The Heart Rate Response to Breathing Maneuvers Predicts Significant Coronary Artery Disease

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ABSTRACT – ENGLISH

Background: A breathing maneuver of hyperventilation (HV) followed by a breath-hold (BH) triggers a strong vasoactive response in the coronary vascular system that can be detected and quantified with Oxygenation-Sensitive Cardiovascular Magnetic Resonance (OS-CMR) imaging. In a recent retrospective study, a blunted heart rate (HR) response to HV was associated with the presence of cardiovascular disease. In the present study, we were interested in its potential as a diagnostic test and assessed its predictive value for the presence of significant coronary artery disease (CAD) in patients with inconclusive stress tests.

Methods: We recruited 57 patients with new-onset angina, regardless of a history of CAD and 14 healthy volunteers. The beat-to-beat HR was recorded while performing a 4-min breathing maneuver, including 2 minutes of normal breathing (NB), followed by 1 min of deep, paced (30 RR/min) HV and a subsequent maximal end-expiratory BH. Significant CAD was defined as an inducible perfusion deficit in first-pass perfusion cardiovascular magnetic resonance (CMR), or in patients undergoing invasive coronary angiography.

Result: Significant CAD was found in 38/57 patients (50% female; 64±10 years). The best-performing HR parameter for differentiating between patients with and without CAD was BH-induced HR recovery (HRR-BH, %) relative to peak HR during HV. Using a cut-off of ≥ 25%, HRR-BH had a sensitivity of 97.4% and a negative predictive value (NPV) of 87.5% for ruling out CAD (area under the ROC curve 0.67; 95% CI: 0.50-0.84), with lower values in females. In patients with intermediate (10%) and high (29%) pre-test probability of CAD, a normal HRR-BH (≥ 25%) decreased the post-test probability to < 5%.

Conclusion: In summary, our preliminary analysis revealed that in patients with suspected coronary artery disease, the heart rate recovery during an expiratory breath-hold after

hyperventilation can be used to predict the risk for significant coronary artery disease. This simple test may serve as a gatekeeper to improve patient selection for further diagnostic testing by correctly reclassifying patients with an intermediate or high pre-test probability but a normal HR response from high-risk to low-risk, thereby avoiding unnecessary stress tests.

ABSTRACT – FRANÇAIS

Contexte : Une manœuvre respiratoire d'hyperventilation (HV) suivie d'une apnée déclenche une forte réponse vasoactive dans le système vasculaire coronaire qui peut être détectée et quantifiée avec l'imagerie par résonance magnétique cardiovasculaire sensible à l'oxygénation (OS-CMR). Dans une étude rétrospective récente, un rythme cardiaque (RC) atténué en réponse à la période de l'HV était associée à la présence d'une maladie cardiovasculaire. Dans la présente étude, nous nous sommes intéressés à son potentiel en tant que test diagnostique et avons évalué sa valeur prédictive pour la présence d'une maladie coronarienne (CAD) significative chez les patients avec des tests d'effort non concluants.

Méthodes : Nous avons recruté 57 patients souffrant d'un angor d'apparition récente, indépendamment d'antécédents de coronaropathie, et 14 volontaires sains. La fréquence cardiaque battement par battement a été enregistrée lors d'une manœuvre respiratoire de 4 min, dont 2 minutes de respiration normale (NB), suivies d'une HV profonde et rythmée (30 RR/min) et puis d'une apnée maximale en fin d'expiration. Une coronaropathie significative a été définie comme un déficit de perfusion inductible lors d'une résonance magnétique cardiovasculaire (RMC) de perfusion de premier passage ou chez des patients subissant une angiographie coronarienne invasive.

Résultat : Une coronaropathie significative a été trouvée chez 38/57 patients (50 % de femmes ; 64 ± 10 ans). Le paramètre HR le plus performant pour différencier les patients avec et sans coronaropathie était la récupération HR induite par l'apnée (HRR-BH, %) par rapport au pic HR à l'HV. En utilisant un seuil de ≥ 25 %, HRR-BH avait une sensibilité de 97,4 % et une valeur prédictive négative (VPN) de 87,5 % pour exclure la coronaropathie (aire sous la courbe ROC 0,67 ; IC à 95 % : 0,50-0,84), avec des valeurs plus faibles chez les femmes. Chez les patients avec

une probabilité pré-test intermédiaire (10 %) et élevée (29 %) de coronaropathie, un HRR-BH normal (≥ 25 %) a réduit la probabilité post-test à < 5 %.

Conclusion : En résumé, notre analyse préliminaire a révélé que chez les patients suspects de maladie coronarienne, la récupération de la fréquence cardiaque pendant une apnée expiratoire après hyperventilation peut être utilisée pour prédire le risque de maladie coronarienne significative. Ce test simple peut servir de gardien pour améliorer la sélection des patients pour d'autres tests de diagnostic en reclassant correctement les patients avec une probabilité pré-test intermédiaire ou élevée mais une réponse du RC normale de haut risque à faible risque, évitant ainsi des tests de stress inutiles.

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CONTRIBUTION OF AUTHORS

The research questions of the present study were developed by Mahya Khaki and the principal investigator, Dr. Matthias Friedrich. Mahya Khaki carried out the study, including recruiting participants, collecting data, data entry, statistical analysis and data interpretation. The other author, Dr. Magdi Sami, helped with patient recruitment by allowing Mahya Khaki to enroll the patients from his clinic. Dr. Mitchel Benovoy developed the MATLAB code for HR data analysis and provided technical guidance. Dr. Judy Luu and Dr. Elizabeth Hillier mentored Mahya Khaki throughout her master's and provided scientific guidance and helpful suggestions. The principal investigator provided conceptual, clinical, and scientific guidance on all aspects of the project.

Mahya Khaki was the primary writer of the thesis. The conceptual and editorial support was provided by Dr. Judy Luu, Dr. Elizabth Hillier and the principal investigator, Dr. Matthias Friedrich.

LIST OF ABBREVIATIONS & ACRONYMS

CAD	Coronary Artery Disease
PCP	Primary Care Physician
ECG	Electrocardiography
CMR	Cardiovascular Magnetic Resonance
OS-CMR	Oxygen-Sensitive Cardiovascular Magnetic Resonance
MRI	Magnetic Resonance Imaging
SPECT	Single-photon Emission Computerized Tomography
HR	Heart rate
BP	Blood Pressure
ANS	Autonomic Nervous System
HRV	Heart Rate Variability
LF	Low Frequency
HF	High Frequency
HF	Heart Failure
HV	Hyperventilation
NB	Normal Breathing
BH	Breath-holding
HV/BH	Hyperventilation followed by Breath-holding
BMI	Body Mass Index
MI	Myocardial Infarction
RCA	Right Coronary Artery
LMCA	Left Main Coronary Artery
LAD	Left Anterior Descending
LCX	Left Circumflex
ACS	Acute Coronary Syndrome
UA	Unstable Angina
FFR	Fractional Flow Reserve
INOCA	Ischemia with Non-obstructive Coronary Arteries
VSA	Vasospastic angina
CMD	Coronary Microvascular Dysfunction
NO	Nitric Oxide

PET	Positron Emission Tomography
MBFR	Myocardial Blood Flow Reserve
AUC	Area Under the Curve
NPV	Negative Predictive Value
PPV	Positive Predictive Value
SNS	Sympathetic Nervous System
PNS	Parasympathetic Nervous System
SA	Sinoatrial
ICNS	Intrinsic Cardiac Nervous System
α-ARs	α-adrenergic receptors
β-ARs	β-adrenergic receptors
EST	Exercise Stress Test
ROC	Receiver Operating Characteristic
RSA	Respiratory Sinus Arrhythmia
PaCO2	Carbon Dioxide Partial Pressure
RPG	Respiratory Pattern Generator
RR	Respiratory Rate
BVP	Blood Volume Pulse
WHR	Waist-to-hip Ratio
ANCOVA	Analysis of Covariance
MPI	Myocardial Perfusion Imaging
CCB	Calcium Channel Blocker
ARB	Angiotensin Receptor Blocker
ACEI	Angiotensin Converting Enzyme Inhibitor
QCA	Quantitative Coronary Angiography

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1. INTRODUCTION

1.1. Rationale

Coronary artery disease (CAD) is a spectrum of pathophysiological entities that include progressive atherosclerotic narrowing of epicardial coronary arteries, transient coronary vasospasm, microvascular dysfunction, and subsequent myocardial ischemia resulting from insufficient oxygen supply to the heart (1). Based on the Canadian Chronic Disease Surveillance System report, about 2.4 million Canadians ≥20 years of age had myocardial ischemia between 2001 and 2013 (2), exerting a significant economic strain on the healthcare system, with a mean annual hospitalization cost of $1,743 CAD per patient at the national level in Canada (3).

Chest pain is a common reason for patients' presentations to primary care practice (4, 5). Primary care physicians (PCPs) are expected to rule out non-cardiac causes of chest pain in a timely manner while avoiding unnecessary investigations (4, 5). The literature, however, has shown a substantial increase in the frequency of unnecessary chest pain investigations, very often due to the clinicians' tendency to overestimate the pre-test probability of CAD (4, 6, 7). Therefore, developing a simple, physiological risk assessment tool may help clinicians to more accurately estimate the pre-test probability of CAD and recognize scenarios where referral to stress testing is unlikely to improve patients' outcomes.

Recently, markers of cardiac autonomic nervous system (ANS) activity, including resting heart rate (HR), heart rate variability (HRV), and the HR response to physiological maneuvers such as breathing, have received growing attention for CAD risk assessment in suspected patients (8-10). A recent study reported that a blunted HR increase in response to hyperventilation (HV) may help differentiate between patients with and without cardiovascular disease (11). Further, the

use of hyperventilation followed by breath-hold (HV/BH) coupled with oxygenation-sensitive cardiovascular magnetic resonance (OS-CMR) imaging technique has been shown to unmask ischemia-induced myocardial oxygenation deficits providing incremental diagnostic information for myocardial ischemia (12-14).

Given the overuse of cardiac stress testing in patients with suspected CAD and the potential of the cardiovascular response to vasoactive breathing maneuvers to reveal cardiovascular diseases, our study aimed to assess whether the use of HR response to HV/BH—as a simple, office-based stress test gatekeeper—could help clinicians rule out CAD in suspected patients, and thus, avoid unnecessary referrals to cardiac stress testing.

1.2. Objective

1.2.1. Primary objectives

1) To determine the discriminative ability of the HR response to a 4-minute breathing maneuver, including 2-minute normal breathing, followed by 1-minute deep, paced (30 RR/min) HV and subsequent end-expiratory maximal voluntary BH (HV/BH) for the presence of significant CAD, compared to the reference standard of stress cardiac magnetic resonance (CMR) perfusion and invasive coronary angiography during the one-year follow-up for detecting CAD.

2) To determine an optimal diagnostic cut-off of the HR response to HV/BH associated with high sensitivity and especially, a high negative predictive value (NPV) for ruling out CAD in patients with equivocal clinical evidence.

1.2.2. Secondary objectives

1) To assess the impact of sex on the discriminative capacity of the HR response to HV/BH and determine the best sex-specific HR cut-offs for ruling out CAD.

2) To assess the impact of HR response to HV/BH on the difference between pre-test probability and post-test probability of CAD. This objective will reveal how likely the HR response is to

provide a clinically meaningful improvement in reclassifying suspected patients from ischemic to the non-ischemic origin, thereby informing clinicians' decision-making for better patient selection for stress testing.

2. LITERATURE REVIEW

2.1. Obstructive coronary artery disease

The coronary circulation consists of medium- to large-sized epicardial arteries (macrovascular system) and arterioles and capillaries (microvascular system). Typically, there are three major epicardial coronary arteries, including the right coronary artery (RCA), left circumflex artery (LCX) and left anterior descending (LAD) (1). Obstructive CAD is defined as a partial or complete blockage of epicardial coronary arteries with impaired blood flow to the heart muscle, leading to a reduced myocardial oxygen supply/demand ratio and subsequent myocardial ischemia (1, 15). This blockage is usually caused by a gradual build-up of plaque in the inner lining of the coronary arteries, called atherosclerotic plaque, consisting of lipids, inflammatory cells, smooth muscle cells, and connective tissue (1, 15) (Figure 1). The turbulent blood flow caused by atherosclerotic plaque contributes to CAD development by impairing the protective function of endothelial cells lining the interior surface of the coronary arteries, increasing a pro-inflammatory state and impairing endothelium-dependent coronary vasodilation (16, 17) (Figure 1). Intravascular thrombosis forming on the surface of either eroding or rupturing coronary plaques can lead to the catastrophic event of myocardial infarction with subsequent heart failure or death (1, 15, 16).

Angina, the most frequent and classic symptom of obstructive CAD (15, 18), is chest discomfort that can be interpreted as chest pain, tightness, pressure, heaviness, burning, or squeezing. Although patients may also report other symptoms of ischemia such as shortness of breath, nausea, fatigue, and palpitations, chest discomfort remains principal to the diagnosis of obstructive CAD in males and females (15, 18). Angina is deemed stable when chronic symptoms

are associated with triggers such as physical exercise or emotional stress (15, 18). Unstable angina is caused by erosion or rupture of an unstable atherosclerotic plaque. It manifests as ischemic episodes that typically are progressive over time, more severe, longer, or occur during rest periods. Unstable angina can be an antecedent to acute myocardial infarction (18).

Conventional coronary angiography (CCA) has been considered a reference standard for detecting obstructive CAD with ≥ 50% diameter reduction as a cut-off value for significant coronary stenosis (19). However, given its invasiveness and the risk of major complications such as thromboembolism and arrhythmia (20), cardiac stress tests are the standard approach for the initial assessment of patients with angina. The exercise stress test (EST) is the most commonly used modality for CAD assessment; it aims to assess the cardiovascular response to exercise-induced stress (21). It can be performed in combination with electrocardiography (Exercise ECG; non-imaging) or with imaging modalities such as echocardiography or single-photon emission computerized tomography (SPECT) (22). In patients unable to perform such a test or to reach the target HR, or those with an intermediate to high risk of CAD, exercise is substituted with pharmacologic agents, such as dobutamine, mimicking the impact of exertion on the heart (22, 23). Alternatively, the coronary vascular capacity is assessed by pharmacological vasodilation with agents such as dipyridamole, adenosine, or regadenoson (24). Pharmacological imaging stress tests include dobutamine stress echocardiography, dobutamine stress cardiovascular magnetic resonance (CMR), first-pass perfusion CMR during pharmacological vasodilation, and myocardial perfusion SPECT during pharmacological vasodilation (22, 24).

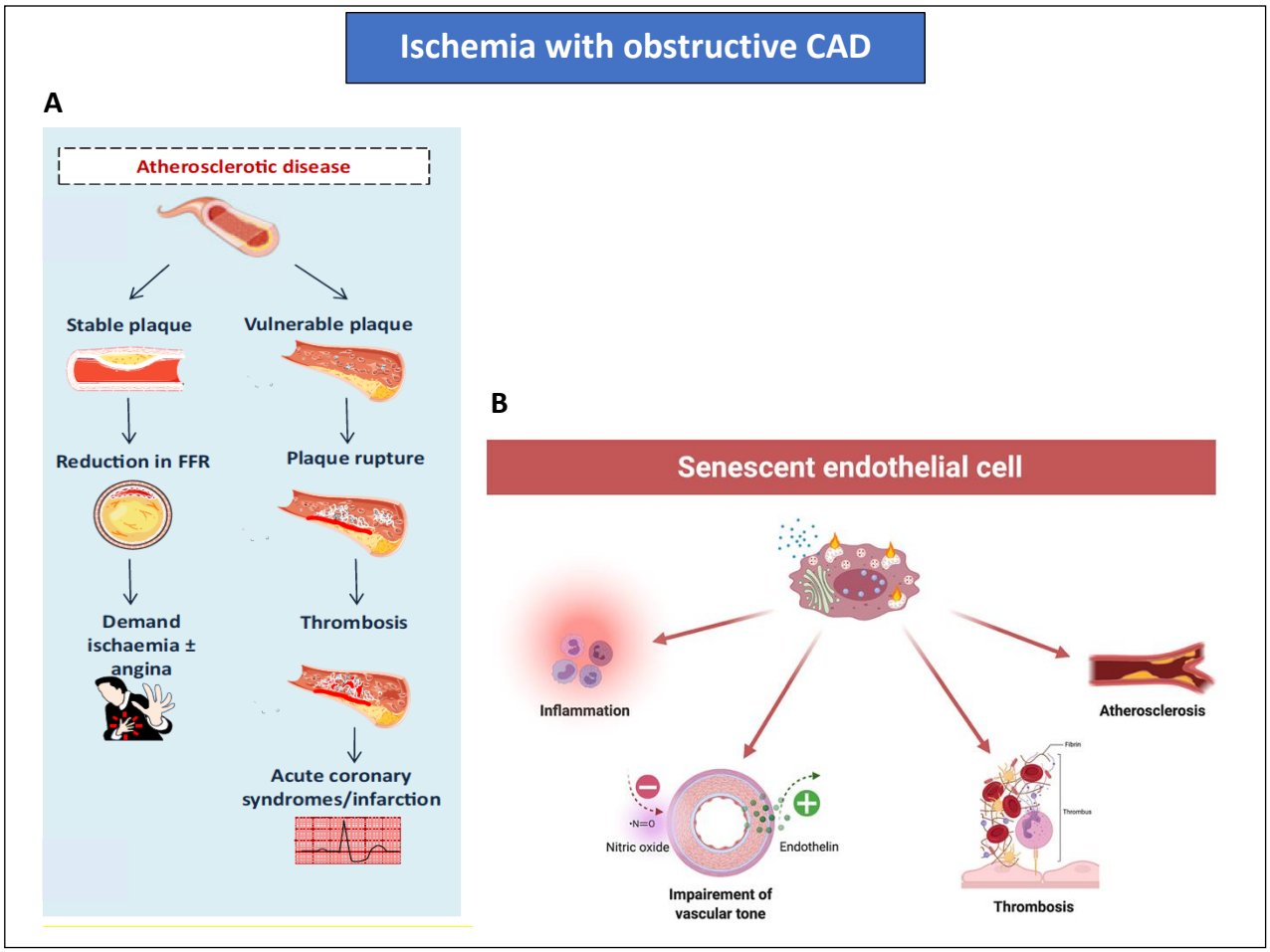


Figure 1. Mechanisms of myocardial ischemia in obstructive CAD.
CAD, coronary artery disease; FFR, fractional flow reserve. Adopted from Kunadian et al. 2020 (25) and Merdji et al. (26).

2.1.1. Cardiovascular magnetic resonance first-pass perfusion

First-pass perfusion CMR has increasingly been utilized to diagnose and risk-stratify patients with suspected CAD (27, 28) due to its safety and other favourable features, including 1) high spatial resolution, allowing for the assessment of subendocardial perfusion deficits; 2) good temporal resolution, allowing for a beat-to-beat visualization of the contrast agent wash-in; and 3) supplementary information on left ventricular function and tissue characteristics (28).

The CE-MARC trial (29) (Cardiovascular magnetic resonance and single-photon emission computed tomography for diagnosis of coronary heart disease) demonstrated the high diagnostic

accuracy of stress CMR perfusion, with a sensitivity of 86.5% and NPV of 90.5% for the presence of stable CAD and its superiority over SPECT compared to the reference standard of invasive coronary angiography. The MR-INFORM trial (30) (MR Perfusion Imaging to Guide Management of Patients With Stable Coronary Artery Disease) compared the impact of stress CMR perfusion on patient management against coronary angiography with fractional flow reserve in 918 symptomatic patients at a high pre-test probability of CAD and reported the capability of stress CMR perfusion to safely manage patients with stable angina with less revascularization but the equivalent patient outcome to an invasive FFR-guided strategy.

First-pass perfusion CMR is performed using intravenous administration of vasodilator agents, typically dipyridamole, adenosine, and, more recently, regadenoson, leading to a 2- to 4-fold increase in coronary blood flow (28). As a blood flow tracer, the injection of a gadolinium-based contrast agent after vasodilator allows for visible perfusion differences between the healthy myocardium and myocardium subtended by significant coronary artery stenosis (28). The perfusion analysis can be performed in a quantitative, semiquantitative, or qualitative fashion. The Society for Cardiovascular Magnetic Resonance recommends qualitative or visual assessment of the CMR perfusion images for the presence of inducible myocardial perfusion deficits in clinical routine (31). In our study, a significant inducible perfusion deficit as reported by qualitative/visual analysis was used as the reference standard for the detection of CAD.

