

Cardiovascular autonomic dysfunction impact on cardiovascular complications across glucose metabolism

PhD dissertation

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1.

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Notes and disclosures

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Preface

The research presented in this dissertation was conducted during my PhD studies at the Department of Public Health, Aarhus University, and Steno Diabetes Center Aarhus, between 2022 and 2025. The project was supported by the Department of Public Health at Aarhus University, Steno Diabetes Center Aarhus, and the European Foundation for the Study of Diabetes/Sanofi European Diabetes Research Programme in Diabetes associated with Cardiovascular Disease. The aim was to better understand the impact of cardiovascular autonomic dysfunction on heart disease in individuals with diabetes or high risk of diabetes. I am grateful for the opportunity to explore how autonomic dysfunction can be demonstrated by investigating variations in heart rate responses across different durations and conditions to gain insights into cardiovascular risk, which remains ongoing issue in current research. It is my hope that the findings of this dissertation will contribute to a deeper understanding of the clinical potential of long-term heart rate variability measures and standardized cardiovascular autonomic reflex tests in identifying individuals at risk for cardiovascular disease across all stages of glucose metabolism. In doing so, I hope this work contributes to the broader effort of improving care for individuals at risk of developing diabetes, as well as those living with diabetes.

Other work and collaboration during the PhD

Much of the work I have been involved in during my PhD is not fully reflected in this dissertation. I would like to take this opportunity to provide an overview and acknowledge the many collaborative establishment that sprouted during my time as a PhD student.

In my work on diabetes epidemiology, I have been deeply curious about the future methods in research. This led me to become a peripheral member of the Hulman Lab, a group with an open heart and a strong foundation in critical thinking, focused on machine learning and clinical prediction. Together with Adam Hulman and Anders Isaksen, we investigated how people perceive the use of artificial intelligence in healthcare. On this project, I am grateful to Lasse Bjerg and Annelli Sandbaek and rest of Health in Central Denmark (HICD) steering committee for integrating our questionnaire into their cohort. I also appreciate Kasper Normann's help with prompt data management. This collaboration resulted in one original research paper and two other submitted manuscript based on wave 2022 and 2024 of data collection from the HICD cohort.

We set out to extend the generalisability of the CANCAN findings to populations without type 2 diabetes. This led us to use data from the Lolland-Falster Health Study (LOFUS). I am deeply grateful to Randi Jepsen for the collaboration on accessing the cohort and for her support in getting the biobank samples analysed. The data is now ready for use in the study. Further appreciation goes to Marie Mathilde Bjerg Christensen, Christian Stevns Hansen, and Jesper Fleischer for updating reference values for CARTs using LOFUS data and for generously sharing their expertise on the measurements.

In my last year of the PhD is was lucky to exchange research environment to Baker Heart and Diabetes Institute. I had the privilege of working alongside a proficient team of epidemiologists consisting of PhD students, postdoctoral researchers, and senior scientists, using data from the PREDICT study involving patients with type 2 diabetes. I was impressed by how closely research and clinical care are integrated. Furthermore, their dedication to utilizing cohorts and organizing multinational data resources was truly admirable. I would like to extend a special thank to Professor Dianna Magliano, Senior Researcher Julian Sacre, and Professor Jonathan Shaw for their valuable input on using questionnaires to screen for heart failure subtypes and for helping to shape a study that will be submitted soon.

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In The Maastricht Study, I would like to thank Marleen van Greevenbroek, Miranda Schram, Carla van der Kallen, and the rest of the team for granting me access to the cohort and for showing me the data collection facilities. Thanks to Marion Feijge for guiding me through the data. I am also grateful to Professor Coen Stehouwer for his sharp insight and deep expertise in diabetes epidemiology. In the ADDITION-PRO study, I would like to thank Anne-Louise Bjerre and Søren Brage for their help in explaining variables, and data manager Marianne at the Department of Public Health for her generous support. In the CANCAN study, I would like to acknowledge Henrik Holm Thomsen and Gitte Jensen for their help with recruitment, and Anne Katrine Møller Gramstrup for extracting clinical data for the study population. I am also deeply grateful to the people who generously agreed to participate in the CANCAN study.

No adventure is truly exciting without cultural exchange—whether through the lovely visits from Peter and Ieva at SDCA or by immersing myself in new research environments. To Professor Dianna Magliano and Professor Jonathan Shaw, thank you for including me in the Epidemiology group at the Baker Heart and Diabetes Institute in Melbourne. It was a truly enriching stay, both scientifically and socially. I would like to extend my sincere thanks to Julian Sacre for his valuable support and insightful contributions in deepening my understanding of the challenges involved in

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Papers in the dissertation

Study I

Cardiovascular autonomic dysfunction is linked with arterial stiffness across glucose metabolism: The Maastricht Study

Jonas R. Schaarup, Lasse Bjerg, Christian S. Hansen, Signe T. Andersen, Marleen van Greevenbroek, Miranda T. Schram, Bastiaan E. de Galan, Coen Stehouwer, Daniel R. Witte (2025). medRxiv 2024.12.03.24317865; doi: <https://doi.org/10.1101/2024.12.03.24317865> (preprint) (under peer-review at BMJ Open Diabetes Research & Care)

Study II

Cardiovascular autonomic dysfunction precedes cardiovascular disease and all-cause mortality: 11-year follow-up of the ADDITION-PRO study

Jonas R. Schaarup, Lasse Bjerg, Christian S. Hansen, Erik L. Grove, Signe T. Andersen, Dorte Vistisen, Søren Brage, Annelli Sandbæk, Daniel R. Witte (2025). medRxiv 2024.12.18.24319131; doi: <https://doi.org/10.1101/2024.12.18.24319131> (accepted at Diabetes, Obesity and Metabolism)

Study III

Cardiovascular autonomic neuropathy and indices of heart failure in type 2 diabetes: The CANCAN Study

Jonas R. Schaarup, Lasse Bjerg, Christian S. Hansen, Daniel R. Witte, Henrik H. Thomsen, Jesper Fleischer, Rodica Pop-Busui, Annelli Sandbæk, Signe T. Andersen. (submitted?)

