other methods is significantly different from HRAT. The coincidence degree is THR method, HRR-method B, and MHR method from high to low. When calculated by the MHR method, more than 90% of CHD patients have a target HR lower than actual HRAT.

When comparing among risk groups, the difference in effectiveness results only appears in the HRR method B and the MHR method, and further pairwise comparison shows that there is only a difference in the MHR method between the moderate- and high-risk group. The formula of the MHR method takes age as a unique variable, so the difference is mainly due to the fact that the age of the high-risk group is larger than that of other groups. There is also a significant correlation between this difference and gender, which may be related to the change in gender composition ratio in the high-risk group. The above results show that in most cases, except for the MHR method, the effectiveness of these methods is less restricted by risk grouping.

Why is HRR method A calculated using age-inferred HRmax more consistent with HRAT than HRR method B using measured HRpeak? The reason may be that the HRAT is normal for most patients, but the measured HRpeak is reduced, as the results have shown. When the same HRR formula is used to calculate the target HR, if the measured HRpeak is used as a variable, the obtained target HR will be lower than the normal HRAT. The HRmax calculated by the age formula is within the range of the normal reference value. When the HRmax is used by the HRR method, the target HR is more consistent with the normal HRAT. This indicates that calculating the target HR using the HRR formula should be based on the expected normal HRmax.

The MHR method also uses the HRmax calculated by the formula. However, which is different from the HRR formula using two variables as HRmax and RHR, HRmax is the only variable in the formula of the MHR method. In fact, when actually measuring HRAT, the HR rise caused by exercise stimulation is also based on the RHR. Actual RHR is affected by different conditions, such as the use of β-blockers. When RHR is not taken into account, it will directly affect the consistency of calculated HR to HRAT. The formula of the HRR method includes the variable of actual RHR, so it can avoid this problem and be consistent with HRAT.

The fourth part of the results is about safety indicators. All participants had no serious adverse events, and the proportion of ECG abnormalities at various risks was less than 10%. There were even no ECG abnormalities in low- and moderate-risk patients. It shows that the overall safety of exercise in CHD patients is high.

Risk of ECG abnormalities only occurred in high-risk patients, of which ECG positive reactions accounted for 12.1% and complex VA accounted for 11.6%. We compare the target HR (calculated by the four methods) and HRAT with the HR range of the above risk of ECG abnormalities in CPET data. The results showed that even in high-risk patients, when exercising according to the target HR set by the above intensity techniques, the proportion of risk of ECG abnormalities was less than 8%.

And most of the abnormal ECG appeared within the HR range set by the HRR method A (e.g., the occurrence rate of complex VA was 7.8%), followed by the measured HRAT and THR method. The frequency of abnormal ECG in HRR method B and MHR method is awfully low. Therefore, from the perspective of safety, there is a higher risk of exercising with the HR set by the HRR method A. If you exercise with the HR calculated by the MHR method, the risk is the lowest.

In our study, compared with the four methods, HRR method A has the best effectiveness, but there are higher risks; the MHR method is the safest, but its effectiveness is low. Previous studies [11] suggested that in the absence of a CPET test baseline, the exercise intensity at the beginning of CR should be set to resting heart rate + 20–30 and the RPE target 11–14. Although it seems vague, it may provide a safe and effective starting point for most patients. The results of our study support this suggestion, and we believe that if we take into account both the effectiveness and safety, the compromise method may be the THR method (i.e., resting heart rate + 20–30).

We analyzed the condition characteristics of patients with risk of ECG abnormalities. Except that 1/3 of them had

ventricular arrhythmia at rest ($P < 0.05$), no significant and characteristic indexes were found to indicate the occurrence of risk of ECG abnormalities. Therefore, when making exercise prescriptions, doctors still need to ponder the disease aspect.

Previous studies [19–21] have shown that the exercise risk of CHD patients is relatively low. Although exercise may trigger cardiovascular (CV) events, developing good exercise habits and CV health can significantly reduce this risk [22]. Previous studies [23] have reported that exercise-induced CV events usually occur in the early stages of participating in CR, which has no correlation with the amount of CR exercise, the level of professionals, and whether ECG monitoring during exercise [24]. The factors that increase the risk of CV events and death are poor compliance with exercise prescriptions [17].

Our data may support the following view: in the early stage of CR aerobic exercise for CHD, it is still necessary to divide patients into risk strata. In low-risk and moderate-risk patients, the four exercise intensity techniques recommended in the guidelines are usually safe. In the absence of a measured anaerobic threshold heart rate, the HRR method A is more effective. For patients with high-risk, if there is no condition to exercise under ECG monitoring, conservative exercise prescription (e.g., MHR or THR method) is recommended. To set the target-heart-rate, the MHR method is the safest, and the THR method is the most effective. Both methods need to be combined with the degree of perceived fatigue, signs or symptoms at the same time. We should emphasize that even though the target-heart-rate set by the MHR method is conservative, it does not require the patient to reach this intensity immediately. It should be done gradually based on the patient's actual physical fitness. Even so, for high-risk patients, when they have the condition to exercise under ECG monitoring, they still should be strongly recommended to ECG monitoring, which will help to minimize the risk of cardiac exercise rehabilitation.

The advantage of this retrospective study is that it records the heart rate changes of patients from rest, warm-up to peak intensity exercise through CPET, and can describe the exercise performance of patients with different intensity and heart rate level, so as to provide basis for the judgment of effectiveness and safety. However, it is worth noting that there may be differences in patients' heart rates and other reactions between the load-increasing exercise program commonly used in CPET and the continuous exercise with a specific load fixed in exercise rehabilitation training. It cannot be confirmed by the current data, and it still needs to be explained by prospective research.

In addition, there is no fixed training mode in the exercise program because many factors (including physical fitness, enthusiasm, and skeletal muscle constraints) will affect the progress speed of patients. The intensity shall be increased timely and moderately within the scope specified in the latest assessment based on the staff's observation and the patient's subjective response. It is equally important to consider psychoeducation for patients in CR to facilitate adherence to physical activity [25].

5. Conclusion

In conclusion, when patients with CHD participate in CR and exercise at the target-heart-rate formulated by the four exercise intensity techniques recommended in the guidelines, unconditionally measuring the anaerobic threshold heart rate, the heart rate calculated by HRR method A is more consistent with the actual AT. The proportion of risk of ECG abnormalities and serious adverse events is very low. All four techniques are safe in low- and moderate-risk patients. In high-risk patients, the use of HRR method A has certain risks. When it is impossible to exercise under ECG monitoring, it is recommended to use the MHR method to set the target-heart-rate. If both effectiveness and safety are considered, the THR method can be selected at the beginning of the CR program.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This work was supported by the Special Fund for Medical and Health Development (inheritance and development of traditional Chinese medicine (TCM)) Scientific Research Project of TCM from Guangdong Bureau of Traditional Chinese Medicine Bureau of Guangdong Province in 2020 [20201154] and in 2018 [20182057].

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Hindawi
Cardiology Research and Practice
Volume 2022, Article ID 6491129, 10 pages
https://doi.org/10.1155/2022/6491129

Research Article

The Metabolomic Characterization of Different Types of Coronary Atherosclerotic Heart Disease in Male

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Received 4 April 2022; Revised 15 May 2022; Accepted 27 June 2022; Published 12 July 2022

Academic Editor: Fanbo Meng

Background. In clinical practice, many patients with coronary atherosclerotic heart disease (CAD) have atypical clinical symptoms. It is difficult to accurately identify stable CAD or unstable CAD early through clinical symptoms and coronary angiography. This study aimed to screen the potential metabolite biomarkers in male patients with stable CAD and unstable CAD. *Methods*. In this work, the metabolomic characterization of the male patients with healthy control ($n = 42$), stable coronary artery disease ($n = 60$), non-ST-elevation acute coronary syndrome ($n = 45$), including prepercutaneous corona intervention ($n = 14$), and postpercutaneous coronary intervention ($n = 31$) were performed by using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). The serum samples of patients were analyzed by multivariate statistics. *Results*. Results showed that 17 altered metabolites were identified to have a clear distinction between the stable CAD group and the healthy subjects. Compared with the stable coronary artery disease group, 15 specific metabolite markers were found in the acute coronary syndrome group. The percutaneous coronary intervention also affected the metabolic behavior of patients with CAD. *Conclusions*. In summary, CAD is closely related to energy metabolism, lipid metabolism, and amino acid metabolism disorders. The different metabolic pattern characteristics of healthy, stable coronary artery disease and acute coronary syndrome are constructed, which brings a novel theoretical basis for the early diagnosis of patients with stable and unstable CAD.

1. Introduction

Cardiovascular disease is the leading cause of death and disability worldwide. The mortality rate of Chinese cardiovascular disease was the highest [1]. Coronary atherosclerotic heart disease (CAD) is still the cardiovascular disease with the highest fatality rate in the world [2]. At present, the "gold standard" for identifying and diagnosing CAD depends on coronary angiography and coronary CT imaging [3, 4]. Some studies indicated that the incidence of acute coronary events in CAD patients was closely related to the stability of coronary plaques and vulnerable plaques (unstable plaques) [5]. Stable coronary artery disease (SCAD) and acute coronary syndromes (ACS) (such as unstable angina, non-ST-elevation myocardial infarction, and ST-elevation myocardial infarction) are significantly different in terms of the treatment process, strategies, and prognostic outcomes. ST-segment elevation acute myocardial infarction can be diagnosed by ECG combined with clinical symptoms, while non-ST-segment elevation ACS is often difficult to distinguish between ECG, clinical symptoms, and SCAD. Although increased troponin is specific for identifying myocardial injury in non-ST-elevation ACS, it is negative in the early stage of non-ST-elevation ACS. As there is no effective conventional technology for the early diagnosis of stable coronary plaques and vulnerable plaques, it is particularly important to find a simple, low-cost, and effective method.

As a new branch of systems biology, metabolomics is an analysis technique that can quantitatively and qualitatively study the relationship between metabolites and pathological changes in the body. It can analyze the overall endogenous metabolites in cells, tissues, and other biological samples, such as blood or urine [6, 7]. Metabolomics research has unique application advantages [8–10]: (1) small changes in gene and protein expression can be amplified on metabolites by catalytic reactions of metabolic enzymes, thus making detection and analysis easier. (2) In addition to genome changes, metabolites are also affected by environmental factors and intestinal flora, which are more dynamic and more sensitive to changes in organisms. (3) Metabolic reactions and metabolic products are similar in the biological systems of all species. Therefore, the metabolomics methodology is more universal. (4) Metabolomics technology can directly detect almost all sample types, including whole blood, plasma/serum, tissue, cell, cell culture supernatant, urine, feces, food, saliva, cerebrospinal fluid, and fat, without establishing whole genome sequencing and mass expression sequence database. Applications of metabolic profiling in coronary heart disease have been developed by using LC-MS or GC-MS. The relationship between circulating blood metabolite levels and coronary heart disease was detected by metabolomics. This technology reveals different potential pathways for the development of coronary heart disease. The occurrence of cardiovascular diseases is associated with the metabolites of amino acids, lipids, peptides, carbohydrates, nucleotides, and xenobiotics [11–13]. These biomarkers are important not only for risk stratification and treatment decision-making but also for improving the understanding of the cardiovascular disease. Metabolomics research is likely to become a new technology and method for the early identification of CAD profiles. As the proinflammatory mediators do not appear to be directly linked to the disease [14], the metabolic markers open up a new diagnosis and treatment target for CAD [15, 16].

Previous studies using metabolomics as a potential diagnostic criterion for SCAD and ACS in human samples are limited, especially in China. In this work, we used metabolomics methods to construct the characteristics of patients' metabolites with SCAD, ACS, and healthy subjects. The pattern characteristics of different conditions were discussed. In addition, we analyzed the influence of PCI on the metabolites of patients with CAD. Through the differential changes and metabolic characteristics, metabolomics is expected to become a novel technology for the early diagnosis of different types of CAD.

2. Method

2.1. Baseline Characteristics and Study Design of Patients. Male participants with ages 40 to 65-year-old were enrolled in the Department of Cardiology (the Daqing Oilfield General Hospital, Daqing, China) between January 2015 and December 2015. As it was not clear that metabolites were the same in different genders under certain conditions, subjects of the same sex were selected to reduce the bias of the results. The inclusion criteria of healthy controls (HCs), SCAD, and non-ST-elevation ACS were confirmed according to American and European guidelines for the diagnosis and treatment of stable coronary heart disease and guidelines for the management of non-ST-segment elevation acute coronary syndrome in ESC [17, 18]. The subjects with no clinical history of the disease, normal electrocardiogram examination, and no uncomfortable symptoms of heavy physical activity were clinically diagnosed as healthy controls. All subjects were excluded from diseases such as hypertension, diabetes, chronic kidney disease, metabolic syndrome, heart failure, COPD, bronchial asthma, connective tissue disease, rheumatic immune disease, tumor, hyperthyroidism, hepatitis, metabolic disease, blood system disease, and severe liver and kidney damage. The baseline characteristics (including urea, Cr, Na, K, blood sugar, blood lipid, smoking history, and BMI) of patients were shown in Table 1. There were no statistical differences in the above indicators among the subjects in experimental groups.