2.2. Chest pain risk assessment in primary care

In the primary care setting, PCPs typically set a working diagnosis and estimate the pre-test probability of CAD using risk assessment tools that incorporate a variety of factors, such as medical history, symptoms, and physical examination (4, 32). Validated risk scores such as Framingham (33), Diamond-Forrester (34), and INTERHEART (35) are used for CAD risk

assessment in cardiology practice. Their performance, however, can be limited in primary care settings, where the prevalence of CAD is lower, and patients are more likely to present with atypical symptoms (6, 7).

A few risk assessment models have been developed to estimate the pre-test probability of stable CAD in the primary care setting (32, 36, 37). The two most extensively tested models include the Marburg Heart Score (32), a prediction model based on age, sex, known vascular disease, pain nature, and patient's perception of pain origin, as well as a modified version of the Marburg Heart Score (37) by adding the cardiovascular risk factors and the chest pain duration and location. Although these models had good diagnostic accuracies for ruling out CAD, their performance was limited by 1) using delayed-type reference standards for detecting CAD, i.e., either imaging stress tests or invasive coronary angiography findings during the 6-month to 1-year follow-up, and 2) a significant drop in their diagnostic performance in the external validation studies.

The lack of an unbiased CAD risk assessment tool and the physicians' need for legal or personal assurance can lead to the overestimation of CAD pre-test probability, especially in a low-prevalence setting, contributing to PCP's low threshold to refer to further, often unnecessary testing (4, 6, 7). A study on the appropriateness of imaging/non-imaging cardiac stress tests ordered by U.S. office-based clinicians revealed a higher proportion of inappropriate tests among PCPs compared to cardiologists (23.8% versus 9.3%) between 2009-2012 (38). Also, other studies in private and academic practices worldwide showed remarkably higher rates of inappropriate referral to nuclear stress tests among PCPs (39, 40).

The overuse of stress testing, especially those involving imaging, has been at the center of debates over rising healthcare costs, unsuitable utilization of healthcare resources, patients' unnecessary exposure to ionizing radiation, and increased risk of false-positive results in low-risk

patients (7, 38, 40, 41). A study on the trend of cardiac stress test referrals in the U.S. reported a 28% growth in imaging stress tests between 1993 and 2010, associated with an additional annual cost of $501 million and 491 possible cancer cases in the future (7).

Given the increasing rate of inappropriate referrals to stress tests and its negative impact on the healthcare system and patients' care, developing a generalizable physiological risk assessment tool might help clinicians appropriately risk-stratify patients with suspected CAD and better recognize scenarios where imaging is unlikely to improve patients' outcomes.

2.3. Autonomic control of the HR

The cardiac autonomic nerve system (ANS) is the principal regulator of cardiac functions, including contractility (inotropy), heart rate (chronotropy), relaxation (lusitropy), and conduction velocity (dromotropy) (42, 43). The ANS consists of two anatomically segregated subdivisions: the sympathetic (SNS) and parasympathetic nervous systems (PNS). The SNS controls the hemodynamics in response to exercise or stress ("fight or flight"); while the PNS mediates the basal autonomic function under normal conditions ("rest and digest") (42). The sympathetic and parasympathetic nerve fibres interact in a reciprocal yet opposing fashion, and increased activity of each component leads to decreased activity of the other (42, 44). The balance between sympathetic and parasympathetic activity is reflected in the heart rate (42, 43).

Heart rate, an important marker of cardiac function and even overall health, is determined by the inherent features of the sinoatrial (SA) node, the natural pacemaker of the heart (42, 43, 45). The SA node produces spontaneous electrical impulses that distribute throughout the atria by cell-to-cell conduction (44, 45). The electrical impulses generated by the SA node are passed to another node called the atrioventricular (AV) node to transmit the generated action potentials from the atria

to the ventricles. The SA and AV nodes are controlled by the interaction between SNS and PNS, dominated by the parasympathetic tone under resting conditions (42, 43) (Figure 2).

The SA node activity is mainly regulated by the following principal signalling cascades (45, 46) (Figure 2):

1) Parasympathetic nervous system or vagal tone: The binding of acetylcholine, the dominant neurotransmitter of the PNS, to muscarinic receptors, especially M2 receptors, causes a decrease in HR (42, 45, 47).

2) Sympathetic nervous system: The sympathetic reflex functions by releasing two main neurotransmitters, norepinephrine and epinephrine and their binding to β-adrenergic receptors. Stimulating these receptors, particularly subtype β1(β1-ARs), increases the SA and AV nodes' firing rates (42, 43).

3) Baroreflex regulation: Arterial baroreflex buffers quick alterations in blood pressure (BP) by inverse HR regulation. The baroreflex control of the HR, called baroreflex sensitivity, is defined as the change in the beat-to-beat interval (IBI) in milliseconds induced by one mmHg change in BP (46).

4) Chemoreflex regulation: The activation of chemoreceptors by changes in blood gas concentrations such as an increase in arterial carbon dioxide enhances sympathetic activity while suppressing parasympathetic tone, leading to an increase in HR. Chemoreflex-induced sympathetic excitation can also enhance the respiration rate and depth to correct the blood chemical imbalance. However, if the respiratory activity does not increase in response to chemoreflex excitation, such as in voluntary breath-holding, the vagal fibres become activated, resulting in bradycardia and coronary vasodilation (42, 48).

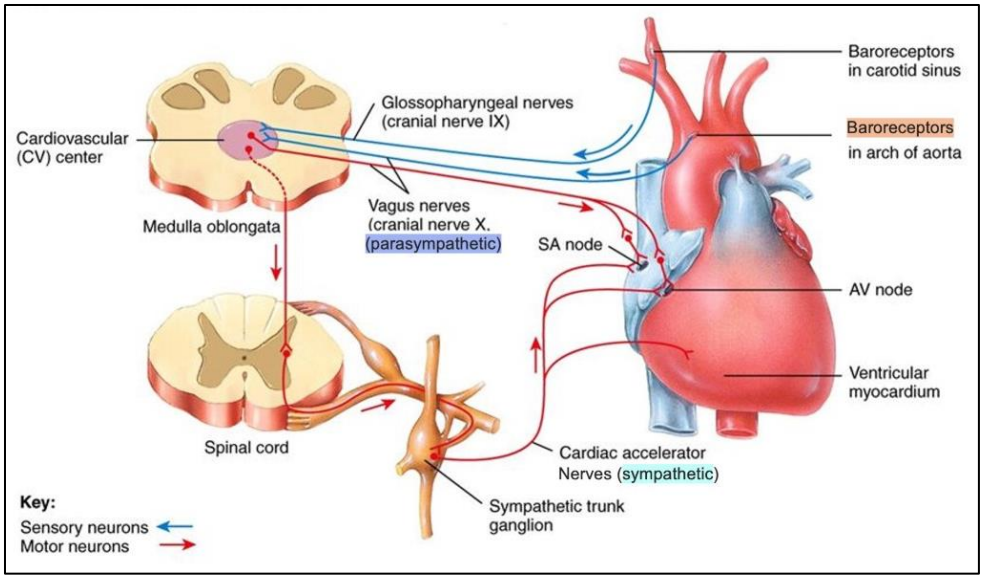


Figure 2. Autonomic Control of the heart rate. Adopted from Marieb et al. (49).

2.3.1. Sex differences in autonomic control of the HR

In recent years, literature reported sex differences in cardiovascular risk factors, as well as in the prevalence of CAD and its presentation (50, 51). Although not fully understood, sex differences in cardiac autonomic modulation may partly explain this observation (52).

Resting HR in females is consistently higher than that of age-matched males between the age of 20 and 45–50 years. The difference seems to gradually decrease and disappear above middle age mainly because of an age-related HR decline in women (53). Studies on the influence of sex on heart rate variability (HRV) using frequency-domain analysis provided more insight into the sex differences in HR control. Most studies revealed a significantly diminished low frequency (LF)/ high frequency (HF) ratio in females suggesting a low sympathetic tone and an overall higher vagally mediated cardiac regulation (54-56).

Since HRV studies suggest a greater parasympathetic influence on the SA node in females, one could expect women to exhibit a higher cardiovagal baroreflex sensitivity. Instead, the opposite is true. Cardiovagal baroreflex sensitivity has consistently been reported to be lower in

females than in males, which could be explained by sex differences in the sensitivity of neural baroreceptors (52, 57, 58).

2.3.2. Autonomic nervous system dysfunction in CAD

The literature suggests a complex two-way relationship between ANS dysfunction, characterized by increased sympathetic activity and/or decreased vagal tone and the development of CAD (8, 17, 59-61).

The ANS dysfunction is linked to CAD progression by contributing to structural and functional abnormalities in coronary arteries. In the presence of endothelial dysfunction, increased sympathetic activity constricts coronary arteries and increases HR, constantly putting stress on the heart by shortening its resting phase and, at the same time, increasing myocardial oxygen demand (17, 60, 62). Furthermore, high HR and BP can accelerate atherosclerotic plaque formation and lead to complications by constant mechanical injury to the vascular wall and local platelet activation (60, 62-64).

Furthermore, myocardial tissue hypoxia caused by relative or absolute myocardial perfusion deficits can impair sympathovagal interaction. If the myocardial oxygen demand exceeds the supply, chemical mediators such as ATP, serotonin (5HT), bradykinin (BK), and adenosine are released (59, 65). These stimulate cardiac afferent sympathetic fibres to relay the ischemia-related information to the dorsal root ganglion situated in the spinal cord, resulting in the reflex excitation of the sympathetic efferent neurones. The resulting sympathetic activation increases the HR and cardiac output with the goal of correcting ischemia-induced hypoxia and washing out metabolites (59, 66, 67).

In patients with repetitive episodes of stress-induced ischemia, however, afferent firing may become exaggerated and persistent, resulting in a chronic pathological sympathoexcitation and, thereby, myocardial hypoperfusion and hypoxia (10, 59, 60, 66). Through a vicious circle,

hypoxia then leads to the further release of ischemic metabolite and sympathetic excitation, causing further deterioration of the oxygen supply-demand mismatch and worsening of myocardial ischemia (59).

2.3.3. The predictive value of the HR for the presence of CAD

Given the ability of the HR to reflect the sympathovagal imbalance and its link with endothelial dysfunction and cardiovascular diseases (10, 64), there is increasing recognition of its diagnostic potential as a simple, office-based parameter for CAD risk assessment (9, 68, 69). Since a single HR measurement in the office setting may not accurately reflect the sympathovagal imbalance (70), studies have aimed to improve its predictive potential using derived measures such as HR variability or the HR response to physiological stressors (9, 11, 69, 71, 72). This section provides an overview of existing HR-related tests and their clinical significance in patients with suspected CAD.

2.3.3.1. Heart rate variability

Heart rate variability, the variation in the duration of successive R-R intervals in ECG, has emerged as a widely used non-invasive method to indirectly quantify the cardiac ANS function (44). Electrocardiography is the method of reference for recording the HRV (44, 73). However, recently, fingertip photoplethysmography (PPG) has been introduced as a practical and convenient alternative to ECG, particularly during ambulatory conditions (74).

HRV can be used in linear, including time-domain and frequency-domain analyses and non-linear metrics of long-term (24 h), short-term (~5 min), and ultra-short-term (<5 min) HR recordings (44, 73). Low HRV indicates increased sympathetic outflow and/or reduced vagal tone (9, 44) and may predict the presence and severity of CAD (8, 9, 75). In a study on patients with angiographically significant CAD, the low-frequency (LF) component of the HRV analyzed from

a 5-min bedside ECG recording was found to be an independent predictor of CAD (adjusted odds ratio [OR] 2.42, p=0.004) with an inverse relationship with its severity (p=0.003) (69). Goldenberg et al. (9) revealed that a cut-off of < 2.6 for non-linear analysis of 1-hour HRV was independently associated with a 2-fold increase in the likelihood of myocardial ischemia (OR 2.00, P=0.01) and had a sensitivity of 71%, specificity of 60%, positive predictive value (PPV) of 11%, and negative predictive value (NPV) of 97% for the presence of myocardial ischemia. The study, however, was limited by the low rate (6%) of myocardial ischemia, likely decreasing the statistical power to detect a statistically significant association between HRV and ischemia.

2.3.3.2. HR response to exercise stress test

In healthy humans, high oxygen consumption during maximal aerobic exercise necessitates adequate oxygen delivery to the working muscles to maintain an aerobic metabolism (22, 76). The ANS contributes to the oxygen supply to the heart and working muscles by 1) increasing the HR, called chronotropic response (76, 77), and 2) coronary vasodilation, mainly due to the release of vasodilating factors from the endothelium and passive relaxation of the coronary smooth muscle cells, avoiding exercise-induced ischemia (22, 76).

The exercise stress test (EST) is a non-invasive test that evaluates the probability and extent of CAD by looking at the cardiovascular response to exertion on a treadmill or stationary bicycle (21). Classically, CAD is diagnosed based on the presence of exercise-induced ST-segment depression in ECG or reduced myocardial perfusion and/or ventricular wall motion abnormalities in imaging (21, 22). Besides the ECG and imaging findings, the incremental diagnostic value of HR response to exercise, including chronotropic response and post-exercise HR recovery, has been increasingly recognized in patients with suspected CAD (68, 71, 72, 78).

2.3.3.3. Impaired chronotropic response to exercise in CAD

Chronotropic response to exercise is defined as an increase in HR resulting from an interplay of parasympathetic withdrawal during the early stage and sympathetic excitation at the later stage of the exercise (76, 77). Chronotropic incompetence, a marker of ANS dysfunction, is most commonly defined as the failure of the HR to reach 85% of the age-predicated maximal HR (220-age) during exertion (77, 79).

Oliveira et al. (80) revealed that chronotropic incompetence during exercise echocardiography was independently associated with angiographically significant CAD (≥ 50% stenosis), with an adjusted OR of 2.62 ($p<0.00001$). Brener et al. (71) demonstrated an independent association between impaired HR responses to exercise, including chronotropic incompetence (OR 1.66 for each drop of 10%, $p <0.001$) and decreased peak HR (adjusted OR 1.42 for each reduction of 10 beats/min, $p <0.001$) and the presence of coronary stenosis. A study on the impact of sex on exercise echocardiography findings demonstrated that only in males, reaching 80% of the target HR during exertion reduced the risk of a false-positive echocardiography result by 81% (OR 0.19, $p= 0.008$) (81).

2.3.3.4. Reduced post-exercise HR recovery in CAD

After the termination of exercise, the HR recovers immediately (rapid phase) due to dominant parasympathetic activity, followed by a gradual decrease (slow phase) owing to sympathetic withdrawal (79, 82). Since blunted HR recovery reflects suppressed vagal tone (68, 72), incorporating HR recovery into EST results may improve patient selection for further investigation. The implementation of HR recovery in clinical practice, however, has been limited by the variations in post-exercise recovery techniques, i.e., passive vs active cool-down, causing the deviation in the cut-off points and clinical value across different protocols (68, 72, 79).

Receiver operating characteristic analysis for diagnostic performance of HR recovery has yielded conflicting results among studies. In a study of patients with suspected CAD, a 1-minute HR recovery cut-off of ≤ 21 bpm (using passive cool-down protocol) was associated with moderate sensitivity (76.1%), low specificity (41.3%) and NPV (48.3%) for predicting CAD (68). Ghaffari et al. (72) reported that a 1-minute HR recovery of less than ≤18 bpm in exercise ECG (using the passive cool-down protocol) had a sensitivity, specificity, and NPV of 48.0%, 83.3%, and 63.4%, respectively for angiographically proven CAD. Gera et al. (83) demonstrated an independent association between 1-minute HR recovery of <=12 bpm (with active cool-down protocol) and composite high-risk myocardial perfusion imaging findings in exercise SPECT(OR 4.3, p <0.001), indicating the importance of further diagnostic evaluation with imaging stress tests in patients with abnormal HR recovery on routine exercise ECG. It is important to keep in mind that post-exercise HR recovery still requires the preceding physical stress and thus comes with all the risks and logistical issues of the exercise stress.

2.4. Cardiovascular responses to respiration

Respiration is a distinctive physiologic function with a reciprocal interaction with the cardiovascular system. It not only has the capability to profoundly affect the cardiovascular system, such as modifying HR and coronary vascular tone , but can, in turn, be modified by ANS dysfunction in cardiovascular diseases (84, 85).

It is well known that the ANS plays a critical role in the interactions between respiratory and cardiovascular systems (84, 85). One of the important physiological cardiorespiratory interactions is respiratory sinus arrhythmia (RSA) (86), the respiration-related HR variability by which the R-R interval decreases during inspiration and prolongs during expiration due to vagal tone changes in the different phases of breathing (86). Another example of cardiorespiratory interaction is the respiration-related oscillations in BP, by which the BP drops during inspiration

and increases during expiration. The underlying mechanism is not fully understood yet, but the baroreflex is assumed to play a role, depending on the respiration rate and intrathoracic pressure (87).

There has been increasing attention toward the clinical application of cardiorespiratory interactions for diagnostic purposes in cardiovascular diseases (11, 14, 88). Recent studies have shown that the use of 1-minute paced (30 RR/min) deep HV and subsequent end-expiratory voluntary BH (HV/BH) can alter the coronary vascular tone (Figure 3), inducing changes in myocardial oxygenation as detected by oxygen-sensitive cardiovascular magnetic resonance (OS-CMR) imaging (12-14). Another study showed that an HR increase in response to the same HV protocol can differentiate patients with cardiovascular disease from healthy subjects (11). Understanding the underlying physiology of interactions between the breathing maneuver and the cardiovascular system, especially those involving the ANS, is vital to growing the screening and diagnostic application of cardiopulmonary interaction for CAD.

2.4.1. Cardiovascular responses to hyperventilation

Hyperventilation (HV), defined as an excessive rate and depth of respiration, is a well-known, robust cardiovascular stimulus, inducing HR acceleration and, if performed for a sufficiently long time, coronary vasoconstriction (11, 13, 89, 90) (Figure 3). Hyperventilation-induced hypocapnia, defined as a drop in carbon dioxide partial pressure (PaCO2) below 35 mmHg (91), seems to be the main contributor to vasoconstriction, but a change of blood CO2 itself, regardless of the absolute value, has been shown to alter coronary vascular tone (92) . The decline of blood CO2 triggers vascular smooth muscle contraction by the intracellular influx of calcium ions (Ca2+) into these cells, leading to coronary constriction and blood flow reduction (91). Further, hypocapnia impairs oxygen diffusion in the myocardial tissue due to its high affinity to blood hemoglobin (91). The diminished coronary blood flow combined with the hypocapnia-induced reduction in

myocardial oxygen diffusion can decrease the oxygen supply to the myocardium (12, 13, 91). It has been shown that the maximum yet safest HV-induced hypocapnia for inducing coronary vasoconstriction is a PaCO2 of about 20 mmHg, resulting in an almost 17% increase in coronary vascular resistance and up to 30% reduction in coronary blood flow (90, 93).

Besides the vascular response, HV can also provoke an increase in HR in healthy humans (89, 94) and, to a lesser extent, in patients with cardiovascular disease (11). Heart rate responses to different durations of HV, including short and long protocols, have been described in multiple healthy subject studies (89, 94). Concerning short protocols, our group reported that in healthy individuals (N=20), 1 minute of paced (30 RR/min) deep HV elicited a significantly higher increase in HR compared to administration of adenosine, a pharmacological vasodilator agent (HR, 25.2 ±14 vs 17.8 ±14.4), suggesting a stronger impact of HV on the cardiovascular system than currently used pharmacological agents (13). With respect to prolonged HV protocols, Alexopoulos et al. (89) investigated the ECG-derived HR response to 5-min hyperventilation at a pace of 30 breaths/minute followed by 10 min recovery in 369 healthy subjects. The prolonged HV increased the HR by 27.4% within the first minute, followed by a further subtle increase during the rest of the hyperventilation, with women showing a less significant overall increase in HR than men. Right after hyperventilation, HR dropped immediately in both sexes by about 20% (Figure 4).

The alteration in the balance between the sympathetic and parasympathetic nervous systems seems to be the principal underlying mechanism for the HR changes during hyperventilation; however, there is a debate as to which component plays the dominant role. One of the proposed mechanisms is the inhibitory effect of hyperventilation on baroreflex control of the HR. Van De Borne et al. (95) investigated the effect of 10-min isocapnic hyperventilation at breathing frequencies of 0.19, 0.27, and 0.32 Hz on arterial baroreflex sensitivity in healthy subjects. The significant BP rise during HV (118 ± 2 to 125 ± 3 mmHg) was associated with

decreased R-R intervals (947 ±18 to 855 ±11 ms) with a more significant reduction at a higher breathing frequency. The study speculated that the excitation of pulmonary stretch receptors through the Hering–Breuer inflation reflex might play a role in the HR response to HV: The extreme stretching of the lungs during deep inspiration stimulates the pulmonary stretch receptors, which in turn, sends inhibitory signals to the cardiac vagal motor neurones located in the lowest portion of the brainstem, decreasing the cardiac vagal tone (84), thereby limiting the baroreflex's capacity to alter the HR in response to BP oscillations. The mediating role of vagal tone in cardiovascular responses to HV was confirmed by Badra et al. (96), who demonstrated the impact of respiratory frequency on vagal activity, as quantified by HRV analysis, indicating the dependence of vagal tone on respiratory activity.