Additional publications

The 2 following original research studies and 2 preprints have been published during the PhD period, but have not been included in the dissertation.

Peer-reviewed

Schaarup JR, Christensen MS, Hulman A, Hansen CS, Vistisen D, Tabák AG, Witte DR, Bjerg L. Autonomic dysfunction is associated with the development of arterial stiffness: The Whitehall II cohort. *GeroScience*, 2023. <https://doi.org/10.1007/s11357-023-00762-0>

Schaarup JR, Aggarwal R, Dalsgaard E-M, Norman K, Dollerup OL, Ashrafi H, Witte DR, Sandbæk A, Hulman A. Perception of artificial intelligence-based solutions in healthcare among people with and without diabetes: A cross-sectional survey from the health in Central Denmark cohort. *Diabetes Epidemiology and Management*, 2023. <https://doi.org/10.1016/j.deman.2022.100114>

Pre-prints

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Anders Aasted Isaksen, **Jonas R. Schaarup**, Lasse Bjerg, Adam Hulman. (2025). Changes in public perception of AI in healthcare after exposure to ChatGPT. *medRxiv* 2025.01.23.25321048; doi: <https://doi.org/10.1101/2025.01.23.25321048> (under review at npj digital medicine)

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Abbreviations

- BMI:** Body mass index
CAN: Cardiovascular autonomic neuropathy
CARTs: Cardiovascular autonomic reflex tests
CD: Carotid artery distensibility coefficient
cf-PWV: Carotid-femoral pulse wave velocity
CI: Confidence interval
CVD: Cardiovascular disease
eGFR: Estimated glomerular filtration rate
FPG: Fasting plasma glucose
GLP1RA: Glucagon-like peptide-1 receptor agonists
HDL: High-density lipoprotein cholesterol
HRV: Heart rate variability
IR: Incidence rate
IRR: Incidence rate ratio
HbA1c: Haemoglobin-A1c
LDL-C: Low-density lipoprotein cholesterol
MACE: Three-point major adverse cardiovascular events
NGM: Normal glucose metabolism
NT-proBNP: N-terminal pro-B-type natriuretic peptide **OGTT:** Oral glucose tolerance test
OR: Odds ratio
PAEE: Physical activity energy expenditure
RPAQ: Recent Physical Activity Questionnaire
SBP: Systolic blood pressure
DBP: Diastolic blood pressure
SGLT2i: Sodium glucose co-transporter type 2 inhibitors
SES: Socioeconomic status
SD: Standard deviation
SDNN: Standard deviation of NN intervals
SDANN: Standard deviation of the averages of NN intervals in 5-minute segments
SDNN index: Mean of the SDs of all NN intervals for all 5-minute segments
pNN50: Proportion of NN intervals differing by more than 50 ms
RMSSD: Root mean square of successive differences between NN intervals
TP: Total power (variance of NN intervals 0.4 Hz)
ULF: Ultra low-frequency range (0.003 Hz)
VLF: Very-low-frequency range (0.003–0.04 Hz)
LF: Low-frequency range (0.04–0.15 Hz)

Abbreviations

HF: High-frequency range (0.15–0.4 Hz)

T2D: Type 2 diabetes mellitus

TC: Total cholesterol

TG: Triglycerides

UACR: Urine albumin-to-creatinine ratio

2. Introduction

Diabetes mellitus is a growing global health concern, posing pressing challenges for public health systems.¹ As prevalence rises, more individuals are exposed to an increased risk of premature mortality and cardiovascular disease (CVD).¹ At the same time, people live longer with diabetes, and therefore endure extended periods under the burden of diabetes-related complications.² Despite advancements in cardiovascular care, coronary artery disease and heart failure are still often detected at more advanced stages, such as during ischemia, major cardiovascular events, or the onset of symptomatic heart failure in patients with diabetes.^[3]^[4]

Over the last decades, cardiovascular autonomic dysfunction has repeatedly gained attention as a risk factor for CVD.⁵ Heart rate variability (HRV) is considered a reliable marker for measuring autonomic function, as it reflects the balance between sympathetic and parasympathetic modulation of heart rate intervals.⁶ Despite its recognition as a CVD risk factor, assessment of cardiovascular autonomic dysfunction has not been implemented in healthcare practice. In diabetes, lower HRV is regarded as an early indicator of cardiovascular autonomic neuropathy (CAN), which is diagnosed using cardiovascular autonomic reflex tests (CARTs).⁷ Signs of autonomic dysfunction, may already be present in individuals with prediabetes.⁸ Despite rising prevalence and increased CVD risk, people with prediabetes often remain outside structured treatment pathways.^[9]^[10] Although diabetes contributes to autonomic dysfunction, it is still unclear at what stage in the diabetes risk spectrum HRV and CARTs become clinically useful for assessing CVD risk.