The selected controls were healthy with no declared history of CAD ($n = 42$), SCAD ($n = 60$), and ACS group ($n = 45$), respectively. The ACS group was divided into pre-percutaneous coronary intervention (PCI) (within 4 h, $n = 14$, PR-ACS group) and post-PCI (within 4 h, $n = 31$, PO-ACS group). The study was performed under the guidance of an institutional ethical committee from Daqing Oilfield General Hospital following the Helsinki Declaration. All subjects agreed to participate in this study, including the blood sample collection. The study design was shown in Figure 1.

2.2. Sample Preparation. Cubital vein blood samples were collected and immediately underwent plasma isolation. The blood samples were centrifuged at 1000 g for 10 min at room temperature. 100 μL of serum was precipitated by adding 300 μL of methanol and vortexed for 30 s. The precipitated proteins were then removed by centrifugation (13,000 g, 15 min) at 4°C. The supernatant was transferred to a microcentrifuge tube and stored at −80°C for further LC-MS analysis. Quality control (QC) samples were prepared by mixing 10 μL of each sample.

2.3. LC-MS/MS Analysis. The separation was performed on an Agilent®1290 Infinity II (Agilent Technologies Inc., USA) using a Waters ACQUITY HSS T3 C18 (100 × 2.1 mm, 1.8 μm). The column oven and the flow rate were set at 30°C and 0.5 mL/min, respectively. In positive mode, the mobile phase contained 0.1% FA in water (A) and 0.1% FA in ACN (B). In negative mode, the mobile phase consisted of 0.5 mM NH_4F in water (A) and ACN (B). The gradient was 0 min, 1% B; 1 min, 1% B; 8 min, 100% B; 10 min, 100% B; 10.1 min, 1% B; 12 min, 1% B.

ABSCIEX® TripleTOF 6600 Plus ultra-performance liquid chromatography-tandem mass spectrometer (UPLC-Q-TOF/MS) was used to acquire the MS/MS spectra on an information-dependent basis during the LC/MS experiment. It was operated in positive and negative mode ion mode under the following operating parameters: GS1: 40 psi; GS2: 80 psi; CUR: 25 psi; TEM: 650°C; ISVF : 5000V (POS), −4000V(NEG), DP : 60V, CE: 35 ± 15. The pooled QC represented the sample matrix and metabolite composition

Table 1: The clinical data for the human plasma samples. Values are presented as mean ± SD. SBP: systolic blood pressure; Cr: creatine. Na: sodium; K: potassium; BMI: body mass index.

Clinical indicator	HC	SCAD	ACS	p value (HC vs. SCAD)	p value (HC vs. ACS)	p value (SCAD vs. ACS)
Sex	Male	Male	Male	—	—	—
Age (year)	52.7 ± 8.4	55.1 ± 7.9	56.5 ± 6.7	0.143	0.180	0.395
SBP (mmHg)	136.8 ± 20.9	132.75 ± 23.7	142.4 ± 20.5	0.381	0.254	0.055
Urea (mmol/L)	5.7 ± 1.4	5.6 ± 1.9	6.2 ± 2.5	0.614	0.294	0.164
Cr (μmol/L)	71.7 ± 12.5	70.7 ± 13.5	73.6 ± 18.2	0.686	0.605	0.384
Na (mEq/L)	141.7 ± 2.3	141.8 ± 3.5	140.6 ± 3.2	0.784	0.099	0.099
K (mEq/L)	4.4 ± 0.4	4.4 ± 0.5	4.4 ± 0.4	0.792	0.832	0.973
GLU (mmol/L)	4.9 ± 0.4	4.9 ± 0.5	5.0 ± 0.4	0.963	0.840	0.884
LDL-c (mg/Dl)	80.7 ± 26.6	89.2 ± 14.6	89.9 ± 16.7	0.746	0.852	0.913
Smoking, n(%)	21 (50%)	40 (66.6%)	22 (48.9%)	0.288	0.747	0.107
BMI (kg/m^2)	25.7 ± 1.6	25.5 ± 1.5	25.7 ± 1.6	0.765	0.967	0.737

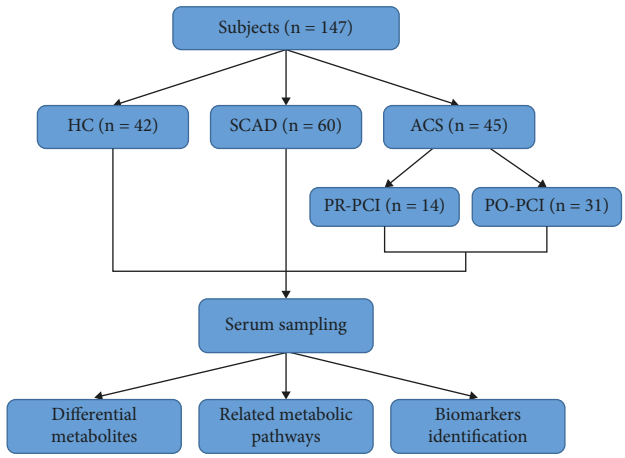

Figure 1: Study design. This study, involving 147 subjects, included 42 healthy controls, 105 patients with SCAD, and 45 patients with ACS. ACS: acute coronary syndrome; HC: healthy control; SCAD: stable coronary atherosclerosis disease; PR-ACS: prepercutaneous coronary intervention; PO-ACS: postpercutaneous coronary intervention.

of the samples. QC was used to construct the calibration curves and to judge precision. Stability and recovery were within the acceptable range. Acquisition software (Analyst TF1.7 software) continuously evaluated full scan survey MS data (m/z 50–1200) as it collected and triggered the acquisition of MS/MS spectra depending on preselected criteria.

2.4. Statistical Analysis. Overall normalization method was employed in this data analysis. The three-dimensional data, including the peak number, sample name, and normalized peak area were analyzed by the SIMCA14.0 software package (Umetrics, Umea, Sweden) for orthogonal projections to latent structures-discriminate analysis (OPLS-DA). To refine this analysis, the first principal component of variable importance projection (VIP) was obtained. The VIP value exceeding 1.0 was first selected as changed metabolites. Results were presented as mean ± SD. An unpaired, two-tailed Student's t-test was used for comparisons between two groups. All analyses were performed using GraphPad Prism 6.0. Differences were considered significant with $p < 0.05$.

3. Results

3.1. LC-MS Data Analysis. A total of 147 samples and 20 QC samples were obtained, of which 4568 peaks were detected for positive mode and 3516 peaks for negative mode. In order to optimize the data, the substance with RSD > 30% of the quality control samples was deleted. Data with a single group of null values or all groups with a null ≤50% were retained. The area normalization method was used to standardize the data. After processing the data, it remained at 925 peaks and 727 peaks, respectively.

3.2. Distinguished Health and CAD Patients by OPLS-DA Analysis. SIMCA software was used to perform OPLS-DA to maximize the differences of the predictive component. The score plot of OPLS-DA(POS) was shown in Figures 2(a)–2(d). HC group was all located to the left of the midline, while the SCAD group was all located to the right (Figure 2(a)). At the latitude of the first principal component, the two groups were well separated. Compared with the principal component score, the separation trend of the two groups was obvious. The samples were all within the 99% confidence interval (Hotelling T2 Ellipse). Similar results were obtained between HC and PR-ACS, SCAD and PR-ACS, PR-ACS, and PO-ACS, respectively, as shown in Figures 2(b)–2(d). The robustness of OPLS-DA was assessed by 200 times permutation tests. The validated model of OPLS-DA was shown in Figures 2(e)–2(h). The R^2 and Q^2 were 0.926 and −0.44 for HC versus SCAD; 0.963 and −0.449 for HC versus PR-ACS; 0.961 and −0.485 for SCAD versus PR-ACS; and 0.773 and −0.421 for PR-ACS versus PO-ACS, respectively. It implied the validation of these OPLS-DA models. The score plot of OPLS-DA(NEG) exhibited similar results as shown in Figures S1(a)–S1(h).

3.3. Differential Diagnosis of Metabolic Biomarkers. Metabolic biomarkers can provide further information on the metabolic mechanism and biochemical pathway of disease [19, 20]. Therefore, screening for differential markers is an important step in metabolomics analysis. The loading plot of the OPLS-DA model (POS) was shown in Figure 3. The load diagram reflects the weight of the variable in the

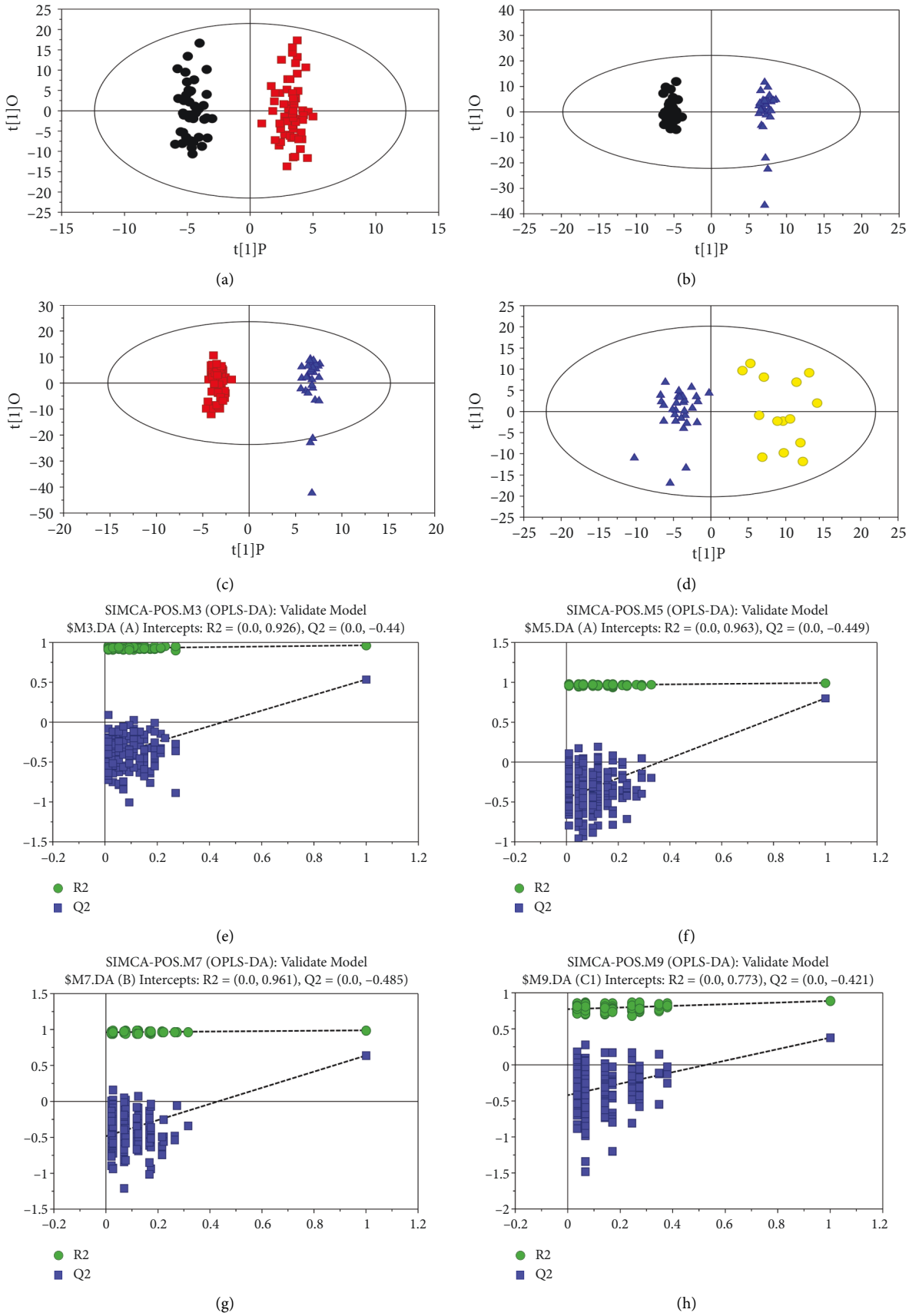

Figure 2: Score plot of and validated model of OPLS-DA obtained from experimental groups (POS). 2(a)–2(d). Score plot of OPLS-DA model obtained from experimental groups. black: HC, red: SCAD, blue: PR-ACS, yellow: PO-ACS. 2(e)–2(h), the validated model of OPLS-DA. 200 times were performed, and the resulting R^2 and Q^2 values were plotted. Green triangle: R^2; blue square: Q^2. The green line represents the regression line for R^2 and the blue line for Q^2.