Sympathetic excitation might be another explanation for the HV-induced increase in HR. Monitoring the cardiac periodic repolarization dynamics (PRD)—an ECG-based biomarker reflecting the cardiac sympathetic activity— before and after 1-min paced HV (RR 30/min) in healthy subjects showed a significant increase in PRD values after HV compared to baseline (3.30 deg2 vs 2.76 deg2, $p = 0.018$), suggesting increased efferent cardiac sympathetic activity during HV (97).

Heart rate response to HV might also be the function of the respiratory activity and the associated changes in $PaCO_2$ and pH (91, 98). Biberman et al. (98) reported that in healthy subjects, hypocapnic hyperventilation induces a more significant decrease in ECG-derived R-R intervals compared to normocapnic hyperventilation (R-R interval in 0.01 sec, 47 ± 05 vs 55 ± 06), suggesting the possible role of hypocapnia and respiratory alkalosis in the sympathetic excitation and HR increase during HV.

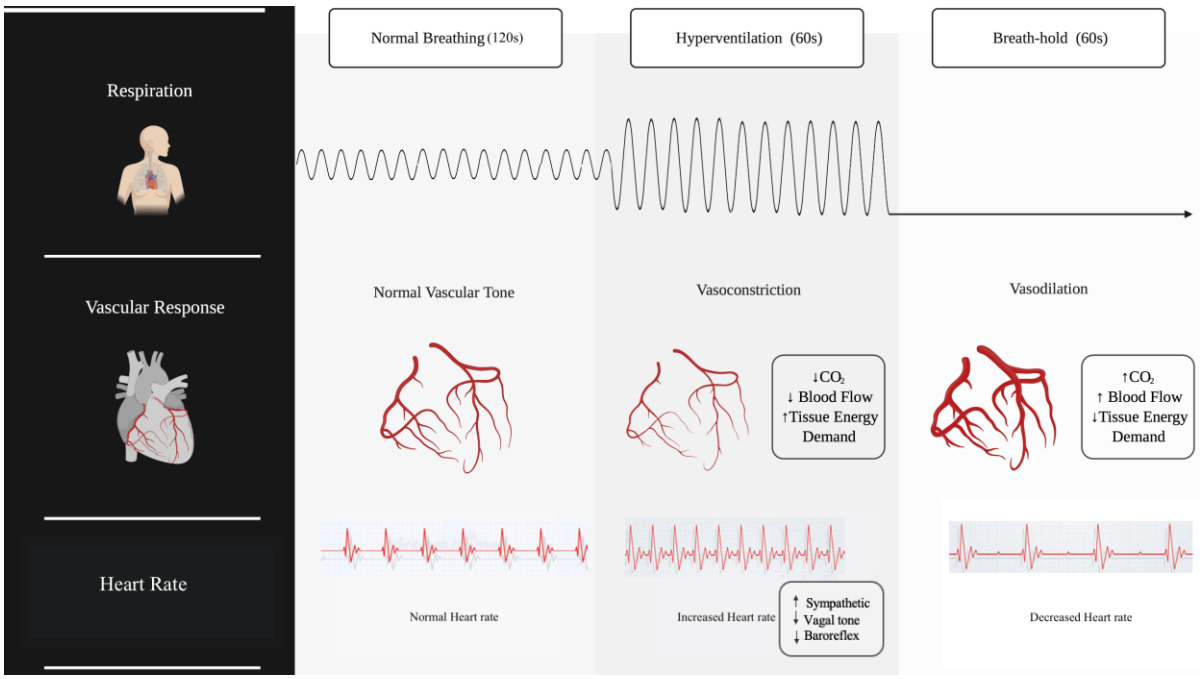


Figure 3. Cardiovascular response to a standardized 4-minute breathing maneuver. 1-min hyperventilation leads to coronary vasoconstriction by decreasing arterial carbon dioxide and heart rate acceleration by sympathetic excitation and reducing vagal tone. Conversely, the following maximal breath-holding increases arterial carbon dioxide and shifts the sympathovagal balance toward vagal activity leading to coronary vasodilatation and heart rate reduction. Created with BioRender.com.

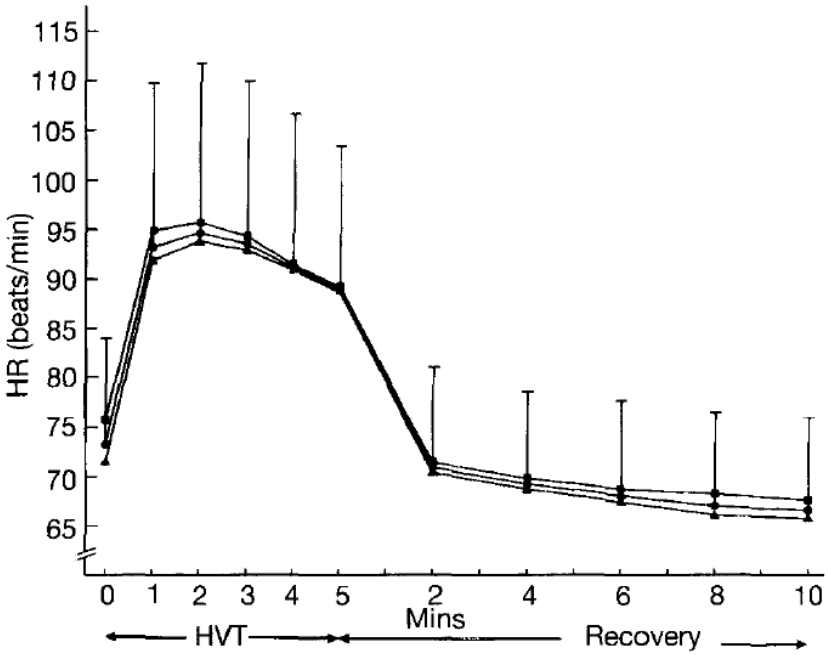


Figure 4. Heart rate changes with hyperventilation in a cohort of healthy controls. HR, heart rate; HVT, hyperventilation. ● = All subjects, ■ = women, ▲ = men. Adopted from Alexopoulos et al. 1995 *(89)*.

2.4.2. Cardiovascular responses to breath-holding

Breath-holding (BH) involves a sequence of dynamic physiological adaptations in the nervous, cardiovascular, and respiratory systems aiming for O2 preservation and redirecting blood flow to vital organs such as the brain and heart (99, 100). It is known that BH can dilate the coronary arteries in healthy individuals, most likely due to 1) the direct relaxation of the coronary vascular smooth muscle cells by the inhibitory effect of increased CO2 or hypercapnia on the voltage-dependent calcium channels and 2) the release of endothelium-derived NO, mediating vascular relaxation (12, 91) (Figure 3). (91). Moreover, BH-induced hypercapnia decreases hemoglobin-oxygen affinity, increasing the oxygen release into the myocardial tissue (91).

The HR change is an immediate cardiovascular response to BH (Figure 3). It is well-known that the HR remarkably decreases during free diving, dropping to 30 bpm in extreme instances, mainly due to parasympathetic stimulation (101-103). One important factor that affects the cardiovascular response to breath-holding is the type of BH, whether it is performed end-inspiratory or end-expiratory. Compared to end-expiratory BH, end-inspiratory BH has been shown to result in a less significant decrease in HR (104), which could be explained by the following reasons: 1) An increase in intrathoracic pressure after inspiration, as in the second phase of the Valsalva maneuver, excites the sympathetic activity to correct the dramatic fall in venous return (105), 2) lung inflation after inspiration stimulates the pulmonary stretch receptors resulting in reflex inhibition of cardiovagal parasympathetic outflow, thereby increasing the HR (84, 104), and 3) an end-expiratory BH leads to a faster increase in blood CO2 than end-inspiratory BH (because of the lower surface available for the diffusion of CO2 into the alveolar space), leading to a greater chemoreceptor stimulation and, thereby, a more significant decrease in HR in case of voluntary BH (106).

Chemoreceptors in the carotid and aortic bodies regulate respiratory activity and cardiovascular autonomic responses to maintain partial pressure of oxygen (PaO2), PaCO2, and pH within normal physiological ranges (85). Once the CO2-sensitive chemoreceptors are activated by BH-induced hypercapnia, they send the sensory information through afferent vagal and glossopharyngeal nerves to Respiratory Pattern Generator (RPG) located in the medulla. The RPG corrects PaCO2 changes by 1) sending action potentials to the diaphragm to start inhalation and 2) sympathetic excitation to increase the HR and the cardiac output. If the RPG-induced inhalation does not happen in the case of voluntary breath-holding, the ANS activates the vagal fibres instead of the sympathetic system, leading to HR decline and coronary vasodilation (84, 85).

Although a decreased PaO2 during BH contributes to the chemoreceptors activation (107), it does not seem to trigger HR reduction or coronary vasodilation, at least not during a short breath-hold (108). Another possible mechanism for BH-induced HR reduction would be the stimulation of the arterial baroreceptors. Breath-holding increases the BP by constricting peripheral blood vessels, which, in turn, activates the baroreflex to correct the high BP by decreasing the HR (99).

2.4.3. Cardiovascular responses to breathing in CAD

Given the bi-directional interaction between respiration and cardiac autonomic function, vascular and HR responses to vasoactive breathing maneuvers have been used for diagnostic purposes for cardiovascular diseases such as CAD (11, 14, 96, 109, 110).

Fischer et al. assessed the feasibility and diagnostic potential of HV/BH, as quantified by signal intensity changes in OS-CMR images, for the presence of CAD in patients with at least one untreated vascular territory with > 50% stenosis. The study demonstrated reduced global myocardial signal intensity changes during hyperventilation (− 9.6 6.8% vs. -3.1 6.5%, $p = 0.012$) and breath-hold (11.3 6.1% vs. 2.1 4.4%, $p < 0.001$) in patients with significant CAD compared to healthy controls, indicating the ability of the HV/BH to reveal an impairment of coronary vascular

response in patients with CAD, without the use of pharmacologic vasodilators or contrast agents (14) (Figure 5). The combination of HV/BH with a novel MRI technique, so-called Fast Strain-ENCoded (fSENC)-CMR imaging, used for myocardial deformation quantification, had a sensitivity and specificity of 81% and 86% for detecting regional hypoperfusion in CAD (111).

Despite various ways to assess the vascular response to HV and BH for CAD detection, only a few studies have assessed the diagnostic significance of the HR response to breathing maneuvers in CAD (11, 112). A retrospective study conducted by our team reported a lower HR increase in response to 1 minute of deep, paced (30 RR/min) HV in patients with CAD and/or HF ($p < 0.001$) than in healthy controls. A cut-off of 15.5 bpm for HR response to HV had a sensitivity of 91%, specificity of 72%, PPV of 73% and NPV of 91% for distinguishing patients with cardiovascular disease from healthy subjects (11) (Figure 6). Greenberg et al. (112) reported that abnormal HR responses to a combined maneuver, including < 15% HR increase in response to standing relative to the sitting position and < 20% HR increase during the post-standing 30-second HV, yielded a sensitivity, specificity, and predictive value of 56%, 92%, and 87%, respectively, for the presence of severe CAD (>70% stenosis). The diagnostic performance of the HR marker was comparable to that of exercise ECG (sensitivity, specificity, and predictive value of 77%, 98%, and 97%, respectively), suggesting the potential of the HR response to standing followed by HV to substitute exercise stress testing in patients unable to perform an adequate exercise.

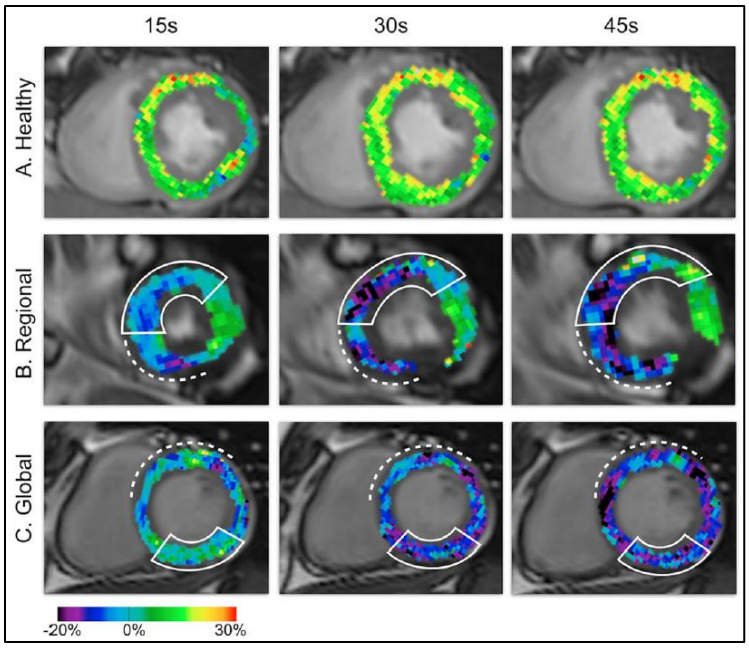


Figure 5. Myocardial oxygenation response throughout a post-hyperventilation breath-hold in OS-CMR. A. Globally increased myocardial oxygenation in a healthy male. B. Regional decrease in myocardial oxygenation in territory subtended to left anterior descending (LAD) stenosis (marked with a solid lined box) in a patient with CAD. C. Globally reduced myocardial oxygenation in a CAD patient with multivessel disease. OS-CMR, oxygenation-sensitive cardiovascular magnetic resonance imaging; CAD, coronary artery disease. Adopted from Fischer et al. 2018 (14).

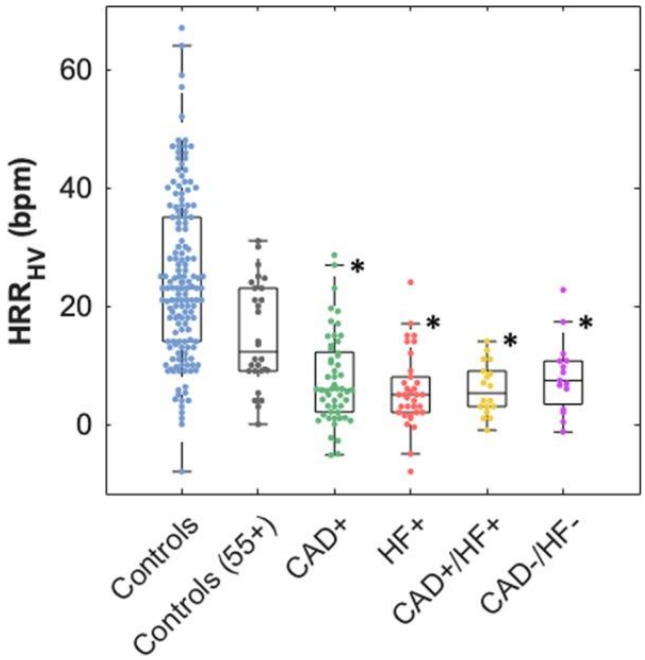


Figure 6. Heart rate response to hyperventilation in patients with cardiovascular diseases. Group medians with the interquartile range are depicted. All cardiovascular patient groups had a significantly attenuated HR response to hyperventilation in comparison to the response of the control sub-group 55 years and older (*$p < 0.05$). CAD, coronary artery disease; HF, heart failure. Adopted from Hawkins et al. 2019 (11).

3. METHODOLOGY

3.1. Study design

This thesis consists of an analysis of data collected in a prospective, observational single-center cohort study conducted at the Royal Victoria Hospital, a part of McGill University Health Centre (MUHC), located in Montreal, Quebec, Canada.

The research proposal of this study was approved by the MUHC Research Ethics Board on June 14th, 2020. The first participant was recruited on July 14th, 2020, and the recruitment is still ongoing to reach the calculated sample size. All participants signed informed consent in compliance with institutional review board regulations of the MUHC. After signing a consent form, participants interested in being enrolled were screened for eligibility criteria.

3.2. Sample size calculation

The primary objective of the study was to determine the discriminative ability of the HR response to the breathing maneuver (HV/BH) for the presence of CAD and to find an HR optimal diagnostic cut-off that is associated with high sensitivity and NPV.

The sample size was calculated to compare the NPV of the HR response to the breathing maneuver with that of the stress CMR perfusion in ruling out CAD among suspected patients. Overall, considering the prevalence of about 10% for myocardial ischemia (2, 113), a total sample size of 83, including 85% cases (N=71), 15% controls (N=12) would achieve a power of 90%, a sensitivity of 99%, a specificity of 100%, and a negative predictive value of 95%. Given the negative impact of the COVID-19 pandemic on the pace of recruitment and the commitment to complete my master's degree within three years, the thesis includes an interim analysis of a smaller patient population (91).

3.3. Cohort selection

3.3.1. Patient population

We prospectively recruited 73 patients with suspected CAD who presented to the cardiology clinic with stable angina (typical or atypical) and were clinically referred to a first-pass perfusion CMR for further clinical decision-making, based on the judgement of their referring cardiologist.

Patients with a known history of CAD were included if the coronary artery lesion was angiographically significant (>50% stenosis) regardless of whether or not the revascularization procedure to treat the blockage was performed. Patients were excluded from the study if they were < 35 years old; had a history of underlying diseases that could impair ANS function, including atrial fibrillation, heart failure, or recent myocardial infarction; or had taken beta-blockers, calcium channel blockers (CCB), or caffeine 12 hours before the CMR exam. Detailed inclusion/exclusion criteria are depicted in Table 1.

3.3.2. Healthy controls

In order to determine the HR response to the breathing maneuver in the healthy population as a normal reference for the patients, we recruited 16 healthy volunteers aged ≥ 35 years through public advertisements in the hospital. Healthy controls were excluded if they had a history of cardiovascular disease, smoked in the last six months, or took caffeine 12 hours before the breathing maneuver exam. Detailed inclusion/exclusion criteria are demonstrated in Table 1.

Figure 7 illustrates the formation of study samples according to inclusion/exclusion criteria.