In the past, measuring HRV needed special instruments like an electrocardiogram. Today, it's easy to track HRV with everyday devices like smartwatches.^[11]^[12] This increased accessibility allows for continuous monitoring and a better understanding of HRV over extended periods and under various free-living conditions.^[13] However, long-term HRV patterns and the relation between specific diurnal responses and risk of cardiovascular complications remain less well understood.

The overall aim of this dissertation is to understand how cardiovascular autonomic dysfunction/CAN affects cardiovascular disease risk (i.e. heart failure, stroke, myocardial infarction) and specific subclinical markers of CVD: carotid-femoral pulse wave velocity and carotid artery distensibility in populations covering the whole glycemic continuum, from healthy glucose metabolism to type 2 diabetes.

3. Background

3.1. Type 2 diabetes and prediabetes

This background introduces the concept of type 2 diabetes (T2D) and its associated cardiovascular risk. It then provides an overview of various cardiovascular complications, including arteriosclerosis, atherosclerosis, and heart failure. Finally, it describes cardiovascular autonomic function (autonomic function) and its potential to enhance our understanding of CVD.

3.1. Type 2 diabetes and prediabetes

The progression from normal glucose metabolism (NGM) to T2D is characterized by sustained elevations in blood glucose levels. T2D is characterized by a progressive decline in beta-cell function, most often as a consequence of chronic insulin resistance.^{14,15} Insulin resistance occurs when certain tissues, such as muscle and liver tissues, lose their sensitivity to insulin.¹⁵ As a result, glucose is not effectively taken up by these tissues and remains in the circulation.¹⁵ Meanwhile, beta-cell function deteriorates, leading to a diminished insulin response to glucose levels.¹⁵ Years before a T2D diagnosis, these changes contribute to rising fasting and postprandial glucose levels.¹⁴

The body regulates glucose through various mechanisms. During fasting, pancreatic alpha cells secrete glucagon, which stimulates hepatic glucose production via glycogenolysis and gluconeogenesis.¹⁵ After a meal, rising blood glucose levels stimulate pancreatic beta cells to release insulin and trigger the secretion of incretins, such as glucagon-like peptide-1 (GLP-1) from the intestines.¹⁵ Insulin and incretins work together to suppress hepatic glucose production, while insulin promotes glucose uptake in muscle and adipose tissue.¹⁵ Excess glucose is primarily stored as glycogen in the liver and muscles, with some converted to triglycerides for long-term storage. Multiple organs, including the pancreas, liver, kidneys, intestines, muscle, and adipose tissue are involved in this coordinated process.¹⁵ The autonomic nervous system plays a supportive role in glucose homeostasis by modulating metabolic activity. Parasympathetic signals tend to reduce glucose production, while sympathetic signals enhance it, especially during hypoglycemia.¹⁵

The World Health Organization (WHO) and American Diabetes Association (ADA) diagnostic criteria for T2D include fasting plasma glucose 7.0 mmol/L, 2-hour plasma glucose 11.1 mmol/L during an oral glucose tolerance test (OGTT), or hemoglobin A1c (HbA1c) 6.5% (48 mmol/mol).^[16]^[17] The OGTT measures glucose levels two hours after the ingestion of a standard 75-gram glucose load in the fasting state^[17]. Progression towards diabetes is a continuous process, with type 2 diabetes defined based on glucose thresholds associated with an increased risk of diabetes-specific microvascular complications, particularly retinopathy^[18]. Many complications of diabetes, such as macrovascular disease, neuropathy, cancer, and cognitive impairment, may start to develop at earlier stages of dysglycemia^[19-21]. This stage is referred to as prediabetes or high risk of diabetes and is defined by fasting plasma glucose levels between 6.1–6.9 mmol/L, 2-hour plasma glucose levels between 7.8–11.0 mmol/L (WHO criteria), and HbA1c levels between 5.7–6.4% (39–47 mmol/mol) (ADA criteria)^[17]. In parallel with the growing prevalence of T2D, the prevalence of prediabetes is also on the rise.⁹

Risk factors for progression to T2D and its complications range from genetic predisposition to lifestyle and socio-environmental factors. The most common risk factor for diabetes is obesity, and in particular central obesity.^[22] The accumulation of diabetes risk factors is linked with a combination

of adverse changes in cardiovascular disease (CVD) risk factors, including increases in low-density lipoprotein (LDL) cholesterol, triglycerides, and systolic blood pressure, along with decreases in high-density lipoprotein (HDL) cholesterol.²³

Diabetes increases the risk of both microvascular and macrovascular complications, which are major contributors to the morbidity and mortality associated with the disease¹⁵. Beyond conventional CVD risk factors, chronic hyperglycemia promotes the formation of harmful byproducts such as reactive oxygen species and advanced glycation end products, which drive oxidative stress and inflammation²⁴. These processes contribute to endothelial dysfunction and vascular damage.²⁴ While the general mechanisms underlying macrovascular complications are well described, the identification of preclinical stages of CVD and the differentiation of CVD risk between individuals at high risk of diabetes and those with established T2D require further clarification.¹⁰