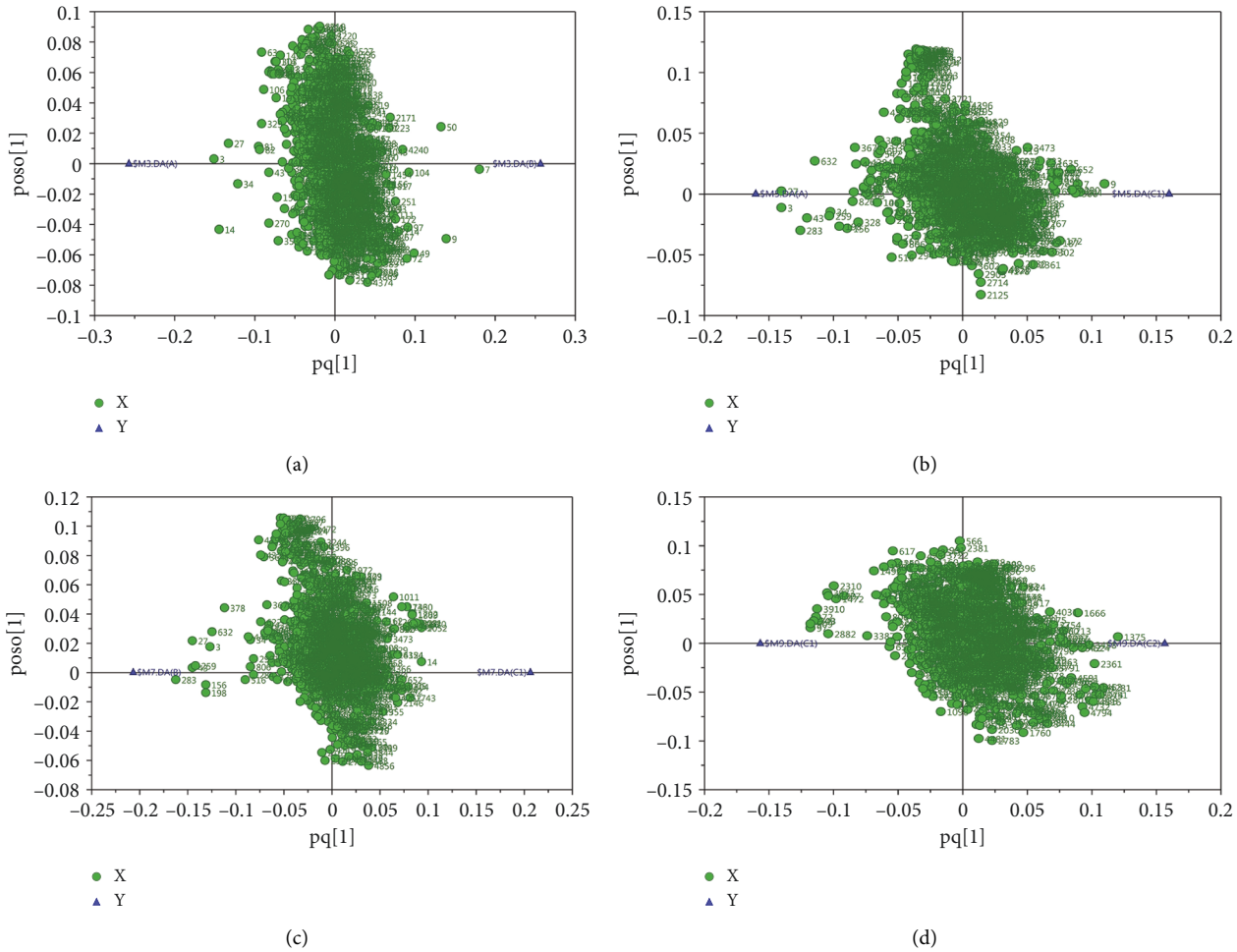

Figure 3: Loading plot of OPLS-DA model obtained from experimental groups (POS). (a) HC versus SCAD, (b) HC versus PR-ACS, (c) SCAD versus PR-ACS, and (d) PR-ACS versus PO-ACS.

principal component. The substances on the left and right sides of the load diagram are the potential altered biomarkers. Results showed that there were specific metabolism biomarkers for health subjects versus CAD patients and SCAD versus ACS. PCI also influenced the metabolites of CAD patients. The mode of loading plot of the OPLS-DA model (NEG) obtained from experimental groups was shown in Figures S2(a)–S2(d).

To evaluate the criteria of metabolomics-based biomarkers, the variable importance in the projection value (VIP) > 1 of the OPLS-DA model and the p value <0.05 adjusted Student's t-test (t-test) were both used to find differential expression of metabolites. In order to identify these metabolites, we further matched the fragments of these metabolites in the MS/MS spectra. The details of metabolic parameters were shown in Table 2. As shown in Table 2, there are 17 altered metabolic biomarkers with a high correlation for HC versus CAD, 15 for SCAD versus PR-ACS, and 7 for PR-ACS versus PO-ACS (POS and NEG).

For identifying the altered metabolites, it is necessary to search the metabolomics database to find the spectrum peak attribution of the possible biomarkers. The KEGG database (https://www.genome.jp/kegg/) was used to screen all the metabolic pathways related to comparison groups. The disturbed metabolic pathways were shown in Supplementary Material 1 based on the KEGG pathway database. Compared with healthy control, we found that the levels of specific metabolites, such as 5-Cholesten-3β, 25(S)-diol, N-Acetyllysine, tyramine, biliverdin, urocanate, phenol, hypoxanthine, L-tryptophane, L-palmitoylcarnitine, were upregulated while the levels of pantetheine, indole, and lecithin were downregulated in CAD patients. Compared with SCAD patients, the levels of α-d-glucose, glycol-cholate, α-tocopherol, inosine, hypoxanthine, L-ornithine, and 5-oxoproline were upregulated in ACS patients. The levels of lecithin were downregulated. Compared with PR-PCI patients, the levels of methacrylyl-CoA, proline, 5-oxoproline, L-proline, primary bile acids, glycine, cholate, adrenosterone, and 1-oleoyl-sn-glycerol 3-phosphate were upregulated and the levels of PE (22 : 5/0 : 0) and bilirubin were downregulated in PO-PCI patients.

3.4. Metabolic Pathway Analysis. MetaboAnalyst 3.0 (https://www.metaboanalyst.ca) performs both metabolic pathway enrichment and topological analysis of different

Table 2: Differentiation of metabolites in experimental groups.

Group	Metabolites	m/z	Retention Time(min)	VIP	p Value	Fold change
HC vs. CAD	N6Acetyl-L-lysine	171.113	0.660	3.620	<0.001	1.847
	Tyramine	120.080	3.011	2.732	<0.001	1.402
	Biliverdin	583.254	4.898	2.45	<0.001	1.936
	25-Hydroxycholesterol	425.340	6.826	1.954	<0.01	1.287
	Phenol	93.034	2.094	2.177	<0.01	1.273
	Urocanic acid	174.988	2.095	1.428	<0.05	1.175
	L-tryptophane	205.097	3.145	2.588	<0.01	1.798
	L-palmitoylcarnitine	422.326	5.880	1.284	<0.05	1.385
	Hypoxanthine	137.045	1.048	1.768	<0.05	1.809
	PE (P-16 : 0/0 : 0)	436.282	6.632	2.466	<0.001	1.212
	PE (P-18 : 1/0 : 0)	462.299	7.579	3.155	<0.001	1.388
	PA (18 : 2/0 : 0)	433.235	5.552	2.272	<0.05	1.739
	PA (20 : 4/0 : 0)	457.235	5.580	1.852	<0.05	1.768
	PC (12 : 0/22 : 2)	758.569	7.152	1.505	<0.05	0.848
	PC (24 : 4/12 : 0)	804.550	7.619	1.764	<0.05	0.866
	Pantothete	220.118	1.444	1.412	<0.05	0.841
	Indole	257.112	1.416	2.068	<0.01	0.846
SCAD vs. PR-ACS	N-Acetyl-L-lysine	171.112	0.660	2.565	<0.05	1.410
	Glycocholic acid	466.328	7.561	2.077	<0.01	1.259
	Alpha-D-Glucose	180.065	3.577	2.243	<0.05	1.247
	N-Acetyl-L-glutamate	265.980	2.871	1.133	<0.05	1.118
	PC (14 : 1/4 : 0)	536.333	4.660	1.841	<0.05	1.291
	α-Tocopherol	431.381	9.694	1.176	<0.05	1.215
	Hypoxanthine	137.045	1.048	2.638	<0.001	2.027
	Ornithine	177.061	0.428	2.022	<0.01	1.309
	PE (P-16 : 0/0 : 0)	436.282	6.632	2.642	<0.05	1.145
	PE (P-18 : 0/0 : 0)	464.314	7.579	2.118	<0.05	1.202
	PA (18 : 2/0 : 0)	433.235	5.552	2.275	<0.01	1.514
	PA (20 : 4/0 : 0)	457.235	5.580	2.083	<0.01	1.561
	PA (22 : 4/0 : 0)	485.266	7.505	2.760	<0.05	1.293
	PC (22 : 5/16 : 1)	828.549	9.560	1.264	<0.05	0.858
	PI (16 : 0/20 : 4)	857.518	8.278	2.147	<0.05	0.738
PR-ACS vs. PO-ACS	L-Proline	116.070	4.212	1.082	<0.01	0.998
	gamma-L-Glutamyl-L-valine	247.128	1.563	2.168	<0.05	1.500
	1-Stearoyl-2-oleoyl-sn-glycerol 3-phosphocholine	786.600	7.686	1.568	<0.01	1.437
	1-Oleoyl-sn-glycerol 3-phosphate	455.259	7.463	1.112	<0.001	1.129
	Glycochenodeoxycholate	430.295	4.037	1.215	<0.05	1.272
	Bilirubin	585.270	4.462	1.812	<0.05	0.784
	PE (22 : 5/0 : 0)	550.289	6.502	2.716	<0.01	0.400

metabolites [21, 22]. The changes in metabolic behavior of different experimental groups in the positive mode were shown in Figures 4(a)–4(d). Compared with HC and SCAD, the metabolisms of glycerophospholipid, linoleic acid, pantothenate, CoA, and primary bile acid biosynthesis were changed. For HC versus PR-ACS, linoleic acid, phenylalanine, tyrosine, and tryptophan biosynthesis, glycerophospholipid were changed. Glutathione, D-arginine, D-ornithine, purine, and glycerophospholipid exhibited different metabolism behaviors compared to SCAD with PR-ACS. For PR-ACS versus PO-ACS, the same metabolic differences of HC versus SCAD in glycerophospholipid, linoleic acid, pantothenate, and CoA were found. Different from HC versus SCAD, arginine proline metabolism was markedly changed compared to PR-ACS with PO-ACS. The metabolic behavior changes of different groups in the negative mode were shown in Figures S3(a)–S3(d). In brief, for HC versus SCAD, the changes in metabolism were histidine, pyrimidine, tyrosine, porphyrin, and chlorophyll; for HC versus PR-ACS, linoleic acid, alpha-linolenic acid, glycerophospholipid, and pyrimidine; for SCAD versus PR-ACS, linoleic acid, alpha-linolenic acid, glycerophospholipid, and arachidonic acid; and for PR-ACS versus PO-ACS, linoleic acid, primary bile acid, fatty acid elongation, and glycerophospholipid. Changes in glycerophospholipid metabolism were found almost in all the comparison groups which suggested that glycerophospholipid had a significant impact on CAD.