Table 1. Inclusion and exclusion criteria

PARTICIPANTS	INCLUSION CRITERIA	EXCLUSION CRITERIA
PATIENTS	1. Aged ≥ 35 2. Written consent in non-emergency situations 3. Clinically indicated referral for first-pass perfusion MRI stress test in subjects with suspected CAD	1. Acute Coronary Syndrome (ACS) in the last 72 hours, 2. Previous myocardial infarction within one month 3. Previous Coronary Artery Bypass Surgery in the last 6 months 4. Clinically unstable condition 5. Significant or uncontrolled arrhythmia Left bundle branch block (LBBB) 6. Established valvular regurgitation or stenosis abnormality above moderate severity 7. Patients with a known history of heart failure (Ejection fraction<40%) 8. MR incompatible devices such as pacemakers, defibrillators, implanted material, or foreign bodies. 9. Vasoactive medication (e.g. nitrate, beta-blocker, calcium channel blocker) during the 12 hours prior to the exam. 10. Consumption of caffeine (coffee, tea, cocoa, chocolate, "energy drink") during the 12 hours prior to the exam 11. Patients who are pregnant
HEALTHY VOLUNTEERS	1. Age ≥ 35 2. Written consent in non-emergency situations 3. No known current or pre-existing problems that would affect the cardiovascular or respiratory system	1. Presence of cardiovascular disease. 2. Regular nicotine consumption during the last 6 months 3. MR incompatible devices such as pacemakers, defibrillators, implanted material, or foreign bodies. 4. Consumption of caffeine (coffee, tea, cocoa, chocolate, "energy drink") during the 12 hours prior to the exam

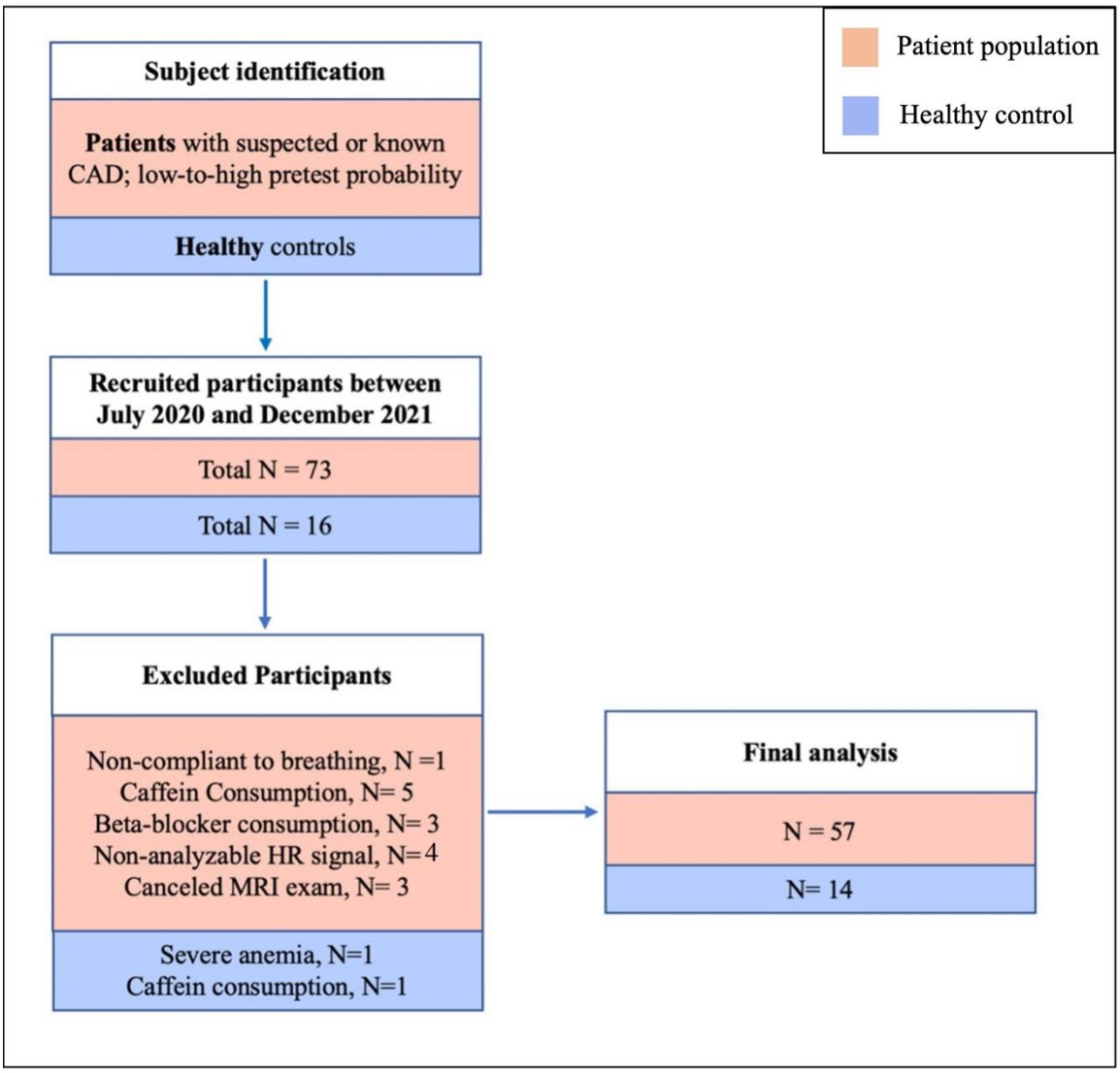


Figure 7. Description of the study sample

3.4. The study procedure and data collection

All participants performed a 4-min breathing maneuver and underwent a cardiac MRI exam the same day. As a standard measure, the patients refrained from consuming caffeine and medications such as beta-blockers and CCBs 12 hours before the MRI appointment. The patients were scheduled for a meeting with the student one hour earlier than their MRI appointment to fill out the research questionnaire and perform the 4-minute breathing maneuver.

To stabilize the HR before the breathing maneuver, the participants were asked to lay down on a bed in the MRI waiting area in a supine position for 10 minutes. Meanwhile, the student

obtained their baseline clinical information using a validated questionnaire to determine their INTERHEART risk score, which includes age, smoking status, the self-reported history of diabetes and hypertension, family history of heart attack, waist-to-hip ratio, psychosocial factors, diet, and physical activity (114) (Appendix A). Afterwards, the student placed ECG and blood volume pulse (BVP) sensors to record the HR and respiration sensors for the respiratory parameters during the maneuver. The participants then watched a short video tutorial instructing how to perform the breathing maneuver with hyperventilation paced by the auditory signal from a metronome. After practicing once with the student, the patients performed the 4-min breathing maneuver, including 2-minute normal breathing, followed by 1-minute deep HV at a pace of 30 RR/min, and a subsequent end-expiratory maximal voluntary BH being guided by a recorded voice instruction and metronome sounds during HV. Respiration and HR parameters were recorded and visualized by the software in a real-time fashion.

Healthy controls were booked for a 60-min appointment with the student at Royal Victoria Hospital and were asked to refrain from caffeine 12 hours before the appointment. They followed the same workflow as the patients, except for the first-pass perfusion CMR.

Figure 8 illustrates the study workflow for patients and healthy controls.

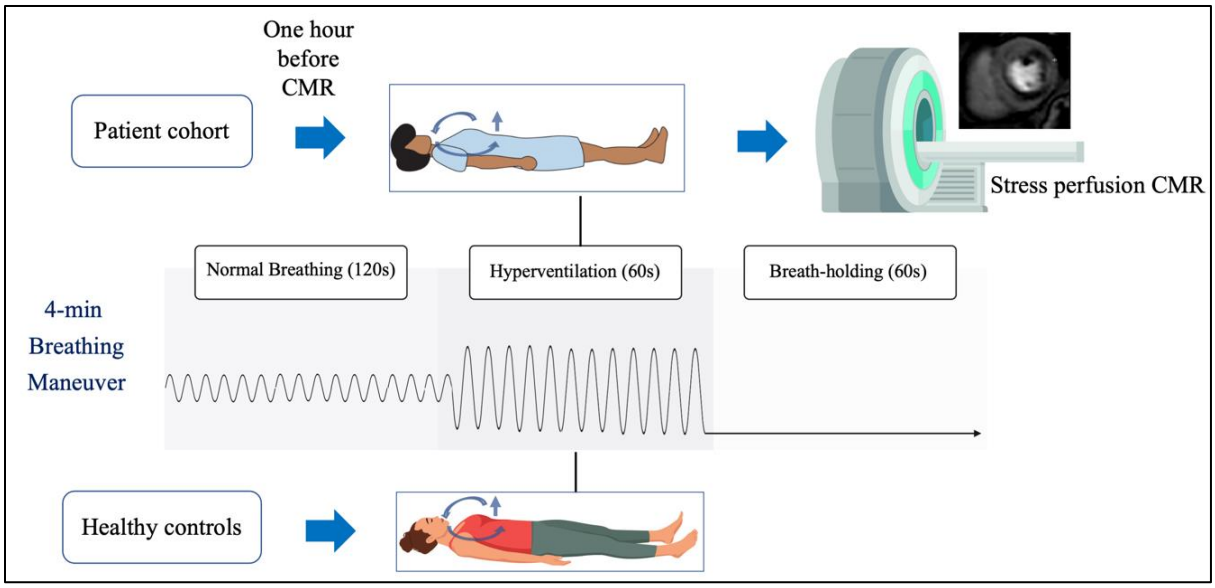


Figure 8. The research workflow in patients and healthy controls

3.5. Modified Diamond-Forrester (MDF) prediction model

In order to estimate the pre-test probability of CAD in the patient population, we used the modified Diamond-Forrester prediction model (115), which was externally validated in a single-center cross-sectional study of 320 patients (116). The model includes age, sex, and nature of symptoms, including typical angina, atypical angina, nonanginal, or dyspnea, defined as shortness of breath.

Based on the updated model, patients with a pre-test probability <5% do not need further diagnostic testing, whereas, in patients with a pre-test probability between 5-15% (intermediate risk), additional diagnostic testing is considered beneficial based on the overall likelihood of CAD and clinicians' clinical judgement. Patients with pre-test probability >15% (high risk) would benefit most from non-invasive diagnostic testing (Figure 9).

	Typical		Atypical		Non-anginal		Dyspnoea[a]	
Age	Men	Women	Men	Women	Men	Women	Men	Women
30–39	3%	5%	4%	3%	1%	1%	0%	3%
40–49	22%	10%	10%	6%	3%	2%	12%	3%
50–59	32%	13%	17%	6%	11%	3%	20%	9%
60–69	44%	16%	26%	11%	22%	6%	27%	14%
70+	52%	27%	34%	19%	24%	10%	32%	12%

Figure 9. The modified Diamond-Forrester Pre-test probability model for obstructive coronary artery disease (115).

3.6. Heart rate data recording

During the 4-minute breathing maneuver, we used FDA-approved sensors for ECG (EKG-Flex/Pro sensor-T9306M, Thought Technology Ltd.; Montreal, Canada), blood volume pulse (BVP) (BVP-Flex/Pro sensor- Thought Technology Ltd.; Montreal, Canada), and respiration (Respiration Sensor -SA9311M, Thought Technology Ltd.; Montreal, Canada) to continuously capture the HR and respiration parameters, at a sampling rate of 256 Hz (Figure 10).

3.6.1. Sensor placement

1. Respiration sensor- SA9311M: The respiration sensor was placed around the upper abdomen, over clothing, using a length-adjustable webbing belt (Figure. 10A). The sensor recorded the respiratory rate and amplitude by measuring abdominal expansion/contraction.

2. EKG-Flex/Pro sensor- SA9306M: The pre-amplified ECG sensor measured the beat-to-beat HR through three ECG leads placed on the forearms (one on the right arm and two on the left arm) (Figure. 10B).

3. BVP-Flex/Pro sensor- SA9308M: The fingertip optical sensor was placed on the palmar side of the first joint of the index finger and was held in position with a Velcro strap. The sensor recorded the HR by measuring the blood volume changes. The BVP sensor data was used as a backup if the ECG sensor did not record the HR correctly (Figure. 10 C).

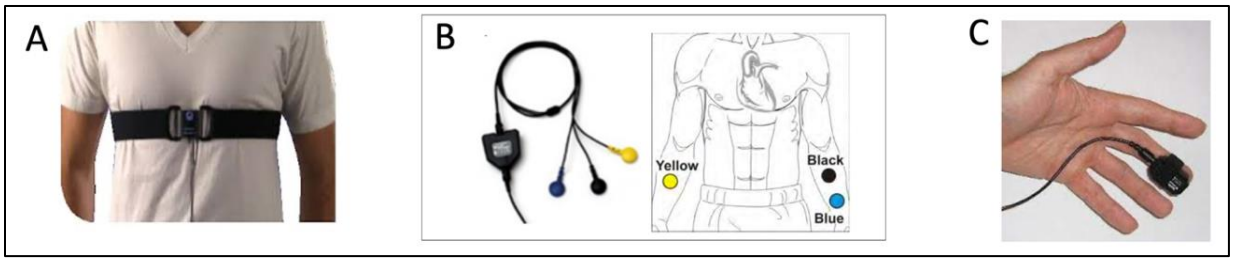


Figure 10. The sensors used for recording the heart rate and respiration parameters. A. The Respiration sensor was placed around the upper abdomen to record the respiration rate and amplitude. B. The electrocardiogram (ECG) sensor measured the heart rate via ECG leads placed on the forearms. C. The fingertip optical sensor for measuring the heart rate.

3.7. Heart rate data analysis

BioGraph Infiniti Software (Thought Technology Ltd.; Montreal, Canada) collected the signal data recorded by sensors and saved them in the form of digital values. Afterwards, a MATLAB-based automated analysis tool was used to analyze the raw ECG, BVP, and respiration data using digital signal processing (DSP). After removing motion artifacts caused by chest movement during hyperventilation, we extracted beat-to-beat HR data during three phases of the breathing maneuver, including normal breathing, hyperventilation, and breath-holding. Also, a MATLAB code was developed to identify important HR markers during each phase of the breathing maneuver, including the average HR during 2-min normal breathing and minimum and maximum HR during HV and BH. The reason for extracting specific HR values was to identify the individual or combined markers with the highest diagnostic performance for CAD. The analysis was conducted while being blinded to the patient information and stress CMR perfusion results. Figure 11 depicts an example of visualized HR and respiration parameters in a healthy control.

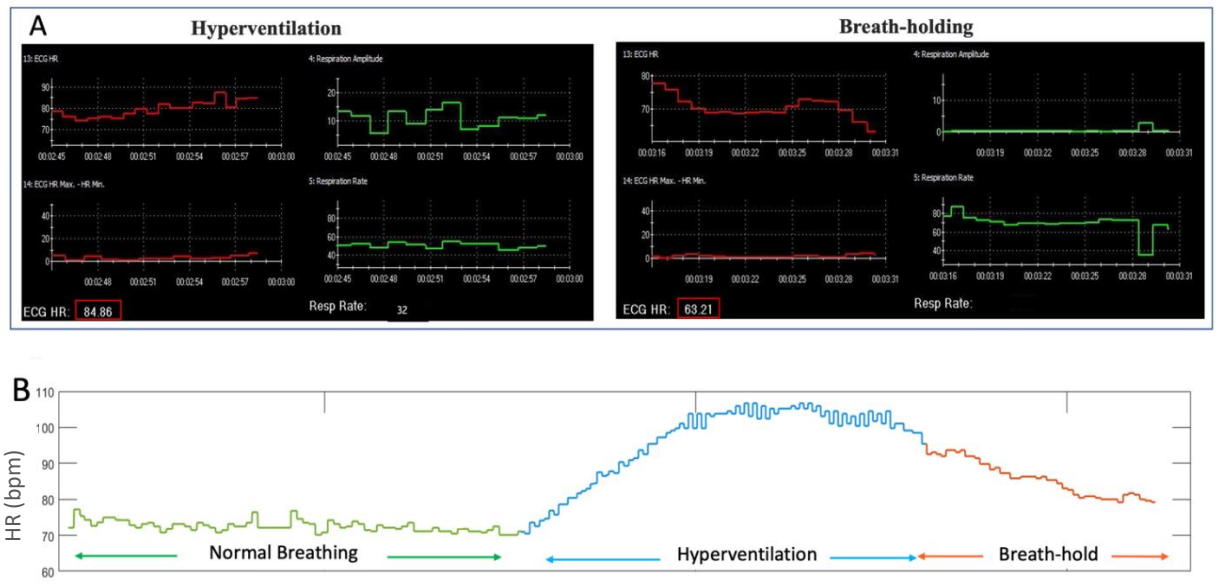


Figure 11. Example of measurements of hemodynamic parameters in a healthy control. A. The display of real-time HR and respiration capturing during the breathing maneuver by BioGraph Infiniti Software. B. The offline extraction and visualization of beat-to-beat HR using MATLAB tool. HR, heart rate; ECG, electrocardiogram.

3.8. Definition of coronary artery disease

In our study, the reference standard for detecting CAD was the presence of an inducible perfusion deficit in a qualitative/visual analysis of first-pass perfusion CMR images acquired as a part of a clinical workup. However, for those patients that underwent invasive coronary angiography during a 1-year follow-up, the visual assessment of coronary stenosis was considered the reference standard (delayed-type reference standard).

3.8.1. First-pass perfusion CMR protocol

The clinical exam was performed using a 3 Tesla MRI system (SIGNA™ Premier, G.E. Healthcare, Waukesha, MS, U.S.). The protocol included cardiac function, stress perfusion and resting perfusion images, and late gadolinium enhancement (LGE). For acquiring the stress perfusion images, the adenosine was infused at a 140 µg/kg/min rate for a maximum of 2-4 min until the patient became symptomatic or had at least a 10% HR increase in response to adenosine. In the case of an adequate response, a bolus of 0.05 mmol/kg gadobutrol, a contrast agent, was

administered at 5 ml/s through a second intravenous line in the other arm. Following a 10-minute washout delay after the stress perfusion acquisition, resting perfusion images were obtained during the second injection of 0.1 mmol/kg of gadobutrol. As a part of a clinical workup in patients with suspected CAD and as per the study design, Dr. Matthias Friedrich and D. Micheal Chetrit, with level 3 certification in CMR, were responsible for conducting the first-pass perfusion CMR exams. While blinded to the HR response, the readers performed a consensus qualitative/visual assessment of the myocardial perfusion during baseline conditions and pharmacological vasodilation.

3.9. Statistical Analysis

Descriptive analyses compared baseline clinical and demographic characteristics between patients with suspected CAD and healthy volunteers using means ± standard deviation (SD) for continuous variables and frequency distributions for categorical variables. An independent t-test or Mann-Whitney U test was used to compare data between groups for continuous variables and chi-square statistic for categorical variables. Backward stepwise logistic regression was applied to choose the best-performing HR variable among different HR parameters recorded during the breathing maneuver. The analysis began with a full model containing HR parameters, and at each step, it gradually eliminated variables from the regression model to find the best-performing HR variable, which was HRR-BH. The analysis of covariance (ANCOVA) was used to compare the HRR-BH between three cohorts of healthy controls, symptomatic patients with no significant CAD, and patients with a diagnosis of significant CAD after controlling for the potential confounding variables, including high BMI, dyslipidemia, and medications such as CCBs, angiotensin-converting enzyme inhibitor (ACEI), and statins. Association between the HRR-BH and age was assessed with a Spearman correlation coefficient.

The receiver operating characteristic (ROC) curve analysis was applied to measure the diagnostic performance of HRR-BH, including sensitivity, specificity, NPV, and PPV and

determine the optimal cut-off value with the highest NPV and sensitivity possible. The area under the ROC curve was calculated as an indicator of the discriminative power of HRR_BH. Fagan's nomogram was applied to illustrate the relationship between the pre-test probability of CAD, calculated from the Modified Diamond-Forrester risk score and the post-test probability, estimated from HRR-BH likelihood ratios.

The main calculations used in the study are as follows:

Sensitivity: The ability of a test to correctly identify patients with a disease.
Sensitivity=True Positive / (True Positive + False Negative)

Specificity: The ability of a test to correctly identify those who do not have a disease
Specificity = True Negative / (True Negative + False Positive)

Negative predictive value: The probability that subjects with a negative screening test truly do not have the disease.
NPV = True Negative / (True Negative + False Negative)

Positive predictive value: The probability that subjects with a positive screening test have the disease.
PPV= True Positive / (True Positive + False Positive)

Negative Likelihood Ratio: the probability of an individual with the disease having a negative test divided by the probability of an individual *without* disease having a negative test.
NLR= (1- Sensitivity)/Specificity

Positive Likelihood Ratio: The probability of an individual with the disease having a positive test divided by the probability of an individual *without* disease having a positive test
PLR= Sensitivity/(1-Specificity)

4. RESULT

4.1. Participants' characteristics

Table 2 depicts the demographics and clinical characteristics of healthy controls and the patient population. Since males and females demonstrated different HR responses to the breathing maneuver**,** Table 2 also illustrates sex-based differences in the patient population's characteristics.

After excluding two healthy controls because of severe anemia and caffeine consumption before the exam, we included 14 controls with an average age of 51 (± 10 yr.), 57% of which were females. Also, 16 patients with suspected or known CAD were excluded from the study. Therefore, the patient population consisted of 57 patients aged 35 to 84, with an average age of 62 (±10 yr.). The proportions of male and female patients were roughly equal in the whole patient population (male (M), 50 % vs female (F), 49%).

Evidence of significant CAD was found in 38/57 patients (CAD group) (63±10 yr., 50% F). There were no significant differences in sex distribution between healthy controls, patients with no significant obstructive CAD (non-CAD group) and patients with significant obstructive CAD diagnosis (p= .85, 95% CI: -0.33-0.52). Patients in both CAD and non-CAD groups were significantly older than controls (p= .002, 95% CI: 4.41-20.36). Unlike the CAD group, females in the non-CAD group were significantly older than males (F, 68 ± 10 vs M, 54 ± 7, p= .004). The systolic and diastolic blood pressure were higher in CAD patients (systolic 138.5 ± 15.9, diastolic 74.6 ± 13.9) than in non-CAD (systolic 136.5 ± 15.5, diastolic 73.9 ± 9.3) and healthy participants (systolic 126.4 ±14.4, diastolic 72.6 ± 7.7). This difference was only significant between the CAD and healthy cohorts (p= .04). The blood pressure was not significantly different between the sexes in non-CAD and CAD cohorts.