3.2. Cardiovascular disease

Globally, CVD remains the leading cause of death. At the population level, CVD risk is primarily attributable to modifiable lifestyle behaviors such as chronic stress, physical inactivity, unhealthy diet, excessive alcohol consumption, and smoking, as well as socio-environmental factors like socio-economic status and air pollution.²⁵ At the individual level, these exposures often manifest through more proximal biological risk factors, including hypertension, hypercholesterolemia, diabetes, and obesity.²⁶ Along the causal pathway, these intermediate conditions tend to cluster, thereby accelerating disease progression. These processes are underpinned by biomolecular mechanisms, including local and systemic inflammation, oxidative stress involving oxidized low-density lipoprotein (LDL), and dysregulated immune responses mediated by pro-inflammatory cytokines and signaling pathways.²⁷ Risk factors contribute to distinct pathophysiological mechanisms across different types of CVD, involving structural, signaling, inflammatory, and hemodynamic changes within the cardiovascular system.²⁷⁻²⁹ Among these, cellular and molecular signaling pathways play a central role in regulating vascular tone, cardiac function, and inflammatory responses. These processes are closely modulated by the autonomic nervous system through sympathetic and parasympathetic nerve branches.³⁰⁻³³

3.2.1. Arteriosclerosis

Evidence emphasizes the role of vascular aging in early disease development, extending beyond the traditional focus on cardiovascular endpoints.²⁹ Arteriosclerosis, commonly referred to as arterial stiffness, is a hallmark of this process. Biologically, the medial layer of large arteries consists of a structured network of vascular smooth muscle cells together with elastic and collagen fibers, forming functional musculoelastic sheets.³⁴ Arterial stiffness arises from progressive remodeling of the arterial wall.^{29,35} This remodeling is driven by changes in the structural interactions between elastin and collagen fibers, along with functional alterations in vascular smooth muscle cells and the accumulation of calcium and advanced glycation end products.³⁴ Remodeling of the arterial wall increases systolic blood pressure and reduces coronary perfusion, thereby contributing to the

development of hypertension and, eventually, cardiovascular disease.³⁶ Additionally, arterial stiffness elevates the pulsatile load on the microcirculation, promoting the progression of chronic kidney disease, vascular dementia, and Alzheimer's disease.²⁹

3.2.2. Atherosclerosis

Atherosclerosis is characterized by the accumulation of cholesterol, lipids, and other substances within the arterial walls, forming plaques that narrow the arteries and reduce blood flow often at specific sites such as the coronary and carotid arteries.³⁷ This chronic process can lead to progressive occlusion of the vessel, contributing to reduced oxygen supply to the heart (ref.), often leading to symptoms of angina.

Atherosclerotic plaques can be classified into stable and unstable types, each with distinct structural characteristics and clinical implications. Stable plaques typically have a thick fibrous cap composed of collagen, a small lipid core, and low levels of inflammation.³⁸ These plaques are less likely to rupture and tend to remain intact over time due to internal remodeling. In contrast, unstable plaques, also known as vulnerable plaques, often contain a large lipid-rich necrotic core, a thin fibrous cap, and infiltration by inflammatory cells such as macrophages.³⁸ A well-recognized subtype of unstable plaque is the thin-cap fibroatheroma, which is particularly prone to rupture. When rupture occurs, the necrotic core becomes exposed to the bloodstream, initiating the formation of a thrombus or blood clot. This acute event can abruptly obstruct the artery, resulting in myocardial infarction (MI).³⁸ Chronic ischemia due to reduced coronary perfusion can lead to myocardial remodeling, impaired contractility, and electrical instability, thereby increasing the risk of arrhythmias and heart failure.^{39,40}

Myocardial infarction

MI occurs due to the rupture of an atherosclerotic plaque in the coronary arteries, triggering thrombus formation that blocks blood flow.⁴¹ This leads to oxygen deprivation (ischemia) and subsequent myocardial injury or necrosis.⁴¹ If untreated, this process can cause extensive cardiac damage and fatal arrhythmias.⁴¹ Over the past decades, the incidence of MI has declined in high-income countries with a marked reduction in MI-related mortality.⁴² These improvements are largely attributed to a combination of public health initiatives and medical advances. On the public health front, a substantial decrease in smoking prevalence has been the most important lifestyle-related factor contributing to the reduction in CVD.^{43,44} Medically, the improved preventive management of hypertension and hyperlipidemia has reduced the burden of atherosclerotic disease.⁴² In acute care, the widespread adoption of evidence-based interventions such as thrombolytic therapy, percutaneous coronary interventions (including stenting), and coronary artery bypass grafting has improved survival and outcomes following MI.⁴⁵ In T2D, the risk of MI is elevated by 72%, with an approximately threefold risk among patients under 60 years compared to age under 60 without T2D.⁴⁶ Similar to the general population, MI incidence and fatality have declined among people with diabetes.⁴⁷ greggedwardw?

Stroke

The majority of strokes are ischemic and result from an obstruction in a cerebral artery. The process often begins with the development of atherosclerotic plaques at the carotid artery bifurcation, which can lead to the formation of emboli⁴⁸. These emboli then travel through the bloodstream and eventually lodge in the cerebral arterial tree, causing ischemic stroke⁴⁸. The second main cause is hemorrhagic stroke, which is characterized as a hypertensive small-vessel disease, leading to small lipohyalinotic aneurysms that subsequently rupture, causing intracerebral bleeding.^{49,50} Ischemic stroke remains one of the global leading contributors to mortality and disability.⁵¹ The incidence, prevalence, and cause-specific mortality of stroke remain high but have stagnated, although some declines have been observed in high-income countries.⁵² Individuals with elevated glucose levels, as measured by fasting plasma glucose, OGTT, or HbA1c, have a 26% higher risk of stroke compared to those with normal glucose levels.^{53,54} In T2D, the ischemic stroke risk is elevated almost two-fold compared with individuals without diabetes.⁴⁶