4. Discussion

The metabolic biomarkers of CAD have been reported in many studies [21]. However, using metabolomics for the early diagnosis of CAD in terms of both stable and unstable plaques is limited, especially in Chinese. The majority of these studies paid more attention to the lipids metabolites [11, 12]. Many altered metabolites with different chemical structures were not presented. In this work, we screened all

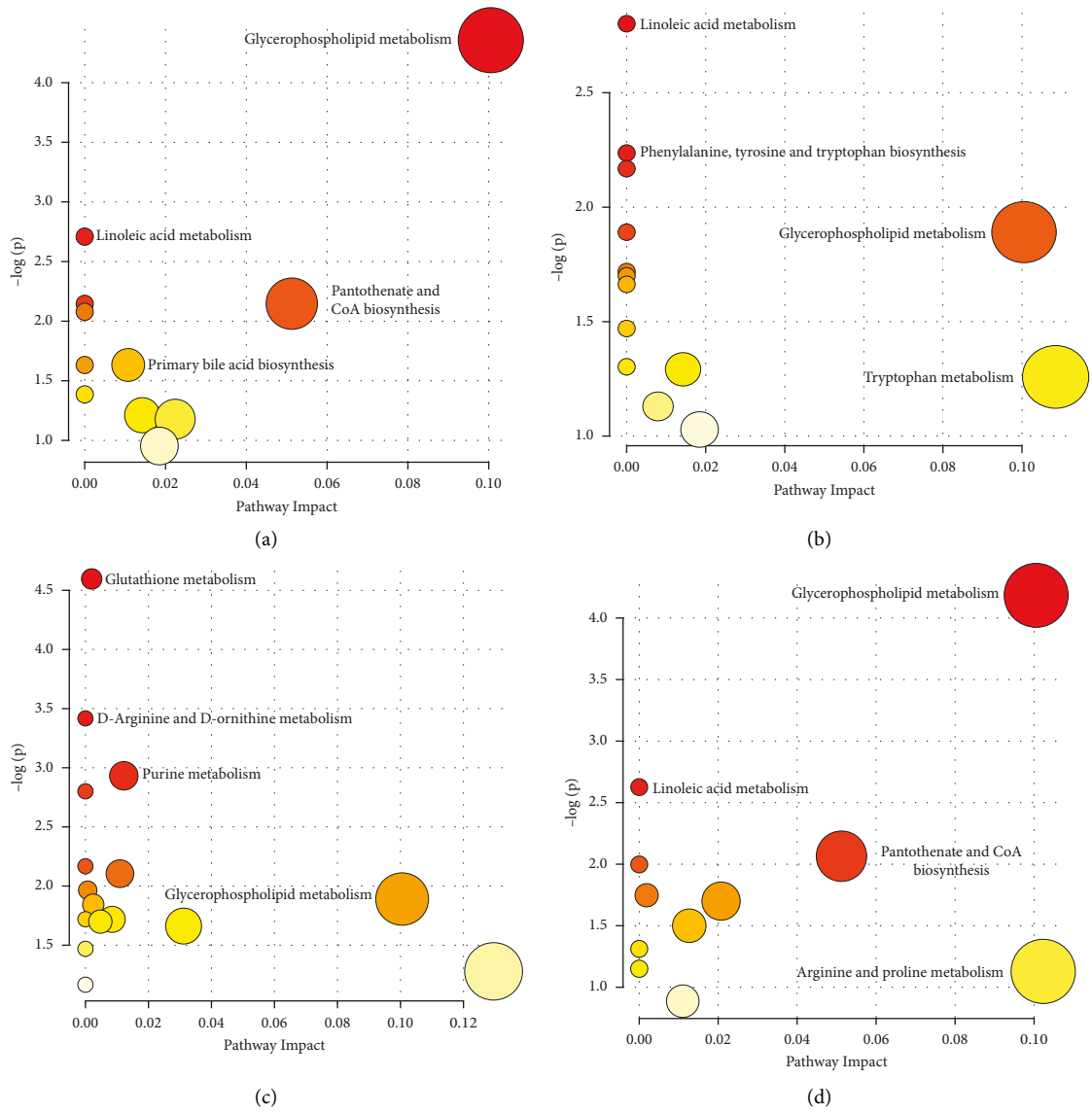

Figure 4: Pathway analysis of experiment group (POS). The larger the circle meant the greater the influence of topology analysis; the redder the color meant the smaller the p value, and vice versa. (a) HC versus SCAD, (b) HC versus PR-ACS, (c) SCAD versus PR-ACS, and (d) PR-ACS versus PO-ACS.

the metabolites in the experimental human groups using a metabolomics approach in an unbiased way. The altered metabolites were selected by OPLS-DA which could reduce the false positives in the data. The identified metabolites were matched with published literature and online resources. The differentiated metabolites of healthy subjects and stable and unstable CAD patients were compared. Furthermore, we focused not only on the metabolism differences between healthy and CAD patients but also on the SCAD and ACS group, PR-PCI and PR-PCI group, which were almost no relevant reports. Our results demonstrated that the accurate model could identify novel biomarkers in different types of CAD.

Previous studies have demonstrated a wide range of metabolites associated with CAD [23–36]. Our study has a high correlation with some of them. Lysine acetylation modification is a reversible posttranslational modification that affects enzyme activity, DNA binding force, and protein stability by changing the charge on lysine residues and the structure of proteins. Wang et al. found higher N-acetyl-threonine levels were identified to be a biomarker associated with heart failure risk [12]. Li et al. reported lysine acetylation was found closely related to CAD [24]. In our studies, N-Acetyl-lysine was upregulated in both SCAD and ACS patients compared with healthy control. This was consistent with previous research. Serum sterols were a risk factor for CAD [25]. Abnormal metabolic pathways of cholesterol to bile acid could lead to cholesterolemia, which was involved in the occurrence and development of CAD [26]. Bhat et al. found that low excretion of bile acids might promote CAD [27]. When compared with healthy control, 5-Cholesten-β, 25(S)-diol, and biliverdin were found upregulated in CAD

patients. It suggested that the primary bile acids were decreased in CAD patients. As an ischemia marker, hypoxanthine had been identified in ACS [28]. As an ATP degradation product, upregulation of hypoxanthine was observed in CAD patients in this study. We found that the energy metabolism was different between healthy and CAD subjects. Tryptophan, an ingredient to generate amino acids, was at high levels in CAD patients. A previous study indicated that activated amino acid biosynthesis was an indicator for CAD [29]. As the most important part of the urea cycle, ornithine was obtained from arginine by arginase. Arginine was negatively associated with CAD risk which had been reported [30]. Compared with patients with normal coronary arteries, patients with CAD downregulated lecithin, phosphatidylcholine, pantetheine, and indole as shown in Supplementary Material 1. Low lecithin cholesterol acyltransferase activity had been linked to CAD [31]. Many phosphatidylcholines (PCs) exhibited a negative association with CAD [32]. Our results showed that PC (12 : 0/22 : 2) and PC (24 : 4/12 : 0) were downregulated in CAD patients, which was consistent with other studies [33]. The changed insulin sensitivity and glycemic control were associated with an increased cardiovascular risk in previous reports [34]. The adenosine, inosine, and hypoxanthine, which were released by the oxygen-deprived heart were AMP catabolites. Inosine is a sensitive and early indicator of wall-thickness changes in the ischemic pig hearts [35]. Vitamin E with its major isoforms α-tocopherol (α-T) and γ-tocopherol (γ-T) and reduced glutathione (GSH) are the main antioxidants in the blood. Supplementation with antioxidant micronutrients could be beneficial for CAD [36]. The levels of lecithin were downregulated in CAD patients. Plasma lipids and fatty acids had been linked to CAD. Linoleic acid deficiency had been proposed as a risk factor for cardiovascular disease. Decreased hexadecanoic suggested an elevated level of fatty acids in the metabolism [37].

Different from other studies, we also obtained some new biomarkers in our study. Our results indicated that amino acid metabolism and biosynthesis could also be used as a new marker to distinguish SCAD from ACS. Tyrosine was increased in CAD patients which suggested the role of amino acid disorder in the CAD process. In addition, L-ornithine and ornithine were also found to significantly increase in the ACS group which had not been reported. PE and PC are two major subclasses of glycerophospholipids. PE is a glycerophospholipid in which a phosphoryl ethanolamine moiety occupies a glycerol substitution site. Though some Lyso PC, PC, and Lyso PE were identified to have a negative association with CAD, different classes of PC and PE might be expressed differently. Xu et al. reported most PE species showed no significant differences between AMI and stable angina patients [32]. It seemed that PEs had a strong negative association with CAD. However, in this study, the levels of PEs (P-16 : 0/0 : 0) and PE(P-18 : 0/0 : 0) were both upregulated in healthy versus CAD and SCAD versus ACS. The results suggested that some PEs might contribute to unstable plaque progress. PA (18 : 2/0 : 0) and PA (20 : 4/0 : 0) exhibited different expression levels compared with the CAD group and healthy control in our studies. However, PA (18 : 2/0 : 0), PA (20 : 4/0 : 0), and PA (22 : 4/0 : 0) were increased in ACS patients (versus SCAD), which could be used as a distinction between ACS and SCAD. PA is rarely reported as a biomarker for CAD diagnosis. As primary bile acids were found to be decreased in CAD patients, the higher level of PA might attribute to the cost of cholesterol in the synthesis of bile acids. The increased α-d-glucose in ACS patients was blood-related to the energy metabolism disorder compared to patients with SCAD. Interestingly, α-tocopherol and 5-oxoproline were upregulated in ACS patients compared with SCAD which suggested the body's autoregulatory function was stronger in ACS. We further investigated the influences of PR-PCI and PR-PCI treatment on patients with ACS. Besides proline, 5-oxoproline, L-proline, primary bile acids, glycine, cholate, the levels of methacrylyl-coA, and adrenosterone of PO-PCI were upregulated compared with PR-PCI patients. PE (22 : 5/0 : 0) and bilirubin were downregulated in PO-PCI patients which alleviated the symptom of CAD. Several new biomarkers were identified from this study with PCI treatment for CAD. These newly found biomarkers enhanced the power for discrimination of different types of CAD.

5. Limitation of This Study

The limitations of this study mainly include the following aspects. Firstly, although the UPLC-MS technology has high detection sensitivity [33, 34], there are still many difficulties in the identification and accurate quantification of trace substances [35]. Secondly, in this study, the number of subjects was relatively small, and the required sufficient samples are not obtained. Whether there are metabolic differences between men and women is not clear. Thirdly, the metabolites of HC, SCAD, ACS, and ACS treated by PCI or not were screened out only by LC-MS. These different metabolites still need to be verified through tracking the related upstream and downstream genes or enzymes in subsequent studies.

6. Conclusion

This study used UPLC-MS for metabolomics analysis in healthy subjects, SCAD, and ACS with PR-PCI or PO-PCI in positive and negative modes. There were 17 different metabolites between the healthy subjects and SCAD, 15 between SCAD and ACS, and 7 between PR-PCI and PO-PCI groups. The results suggested that CAD was closely related to energy metabolism, lipid metabolism, and glucose metabolism disorders. In summary, the altered metabolites can be used as special metabolic biomarkers for patients with different types of CAD in the early diagnosis.

Abbreviations

ACS: Acute coronary syndrome
BMI: Body mass index
CT: Computed tomography
CAD: Coronary atherosclerotic heart disease
GSH: Glutathione

HC: Healthy controls
IVUS: Intravascular ultrasound
NEG: Negative mode
OPLS-DA: Orthogonal projections to latent structures-discriminate analysis
PCI: Percutaneous coronary intervention
PA: Phosphatidic acid
PE: Phosphatidyl ethanolamines
PC: Phosphatidylcholine
POS: Positive mode
PCA: Principal component analysis
QC: Quality control
SBP: Systolic blood pressure
SCAD: Stable coronary artery disease
3D: Three-dimensional
2D: Two-dimensional
UPLC-MS: Ultra-performance liquid chromatography-mass spectrometry
VIP: Variable importance projection.

Data Availability

The data in the used to support the findings of the study are available in Supplementary Information files.

Consent

The study was performed under the guidance of an institutional ethical committee from Daqing Oilfield General Hospital following the Helsinki Declaration. Informed consent was obtained from all patients being included in the study.

Conflicts of Interest

The authors declare no conflicts of financial interests.

Acknowledgments

This study was supported by Daqing Oilfield Co. Ltd. Science and Technology Projects.

Supplementary Materials

Supplementary Material 1: metabolic pathways analysis based on the KEGG pathway database in all experimental groups. Figure S1: Figure S1(a)-S1D. Score plot of OPLS-DA model obtained from experimental groups (NEG). black: HC, red: SA, blue: PR-ACS, yellow: PO-ACS. Figure S1(e)-S1H, the validated model of OPLS-DA. 200 times were performed, and the resulting R2 and Q2 values were plotted. (Green triangle): R2; (blue square): Q2. The green line represents the regression line for R2 and the blue line for Q2. Figure S2: loading plot of OPLS-DA model obtained from experimental groups (NEG). A: HC versus SCAD, B: HC versus PR-ACS, C: SCAD versus PR-ACS, D: PR-ACS versus PO-ACS. Figure S3: pathway analysis of experiment group. The larger the circle meant the greater the influence of topology analysis; the redder the color meant the smaller the p value, and vice versa. A: HC versus SCAD, B: HC versus PR-ACS, C: SCAD versus PR-ACS, D: PR-ACS versus PO-ACS (NEG). (*Supplementary Materials*)

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Hindawi
Cardiology Research and Practice
Volume 2022, Article ID 5108389, 8 pages
https://doi.org/10.1155/2022/5108389

Research Article

Radial Artery Calcification in Predicting Coronary Calcification and Atherosclerosis Burden

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Received 14 March 2022; Revised 3 May 2022; Accepted 17 May 2022; Published 31 May 2022

Academic Editor: Fanbo Meng

Background. Atherosclerosis is a systemic arterial disease with heterogeneous involvement in all vascular beds; however, studies examining the relationship between coronary and radial artery calcification are lacking. The purpose of this study was to assess the relationship between the two sites and the prognostic value of radial artery calcification (RC) for coronary artery disease. *Methods*. This is a single-center, retrospective cross-sectional study based on Doppler ultrasound of radial artery (RUS) and coronary artery angiography (CAG). We included a total of 202 patients undergoing RUS during distal radial access and CAG at the same procedure, between December 2020 and May 2021, from which 103 were found having RC during RUS (RC group) and 99 without (NRC group). Coronary calcifications were evaluated either by angiography examination (moderate and severe), positive CT (>100 Agatson units), or intracoronary imaging (IVUS, OCT). *Results*. A significant correlation was observed between radial calcification and coronary calcification variables (67.3% vs. 32.7%, $p = 0.001$). The correlation between risk factors such as age, smoking, chronic kidney disease, and diabetes mellitus was higher while sex did not play a role. The need of PCI and/or CABG was higher in the RC group (60% vs. 44%, $p = 0.02$). RC, therefore, predicts the extent and severity of coronary artery disease. *Conclusion*. RC may be frequently associated with calcific coronary plaques. These findings highlight the potential beneficial examination of radial arteries whenever CAD is suspected.