Regarding cardiovascular risk factors, the BMI was significantly higher in the CAD group than in the control group (mean 28.3 ± 5.6. vs 23.1 ± 2.3, p= .005, 95% CI: 1.49-8.91). The BMI was not significantly different between CAD and non-CAD patients (mean 28.3 ± 5.6 vs 27.3 ± 4.4, p >.05) but was higher in females in both groups (p >.05). Waist-to-hip ratio (WHR) was not significantly different between controls, non-CAD, and CAD groups (p= .33, 95% CI: -0.03-0.16). Females had a lower WHR than males in both CAD and non-CAD groups, with a statistically significant difference in the CAD group (F, 0.90 ± 0.07 vs M, 1.01 ± 0.06, p < .001).

The prevalence of diabetes was not significantly different between CAD and non-CAD groups, but was lower in female patients in both groups, which was more significant in the CAD group (F, 11% vs M, 32%, p= .001). Dyslipidemia was more prevalent in the CAD than in the non-CAD group (63% vs 58%, p= .008, 95% CI: 0.06-0.39), with a lower prevalence in female patients in both groups, more significantly in the CAD group (F, 42.1% vs M, 84.2%, p= .049). The proportion of patients with hypertension was higher in the CAD than in the non-CAD group (71% vs 53%, p< .001, 95% CI: 0.13-0.46), with a higher prevalence in females in the non-CAD group (F, 67% vs M, 40%, p= .47).

Compared to the non-CAD group, a higher proportion of patients in the CAD group had a history of known CAD (11% vs 18%, p >.05, 95% CI: -0.01-0.18). In both groups, the history of known CAD was less prevalent in females, more significantly in the CAD group (F, 5% vs M, 32%, p= .048). The percentage of current and former smokers and participants with a family history of CAD was not significantly different between control, CAD, and non-CAD groups, regardless of sex differences (p > .05).

Patients with CAD had a significantly lower HRR-BH (11.9±6.5%) than healthy controls (26.5±11.1%, p<.001, 95% CI: -22.2_ -6.8) and non-CAD cohort (20.1±14.7%, p= .01,95% CI:-14.79_ -1.54) but this difference was not significantly different between healthy controls and patients in the non-CAD group (p = .18, 95% CI: -14.67_1.92). The impact of sex on HRR-BH is explained in the next sections. Since the participants performed the hyperventilation in sync with a metronome at a pace of 30 RR/min, the respiratory rate was not significantly different between healthies, non-CAD, and CAD cohorts.

Based on the MDF risk score, the proportion of patients with a high pre-test probability of CAD was significantly higher in the CAD than in the non-CAD group (45 % vs 32%, p= .002, 95% CI: 0.04-0.33), whereas a larger number of patients in the non-CAD group had low (16% vs

11%, p=.11, 95% CI: -0.01-0.12) and intermediate (non-CAD 53% vs CAD 45%, p=.16, 95% CI: -0.04-0.25) pre-test probability of CAD. With regards to sex differences, in the non-CAD group, all females had an intermediate pre-test probability of CAD (100%), while the majority of males were in the high-risk category (60%), followed by low-risk (30%) and intermediate-risk (10%) categories. In the CAD group, a significant number of females had intermediate pre-test probability (74%) of CAD, followed by low (21%) and high (5%) pre-test probability.

Concerning the medications, there was no difference in the use of beta-blockers between CAD and non-CAD groups (37% in both groups, p= .092, 95% CI: -0.02-0.26). Compared to the non-CAD group, a significantly larger number of patients in the CAD group were on CCBs (37% vs 16%, p= 0.004, 95% CI: 0.06-0.32), ACEI (32% vs 16%, p= .012, 95% CI: 0.02-0.23), anti-platelets (53% vs 26%, p< .001, 95% CI: 0.11_0.41), or statins (63% vs 47%, p= 0.002, 95% CI: 0.10-0.42). With regards to sex differences in medication use, compared to males in non-CAD group, a smaller number of females were on beta-blockers (F, 33% vs M, 40%, p= .68), CCBs (F, 11% vs M, 20%, p=.56), statins (F, 44% vs M, 50%, p= .71), or anti-platelet agents (F, 11%, vs M, 40%, p=.15). In the CAD group, the proportion of females taking beta-blockers (F, 42% vs M, 32%, p=.56) and angiotensin receptor blocker (ARB) (F, 21% vs M, 16%, p= .13) was higher than that of males while the use of other medications, including CCB, ACEI, statins, and anti-platelet agents was more prevalent in males (p> .05).

Table 2. Participants' characteristics

	Healthy Control (n=14)	Stress Perfusion CMR + Coronary Angiography									
		[1] Non-CAD patients (n=19)				CAD patients (n=38)				[3]P-value (total)	95% CI
		Total (n=19)	Females (n=9)	Males (n=10)	[2]p-value (M vs F)	Total (n=38)	Females (n=19)	Males (n=19)	[2]P-value (M vs F)		
Demographics & Cardiovascular Risk Factors											
Sex (Female)	8 (57%)	9 (47%)	9 (100%)	0 (0.0%)	-	19 (50%)	19 (100%)	0 (0.0%)	-	0.85	-0.33-0.52
Age (years)	51 ± 10	60 ± 11	68 ± 10	54 ± 7	**0.004**	64 ± 10	61 ± 12	66 ± 7	0.15	**0.002 ✦**	4.41-20.36
BMI (kg/m2)	23.1 ± 2.3	27.3 ± 4.4	27.9 ± 5.3	26.7 ± 3.7	0.60	28.3 ± 5.6	29.1 ± 6.8	27.6 ± 4.5	0.41	**0.005 †**	1.49-8.91
Systolic BP	126.4 ±14.4	136.5 ± 15.5	143.4 ± 16.2	130.3 ± 12.6	0.17	138.5 ± 15.9	137.4 ± 13.2	139.5 ± 18.6	0.3	0.8 †	-8.50-12.45
Diastolic BP	72.6 ± 7.7	73.9 ± 9.3	72.8 ± 3.8	74.9 ± 12.6	0.93	74.6 ± 13.9	74.1 ± 14.7	75.2 ± 13.4	0.8	0.9	-7.24-8.71
Waist to hip ratio	0.89 ± 0.2	0.94 ± 0.12	0.89 ± 0.11	0.98 ± 0.11	0.09	0.95 ± 0.08	0.90 ± 0.07	1.01 ± 0.06	**<0.001**	0.33	-0.03-0.16
Diabetes	-	5 (26%)	2 (22%)	3 (30%)	0.64	8 (21%)	2 (11%)	6 (32%)	**0.001**	0.38	-0.06-0.17
Dyslipidemia	-	11 (58%)	5 (56%)	6 (60%)	0.72	24 (63%)	8 (42%)	16 (84%)	**0.049**	**0.008**	0.06-0.39
Hypertension	-	10 (53%)	6 (67%)	4 (40%)	0.47	27 (71%)	13 (68%)	14 (74%)	0.81	**<.001**	0.13-0.46
Smoking											
- Current	0	2 (11%)	1 (11%)	1 (10%)	0.60	3 (8%)	1 (5%)	2 (11%)	0.93	0.65	-0.05-0.09
- Former	6 (43%)	6 (32%)	3 (33%)	3 (30%)	0.85	15 (40%)	7 (37%)	8 (42%)	0.35	0.78	-0.52-0.30
[4]History of known CAD	-	2 (11%)	0 (0%)	2 (20%)	0.56	7 (18%)	1 (5%)	6 (32%)	**0.048**	0.08	-0.01-0.18
Family history of CAD	8 (57%)	8 (42%)	3 (33%)	5 (50%)	0.78	19 (50%)	11 (58%)	8 (42%)	0.51	0.68	-0.57-0.27
[5]HRR-BH (%)	26.5 ± 11.1	20.1 ± 14.7	18.2 ± 13.7	21.8 ± 16.2	0.62	11.9 ± 6.5	13.8 ± 6.4	10.1 ± 6.3	0.08	**<0.05 †**	-15.07_ -1.26
[6]RR during HV	30.0 ± 3	29.0 ± 2	30 ± 2	28 ± 3	0.71	29 ± 1	28 ± 2	30 ± 1	0.65	0.75	-0.43-0.28
MDF Risk Score											
Low risk	-	3 (16%)	0 (0%)	3 (30%)	0.07	4 (11%)	4 (21%)	0 (0%)	0.040	0.11	-0.01-0.12
Intermediate risk	-	10 (53%)	9 (100%)	1 (10%)	**0.002**	17 (45%)	14 (74%)	3 (16%)	**0.002**	0.16	-0.04-0.25
High risk	-	6 (32%)	0 (0%)	6 (60%)	**0.006**	17 (45%)	1 (5%)	16 (84%)	**<0.001**	**0.002**	0.04-0.33
Medications											
BB	-	7 (37%)	3 (33%)	4 (40%)	0.68	14 (37%)	8 (42%)	6 (32%)	0.56	0.092	-0.02-0.26
CCB	-	3 (16%)	1 (11%)	2 (20%)	0.56	14 (37%)	5 (26%)	9 (47%)	0.24	**0.004**	0.06-0.32
ARB	-	3 (16%)	2 (22%)	1 (10%)	0.92	7 (18%)	4 (21%)	3 (16%)	0.13	0.18	-0.03-0.17
ACEI	-	3 (16%)	3 (33%)	0 (0%)	0.07	12 (32%)	4 (21%)	8 (42%)	0.21	**0.012**	0.02-0.23
Statins	-	9 (47%)	4 (44%)	5 (50%)	0.71	24 (63%)	9 (47%)	15 (79%)	0.14	**0.002**	0.10-0.42
Anti-platelets	-	5 (26%)	1 (11%)	4 (40%)	0.15	20 (53%)	8 (42%)	12 (63%)	0.30	**<.001**	0.11-0.41

Continuous variables are reported as mean ± SD. Categorical variables are reported as the number of occurrences in each group (n (%)).
✦ Indicates a significant difference between healthy controls and patients in both non-CAD and CAD groups.
† Indicates a significant difference between healthy controls and only the CAD group.
[1] Non-CAD: No significant obstructive CAD in patients presenting with stable angina who had either 1) less than 50% stenosis in invasive coronary angiography, regardless of the result of stress CMR perfusion, or 2) normal perfusion in stress CMR perfusion in patients who did not undergo coronary angiography afterwards.
[2] p-value (M vs F): The difference between males and females in the non-CAD and CAD groups.
[3] p-value (total): The difference between patients in non-CAD and CAD groups, regardless of sex
[4] History of known CAD: Patients with a previous history of angiographically significant coronary artery stenosis (>50% stenosis) regardless of whether a revascularization procedure to treat the blockage was performed or not.
[5] HRR-BH: The percentage of heart rate recovery during breath-hold relative to peak heart rate during hyperventilation
[6] RR during HV: respiratory rate during hyperventilation
CAD, coronary artery disease; BP, blood pressure; BMI, body mass index; MDF, modified Diamond-Forrester; BB, beta-blocker, CCB, calcium channel blocker; ARB, angiotensin receptor blocker; ACEI, angiotensin-converting enzyme inhibitor; CI, confidence interval.

4.2. The pattern of the HR response to the breathing maneuver

Figure 12 depicts the pattern of HR changes during 1-minute deep, paced (30RR/min) HV followed by end-expiratory maximal voluntary breath-hold in control, non-CAD, and CAD groups. Each breathing phase contained four important HR markers, including 1) HR at the start of HV and BH, 2) HR at the middle of HV and BH, 3) HR at the end of HV and BH, and 4) max HR at HV and min HR at BH.

Hyperventilation caused an increase in HR in all groups ($p < .001$ compared to their baseline), peaking in the second half of HV in the majority of participants. The CAD group, however, demonstrated a lower HR response to HV compared to non-CAD patients ($p = .007$) and healthy controls ($p > .05$). After peaking, the HR dropped slightly toward the end of HV in most participants.

During the subsequent breath-hold, in most patients, the HR decreased and reached a minimum in the second half, followed by a minimal HR increase toward the end of BH. In all groups, the HR recovered significantly relative to the peak HR at HV ($p < .001$). The CAD group exhibited a blunted HR recovery compared to non-CAD and healthy controls ($p < .05$).

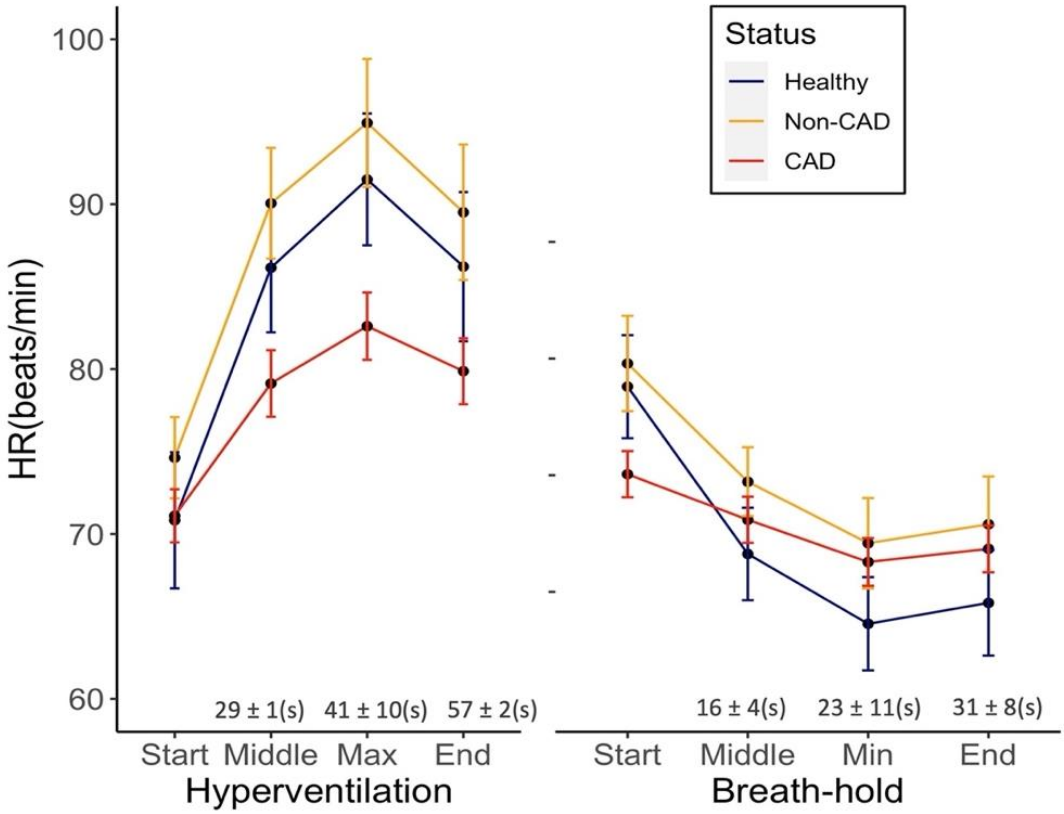


Figure 12. The pattern of HR changes with hyperventilation and breath-hold in three cohorts of participants, including healthy controls, non-CAD, and CAD groups. The HR changes are reported as mean ± SE. Compared to their baselines, all three cohorts exhibited an increase in HR during hyperventilation and HR recovery during the following breath-hold. Patients with CAD had a lower HR change during hyperventilation and breath-hold than controls and non-CAD groups. HR, heart rate; CAD, coronary artery disease.

Table 3 provides a numerical demonstration of Figure 12 with sex-specific HR response to the breathing maneuver. Females in the control and CAD cohorts demonstrated a higher peak HR during HV and min HR during the BH than males, which however was statistically significant only in the control group ($p < .05$). In the non-CAD cohort, males demonstrated a higher peak HR during HV than females with similar min HR during BH when compared to females ($p > .05$). The HRR-BH was lower in females in the control and non-CAD cohorts, with statistical significance in the control cohort ($p < .05$). CAD females exhibited a higher HRR-BH than males ($p > .05$).

Table 3. The heart rate response to breathing maneuver

	Healthy			Non-CAD			CAD			
	Total	Females	Males	Total	Females	Males	Total	Females	Males	P-value ▫
[1] NB_HR_MEAN (bpm)	66.4 ± 12.1	72.8 ± 12.3*	57.7 ± 3.3	71.5 ± 9.4	72.6 ± 10.9	70.5 ± 8.4	69.4 ± 9.9	72.0 ± 8.6	66.8 ± 10.7	0.37
[2] HV_HR_MAX (bpm)	91.5 ± 14.9∞	96.5 ± 16.4*	84.7 ± 10.3	94.9 ± 16.8∞	91.7 ± 16.0	97.8 ± 17.9	82.6 ± 12.5∞	86.1 ± 11.9	79.0 ± 12.4	0.007☆
[3] BH_HR_MIN (bpm)	67.2 ± 15.5∞	77.1 ± 13.0*	54.0 ± 5.2	74.1 ± 12.0∞	74.1 ± 15.9	74.1 ± 7.9	72.5 ± 11.3∞	74.2 ± 11.9	70.8 ± 10.8	0.27
[4] HRR-BH (bpm)	24.2 ± 10.8	19.3 ± 6.3*	30.6 ± 12.7	20.7 ± 18.9	17.5 ± 14.5	23.6 ± 22.6	10.0 ± 6.1	11.9 ± 5.9	8.1 ± 5.9	<.001☆☊

Mean ± SD of HR changes during phases of the breathing maneuver, including normal breathing (NB), hyperventilation (HV), and breath-holding (BH).
▫ Indicates the difference between participants in control, non-CAD and CAD groups, regardless of sex.
∞ Indicates a significant change in HR in each group relative to the previous breathing phase.
* Indicates a significant difference between males and females. ☆ Indicates a significant difference between non-CAD and CAD groups.
☊ Indicates a significant difference between CAD patients and healthy controls.
[1] NB_HR_MEAN represents the average heart rate during two-minute NB.
[2] HV_HR_MAX represents the peak heart rate during 1-minute HV.
[3] BH_HR_MIN represents the minimum heart rate during maximal voluntary BH.
[4] HRR-BH represents the absolute decrease in HR during breath-hold relative to peak HR at HV.
bpm, beats per minute; NB, normal breathing; HV, hyperventilation; BH, breath-holding; HRR-BH, breath-hold heart rate recovery.

4.3. The best-performing HR marker for CAD, HRR-BH

In order to identify the HR marker with the highest discriminative ability and accuracy, including sensitivity and specifically negative predictive value, to distinguish between patients with and without CAD, a backward stepwise regression model was applied to various HR variables acquired during the breathing maneuver. As illustrated in Figure 13, the best-performing marker chosen by the regression model was BH-induced HR recovery (HRR-BH, %), defined as the percentage of HR recovery during BH relative to peak HR during HV (with an Akaike's Information Criteria (AIC) of 70 and an area under the ROC curve of 0.67, being the highest among candidate HR markers). The equation used for calculating HRR-BH is shown in Figure 13. The rest of the statistical analyses are performed based on HRR-BH.

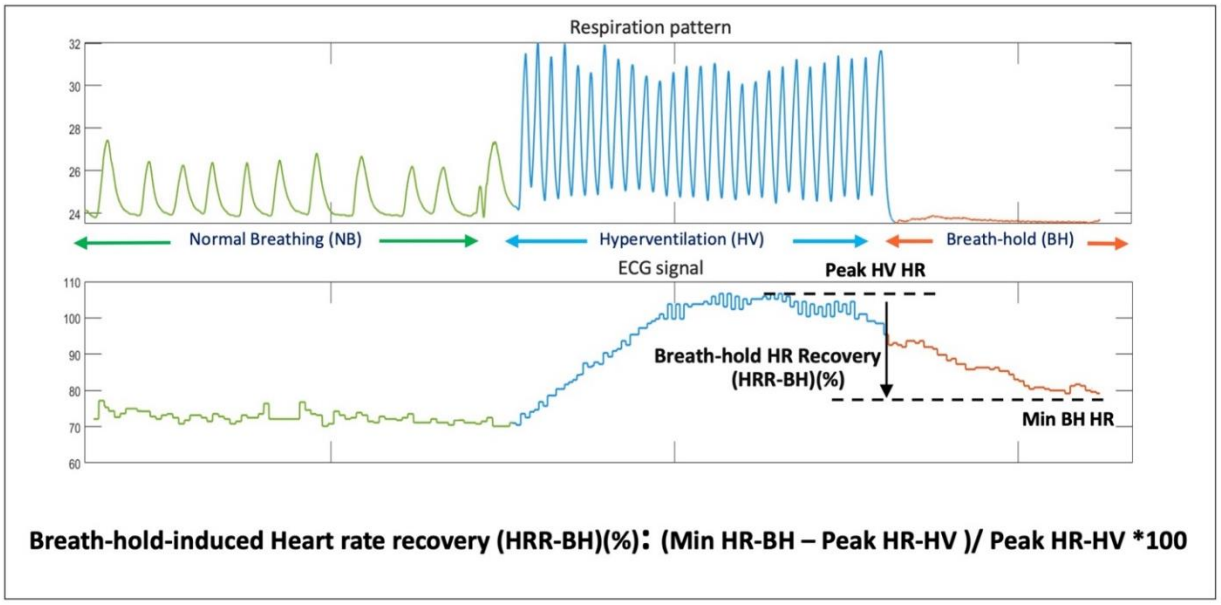


Figure 13. The HR marker of interest with highest diagnostic performance was BH-induced HR recovery (HRR-BH).