3.2.3. Heart failure

Heart failure develops gradually with age and often accelerates with the progression of T2D. As prevention and treatment of CVD have improved survival in recent years, the prevalence of heart failure has increased, while the incidence remains stable, but may rise with aging populations.⁵⁵

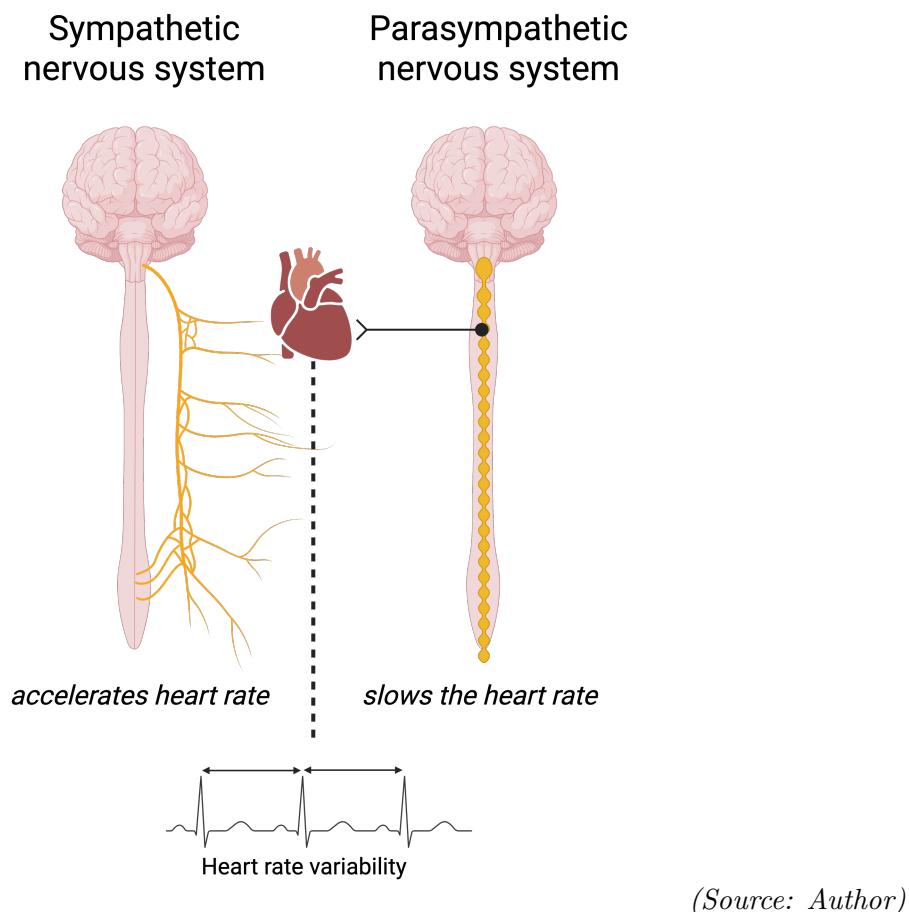
Heart failure may arise as a consequence of atherosclerosis, arteriosclerosis, or both, contributing to myocardial ischemia, pressure overload, and structural cardiac changes.⁵⁶ Heart failure can be defined hemodynamically as the inability to maintain adequate cardiac output at rest or during exertion, or the ability to do so only with elevated cardiac filling pressures.⁵⁶ It is a complex cardiovascular disease caused by structural and functional changes in the heart musculature, affecting systolic and/or diastolic pumping function.⁵⁶ Heart failure is generally classified into two subtypes: heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF).⁵⁶ Both subtypes involve cardiac remodeling but are defined by left ventricular ejection fraction (LVEF).⁵⁶ HFrEF is defined by an LVEF < 40%, while HFpEF is characterized by an LVEF

50% along with structural or functional cardiac abnormalities, as assessed by echocardiography.⁵⁶ HFrEF is often a consequence of repeated, non-fatal MIs. These events can leave behind scar tissue in the myocardium, impairing the heart's ability to contract effectively and leading to progressive systolic dysfunction.[@campbell2024]

The most common feature of HFpEF is left ventricular diastolic dysfunction, caused by impaired relaxation and increased stiffness, leading to elevated left atrial pressure and reduced diastolic reserve.⁵⁷ Over the past decades, the prevalence of HFpEF has increased with an aging population and more people living with conditions such as hypertension, diabetes, and obesity.⁵⁸ It is diagnosed based on structural or functional abnormalities identified through echocardiographic measures, such as left ventricular hypertrophy, left atrial enlargement, or elevated filling pressures.⁵⁸ The diagnosis may seem straightforward, but it is often challenging in community settings, as patients frequently present without typical heart failure symptoms (e.g., shortness of breath) and are not routinely assessed with biomarkers like N-terminal pro-B-type natriuretic peptide (NT-proBNP) or brain-natriuretic-peptide (BNP).⁵⁸ As a result, HFpEF is commonly underdiagnosed and consequently detected at more severe stages, leading to hospitalization .[⁵⁸]⁵⁸