1. Introduction

Asymptomatic individuals with significant coronary artery disease (CAD) are at risk of unanticipated cardiac events including myocardial infarction (MI). Laboratory studies, stress tests, and coronary artery imaging including coronary artery calcification (CAC) scoring are used for evaluating at-risk individuals. CAC scoring has been demonstrated to not only show current coronary disease but also predict future cardiac events [1–3]. Coronary artery calcification and cardiac valve calcific deposits correlate well and predict mortality in the general population [4, 5]. There also seems to be a strong association between carotid and coronary stenosis [6–9]. While carotid examination in CAD and vice versa has become of clinical importance in order to accurately identify patients who could benefit from aggressive preventive therapies as well as timely treatment, no relationship between radial and coronary arteries has been investigated. Based on the shared underlying atherosclerosis pathology in the two arterial systems, this study aimed to explore whether the extent of calcifications in the two arteries is correlated and if RC is a parameter for predicting CAD.

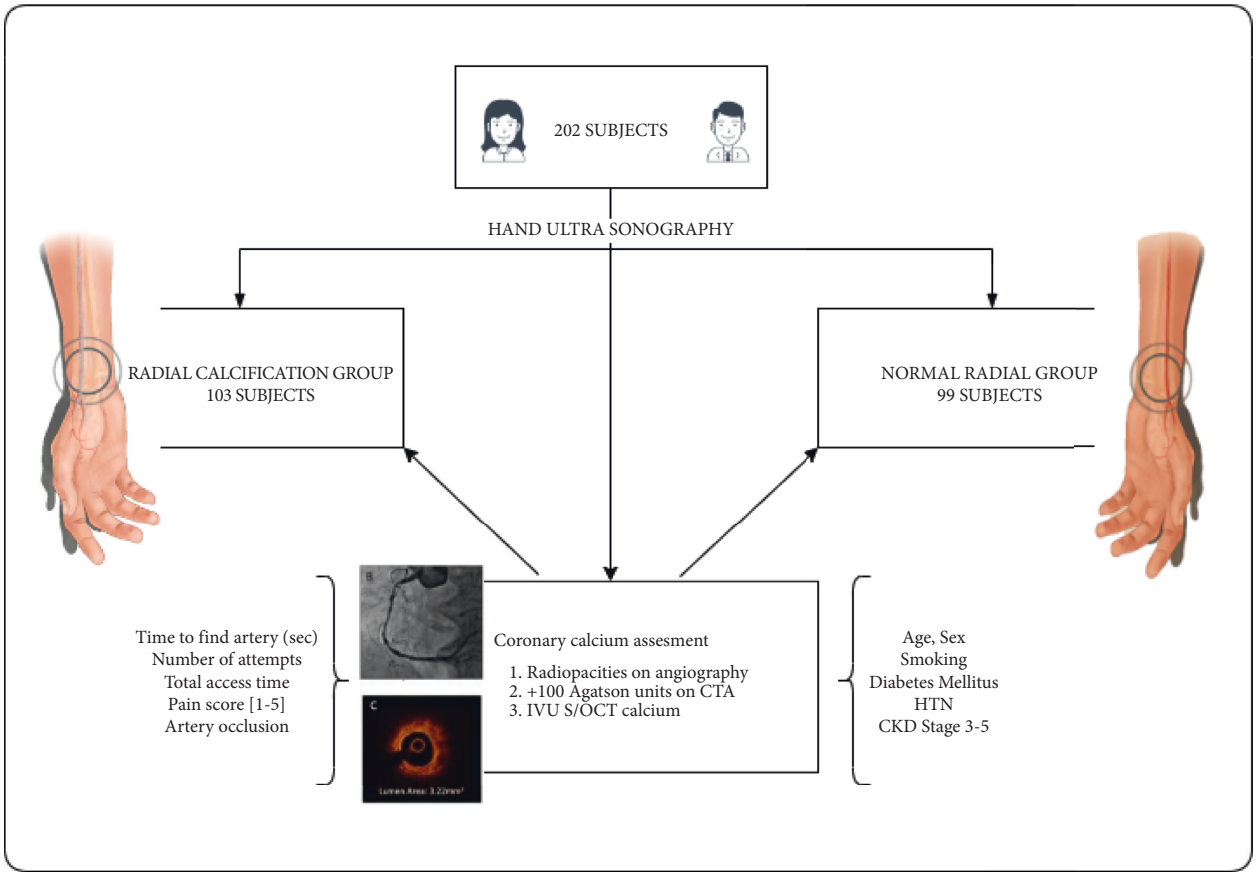


Figure 1: Study design and patient selection. Inclusion in each group was done blindly and retrospectively.

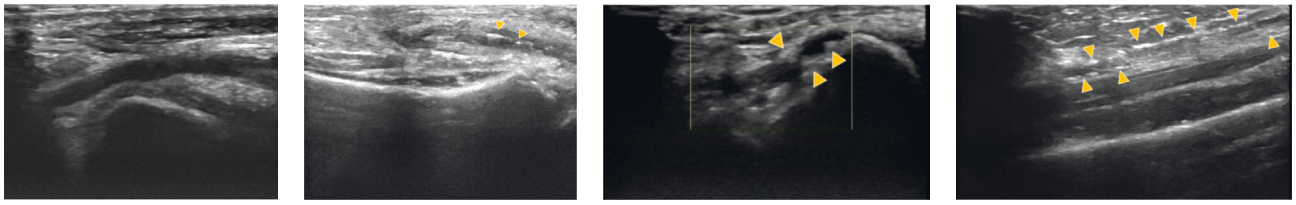
2. Methods

2.1. Study Population and Design. From December 2020 to May 2021, 202 consecutive patients who underwent coronary angiography and who required radial Doppler ultrasound examination were recruited in the study. All patients from this period who came to our catheterization laboratory for various transcatheter procedures were included, in the context in which they received standardized pre- and postoperative RUS evaluation of the radial artery [10–12]; the only inclusion criterion was therefore the invasive cardiovascular evaluation, where the ultrasound images were clear, conclusive, and could be noted retrospectively. Design of the study is presented in Figure 1. The 2 groups were divided, according to the sonographic result at the level of the radial artery. Coronary status was analyzed as a follow-up. A correlation was made between the two arterial systems, with emphasis on the most relevant risk factors and the coronary outcome.

2.2. Calcification Assessment. All patients underwent RUS-assisted distal radial puncture, as per center's protocol, scanning the artery at the anatomical snuffbox area, using a 7.5 MHz probe. Cross sections of the radial artery were assessed using the following factors: lumen diameter, vessel diameter, plaque distribution, and percent plaque area, with particular attention given to the type and extent of calcium deposition (diffuse vs. nodular, medial vs. intimal). RC was visually assessed accordingly, assigning scores in each of two calcification categories based on ultrasound findings, as follows: longitudinal involvement, 0 = no calcification, 1 = focal calcification, and 2 = diffuse calcification; density, 0 = no calcification, 1 = light calcification, and 2 = dense calcification. The designation of light versus dense calcification was purely qualitative. A calcification index was derived and patients with a score of minimum 2 pcts were considered positive and included in the RC group. Only clear echoreflective areas with acoustical shadowing associated with calcific plaques, as exemplified in Figure 2, were included.

As step two, quantitative analysis of the angiographic images was performed by a single individual blinded to the ultrasound results. Positive coronary calcification was defined as one of the following: (1) on angiography, radiopacities readily visible but mild degree and/or obvious, heavy calcification seen without cardiac motion, before contrast injection; (2) on cardiac CT, calcium score above 100 Agatson units; (3) during intracoronary imaging (IVUS, OCT), the presence of an arc of calcium >180°, length >5 mm, and calcium thickness >0.5 mm.

Significant CAD was defined by the need of PCI and/or CABG. Additional risk factors (age, sex, smoking, diabetes mellitus, primary hypertension, and renal failure) and radial



(a) (b) (c) (d)

Figure 2: Ultrasound scanning of the distal radial artery, showing normal aspect (a) and calcific deposits within the vessel wall (yellow arrows), organized as calcific nodules (b), calcific plaques (c), and diffuse mediocalcinosis (d).

access performance indexes (time to find artery [sec], number of attempts, access time [sec], pain score [1–5], and artery occlusion) were analyzed.

2.3. Statistical Analysis. Continuous variables were expressed as mean ± standard deviation. Statistical analyses were performed using IBM SPSS v26.0 (Chicago, IL, USA). Correlations between dichotomious variables were performed using the Pearson Chi Squared test, or Fisher's test. Median values between the two groups were compared using Mann–Whitney U test. A multivariable logistic regression analysis was performed to identify independent predictors of RC. All p values were two-sided, and $p < 0.05$ was considered statistically significant.

Written informed consent was obtained from all patients, and the Institution's Ethics Committee approved the study.

3. Results

Baseline characteristics are presented in Table 1. There was no difference in sex across the two groups, but the mean age of the RC group was significantly higher (69.24 ± 9.80 years vs. 63.35 ± 11.59 years, $p = 0.001$). The full spectrum of patients was included but the main indication for coronary angiography remained to be stable angina (40%).

Representative duplex ultrasound images of normal and calcified radial arteries are shown in Figure 2. The normal artery (Figure 2(a)) is characterized by a thin, homogeneous wall and a smooth, luminal surface. Calcifications (Figure 2(b)–2(d)) appear as echoreflective areas within the vessel wall (not to be confused with tissue streaking seen in the soft tissues of both normal and calcific studies) and are associated with acoustical shadowing. The calcified vessel in Figures 2(b)–2(d) is narrower in caliber and exhibits an irregular luminal surface.

There was a statistically significant association between the presence of radial calcinosis and coronal calcification ($p = 0.001$). The usage of PCI and/or CABG was significantly higher in the patients with radial calcinosis ($p = 0.02$) (Table 2).

Several comorbidities were evaluated. An unadjusted analysis was performed to establish the risk factors involved in the presence of the radial calcinosis (Table 3). Out of a total of 19 smokers, 16 (84.21%) of them presented radial calcinosis ($p = 0.001$). Patients with renal failure had a higher frequency of renal calcinosis (69.23%, 45/65) than the patients without renal failure (42.33%, 58/137), the difference being statistically significant ($p = 0.001$). A statistically significant correlation was established between the presence of renal calcinosis and diabetes (55.97%, 89/159 vs. 32.55%, 14/43, $p = 0.001$). No statistically significant correlations between either hypertension or artery occlusion and the presence of radial calcinosis were found.

Afterwards, a multivariable logistic regression analysis was performed (Table 4), demonstrating that age over 60 ($p = 0.001$, OR 3.4, 95% CI), smoking ($p = 0.03$, OR = 4.9, 95% CI), renal failure ($p = 0.01$, OR = 2.3, 95% CI), and diabetes ($p = 0.03$, OR = 2.3, 95% CI) were independently associated with radial calcinosis.

A series of parameters involved in the performance of the radial puncture were compared between the two groups. The mean value of the time to find artery was significantly higher in the patients who presented radial calcinosis (median time 3 minutes vs. 2 minutes, $p = 0.01$). There were no statistically significant differences regarding the number of attempts, access time, or pain score (Table 5).

4. Discussion

The main findings of our study were (1) significant correlation between radial and coronary calcification in adults presenting with angina symptoms and associated risk factors and (2) the rate of revascularization treatment was higher in this population, suggesting the potential of radial artery calcification to become a new marker of prediction of severe coronary artery disease.

Based on our study, we suggest that incidental findings of upper extremity artery calcification on routine radiographs or Doppler ultrasound may warrant systemic evaluation for atherosclerosis in other areas of the body, especially screening for CAD. Increasing RC occurrence correlated with CAC, but more importantly with more advanced CAD (60% rate of PCI/CABG in the RC group vs. 44% in the NRC group). Latest European prevention guidelines state that CAC scoring may be considered to improve risk classification, and plaque detection by carotid ultrasound is an alternative when CAC scoring is unavailable or not feasible (level of recommendation IIb) [13]. Thus, the theory of including RUS as another alternative is attractive.