4.4. The between-group comparison of the HRR-BH

As depicted in Figure 14, we used ANCOVA to compare the HRR-BH between three groups of participants after controlling for the potential confounding variables. High BMI, dyslipidemia and medications such as CCBs, ACEI, and statins were potential confounders as they were significantly more prevalent in patients with CAD (independent variable) and correlated with HRR-BH (dependent variable). As shown in Figure 14, the analysis revealed that patients with CAD had a significantly lower HRR-BH (11.9±6.5%) than healthy controls (26.5±11.1%, p< .001, 95% CI: -21.91- -7.17) and patients without CAD (20.1±14.7%, p= .01, 95% CI: -14.79- -1.54). The HRR-BH was not significantly different between healthy controls and patients in the non-CAD group (p= .18, 95% CI: -14.67-1.92).

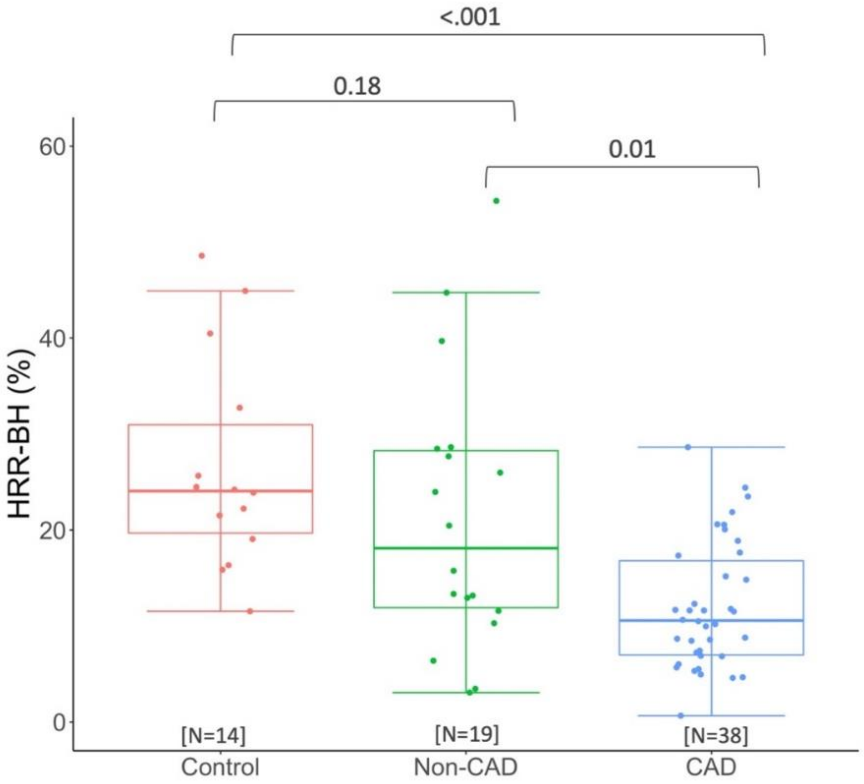


Figure 14. The comparison of the HRR-BH between three groups. Using one-way ANCOVA with Post Hoc test demonstrated that patients in the CAD group had a lower HRR-BH compared to non-CAD patients (p=0.01) and healthy controls (p<.001). CAD, coronary artery disease; HRR-BH, breath-hold heart rate recovery

4.5. Impact of sex and age on the HRR-BH

Figure 15 demonstrates the impact of sex on HRR-BH. While females had a lower HRR-BH than males in control (F, 20.0± 4.8% vs M, 35.2±11.4%, p= 0.02) and non-CAD groups (F, 18.2±13.7% vs M, 21.8±16.2%, p> .05), in the CAD group, they exhibited a greater HRR-BH (F, 13.8±6.4% vs M, 10.1±6.3%, p> .05), which was not statistically significant.

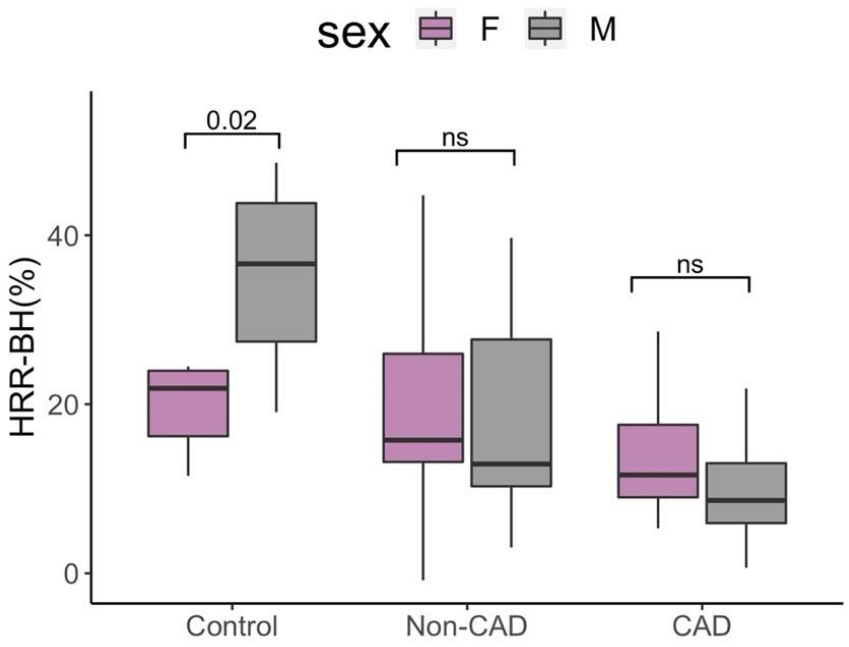


Figure 15. The comparison of the HRR-BH as a function of sex. The comparison of the impact of sex on HRR-BH showed no significant difference between sexes in non-CAD and CAD groups but a significantly higher HRR-BH in males than females in the control group (p=.02). HRR-BH, breath-hold heart rate recovery; ns, non-significant.

The impact of age on HRR-BH is illustrated in Figure 16. Overall, considering all participants as one cohort, we observed a statistically significant inverse correlation between age and HRR-BH (r= -0.31, p= .0086, 95% CI: -0.53- -0.09, Fig. 16A). However, the correlation became non-significant after subgroup analysis in healthy control (r= -0.092, p= .76, 95% CI: -0.59-0.46, Fig. 16B), non-CAD (r= -0.13, p=.59, 95% CI: -0.55-0.34, Fig. 16B), and CAD groups (r=-0.21, p= .2, 95% CI= -0.49-0.11, Fig. 16B).

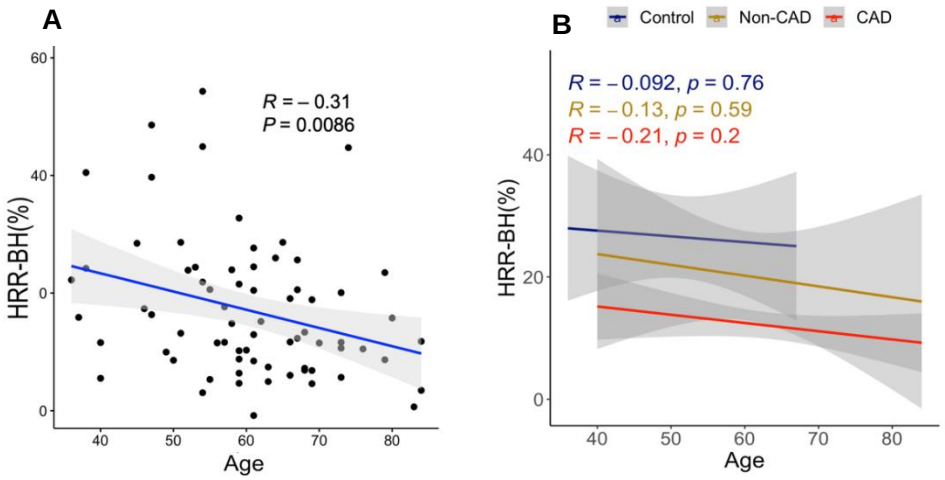


Figure 16. Impact of age on HRR-BH. A. Overall, considering all participants as one cohort, age showed an inverse correlation with HRR-BH. B. At the sub-group level, the negative relationship between age and HRR-BH became insignificant. The ribbon around the fitted lines indicates 95% confidence interval. HRR-BH, breath-hold heart rate recovery

4.6. The impact of antihypertensive medications on HRR-BH

Figure 17 depicts the correlation between HRR-BH and antihypertensive medications, including beta-blockers, CCBs, ARBs, and ACEIs. The comparison of HRR-BH between patients who were not prescribed beta-blocker at the time of the study and those who were on beta-blockers but stopped the medication 12 hours before the MRI appointment and breathing maneuver showed no significant difference in HR response (mean HRR-BH, 14.8% vs 14.6%, p= 0.96, 95% CI: -6.08-5.85, Fig. 17A). Similiarly, no significant difference was found fo patients who were on ARBs (mean HRR-BH, 16.3% vs 14.4%, p= 0.99, 95% CI: -9.15-9.07, Fig. 17D). Conversely, patients who withheld CCBs 12 hours before the MRI exam had a significantly lower HRR-BH than those who were not prescribed the medication at all (mean HRR-BH 9.7% vs 16.8%, p= .002, 95% CI: -11.66- -2.59, Fig. 17B). However, this difference became non-significant after controlling for confounding factors such as age, waist-to-hip ratio, and hypertension in ANCOVA analysis (P= .37). Like CCBs, patients who were on ACEIs had a significantly lower HRR-BH compared to those who were not on the medication at all (mean HRR-BH 10.6% vs 16.1%, p= .02, 95% CI:

10.6-16.1, Fig. 17D). This difference, however, became non-significant after controlling for hypertension in ANCOVA analysis (P= .22).

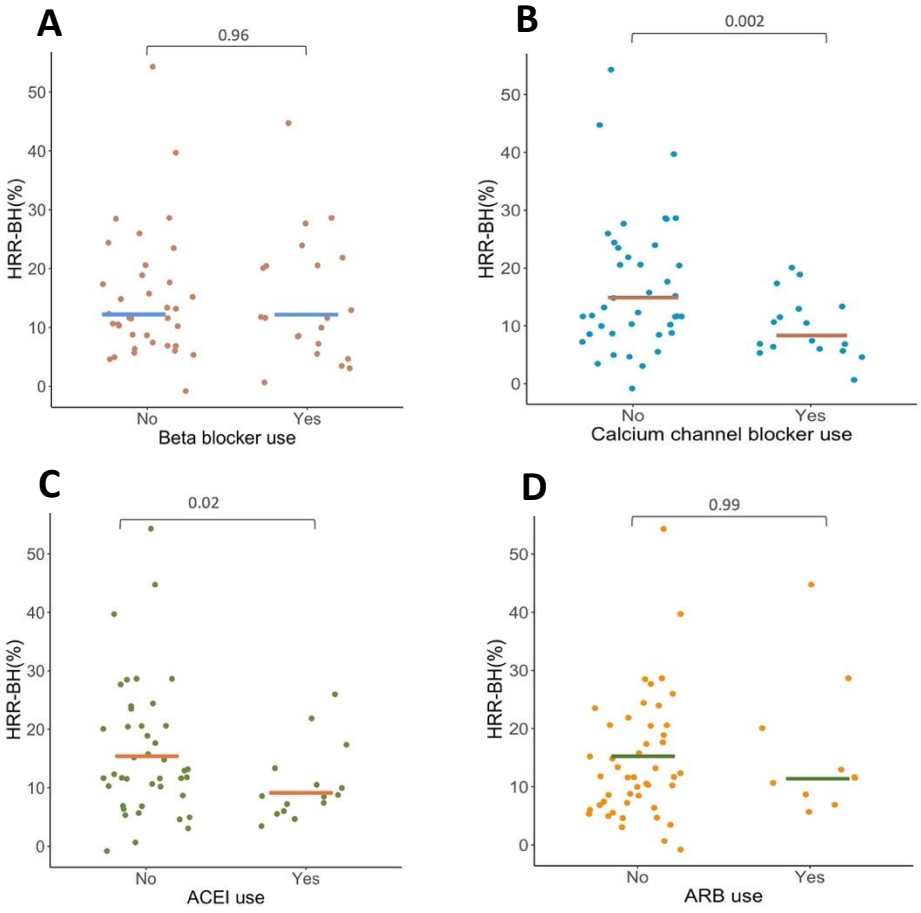
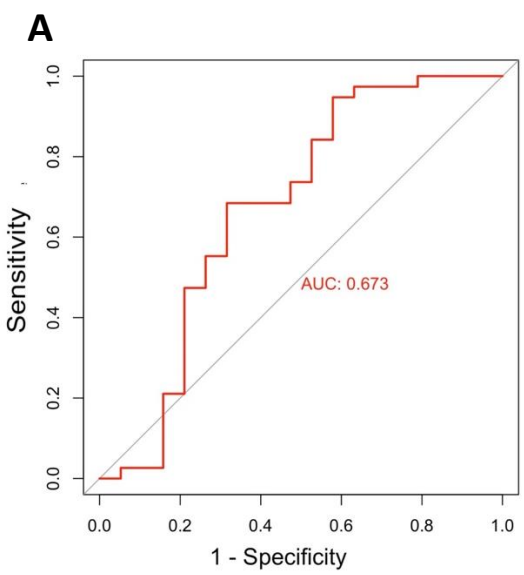


Figure 17. Impact of HR-lowering medications on the HRR-BH. A. The use of beta blockers did not affect the HRR-BH significantly. B. The comparison of the HRR-BH as a function of calcium channel blocker use showed a significant difference between groups. C. The use of ACEI decreased the HRR-BH significantly. D. The use of beta ARBs did not affect the HRR-BH significantly. HRR-BH, breath-hold heart rate recovery; ACEI, and angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker.

4.7. ROC curve analysis of HRR-BH

The area under the ROC curve (AUC) was used to measure the discriminative ability of HRR-BH for the presence of CAD, as detected by either stress CMR perfusion or invasive coronary angiography. As demonstrated in Figure 18, the AUC for HRR-BH was 0.67 (95% CI: 0.50-0.84, Fig. 18A). With ROC analysis, we also identified an optimal cut-off in the curve with the best diagnostic performance possible. Because the main goal of the HRR-BH risk assessment tool is to

help clinicians rule out CAD in suspected patients, we looked for cut-off points on the ROC curve with the highest NPV and sensitivity possible. Using HRR-BH cut-off of 25% was associated with a sensitivity of 97.4% (95% CI: 0.86-1.00), specificity of 37% (95% CI: 0.16-0.62), a negative predictive value of 87.5% (95% CI: 0.47-1.00), and a positive predictive value of 75.5% (95% CI: 0.61-0.87) (Fig. 18B). The comparison of HRR-BH and stress CMR perfusion findings in two patients with suspected CAD are shown in Appendix B.



B

HRR-BH Cut-off= 25%		Stress CMR Perfusion OR Coronary Angiography		
		CAD (n=38)	No CAD (n=19)	
HRR-BH	+	tp= 37	fp= 12	PPV= 75.5%
	-	fn= 1	tn= 7	NPV= 87.5%
		Sens= 97.4%	Spec= 36.8%	

Figure 18. The ROC curve represents the diagnostic performance of HRR-BH. A. The AUC as measure of the predictive power of HRR-BH for CAD risk assessment was 0.67. B. HRR-BH cut-off of ≥ 25% was associated with a sensitivity of 97.4% and NPV of 87.5% for the presence of CAD. ROC, receiver operating characteristic; AUC, area under the ROC curves; CAD, coronary artery disease; HRR-BH, breath-hold heart rate recovery

4.7.1. Sex-specific ROC curve analysis of HRR-BH

As illustrated in Figure 19, sex-specific analysis of AUC demonstrated a lower discriminative power of HRR-BH for the presence of CAD in females than in males (F, 0.61 vs M, 0.73, Fig. 19B). When assessing the diagnostic thresholds in the ROC curves for both sexes, sex-based differences in the optimal cut-off and their diagnostic performance were observed. The optimal sex-specific cut-offs of HRR-BH for females and males were 24% and 22%, respectively (Fig.

19A & C). Compared to males, the female-specific HRR-BH cut-off ≥ 24% was associated with lower specificity (F, 33.3% vs M, 50%), positive predictive value (F, 75% vs M, 78.2%), and negative predictive value (F, 75% vs M, 83.3%) for differentiating between patients with and without CAD. Using a sex-specific cut-off did not affect the sensitivity (97.4%) of HRR-BH in both sexes.

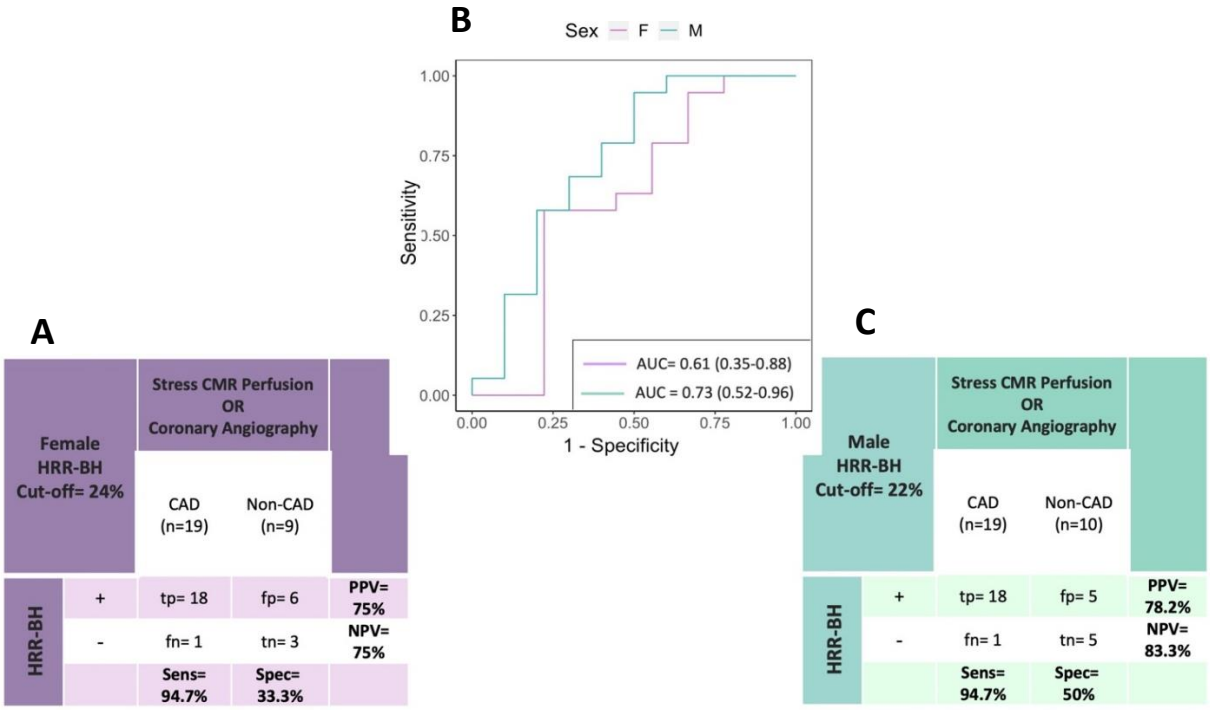


Figure 19. ROC curve represents the sex-specific diagnostic performance of HRR-BH. A&C. Female-specific cut-off of 24% HRR-BH had a lower specificity, PPV, and NPV for CAD when compared to cut-off of 22% in males. B. The AUC was lower in females than in males. ROC, receiver operating characteristic; AUC, area under the ROC curves; PPV, positive predictive value; NPV, negative predictive value; CAD, coronary artery disease; HRR-BH, breath-hold heart rate recovery

4.8. Impact of HRR-BH on pre- and post-test probability

Figure 20 depicts the relationship between pre-test and post-test probability in Fagan's nomogram. As mentioned in the method section, we used the Modified Diamond-Forrester prediction model to estimate the pre-test probability of CAD. This model categorized patients as low (<5%), intermediate (5–15%), or high (>15%) pre-test probability of CAD based on their age, sex, and

nature of symptoms. In our study, a negative likelihood ratio of 0.07 for a negative HRR-BH result, i.e., HRR-BH of ≥ 25%, decreased the risk of CAD from a pre-test probability of 10% (intermediate-risk) to the post-test probability of< 1% (low-risk) (Fig. 23A). Similarly, in patients with a high pre-test probability of CAD (29%), the post-test probability of CAD given negative HRR-BH dropped to 2.7%, which would be considered as low-risk (Fig. 23B). This result indicates that the HRR-BH has the potential to serve as a gatekeeper and a guide for better patient selection for further testing.