3.3. Cardiovascular autonomic dysfunction

The cardiovascular system is regulated by the autonomic nervous system, which influences heart rate and vasoconstriction through the sympathetic and parasympathetic nerves.³² Sympathetic activation increases heart rate and myocardial contractility by stimulating the sinoatrial (SA) node, atrioventricular (AV) node, and ventricular myocardium. In contrast, parasympathetic activation primarily reduces heart rate by directly modulating SA node activity through vagal stimulation.³² It also slows AV nodal conduction, predominantly via the left vagus nerve, thereby prolonging atrioventricular conduction time.³² Afferent nerves mainly carry sensory information (e.g., baroreceptor input from the carotid sinus and aortic arch) to the brain, which then adjusts efferent autonomic output to regulate arterial tone. Hence, the autonomic nervous system dynamically regulates heart rate and blood pressure to maintain homeostasis in response to physiological demands, such as rest, stress, eating and physical activity.³²



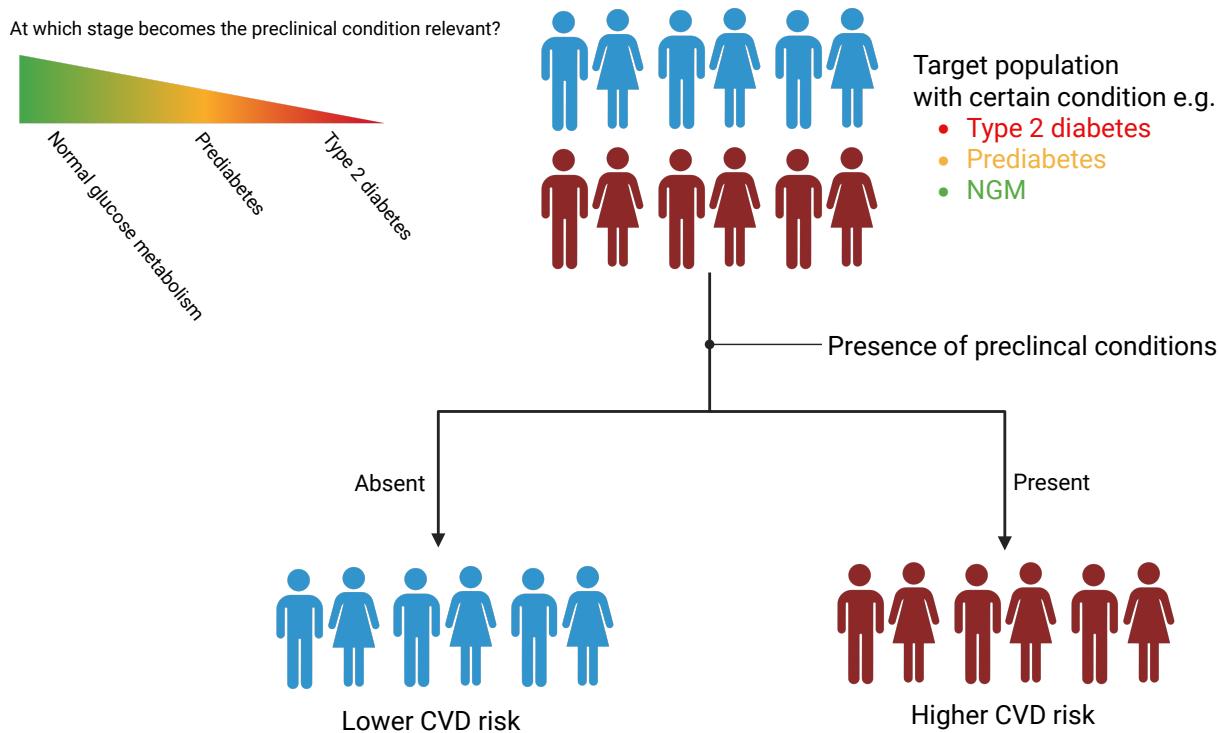
In youth, the autonomic nervous system is highly adaptive and responsive to living conditions, maintaining autonomic balance. However, with aging, there is a gradual decline in parasympa-

thetic function and an increase in sympathetic activity. Additionally, metabolism-related conditions such as obesity and diabetes have been shown to further contribute to cardiovascular autonomic dysfunction (autonomic dysfunction).⁵⁹ Autonomic dysfunction reflects a stressed cardiometabolic environment, as both dysfunction in lipid and glucose metabolism are associated with increased sympathetic activity.⁵⁹ This dysfunction may result from cumulative neural damage mediated by mechanisms such as hyperinsulinemia, insulin resistance, and elevated levels of adipokines. At the same time, autonomic dysfunction is known to disrupt lipid and glucose metabolism.⁵⁹ Therefore, the relationship between autonomic dysfunction and cardiometabolic factors is likely a vicious cycle.⁶⁰ The consequences can lead to autonomic dysfunction/neuropathy (CAN), resulting in dysregulation in heart rate and vascular dynamics. CAN prevalent in 12-73% in individuals with T2D is linked to CVD diabetic kidney disease, and all-cause mortality.^{[7;61]62} In this dissertation, ‘autonomic dysfunction’ will be used as the broader term, while ‘CAN’ will refer specifically to autonomic dysfunction resulting from neuropathy in diabetes.