Risk factors seem to play a role for arterial calcification. Our study confirmed that radial calcinosis is more frequently found in population above 60 years, smokers, diabetics,

Table 1: Baseline characteristics of all 202 patients.

Demographic features	Mean ± SD/N (%)		p value
	RC group ($n = 103$)	Non-RC group ($n = 99$)	
Age (years)	69.24 ± 9.80	63.35 ± 11.59	0.07
Gender: female/male, % (n)	43.6% (45)/56.3% (58)	40.4% (40)/59.6% (59)	0.44
Height (cm)	169.4 ± 8	169.05 ± 5	0.92
Weight (kg)	84 ± 15	87 ± 16	0.23
Prior Comorbidities			
Atrial fibrillation	17 (16.5%)	21 (21.2%)	0.39
Renal failure	45 (43.6%)	20 (20.2%)	0.003
Diabetes mellitus	89 (86.4%)	70 (70.7%)	0.006
Hypertension	45 (43.6%)	33 (33%)	0.13
Smoking	16 (15.5%)	3 (3.03%)	0.002
Family History	14 (13.6%)	11 (11.1%)	0.59
Dyslipidemia	29 (28.1%)	23 (23.2%)	0.42
Previous MI	12 (11.65%)	10 (10.1%)	0.72
Previous CABG	8 (7.7%)	3 (3.03%)	0.13
Indication for Catheterization			
Stable angina	42 (40.7%)	40 (38.8%)	0.95
Unstable angina	12 (11.6%)	8 (8.08%)	0.39
NSTEMI	22 (21.3%)	17 (17.1%)	0.45
STEMI	8 (7.7%)	14 (14.1%)	0.14
Heart failure	3 (2.9%)	2 (2.02%)	0.92
Severe aortic stenosis	5 (4.8%)	7 (7.07%)	0.45
Peripheral interventions	6 (5.8%)	8 (8.08%)	0.55
Other	9 (8.7%)	3 (3.03%)	0.32

CABG: coronary artery bypass graft; MI: myocardial infarction; NSTEMI: non-ST elevation myocardial infarction; RC: radial artery calcification; SD: standard deviation; and STEMI: ST elevation myocardial infarction.

Table 2: Association between the presence of coronary calcification and presence of radial calcinosis (top). Association between the usage of PCI and the presence of radial calcinosis (bottom).

Parameters		Radial calcinosis	No radial calcinosis	p value
Coronary calcification	Present	68	33	0.001
	Absent	35	66	
PCI/CABG	Used	62	44	0.02
	Not used	41	55	

CABG: coronary artery bypass graft; PCI: percutaneous coronary intervention.

Table 3: Unadjusted analysis of the risk factors involved in the presence of radial calcinosis.

Parameters		Radial calcinosis	No radial calcinosis	p value
Smoking	Smoker	16	3	0.001
	Non-smoker	87	96	
Renal failure	Absent	58	79	0.001
	Present	45	20	
Diabetes	Absent	14	29	0.001
	Present	89	70	
Hypertension	Absent	58	66	0.08
	Present	45	33	

Table 4: Multivariable logistic regression analysis of the risk factors involved in the presence of radial calcinosis.

	B	S.E.	Wald	df	Sig.	Odds ratio	Confidence interval
Age over 60	1.236	0.371	11.091	1	0.001	3.443	3.102–3.774
Smoking	1.453	0.669	4.711	1	0.03	4.875	3.921–6.118
Renal failure	0.855	0.346	6.095	1	0.014	2.352	2.091–2.797
Diabetes	0.845	0.402	4.424	1	0.035	2.328	1.762–3.111
Hypertension	0.579	0.311	3.294	1	0.07	1.764	1.394–2.122

B = beta coefficient; S.E. = standard error; Wald = the Wald test; df = degrees of freedom; and sig = statistical significance.

Table 5: Median values of the parameters involved in the performance of the radial punction (interquartile ranges).

Parameters	Radial calcinosis	No radial calcinosis	p value
Time to find artery (minutes)	3 (2, 10)	2 (1, 5)	0.01
Number of attempts	2 (1, 3)	2 (1, 2)	0.09
Access time (minutes)	37 (20, 60)	35 (20, 50)	0.16
Artery occlusion			
Absent	73	97	0.4
Present	4	2	

Between the two groups, artery lumen patency at 48 h follow-up, documented by RUS examination, showed a numerically higher occlusion rate in the RC group, which was not statistically significant (5.19% vs. 2.02%, $p = 0.4$).

hypertensives, and chronic kidney disease patients, with a strong emphasis on smoking (4.8 times higher risk).

Our findings are clinically important for several other reasons. First, RUS may serve as a pre- and peri-procedural adjuvant tool for the interventionist, facilitating a "per primam" selection of coronary calcium debulking technique, intuiting stent underexpansion, and preparing the interventionist to expect a more difficult sheath placement or even radial access failure, with a longer, more complex procedure. Not losing the radial access advantages in complex PCIs of severe calcific disease is of paramount importance [14]. Second, RUS may be useful to cardiovascular surgeons, since the radial artery is commonly used as a conduit for coronary artery bypass and the presence of calcifications may reduce suitability of this graft. Third, the strong relationship we found between RC and severity of coronary artery disease and stenosis not only serves to predict the presence of severe disease, but also aids in the identification of patients demonstrating established arterial disease who need intensive risk factors control and follow-up management.

For many decades, vascular calcification has been noted as a consequence of aging. Studies now confirm that vascular calcification is an actively regulated process and shares many features with bone development and metabolism. It occurs in two sites, the tunica intima and the tunica media, with different disease association and outcomes (Figure 3).

The intimal layer of the vessel wall is normally composed of endothelial cells and a small amount of subendothelial connective tissue. In atherosclerosis, the intima becomes greatly inflamed and thickened and calcification occurs. Natural history is that microcalcifications may arise inside lipid pool following the apoptosis of smooth muscle cells or macrophages. They coalesce into larger mases over time to form speckles, further progressing to calcified sheets or plates. Fragmentation of these sheets leads to nodules that may extend to the lumen and become protuberant with discontinuation of the endothelium [15]. Calcification of coronary arteries is an excellent predictor of atherosclerotic plaque burden and may contribute to atherosclerotic plaque rupture, though the connection between atherosclerotic plaque calcification plaque rupture is heavily debated. Several studies show a link between high CAC and risk of cardiac events and mortality, yet some studies have suggested that the most calcified plaques may be more stable, and that the plaques most vulnerable to rupture may be those which have a mixed composition of calcified and uncalcified tissue [16]. Indeed, unstable lesions are associated with focal calcium deposits that may be related to fibrous cap disruption [15]. Calcium in a spotty distribution has previously been observed, pathologically, in sudden coronary death victims [17]. While spotty calcification was more commonly associated with unstable plaques, extensive calcification was more common with stable plaques [17].

The medial layer of the vessel wall is composed of smooth muscle cells and elastin-rich extracellular matrix. Calcification of the media occurs preferentially along the elastic lamina, as opposed to the diffuse localization seen in intimal calcification, and is associated with diabetes, kidney disease, hypertension, and osteoporosis (also referred to as Monckeberg's sclerosis). The result of medial calcification is a stiffening of the artery wall, with an associated rise in blood pressure, and a higher risk of cardiovascular mortality than that of intimal artery calcification, because left ventricular strain, hypertrophy, and decreased myocardial perfusion during diastole appear as maladaptive mechanisms [16, 18, 19].

At the same time, both layers can be affected simultaneously, with exponential harmful effect [20]. RUS can detect both forms of vascular calcification, as illustrated in Figure 2. Forearm fluoroscopy can also very obviously detect mediocalcinosis. An illustrative example is Figure 4, which shows how pregnant mediocalcinosis is and how distinctly it can be seen on a forearm X-ray. Such diffuse changes are most common in end-stage kidney disease. Our center is a dedicated ultrasound-assisted distal radial access center, having switched to this approach since 2019 [10–12]. Duplex US was used in the operating room to investigate all forearm arteries. RA diameter and peak systolic velocity were measured at the wrist level. We believe the use of ultrasound guidance enables the operator to identify important anatomical landmarks and avoid injuring adjacent structures. US can be also used to determine whether the lumen is large enough to accommodate the necessary sheath and check for calcifications that can block the equipment delivery. Therefore the RC aspect is also relevant for the operator's success as it can affect performance index. In our study, time of puncture and the number of attempts were similar across the two groups, but the total time to find the artery by US as well as the artery occlusion rate was higher in the radial calcification population (Table 5).

Vascular ultrasound-based imaging techniques allow relatively inexpensive and nonevasive widely available means to detect VC and to differentiate between

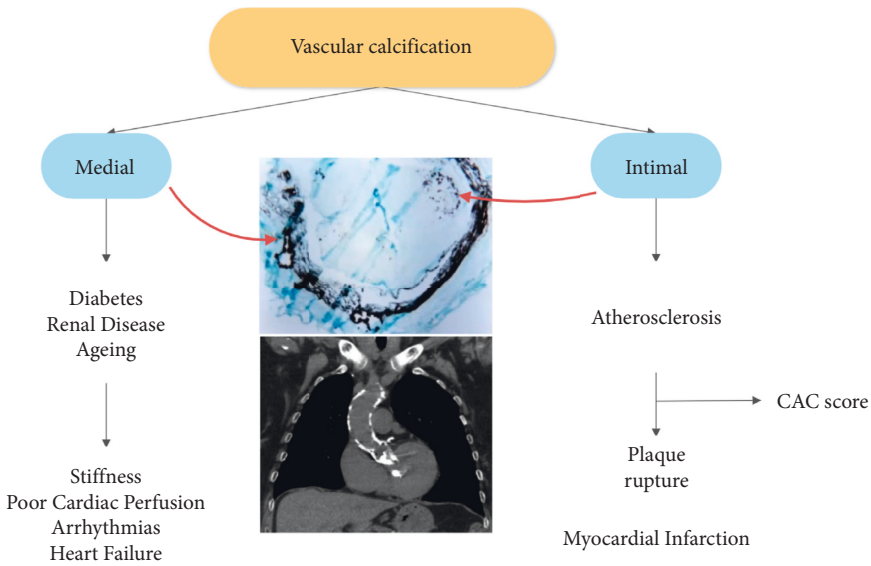


Figure 3: Site-specific phenotype of calcific lesions according to their location within the arterial wall.

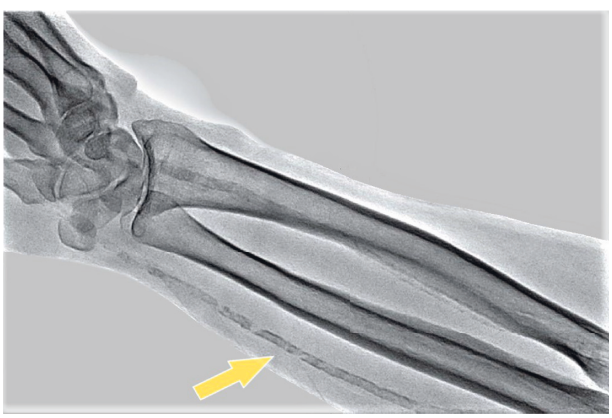


Figure 4: Forearm radiography: diffuse mediocalcinosis along the entire length of the radial artery (arrow).

Mönckeberg's medial calcific sclerosis and the atherosclerosis-related lesions and assess arterial wall abnormalities, such as intima-medial wall thickening and endothelial dysfunction [21]. This has been well described within peripheral arterial disease, predominantly chronic limb threatening ischemia [22] and carotid atherosclerosis [23] where US is a valuable tool for disease and risk assessment, indicated by the guidelines. Our findings are in line with the consistent evidence that VCs affects the entire arterial tree, adding another vessel to the puzzle and draws attention upon careful radial artery evaluation, especially when US is performed before and during cannulation anyhow.

4.1. Study Limitations

(1) Although detecting subclinical atherosclerosis is valuable in risk stratification, we must acknowledge that direct proof that such detection translates into a better outcome is lacking, although several reports suggest that the frequency of use of risk-modifying interventions is increased when subclinical atherosclerosis is detected.

(2) Although obtained in a small sample, these results indicate the usefulness of radial ultrasound as a further screening tool to identify patients who deserve consideration for a coronary noninvasive test. It is important to note, however, that this study was performed in patients who had undergone cardiac catheterization because of suspected or proved heart diseases; thus, whether our results can be extended to patients without a cardiovascular history remains to be defined.

(3) Significant ischemic coronary disease was defined by the decision to continue with/history of PCI or/and CABG. Although coronary revascularization is a medical decision based on proven myocardial ischemia, not all significant coronary stenoses are followed by correct treatment and some nonsignificant coronary stenoses are overtreated.