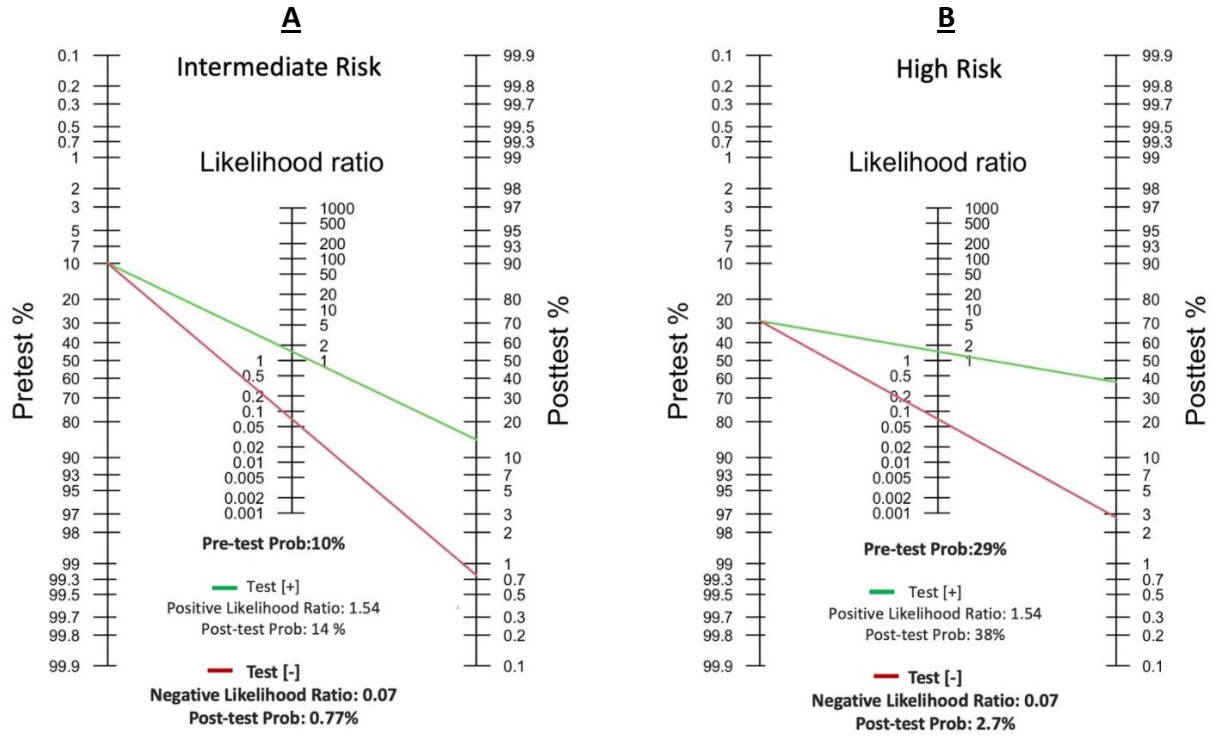


Figure 20. Fagan's nomogram for HRR-BH. Fagan's nomogram depicts the relation between pre-test and post-test probabilities for positive and negative likelihood ratio values. The red line crosses the likelihood ratio for a negative test, and the green line corresponds to the likelihood ratio for a positive test. In patients with an intermediate (10%) and high (29%) pre-test probability of CAD, a negative likelihood ratio of 0.07 for negative HRR-BH (HRR-BH ≥ 25 %) decreased CAD post-test probability to 0.77% and 2.7%, respectively.

5. DISCUSSION

The analysis of 14 healthy volunteers and 57 patients with known or suspected CAD presenting with new-onset angina revealed that the HR response to the breathing maneuver, i.e. BH-induced HR recovery relative to peak HR during HV (HRR-BH), had an area under the ROC curve of 0.67 for differentiating between patients with and without CAD compared to the reference standard of stress CMR perfusion and invasive coronary angiography during 1-year follow-up. An HRR-BH cut-off of ≥ 25% was associated with high sensitivity (97.4%) and negative predictive value (87.5%) and low negative likelihood ratio (0.07) for the presence of CAD, indicating its potential to serve as an efficient tool to rule out CAD for clinicians, thereby avoiding unnecessary investigations.

Compared to the 2019 Modified Diamond-Forrester risk score, HRR-BH could provide clinically meaningful improvements in CAD risk assessment by correctly reclassifying patients from high (29%) and intermediate-risk (10%) categories to the low-risk (<5%) category, informing clinicians' decision-making for better patient selection for further testing and referring the right patient to the right diagnostic test.

5.1. Comparison with previous studies

The present study added to the body of evidence from the previous study on the diagnostic performance of HR response to HV for cardiovascular disease.

Hawkins et al. (33) retrospectively studied the HR response to 1 minute of deep, paced (30 RR/min) HV (HRR_{HV}) in healthy controls and four patient groups classified as HF, CAD with at least one untreated coronary stenosis ≥50% of the diameter as evaluated by angiography, patients with both HF and CAD (CAD+/HF+), and symptomatic patients with no history of HF and significant CAD (CAD-/HF-). Patients in all cohorts had a lower HRR_{HV} compared to healthy

subjects (healthy: 15 ± 9, HF: 6 ± 6, CAD: 8 ± 8, CAD+/HF+: 6 ± 4, CAD-/HF-: 8 ± 6 bpm, $p < 0.001$) with no significant difference between disease groups. The ROC analysis showed that an HRR_{HV} cut-off of 15.5 bpm was associated with a sensitivity of 91%, a specificity of 72%, a positive predictive value of 73% and a negative predictive value of 91% for distinguishing patients with cardiovascular disease from healthy subjects.

One of the major methodological differences between the study of Hawkins et al. and our study is the breathing protocol and the specific HR marker related to the recovery that performed better than the mere HR increase during HV. Hawkins et al. investigated the HR changes only during HV, specifically the HR response to HV relative to resting HR during preceding normal breathing (HRR_{HV}). In our study, the 1 minute period of HV was followed by maximal voluntary BH, and the main HR parameter was the BH-induced HR recovery relative to peak HR during preceding HV(HRR-BH). Given the variation of the HR during HV, the protocol we used in this study is more controlled and likely more reproducible concerning the measured values.

The other difference is the method of HR cut-off calculation. Hawkins et al. reported the absolute HR change by calculating the difference between HV and normal breathing HR, while in the present study, we reported the relative HR change by calculating the percentage of HR recovery during BH relative to peak HR at HV (HRR_{HV} 15.5 bpm vs HRR-BH 25%). Using different methods of effect measurement, either absolute or percent change, has been shown to yield different diagnostic accuracies depending on baseline data distribution and the correlations between baseline and post-exposure data (117, 118).

Other than specificity that was higher for HRR_{HV} compared to HRR-BH (72% vs 36.8%), the rest of the diagnostic performance indices were comparable between HRR_{HV} and HRR-BH (HRR_{HV} vs HRR-BH: sensitivity 91% vs 94.7%, NPV 91% vs 87.5%, PPV 73% vs 75.5%). Combining the HF and CAD groups into a single group in Hawkins et al. study allowed performing

the ROC analysis on larger sample size, leading to greater statistical power and the ability to perform age-stratified analysis.

Like the CAD-/HF- group in Hawkins et al. study, our study showed that patients with new-onset angina but without significant CAD in reference standard had a blunted HRR-BH. Although these patients had no evidence of significant CAD, they are not considered healthy because of the underlying cardiovascular risk factors and abnormal ECG stress tests. Similar to Hawkins et al., we found that the use of beta-blocker did not reduce the HRR-BH in the patient population. However, we could show that taking CCBs significantly decreases HRR-BH.

5.2. Pathophysiology of a blunted HR response to breathing maneuver

5.2.1. Pathophysiology of a blunted HR response to hyperventilation

As mentioned before, the literature is inconsistent as to which component of ANS plays the dominant role in cardiovascular response to HV (95-98). Given the mediating role of increased sympathetic and cardiovagal tone in coronary vasodilation in healthy individuals (42, 47, 119) and the presence of coronary vasoconstriction in response to HV in healthy volunteers, as evidenced by OS-CMR (12-14), it appears likely that vagal withdrawal plays a more critical role than sympathetic activity in mediating the cardiovascular responses to HV. In patients with CAD, however, the extent and spectrum of sympathovagal balance might vary, depending on the location and extent of ischemia (75, 120). This section reviews the possible pathomechanisms for blunted HR response to HV in CAD patients.

1. Sympathetic desensitization:

The constant exposure to sympathetic excitation in patients with CAD decreases the sensitivity of the SA node to beta-adrenergic stimulation—as an adaptative process—thereby reducing the HR response to sympathetic activation during provocative maneuvers (121-123). Our speculation on the pronounced contribution of vagal withdrawal to cardiovascular responses to HV does not

exclude a significant role of sympathetic activity in cardiovascular responses to HV. In the present study, the reduced SA node sensitivity to HV-induced sympathetic excitation might explain the blunted HR response in CAD patients. Measuring other markers of ANS activity such as heart rate variability may provide more insight into the underlying role of sympathetic desensitization.

2. The protective anti-ischemic mechanism:

It is well-established that most of the blood inflow into the myocardium occurs during diastole and that, as the HR increases, the duration of diastole decreases from about 70% of the cardiac cycle at rest to approximately 20% at a maximum HR (124). In patients with CAD, as a protective response to increased sympathetic outflow to the heart in stressful conditions, the autonomic control of the SA node reduces the HR response by compensatory parasympathetic stimulation, allowing more time for myocardial perfusion during more prolonged diastole (124, 125).

In the present study, the compensatory vagal stimulation may explain the blunted HR response to HV in CAD patients: The addition of HV-induced coronary vasoconstriction to the already narrowed coronary arteries in CAD patients further decreases the blood supply to the heart, triggering vagal excitation as a protective mechanism to increase the myocardial perfusion time and decrease oxygen demand by suppressing the HR increase in response to HV. Measuring beat-to-beat BP during the breathing maneuver might have helped us understand the role of the protective mechanism by estimating the rate pressure product as an indirect measure of myocardial oxygen demand.

3. The impact of resting vagal tone:

As mentioned before, we hypothesize that vagal withdrawal might be the main contributor to cardiovascular responses to HV. In the present study, the low resting vagal tone in patients with CAD, as manifested by higher resting HR during normal breathing than in healthy controls, may limit the ability of the ANS to further reduce the vagal tone during HV, resulting in a blunted

increase in HR in these patients. Performing time-domain and frequency-domain HRV analysis in the next steps will help to better understand the role of vagal tone in the HR response.

5.2.2. Pathophysiology of a blunted HR response to breath-holding

The recovery of the HR at the same time with coronary vasodilation during BH in healthy individuals (13, 100) suggests a dominant role of increased cardiovagal tone in mediating the cardiovascular response to BH in healthy people. Hence, we speculate that in the present study, the low cardiovagal tone in CAD patients may be the main contributor to the blunted HRR-BH. Further, it is conceivable from the study results that the extent of BH-induced vagal withdrawal depends on the vagal tone during the preceding HV; the low magnitude of vagal withdrawal during HV in CAD patients, as explained in the previous section, might have negatively affected the ability of the ANS to increase vagal activity and recover the HR during BH, contributing to blunted HR recovery.

5.3. Impact of sex on the HRR-BH

In the present study, the sex-specific ROC analysis deteriorated the AUC (F, 0.61 vs M, 0.73) and diagnostic performance of HRR-BH in females compared to males [specificity (F, 33.3% vs M, 50%), PPV (F, 75% vs M, 78.2%), and NPV (F, 75% vs M, 83.3%)] for differentiating between patients with and without CAD.

The sex differences in cardiovascular responses to breathing maneuvers have not been extensively studied in the literature. In a study of hemodynamic response to HV in 369 healthy subjects, Alexopoulos et al. (89) reported a slightly lower HR increase in response to 5 minutes of HV and an HR drop during the subsequent 10-min recovery in females compared to males. Conversely, Hawkins et al. showed no significant sex differences in the HRR_{HV} in healthy controls and HF, CAD, CAD+/HF+, and CAD−/HF− cohorts.

In the present study, in general, females exhibited a blunted HRR-BH compared to males, and among the female population, the HRR-BH was not significantly different between the three cohorts of healthy, non-CAD and CAD. It appears from the literature that women have a higher baseline parasympathetic activity than males (54-56). The higher vagal tone might have attenuated the extent of HR increase during HV, which, in turn, influenced the amount of HRR-BH during the subsequent BH. Furthermore, the lower baroreflex sensitivity in females (52, 57, 58) could be another explanation for blunted HR response to the breathing maneuver. This observation also confirms our speculation that the magnitude of BH-induced vagal restoration depends on the extent of HV-induced vagal withdrawal.

Our analysis showed that the sex differences in HRR-BH were less significant in patients with CAD diagnosis. The structural and functional changes in ANS in patients with CAD possibly blur the sex differences in the HR response to the breathing maneuver. The underlying pathophysiological mechanisms in CAD, such as decreased SA node sensitivity to sympathetic stimulation (121-123) and vagal excitation as a protective mechanism to increase the myocardial perfusion time (124, 125) during hyperventilation, might have decreased the sex differences in ANS function between males and females, who have been reported to have a higher baseline parasympathetic activity, leading to blunted HR changes during the breathing maneuver in both sexes. Assessing the sex disparities in HRV analysis, a well-known marker of ANS function, and its comparison with quantitative perfusion will provide us with a better understanding of the sex differences in ANS function and its association with perfusion deficit.

Another possible explanation for the different HR responses could be the role of female-specific risk-enhancing factors. The female-specific risk factors such as polycystic ovarian syndrome (PCOS), premature menopause, and hormone therapy have been shown to be associated

with the sympathovagal imbalance and increased incidence of CAD (50, 126, 127). In the present analysis, we did not collect information on female-specific risk factors.

5.4. Impact of age on the HRR-BH

We found an inverse association between HRR-BH and ageing in all cohorts of healthy controls, non-CAD, and CAD groups (R= -0.31, p= 0.0086).

Normal ageing is associated with changes in the autonomic control of the heart (128). The blunted post-exercise HR recovery with ageing suggests an age-related decline in cardiovagal activity (79). The frequency-domain analysis of short-term HRV in healthy males and females aged 6 to 55 years revealed a significant decrease in HF and LF/HF ratios, indicating a declined vagal responsiveness and increased dependency on sympathetic control of cardiac function. (129). In addition, baroreflex dysfunction and increased circulating levels of norepinephrine may contribute to age-associated reduced vagal modulation (130).

Our findings are in concordance with the literature. As mentioned before, we speculate that vagal restoration plays a central role in BH-induced HR recovery or HRR-BH. Therefore, the reduced vagal responsiveness with ageing seems to explain the attenuation of HRR-BH with ageing in our study.

5.5. Impact of hypertension and medications on the HRR-BH

The respiration-related oscillations in BP are an important component of cardiorespiratory interaction (87). However, the studies have demonstrated inconsistent findings on the impact of respiration on blood pressure, with the majority reporting a drop in BP during HV and BP increase during BH (131, 132). In the present study, we did not objectively measure the participants' blood pressure during the breathing maneuver, limiting the ability to explore the relationship between

blood pressure and HRR-BH. Acquiring the beat-to-beat blood pressure in future studies on the breathing maneuver will allow us to investigate this association.

In the present study, we recorded the self-reported history of hypertension, and the comparison of the HRR-BH as a function of the history of hypertension demonstrated no significant difference between groups. It is well-known that hypertension is associated with ANS impairment characterized by increased sympathetic activity and decreased vagal tone (133). Individuals with hypertension have been reported to have lower HR variability and higher resting HR (134). The association between self-reported hypertension and ANS impairment has not been extensively studied in the literature. However, a systematic review of the accuracy of the self-reported HTN showed an overall sensitivity of 42.1% (95% CI 30.9– 54.2) with high heterogeneity ($I^2 > 99\%$; $P < 0.001$) (135), indicating the limited accuracy and applicability of self-reported HTN and its limited clinical value for correlation with the pathophysiological mechanisms.

5.5.1. Impact of beta-blockers on the HRR-BH

The present study demonstrated no significant difference in HRR-BH between patients who were not prescribed beta-blocker at the time of the recruitment and those who were on beta-blockers but stopped the medication 12 hours before performing the breathing maneuver and undergoing MRI.

The HR-lowering agents are common confounders that can affect the HR profile during the stress test. Beta-blockers can block the effects of sympathetic neurotransmitters by binding to β-ARs, especially β1-ARs located in the SA node, reducing the HR and BP (136). Given the main effect of beta-blockers on sympathetic activity, they are expected to not significantly influence the vagally-mediated HR responses. In patients with a negative exercise stress echocardiography, the post-exercise HR recovery, reflective of the parasympathetic restoration, was reported not to be affected by the use of beta-blockers (137). Another study, however, reported the negative impact of beta-blockers on post-exercise HR recovery in patients with CAD, which was suggested not to

be directly related to beta-blocker but to be due to the high dependence of the HR recovery on the chronotropic response to exercise, which was blunted by beta-blockers. (138).

The lack of impact of beta-blockers on HRR-BH in the present study confirmed our speculation on the prominent role of the vagal tone in mediating the HR response to both HV and BH. This finding is in concordance with Hawkins et al.(11), who reported no significant difference in the HR response to HV based on beta-blocker status, whether they were taken, withheld, or not prescribed at all.

5.5.2. Impact of calcium channel blockers on the HRR-BH

As the first study exploring the impact of CCBs on the HR response to the breathing maneuver, we demonstrated a significantly lower HRR-BH in patients who withheld CCBs 12 hours before the MRI than in those who were not prescribed the medication at all.

Calcium channel blockers are another widely used medication for cardiovascular diseases, including hypertension, coronary spasm, angina pectoris, etc. (139). The dihydropyridine class of CCBs is more vascular selective, reducing the BP by vasodilation, while the non-dihydropyridine class primarily acts on the myocardium and tends to reduce the HR by exerting inhibitory effects on the SA and AV nodes (139, 140).

In contrast to the literature where dihydropyridine type of CCBS has been shown to have minimal effect on HR (140, 141), the majority of the patients in the present study were on dihydropyridine type of CCBS. Therefore, it is conceivable that the reduced HRR-BH in patients on CCB may not be directly related to the medication but to the confounding effect of age, waist-to-hip ratio, and a higher proportion of patients with hypertension in this group.

5.5.3. Impact of ACEIs on the HRR-BH

The ACE inhibitors modulate blood pressure by inhibiting the conversion of angiotensin I to angiotensin II, resulting in vasodilation and loss of sodium and water (142). One of the hallmarks

of ACE inhibitors is that they decrease peripheral vascular resistance without inducing a compensatory increase in HR, likely by reducing central sympathetic outflow, increasing vagal tone, and restoring baroreflex control (142-144).

Given the parasympathomimetic effect of ACEI inhibitors, HRR-BH is expected to be greater in patients who were on ACEI inhibitors. However, in the present study, patients on ACEI inhibitor treatment showed a significantly lower HRR-BH than those who were not prescribed the medication at all. It is important to note that after controlling for history of hypertension, the difference in HRR-BH based on ACEI inhibitor status became non-significant.

5.6. Reproducibility of cardiovascular response to breathing maneuver

In order to implement the HRR-BH in clinical practice as a rule-out risk assessment tool for CAD, the knowledge of its reproducibility is essential. In the present study, we did not assess the reproducibility of the HRR-BH, but a recent work conducted by our team studied the reproducibility of coronary vascular response to the same breathing maneuver, i.e., normal breathing followed by deep, paced (30 RR/min) HV and subsequent maximal voluntary BH, as quantified by OS-CMR (145). This study recruited 21 patients with HF and 21 healthy participants who performed repeated breathing maneuvers with the same methodology inside MRI. The global myocardial oxygenation, as characterized by signal intensity changes in OS-CMR, was not significantly different after hyperventilation (HV1: -7.82±5.2; HV2: -7.89±6.4, p=0.9) or breath-hold (BH1: 5.34±3.1; BH2: 6.0±3.3, p=0.5) between the repeated breathing maneuvers, suggesting consistent myocardial oxygenation changes during repeated hyperventilations and breath-holds. Overall, the study revealed that the breathing maneuver is a robust vasoactive stimulus that induces reproducible cardiovascular responses in both healthy individuals and patients.