Autonomic function can be assessed using heart rate variability (HRV) indices, which measure the variation in successive normal RR intervals in milliseconds. HRV provides time- and frequency-domain estimates of the balance between sympathetic and parasympathetic activity.⁶ High HRV reflects an autonomic nervous system with strong adaptability to the body’s demands, whereas low variation indicates poor adaptation to changing conditions. HRV changes in response to different physiological or environmental conditions (e.g., sleep, stress, posture, physical activity), and these changes can be observed in its natural 24-hour (circadian) pattern.¹³ Most studies have examined autonomic function using short-term ECG recordings at rest.⁵ However, extended HRV recordings across the circadian cycle may offer deeper insights into the influence of lower-frequency variability sources, such as very-low frequency (0.003–0.04 Hz) and ultra-low frequency (0.003 Hz).⁶ HRV has been applied across several research domains. For example, in psychology as a marker of mental stress, in exercise physiology as an indicator of recovery, in cardiovascular research as a marker of autonomic dysfunction due to cardiac complications, and in diabetes research as a marker of autonomic neuropathy.^{7,63–65} T2D alters the expression of sympathetic bursts, as measured by resting muscle sympathetic nerve activity (MSNA). MSNA is elevated in individuals with both T2D and hypertension, compared to those who are normotensive, regardless of whether they have diabetes or not.⁶⁶ Parasympathetic activity is also impaired in individuals with high cardiometabolic risk and T2D, as reflected by reduced baroreflex sensitivity and lower HF and RMSSD short-term HRV.⁶⁷ Before onset of diabetes and during progression of diabetes long-term (24-hour) HRV has shown to be lower compare to those with .^{8,60} Cardiovascular autonomic reflex tests (CARTs) and orthostatic hypotension are considered the gold standard for assessing CAN.⁶⁸ The diagnosis includes assessing pulse rate ratio under test conditions, such as the deep breathing test, the lying-to-standing test, and the Valsalva maneuver.⁶⁸ Both HRV and CARTs have shown to be associated with cardiovascular disease, heart failure, and all-cause mortality, primarily in populations with T2D or established cardiovascular disease.^{[5]61,69} However, it remains unclear at which stage in the progression of diabetes risk to pre-diabetes to diabetes these measures begin to influence the risk of cardiovascular complications.

3.4. Risk-stratification

Current cardiopreventive guidelines place strong emphasis on prevention and treatment of T2D. The 2022 ADA/EASD guidelines for the management of hyperglycemia in T2D recommend, cardio-protective medication (glucagon-like peptide-1 receptor agonists [GLP-1RA] and Sodium-Glucose Transport Protein 2 [SGLT2] inhibitors) as first-line options for individuals at high cardiovascular risk⁷⁰. Due to their benefits in heart failure, SGLT2 inhibitors are specifically recommended for patients with documented HFrEF or HFpEF. High cardiovascular risk is defined as the presence of at least two risk factors at age >55 years, such as obesity, hypertension, smoking, dyslipidemia, or albuminuria⁷⁰. However, no additional preclinical markers are recommended to identify individuals at higher CVD or HF risk or for younger individuals. Despite their increased risk of cardiovascular complications, individuals at high risk of developing diabetes remain outside structured treatment options, even though diabetes risk and cardiometabolic markers can be successfully modified through lifestyle interventions and medication such as GLP-1 analogues [71]⁷². During the progression and following the onset of T2D, preclinical indicators of CVD risk become apparent, providing potential opportunities for early risk stratification. Risk stratification is the process of classifying or ranking individuals in increasing order of estimated risk, based on risk scores, biomarker levels, omic data (metabolomic, proteomics, and genomic) or clinical characteristics⁷³. This approach aids in identifying patients at highest risk for further prognostic or diagnostic purposes, identifying subgroups that require further evaluation, specific treatment, or lifestyle modifications⁷³.



(Source: Author)

3. Background

Autonomic dysfunction despite its relationship with cardiovascular complication has not been used in clinical practice in Denmark. Larger epidemiological cohort studies encompassing various stages of diabetes risk, from prediabetes, onset of T2D, and longer term progression of T2D, serve as valuable resources for identifying risk-stratification opportunities. Epidemiological studies provide a broad representation of the target population, enabling an understanding of the relationship between autonomic dysfunction and cardiovascular complications across different levels of care: public health, primary care, and secondary care. By utilizing observational cohorts, we have the potential to determine when, along the trajectory of diabetes progression, autonomic function becomes a meaningful factor for cardiovascular risk stratification.⁷³

4. Aim and hypothesis

4. Aim and hypothesis

The overarching hypothesis of this dissertation are that:

- CAN and autonomic dysfunction are associated with CVD and act as an early risk factor for heart failure and other cardiovascular complications, including stroke, and myocardial infarction in patients with prediabetes and/or T2D.
- Autonomic dysfunction is associated with higher levels of sub-clinical measures of CVD such as carotid-femoral pulse wave velocity and carotid artery distensibility.

This dissertation investigates the hypothesis by addressing the following three aims:

Study I: Quantify the cross-sectional association between 24-hour HRV and subclinical markers of cardiovascular complications: carotid-femoral pulse wave velocity and carotid artery distensibility, in participants with NGM, prediabetes or T2D.

Study II: Quantify the longitudinal association of multiday and hourly HRV with incidence of ischemic-related CVD, heart failure, and all-cause mortality in a population with high-risk of diabetes.

Study III: Quantify the cross-sectional association between CAN and heart failure. Heart failure will be defined by clinical measures i.e. N-terminal-pro-BNP (NT-proBNP), WATCH-DM risk, and New York Heart Association (NYHA) classification scores among individuals with T2D.