(4) It should be emphasized that the calcification scoring and evaluation is operator dependent, therefore subjective; however, we have adopted a policy of not exploring arteries that appear "borderline" or poor-quality images, projections, and so on; only clear calcific disease was counted as positive. Coronary angiography has low-moderate sensitivity compared to IVUS and CT for detection of CAC but is very specific (high positive predictive value) [24, 25].

(5) Another limitation of the current study is the lack of histologic data to correlate with duplex findings. Histologic data would be helpful because the precise level of calcifications within the vascular wall cannot be determined by the imaging technique used, and the underlying pathology (atherosclerosis vs. Monckeberg's sclerosis), therefore, cannot be determined either.

5. Conclusion

RC may be associated with calcific coronary plaques frequently. These findings highlight the potential beneficial examination of radial arteries whenever CAD is suspected.

Abbreviations

RC: Radial calcification
RUS: Radial ultrasound
CAG: Coronary artery angiography
CAD: Coronary artery disease
CT: Computer tomography
MI: Myocardial infarction
CAC: Coronary artery calcification
PCI: Percutaneous coronary intervention
CABG: Coronary artery bypass grafting
IVUS: Intracoronary vascular ultrasound
OCT: Optical coherence tomography.

Data Availability

The patient data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The authors report no financial relationships or conflicts of interest regarding the content herein. Current study received the proper ethical oversight.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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Hindawi
Cardiology Research and Practice
Volume 2022, Article ID 7981637, 7 pages
https://doi.org/10.1155/2022/7981637

Research Article

Association of Low Expression of *NUMB* in Peripheral Blood with Acute Myocardial Infarction

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Received 16 February 2022; Accepted 12 April 2022; Published 26 April 2022

Academic Editor: Wei Jiang

Objective. Our study's goal was to find out acute myocardial infarction (AMI) patients' *NUMB* gene expression patterns and to evaluate its role as a diagnostic marker for AMI detection. *Methods*. Peripheral blood was drawn from 124 individuals who had an AMI and 115 patients who had stable coronary artery disease (SCAD). The real-time quantitative polymerase chain reaction was used to measure the mRNA expression level of the *NUMB* gene in peripheral blood. *Results*. The AMI group's *NUMB* gene expression was 0.906 (0.181–0.954), whereas the SCAD group's expression was 1.024 (0.207–1.127). However, the AMI group had 0.885 times lower *NUMB* mRNA expression than the SCAD group ($P < 0.05$). *Conclusion*. Multivariate logistic regression evaluation found that lower *NUMB* expression was correlated with an increased risk of coronary artery disease. However, age and fasting plasma glucose levels were not associated with decreased *NUMB* expression.

1. Introduction

Although cardiovascular disease mortality is declining, it is still the leading cause of death globally [1]. About 20% of all fatalities in Europe and the United States are attributed to coronary artery disease (CAD), which kills more than 1.7 million people annually. IHD is still the largest cause of death in the world, despite a 22% drop in mortality from ischemic heart disease (IHD) during the previous 30 years [2]. One of the major causes of mortality in IHD is acute myocardial infarction (AMI). The incidences of AMI and post-AMI mortality are declining in most countries, especially in developed countries with high per capita income [3–5].

Treatment options such as coronary intervention, coronary bypass surgery, and drugs have improved the prognosis of AMI over the past few decades. However, the reduction in mortality has, at best, remained modest. Therefore, early identification and risk stratification of AMI is important to accelerate early intervention [6, 7]. Although highly sensitive biomarkers such as troponin T and I and creatine kinase-MB have long been used in a clinical setting to detect and diagnose AMI, the emergence of novel biomarkers may considerably improve the accuracy of early diagnosis. Advances in genome-wide analysis, especially microarray analysis, play an important role in discovering the novel clinical biomarkers of AMI [8, 9]. Whole blood gene expression profiling can provide information on the dynamics of disease states and shed light on the underlying disease mechanisms [10]. Clinically, many common diseases such as AMI [11–14] and different types of atherosclerosis [15] exhibit a characteristic gene expression profile.

First discovered in Drosophila, *NUMB* is an endocytic adaptor protein [16] that has been extensively studied as an

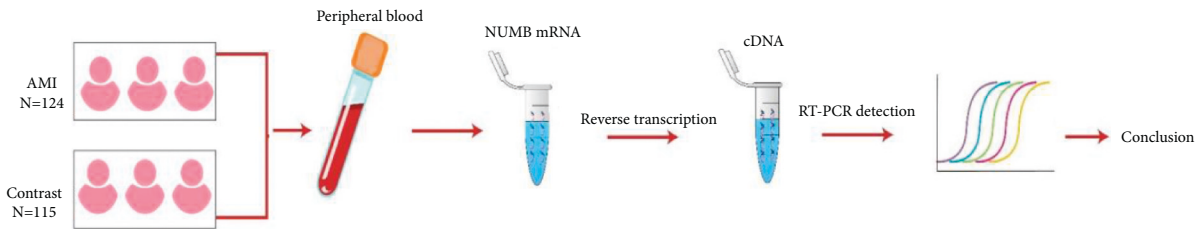

Figure 1: Schematic diagram depicting the research technology route. See research subjects and methods for details.

inhibitor of the Notch receptor signaling pathway [17]. *NUMB* is an evolutionarily conserved protein that regulates endocytosis, adhesion, cell division, cell fate, migration, various signaling pathways (i.e., Notch, Hedgehog, and p53), and ubiquitination of certain substrates [18]. According to recent research, inhibition of the aforementioned *NUMB*-dependent processes is important in cancer treatment in addition to controlling physiological developmental processes. For instance, *NUMB* is significantly expressed in cervical squamous cell carcinoma [19] and astrocytoma [20], while downregulated in salivary gland cancer [21], non-small-cell lung cancer [22], and breast cancer [23]. However, no study has been performed on the correlation between AMI and *NUMB* gene expression. According to recent research, it has been involved in the JIP2/LAMP1/JNK pathway's control of ischemia-reperfusion. The expression of *NUMB* can improve myocardial injury and left ventricular function and reduce cardiomyocyte apoptosis [24].

Gene chip detection was performed in our prior work, and we observed that the AMI group's peripheral blood *NUMB* gene expression levels were significantly lower compared to the control group. This study, therefore, aims to validate the findings of the gene chip detection of *NUMB* expression and to investigate whether the *NUMB* gene may be employed as a molecular marker for the early identification of AMI.

2. Materials and Methods

2.1. Study Technology Route. The overall methodology used in this study is depicted in Figure 1. There were 239 patients, 124 among them participated in the experimental group and 115 of whom participated in the control group. RT-qPCR was used to evaluate the relative mRNA expression level of total RNA collected from peripheral blood. SPSS software was used to conduct the statistical analysis.

2.2. Research Subjects. All study participants were admitted to the Department of Cardiovascular Medicine at the China-Japan Union Hospital of Jilin University from March to May 2018. The experimental group consisted of 124 individuals who had been recognized as having AMI in accordance with the 2018 update to the global definition of MI [25]. If there was clinical evidence of acute myocardial damage and acute myocardial ischemia, the inclusion criterion for the AMI experimental group would be based on an increase and/or decrease in cTn values, at least one of which was greater than the upper reference limit of 99%. The presence of at least one of the following signs or symptoms is considered clinical evidence of acute myocardial ischemia, such as pathological Q wave development on new electrograms, coronary angiography or autopsy confirming the existence of coronary-arterial thrombosis (CAT/PAT), new ischemic electrocardiogram alterations, and imaging data revealing a new loss of myocardial activity or new regional wall motion abnormalities, respectively. Moreover, the control group consisted of 115 SCAD patients as per the 2019 ESC Guidelines for the Diagnosis and Management of Chronic Coronary Syndrome [26], based on the following inclusion criteria, i.e., patients with new heart failure or heart disease, patients with "stable" angina and dyspnea, patients who have been asymptomatic or had stable symptoms for more than a year after their initial diagnosis of coronary heart disease or after their most recent revascularization, patients who were recently revascularized or were asymptomatic and asymptomatic people with coronary heart disease who were found while screening for angina pectoris, probable vasospasm, or microvascular disease. Detailed clinical data, including gender and age, low-density lipoprotein, hypertension and diabetes, triglyceride, troponin levels, high-density lipoprotein, total cholesterol, and smoking history of all individuals, were recorded. Patients' informed permission was obtained before any samples or clinical data could be gathered from them.

The following were the exclusionary criteria: coronary artery bypass surgery (CABG) or percutaneous coronary intervention (PCI) may cause myocardial infarction. Second, MI is associated with a blood supply imbalance. Third, MI with cardiac or noncardiac surgery; fourth, multiple factors or unknown myocardial damage caused by uncertain diseases such as severe stress cardiomyopathy, severe pulmonary embolism, heart failure, or pulmonary hypertension, serious infectious diseases, malignant tumor complications, and other severe neurological diseases.

2.3. Study Procedures

2.3.1. Harvesting of Lymphocytes. Six millilitres of blood from each individual were taken in the morning and kept at 4°C in EDTA anticoagulation tubes. A peripheral blood lymphocyte isolation kit was used to separate lymphocytes within four hours after sample collection. The equal volume of anticoagulant mixed with 0.9% NaCl was added to an equal volume of lymphocyte-isolated medium. The plasma layer, clear separation red blood cell layer, and milky white lymphocyte layer were separated from

Table 1: RT-qPCR primer sequences.

Genes		Genes primer sequence (5′-3′)
NUMB	F[a]	TCAGCAGATGGACTCAGAGTT
	R[b]	AGGCTCTATCAAAGTTCCTGTCT
GAPDH	F[a]	TGTGGGCATCAATGGATTTGG
	R[b]	ACACCATGTATTCCGGGTCAAT

F[a]: forward primer; R[b]: reverse primer.

the sample after centrifugation at 3000 rpm for 20 minutes. After aspirating the lymphocyte layer, it was utilized in subsequent studies.

2.3.2. Lymphocyte cDNA Synthesis. To extract total RNA, we used an extraction kit for blood total RNA (Blood Total RNA Kit, Xinjing Biological Reagent Development Co., Ltd., Hangzhou). To prevent RNA degradation or contamination, the extraction procedure was carried out in accordance with the kit's instructions. Polyacrylamide gel electrophoresis was used to check the quality of the RNA solution, and the visible brightness of the 28S rRNA band was nearly twice that of the 18S rRNA band. A microplate reader was used to measure the concentration and absorbance of RNA from samples that met the standards. The concentration and absorbance of RNA samples meeting the standards were determined using a microplate reader. The 260/280 value should be 1.7–2.1, and the A260/A230 value is >2. RNA samples were reverse transcribed following the manufacturer's instructions and satisfying the specifications of the reverse transcription kit (rapid one-step genomic cDNA first-strand synthesis premix, Tiangen Biochemical Technology Co., Ltd., Beijing). Each sample had the same RNA concentration. For the following phase of the investigation, a fluorescent quantitative polymerase chain reaction (PCR) was performed on the cDNA samples stored at −80°C.

2.3.3. Real-Time Quantitative PCR (RT-qPCR). An SYBR RT-qPCR kit (Sangon Fluorescence Quantitation Kit, Taq qPCR Synthesis Premix Reagent, Shanghai) was used to amplify the cDNA samples after a 20-fold dilution. As previously mentioned, the 20 μL reaction solution included the following components: 10 μL of 2 × SG Fast qPCR reaction mix; 2 μL of DNF buffer; 0.4 μL each of the forward and backward primers (concentration 10 μ mol/L); 6.2 μL of sterilized double-distilled water; and 1 μL of the cDNA sample. Furthermore, predenaturation at 95°C for 5 minutes was followed by 40 cycles of denaturation at 95°C for 3 s, annealing at 60°C for 30 s, and extension at 72°C for 20 s under the reaction conditions described previously. The melting and amplification curves in the 60°C to 95°C temperature range were recorded after the process. Based on the ABI-FAST7500 dissociation curve, amplification conditions were found to be highly specific for GAPDH and *NUMB*, respectively. All samples are represented as the relative expression level $2^{-ct}2^{-\Delta ct}$ (Δct = target gene ct value−reference gene ct value), which is the difference between the target gene's and the reference's cycle thresholds (ct). Table 1 lists the primers used in this investigation.

2.4. Statistical Analysis. SPSS 25.0 was used to conduct the statistical analysis. The measurement data were subjected to a normality test. $X \pm S$ was used to statistically characterize data that followed a normal distribution ($P > 0.1$), and the differences between groups were examined using a two independent samples t-test. The median and interquartile ranges were used to statistically characterize data that did not follow a normal distribution ($P \leq 0.1$), and differences between two independent groups were evaluated using the nonparametric rank-sum test. For statistical analysis, count data were characterized by differences between groups, and frequency was examined using the chi-square(x^2) test. AMI-related risk variables were studied using binary logistic regression analysis. The link between *NUMB* gene expression and troponin I was studied using bivariate correlation analysis. At a two-sided test $P \leq 0.05$, statistical findings were considered statistically significant.