5.7. Validation Steps of the HRR-BH Method

The groundwork for the present study was laid by the retrospective proof-of-concept study by Hawkins et al.(11), which allowed us to develop the concept of the discriminatory potential of the HR response to the breathing maneuver for significant CAD prospectively in the present study in a clinical setting.

In the next phase, we intend to assess the diagnostic performance of the selected HRR-BH threshold compared to invasively measured markers for the severity of coronary artery stenosis. To this end, we plan to analyze the HR data collected from the B-More (Breathing-Maneuver-Induced Myocardial Oxygenation Reserve) pilot study. The B-more study was conducted at McGill University Health Centre and aimed to assess the diagnostic accuracy of vasoactive breathing maneuvers coupled with Oxygenation-Sensitive Cardiac Magnetic Resonance Imaging (OS-CMR) in diagnosing coronary artery stenosis compared to the current clinical reference standard of fractional flow reserve (FFR) or quantitative coronary angiography (QCA) in 65 patients with suspected CAD and 27 healthy volunteers. The HR changes during the breathing maneuver were continuously captured during the MRI exam. Using the HR data, we will determine the performance measures of HRR-BH, including discriminative capability, calibration, sensitivity, specificity, negative and positive predictive values, and negative and positive likelihood ratios for the presence of significant CAD. This analysis will also help us further validate the clinical features that may influence the diagnostic performance measures, requiring different thresholds for selected sub-groups, e.g., females, patients on medication, or caffeine. Further, for internal validation, the bootstrap technique will be applied to the results of the present study and B-More pilot study to provide valid estimates of predictive performance with low bias.

A potential barrier to the utility of the HRR-BH method in real-world practice might be the need for a medical device to record the HR and respiratory information, which are often expensive

and difficult to maintain. Hence, we plan to develop a mobile medical application to compute and track HR changes during guided breathing maneuvers in real-time using a smartphone photoplethysmography (PPG) sensor. In the verification step, we will evaluate whether the software meets the design specifications, including sufficient photoplethysmography signal quality and accurate detection of maximum and minimum HR plateau during hyperventilation and breath-hold compared to an FDA-approved medical device. Once the HRR-BH is internally validated, external validation studies involving several centers with large sample sizes should be performed to validate the performance of the software-derived HRR-BH threshold and detect clinically important changes in performance compared to the internally validated estimate.

5.8. Application of HRR-BH in daily clinical practice

Due to the wide differential diagnosis for chest pain and the growing financial burden of over-investigation on the healthcare system, accurate stratification is critical, especially for preventing low-risk patients from undergoing costly and unnecessary medical tests. The greatest potential clinical utility of software-derived HRR-BH is in-office risk stratification of patients with angina presentation for the presence of significant CAD and identifying those who may not benefit from further testing. **Figure 10** depicts the suggested workflow for evaluating patients with stable angina using HRR-BH. Having said that, this proposed workflow has to be tested prospectively in larger clinical studies. Besides the office-based application of the HRR-BH method for CAD risk stratification, it has the potential to be incorporated into the telemedicine strategy for remote assessment of patients' risks.

In addition to the utility of HRR-BH as a single marker, it can be used as a complementary marker in combination with stress perfusion or breathing-enhanced CMR protocols. Particularly, the addition of HRR-BH to OS-CMR results might provide incremental diagnostic information for the presence of CAD. B-more pilot data indicates that a composite marker of (with at least two out

of three markers positive for disease state) myocardial oxygenation reserve, strain, and HR changes during breathing maneuver can identify patients with significant CAD with a sensitivity of 94.4%, the negative predictive value of 97.4%, and overall diagnostic accuracy of 84.4% (unpublished data).

Further, the use of HRR_BH as a stress testing gatekeeper in the primary care setting helps to improve the accessibility of healthcare services for patients with a more critical need for cardiac testing and reduces the economic burden and the waste of resources caused by over-referrals to stress tests.

For rural, remote or isolated regions, the HRR-BH method could positively impact several aspects of healthcare services delivery; it will help family physicians overcome healthcare access barriers by ruling out significant risk for CAD in suspected patients in their office without the need to make the patients travel extraordinary distances for unnecessary cardiac testing. This will save travel costs for patients and professionals and help deliver specialized services in a timely fashion for patients with a more critical need for secondary assessments.

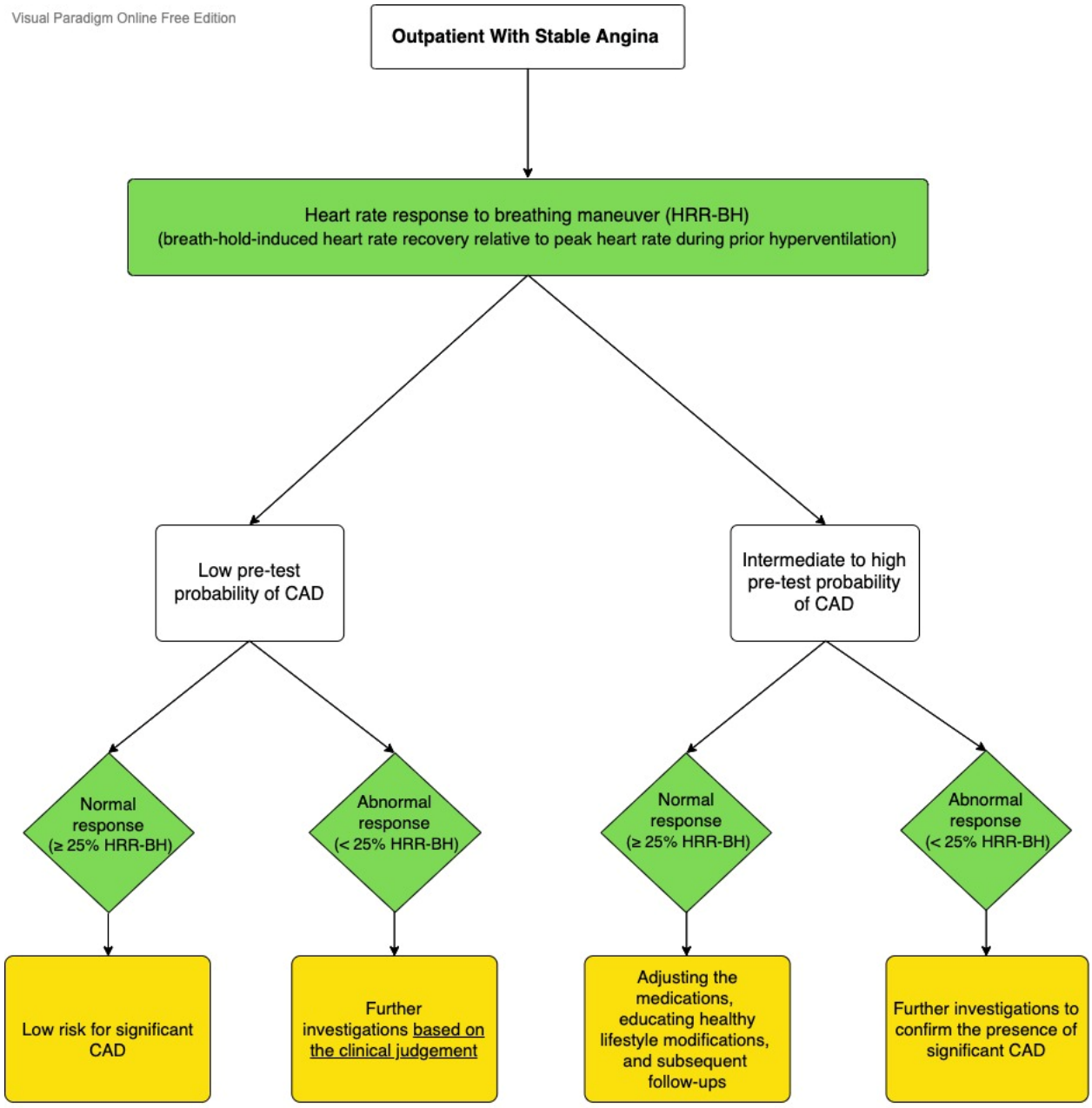


Figure 21. Evaluation algorithm for outpatients with stable angina using HRR-BH. Created with Visual Paradigm Online Free Edition.

5.9. Limitation

There are some limitations to our study:

1. The study design may not allow the generalizability of the findings to other clinical settings: The present study was conducted at a single tertiary-care referral center and thus was open to biases in patient selection and referral patterns for stress CMR perfusion and coronary angiography. This limitation might also be a potential strength by simulating actual clinical application and real-world clinical practice. We left the decision for patients' referral to the physicians' judgement, and no attempt was made to influence their decision by providing a guideline and strict criteria.

2. We could not exclude differential verification (or work-up) bias as the reference standard for CAD diagnosis was not consistent among all patients. The reference standard for detecting CAD was the presence of inducible myocardial perfusion deficits, as evidenced by qualitative/visual analysis of stress CMR perfusion. However, for those patients that underwent invasive coronary angiography during a 1-year follow-up, the visual assessment of coronary stenosis was considered the reference standard.

3. The limited sample size did not allow us to conduct subgroup ROC and diagnostic performance analyses. In a larger sample size, different thresholds for age categories and patients on medications may need to be developed.

4. In this study, we could not control or account for potential differences in performing the breathing maneuver between individuals, such as the depth of HV or performing the Valsalva maneuver during the BH. However, a work conducted by our team using OS-CMR reported the reproducibility of the coronary vascular responses to both HV and BH in healthy individuals and patients with HF (145).

5. In the present study, the diagnosis of CAD in stress CMR perfusion was based on the qualitative/visual assessment of images. While qualitative assessment has been shown to have a

good diagnostic accuracy compared to invasive coronary angiography (27, 30), it may avoid a more complex subgroup analysis according to the severity of CAD, leading to misrepresentation of the atherosclerotic burden, especially in patients with microvascular dysfunction and diffuse coronary disease. Also, the qualitative assessment is subjected to significant inter-observer variability. Blinding the clinical readers to the HR response to the breathing maneuver might have reduced the bias. As the next step in our study, we will be performing a quantitative perfusion analysis to substantiate our hypothesis with respect to the severity of coronary involvement and to see whether it will produce better diagnostic performance indices for HRR-BH.

6. Another potential limitation of our study is the lack of beat-to-beat BP data, which could shed light on the peripheral vascular response to the breathing maneuver and the mediating role of the baroreflex in the HR response to HV and BH.

6. CONCLUSION

In summary, our preliminary analysis revealed that patients with coronary artery disease (CAD) had a lower breath-hold-induced heart rate (HR) recovery relative to peak HR during hyperventilation (HRR-BH) compared to symptomatic patients without CAD diagnosis and healthy participants. The HRR-BH was the best-performing HR parameter with a high negative predictive value and sensitivity for ruling out CAD in the office setting, albeit with a lower performance in women. Given a normal HR response, the low negative likelihood ratio of HRR-BH decreased the pre-test probability of CAD from intermediate or high to low post-test probability.

The study findings suggest that the HRR-BH may serve as a simple, readily available, and inexpensive stress test gatekeeper by correctly reclassifying patients with an intermediate or high pre-test probability but a normal HR response from high-risk to low-risk, thereby improving

patient selection for further diagnostic, typically stress testing but also possibly avoiding invasive diagnostic procedures.

In order to implement HRR-BH as a pre-imaging screening test in clinical practice, a prospective study should be conducted with a larger sample size—a separate sample from that used to initially derive the HRR-BH cut-off—to assess its external validity and its classification and calibration indices (how closely the predicted outcome matches the actual outcome) for providing accurate estimates of CAD risk in suspected patients. Future assessments would aim to develop mobile medical software for clinicians with the capacity for real-time monitoring and analysis of the HR changes during the breathing maneuver, aiding clinical decision-making for further investigation in patients with suspected CAD.

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8. DECLARATIONS

Dr. Matthias Friedrich is listed as a holder of: United States Patent No. 14/419,877: Inducing and measuring myocardial oxygenation changes as a marker for heart disease; United States Patent No. 15/483,712: Measuring oxygenation changes in tissue as a marker for vascular function; United States Patent No 10,653,394: Measuring oxygenation changes in tissue as a marker for vascular function; Canadian Patent CA2020/051776: Method and apparatus for determining biomarkers of vascular function utilizing bold CMR images.

9. APPENDIX

9.1. Appendix A

The "Non-Laboratory" Based INTERHEART Modifiable Risk Score*

Risk factor	Question	Points for the answer		Points for each section
Age	Are you a man 55 years or older OR woman 65 years or older?		2	**Points:**
	OR Are you a man younger than 55 years or woman younger than 65 years		0	
Smoking. Pick the description which matches you best:	I never smoked		0	**Points:**
	OR I am a former smoker (last smoked more than 12 months ago)		2	
	OR I am a current smoker or I smoked regularly in the last 12 months, and I smoke…	1-5 cigarettes per day	2	
		6-10 cigarettes per day	4	
		11-15 cigarettes per day	6	
		16-20 cigarettes per day	7	
		More than 20 cigarettes per day	11	
Second hand smoke	Over the past 12 months, what has been your typical exposure to other people's tobacco smoke?	Less than 1 hour or exposure per week or no exposure	0	**Points:**
		OR One or more hours of secondhand smoke exposure per week	2	
Diabetes	Do you have diabetes mellitus?	Yes	6	**Points:**
		No or unsure	0	
High Blood Pressure	Do you have high blood pressure	Yes	5	**Points:**
		No or unsure	0	
Family history	Have either or both of your biological parents had a heart attack?	Yes	4	**Points:**
		No or unsure	0	
Waist to hip ratio	Pick one only:	Quartile 1: Less than 0.873	0	**Points:**
		Quartile 2 &3: 0.873 - 0.963	2	
		Quartile 4: greater than or =0.964	4	
Psychosocial factors	How often have you felt work or home life stress in the last year? Pick one only	Never or some periods	0	**Points:**
		OR Several periods of stress or permanent stress	3	
	During the past 12 months, was there ever a time when you felt sad, blue, or depressed for two weeks or more in a row?	Yes	3	**Points:**
		No	0	
Dietary factors. Pick one answer for each food group mentioned	Do you eat salty food or snacks one or more times a day	Yes	1	**Points:**
		No	0	
	Do you eat deep fried foods or snacks or fast foods 3 or more times a week?	Yes	1	**Points:**
		No	0	
	Do you eat fruit one or more times daily?	Yes	0	**Points:**
		No	1	
	Do you eat vegetables one or more times daily?	Yes	0	**Points:**
		No	1	
	Do you eat meat and/ or poultry 2 or more times daily?	Yes	2	**Points:**
		No	0	
Physical activity	How active are you during your leisure time?	I am mainly sedentary or perform mild exercise (requiring minimal effort)	2	**Points:**
		OR I perform moderate or strenuous physical activity in my leisure time	0	

Figure 22. Non-laboratory INTERHEART questionnaire

9.2. Appendix B

9.2.1. Case Presentation 1

A 49-year-old man with a history of smoking and a family history of CAD and sudden cardiac death presented to the cardiology clinic with exertional pleuritic chest pain, resolving gradually in 45 min while resting with no dyspnea or palpitation complaints. Since the exercise ECG for CAD risk assessment was inconclusive, the patient was referred to stress CMR perfusion for further investigation.

The patient performed the 4-min breathing maneuver before undergoing the MRI exam. As depicted in Fig. 24A, the HR increased significantly during HV and recovered by 27% during the subsequent breath-hold, which was more than the HRR-BH cut-off of 25%. After performing the breathing maneuver, the patient underwent stress CMR perfusion, which showed no evidence of inducible perfusion deficit (Fig. 24B). There negative stress CMR perfusion and normal HRR-BH results in this patient with intermediate risk of CAD represent an example of the potential of the breathing-driven HRR-BH to avoid unnecessary testing.

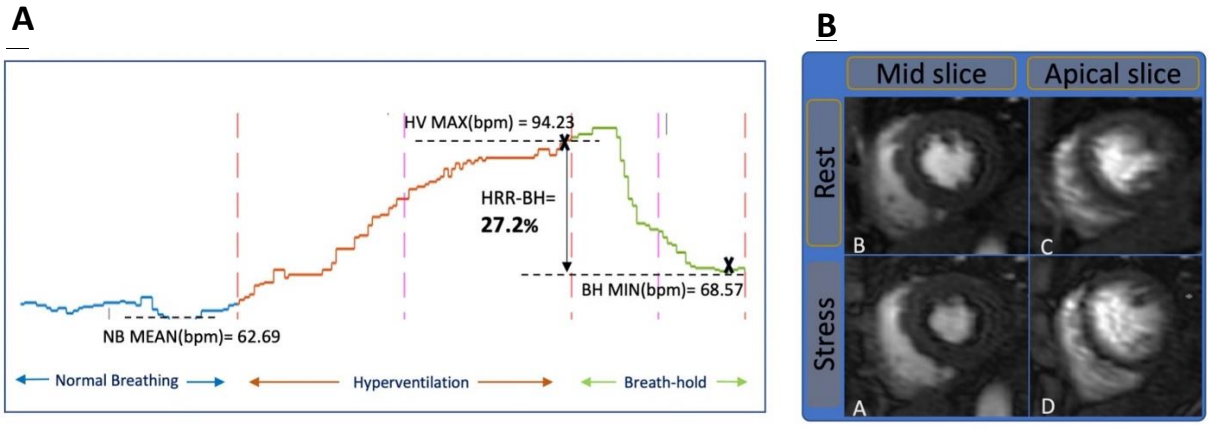

Figure 23. The comparison of the HRR-BH with stress CMR perfusion findings in a patient. A. The breathing-driven ECG signal shows an HRR-BH of 27%. B. The visual comparison of rest and stress myocardial perfusion imaging showed no inducible perfusion deficit.

9.2.2. Case presentation 2

A 57-year-old woman with a history of smoking, diabetes, hypertension, and human immunodeficiency virus (HIV) since 1995 presented to the cardiology clinic with sporadic non-exertional chest pain for 5 to 10 minutes and exertional dyspnea after climbing one flight of stairs. The exercise ECG was clinically and electrically positive. The patient was, therefore, referred to stress CMR perfusion CMR for a more definite diagnosis. During the breathing maneuver, the patient demonstrated an HRR-BH of 18%, which was lower than the cut-off of 25% (Fig. 25A). Visual analysis of perfusion CMR images revealed an inducible perfusion deficit in the right coronary and left circumflex arteries (Fig. 25B).

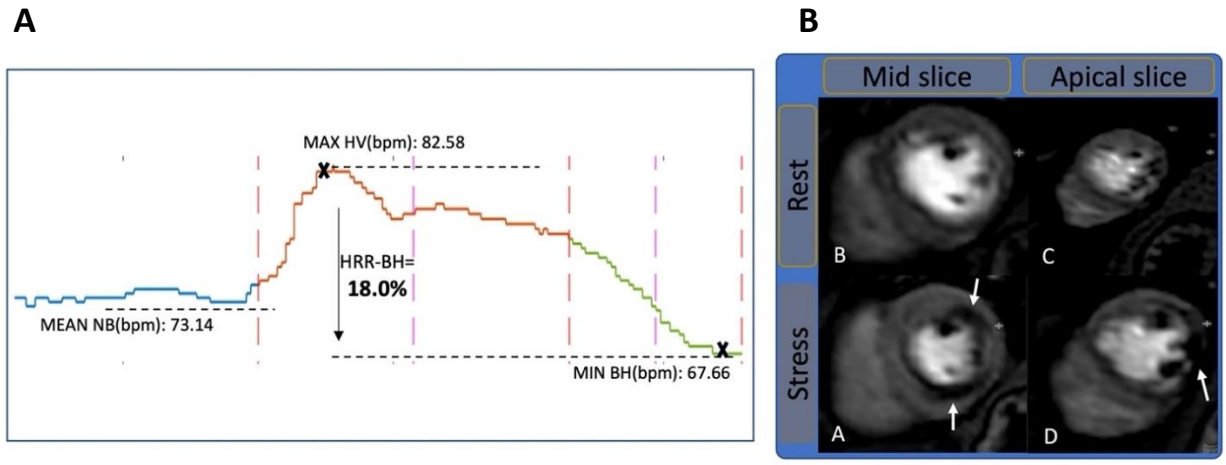

Figure 24. The comparison of the HRR-BH with stress CMR perfusion findings in a patient. A. The breathing-driven ECG signal shows an HRR-BH of 18%. B. The visual comparison of rest and stress myocardial perfusion imaging showed inducible perfusion deficit in the right coronary and left circumflex territories.