3. Results

3.1. Clinical Data Analysis of Research Subjects. AMI patients were found to be considerably older than those in the control group ($t = -2.318$, $P = 0.020$) and have significantly higher fasting plasma glucose levels ($Z = -2.505$, $P = 0.012$) after clinical data analysis. However, there were no significant variations in the other indices (Table 2).

3.2. RT-qPCR for the Detection of NUMB Amplification Products. It was found that the amplification curve of the *NUMB* gene exhibited an apparent and smooth "S shape" utilizing peripheral blood RNA. There was only a single peak in the dissociation curve, and the amplification product showed a high level of specificity.

3.3. The mRNA Levels of the NUMB Gene Comparison between the AMI and SCAD Groups. The Δct values were obtained using RT-qPCR represent the average of 3 replicate measurements per sample. The results showed that the $2^{-\Delta ct}$ of the AMI group was 0.906 (0.181–0.954) and that of the stable CAD group was 1.024 (0.207–1.127). There was a significant difference between the two groups ($P < 0.05$). The mRNA level of *NUMB* gene expression in the peripheral blood of patients in the AMI group was significantly lower than that of patients in the SCAD group, with a relative expression that was 0.885 times that in the SCAD group (Figure 2).

3.4. The Correlation Analysis between NUMB Gene Expression Levels and Patient Features. Our results demonstrated variations in the expression of the *NUMB* gene, age, and fasting plasma glucose levels between the AMI and SCAD groups. We also investigated to observe if mRNA expression of the *NUMB* gene was associated with fasting plasma glucose levels or age [27]. Subjects were divided into two groups based on their fasting plasma glucose levels, with the normal group having a level (≤5.6 mmol/L) and the increased group having a level >5.6 mmol/L. The standard age grouping split the participants into older (65 years of age and

Table 2: Clinical data analysis of subjects in the AMI and SCAD groups.

Clinical indicators	AMI group ($N = 124$)	SCAD group ($N = 115$)	$t/x^2/z$	P
Sex				
Male, n (%)	85 (68.6)	84 (73.0)	0.582	0.445
Female, n (%)	39 (31.4)	31 (27.0)		
Age	65.000 (57.000–74.000)	61.660 ± 8.778	−2.318	0.020
Hypertension, n (%)	57 (0.460)	58 (0.504)	0.477	0.490
Smoking history, n (%)	56 (0.452)	48 (0.417)	0.284	0.594
Type 2 diabetes, n (%)	38 (0.306)	28 (0.243)	1.184	0.277
TG (mmol/L)	1.570 (1.115–2.503)	1.660 (1.200–2.450)	−0.433	0.665
TC (mmol/L)	4.515 (3.810–5.188)	4.303 ± 0.992	−1.784	0.074
HDL-C (mmol/L)	0.950 (0.833–1.138)	0.971 ± 0.219	−0.706	0.480
LDL-C (mmol/L)	3.041 ± 1.011	2.817 ± 0.829	1.793	0.074
Fasting plasma glucose (mmol/L)	6.430 (5.510–9.280)	5.725 (5.198–7.375)	−2.505	0.012

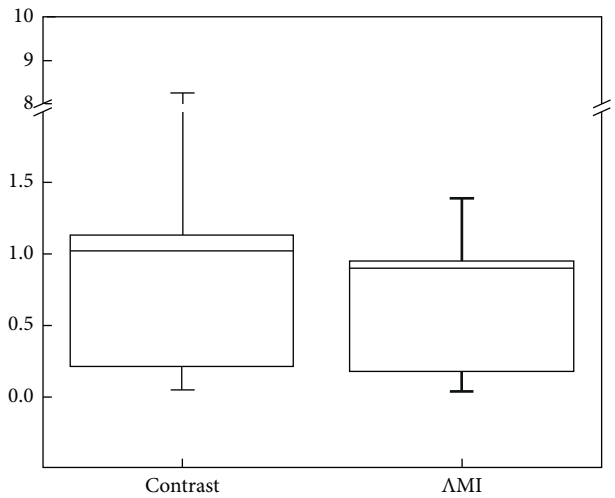

Figure 2: Relative expression of *NUMB* gene mRNA. The abscissa is a grouping, the ordinate is a relative expression, and the ordinate is an unequal distance coordinate. AMI, acute myocardial infarction.

Table 3: Correlation analysis of *NUMB* gene expression with fasting plasma glucose level and age.

Groups	No	*NUMB* relative expression	Z	P
Fasting plasma glucose normal	81	0.939 (0.775–1.281)	−0.596	0.551
Fasting plasma glucose elevated	138	1.015 (0.775–1.480)		
Younger age	132	1.001 (0.734–1.407)	−0.461	0.645
Older age	107	1.007 (0.796–1.500)		

older) and younger (65 years of age and younger) groups. For each individual, the mRNA expression of the *NUMB* gene was expressed as $2^{-\Delta ct}$ ratio, and the association between each group and *NUMB* expression was examined accordingly. The results showed no differences in the expression of the *NUMB* gene mRNA between the fasting plasma glucose normal and elevated groups ($P = 0.551$). Additionally, no change was observed in the *NUMB* gene mRNA expression between the young and elderly ($P = 0.645$) (Table 3).

3.5. Logistic Regression Analysis. The cutoff values for the relative expression of the *NUMB* gene were used to separate all participants into high ($2^{-\Delta ct} > 0.662$) or low gene expression groups ($2^{-\Delta ct} \leq 0.662$). Based on the results of the binary logistic regression analysis, we were able to determine how age, fasting plasma glucose, and AMI all correlate with the mRNA expression of the *NUMB* gene as shown in Table 4 ($P = 0.007$). Decreased *NUMB* gene expression was an independent risk factor for AMI. Low *NUMB* gene expression increased the risk of AMI by 3.287 times when compared to high *NUMB* gene expression. In addition, older age ($P = 0.030$, Table 4) was an independent risk factor for AMI by 1.853 times. Moreover, elevated fasting plasma glucose levels were not an independent risk factor for AMI ($P = 0.098$, Table 4).

3.6. Bivariate Correlation. The AMI group had a troponin I concentration of 1.390 ng/mL (0.060–2.390). Myocardial infarction is measured by troponin I concentration. *NUMB*

Table 4: Logistic regression analysis of the independent risk factors for AMI.

	B	SE	WALD	Degrees of freedom	P	OR	95%CI
Low expression of *NUMB* gene	1.190	0.437	7.404	1	0.007	3.287	1.395–7.744
Fasting plasma glucose elevated	0.482	0.291	2.744	1	0.098	1.619	0.915–2.865
Older age	0.617	0.283	4.737	1	0.030	1.853	1.063–3.230

gene expression in peripheral blood does not have any correlation with serum troponin I concentration in a bivariate correlation analysis ($r = -0.027$, $P = 0.707$), indicating there was no correlation between the expression level of *NUMB* gene in peripheral blood and the severity of acute myocardial infarction.

4. Discussion

In this study, we compared the expression of *NUMB* gene mRNA in peripheral blood between the AMI and SCAD groups and found that *NUMB* gene expression in the AMI group was significantly lower than that in the SCAD group and that its relative expression was 0.885 times that in the SCAD group.

Atherosclerosis is a pathological condition in which monocytes and lymphocytes adhere, migrate, and aggregate under the damaged intima to form foam cells due to intimal injury, which further leads to the development of lipid streaks in atherosclerotic plaque lesions [28]. Atherosclerosis is the most prevalent cause of CAD, which is also a widespread disorder that poses a major danger to human health globally. Studies have shown that the endocytic adaptor protein *NUMB* plays an important role in migrating cell-directed integrin transport mechanism [29]. Integrins are the main family of migration-promoting receptors that regulate cell migration and promote the development of atherosclerosis by mediating cells and the extracellular matrix [30], whereas integrin-stimulated cell migration requires the participation of *NUMB* [29]. However, to our knowledge, the role of the *NUMB* gene has not yet been studied in correlation to cardiovascular disease development and progression.

Studies have shown a strong, positive, independent relationship between serum total cholesterol and the incidence of CAD across a wide range of cholesterol levels, including normal and mildly elevated levels [31]. Lowering plasma cholesterol levels by inhibiting the absorption of exogenous cholesterol may prevent the development of atherosclerotic cardiovascular disease [32]. *NUMB* is a key regulator of cholesterol homeostasis in humans, mediating cholesterol absorption in the stomach and hepatic bile reabsorption in the liver [33]. Ablation of *NUMB* in the mouse intestine significantly reduces the absorption of dietary cholesterol and decreases plasma cholesterol levels [34]. Although overall blood cholesterol levels did not vary significantly between the two groups, we discovered that the level was greater in the low expression *NUMB* group. As a consequence of acute inflammation's inhibitory influence on lipoprotein concentrations [35], serum cholesterol levels decreased by 10.6% from days 1 to 2–4 after AMI by a mean of 0.55 mmol/L [36].

Fasting plasma glucose levels in the AMI and SCAD groups were substantially different, i.e., the AMI group was significantly higher than that of the SCAD group. Furthermore, there was no correlation observed between low *NUMB* gene expression and high levels of fasting plasma glucose. Although logistic regression analysis found that an increase in fasting plasma glucose levels was not an independent risk factor for AMI, the OR was still 1.619 ($P > 0.05$). We hypothesized that the stress reaction after an AMI may have triggered an increase in fasting plasma glucose levels.

AMI mortality and prevalence have been shown to rise with age [37]. In patients with AMI, age is a significant independent predictor of death in the hospital [38]. This research found that the AMI and SCAD groups were significantly different in age. Furthermore, we discovered that aging did not affect the *NUMB* gene's level of expression. Low *NUMB* gene expression was shown to be an independent risk factor for AMI in a binary logistic analysis (OR: 3.287, $P = 0.007$), as was age (OR: 1.853, $P = 0.030$). Patients with stable CAD having a low expression of the *NUMB* gene had 3.287 times the risk of an AMI, regardless of age or other variables. Elderly people have a 1.853-fold greater chance of having a heart attack. Therefore, it is speculated that elderly patients with lower *NUMB* expression are more prone to AMI.

Myocardial filaments and skeletal muscle contain troponin complexes. The troponin subunits I, T, and C make up this protein. Involved in muscle function, it relates changes in intracellular Ca^{2+} concentration to contraction. Troponin I and T have been frequently employed in the diagnosis of cardiomyocyte mortality (myocardial trauma, myocardial infarction, etc.) over the last 35 years [39]. The relationship between *NUMB* and troponin I was established by CTRP3. The downregulation of CTRP3 will lead to the upregulation of troponin I and increase the injury of myocardial ischemia-reperfusion. Research shows that *NUMB* is important in the control of ischemia-reperfusion in the LAMP1/JIP2/JNK pathway [24].

Our study has some shortcomings. Chemotaxis, adhesion, aggregation, lipid metabolism, and other biological processes of cells in the human body are regulated by various factors. The combined analysis of multiple genes and the overall study of each influencing factor may provide sufficient evidence for the genetic diagnosis and treatment of AMI. Although this study does not prove whether the low expression of *NUMB* is directly related to the occurrence of AMI, it can be speculated that the low expression of *NUMB* is one of the reasons for the occurrence of AMI. Therefore, we believe that prospective studies are an effective means of verifying a direct relationship.

5. Conclusions

Precisely, individuals with AMI had lower levels of *NUMB* gene expression in their peripheral blood than patients with SCAD. Peripheral blood *NUMB* gene expression was associated with an increased risk of acute myocardial infarction (AMI). *NUMB* gene expression in peripheral blood may be a molecular diagnostic for early detection of AMI.

Data Availability

The PCR data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

Ethical approval was obtained from the Ethics Committee of China-Japan Union Hospital of Jilin University. All procedures have been performed in accordance with the Declaration of Helsinki.

Consent

Written informed consent was signed by all participates.

Disclosure

Some contents of the abstract were published in "Great Wall International Congress of Cardiology 2020/Asian Heart Society Congress 2020." Some authors did not participate in the follow-up research work, so they did not sign, and the missing authors agreed with the signature and signature order of the current article.

Conflicts of Interest

The authors state that there are no conflicts of interest to disclose.

Authors' Contributions

Heyu Meng took part in research design and manuscript editing. Lihong Li was responsible for experiment work, data analysis, and manuscript writing. Jianjun Ruan, Yanqiu Chen, Zhaohan Yan, Jinsha Liu, and Xiangdong Li contributed to clinical studies. Ping Yang was responsible for study concepts. Heyu Meng and Lihong Li contributed equally to this paper. All authors read and approved the final manuscript.

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