5. Materials and methods

5.1. Overview of the studies

Table 5.1.: Table 1: Overview of studies

	Study I	Study II	Study III
Title	Cardiovascular autonomic dysfunction is linked with arterial stiffness across glucose metabolism: The Maastricht Study	Cardiovascular autonomic dysfunction precedes cardiovascular disease and all-cause mortality: 11-year follow-up in the ADDITION-PRO study	Cardiovascular autonomic neuropathy and subclinical heart failure in T2D: The CANCAN study
Design	Aetiological cross-sectional study	Aetiological prospective cohort study	Descriptive cross-sectional study
Cohort	Maastricht study	ADDITION-PRO study	CANCAN study
Study population	3673 individuals with NGM, prediabetes, or T2D	2082 individuals with high risk of diabetes	173 patients with T2D visiting outpatients clinics
Data sources	Population-based cohort sources from The Maastricht Study in the Netherlands	Cohort study of selected individuals based on having high risk of diabetes	Clinical cohort study
Determination	24-hour HRV	Multiday and hourly HRV	Cardiovascular autonomic reflex test
Primary outcome	Arterial stiffness	Major adverse cardiovascular events, heart failure, and all-cause mortality	NT-proBNP, NYHA classification, and WATCH-DM risk score
Statistical analysis	Linear regression	Poisson regression	Logistic regression

Missing Complete case analysis data

Multiple imputation of chained equations for confounders

Complete case analysis and multiple imputation of chained equations for CART and confounders

5.1.1. Study population

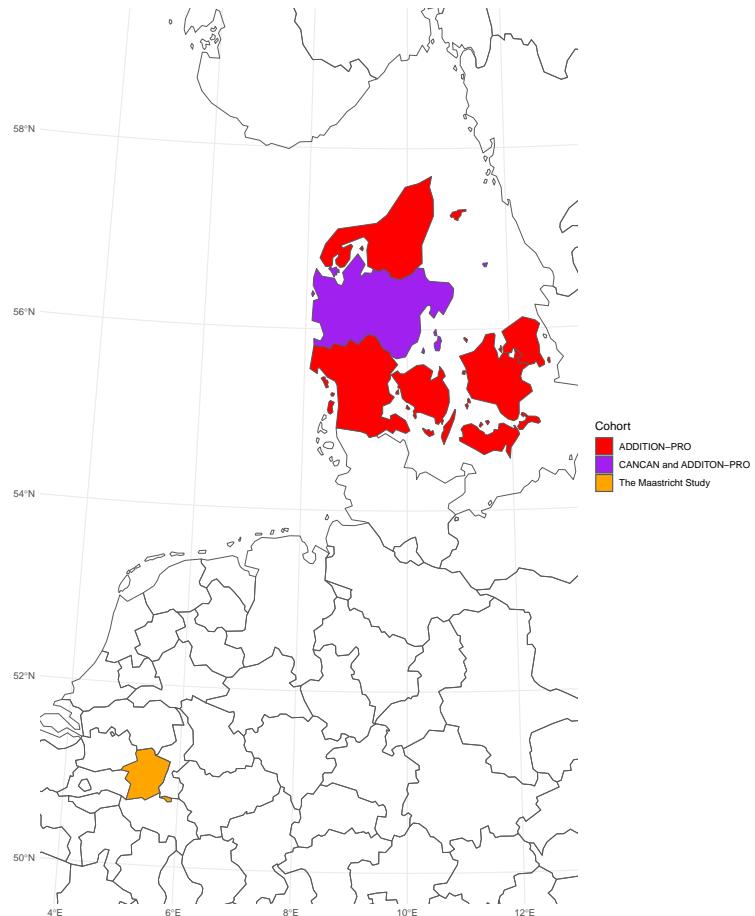


Figure 5.1.: Study populations

5.1.1.1. Study I - The Maastricht Study

The Maastricht Study is a prospective observational population-based study of the general population of the province of Limburg, in the southern part of the Netherlands. The study emphasized the recruitment of individuals with T2D, through the regional Diabetes Patient Registry, to extensively

phenotype individuals with T2D and those in intermediate stages of the disease. The eligibility criteria included an age range of 40–70 years. Participants were recruited through mass media campaigns and mailings from municipal registries (Gemeentelijke Basis Administratie; GBA).⁷⁴ In the analysis of Study I, the study among 7449 individuals included participants with measurements of 24-hour HRV and at least one measure of arterial stiffness (carotid-femoral pulse wave velocity or carotid artery distensibility), both of which were completed within a three-month period between November 2010 and December 2020. The study has been approved by the institutional medical ethics committee (NL31329.068.10) and the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All participants gave written informed consent.⁷⁵

5.1.1.2. Study II - ADDITION-PRO

The ADDITION-PRO study is a prospective, population-based cohort nested within the Danish arm of the ADDITION-Europe study. ADDITION was originally designed as a stepwise screening program for T2D in general practice, aiming to identify individuals with screen-detected T2D for recruitment into the ADDITION trial. ADDITION-PRO aims to investigate early markers of CVD and metabolic dysfunction in individuals in different tiers of diabetes risk.

The ADDITION-Europe screening program identified a large number of individuals with impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and normoglycemia despite having risk factors for diabetes and CVD. Participants for ADDITION-PRO were recruited from the original ADDITION-DK screening cohort, which included individuals from 190 general practices across Denmark. The recruitment strategy focused on individuals at high risk of diabetes without T2D, identified through a stepwise screening program that incorporated the Danish diabetes risk score from the Inter99 study⁷⁶. This assessment, conducted between 2001 and 2006, considered factors such as age, sex, history of gestational diabetes, family history of diabetes, known hypertension, BMI, and physical activity. High-risk individuals were further screened for T2D using blood measurements, including HbA1c, random blood glucose, FPG, and OGTT. Those with screen-detected diabetes, confirmed by a second OGTT, were invited to participate in the ADDITION trial. High risk individuals without T2D were further considered in as the sampling frame for ADDITION-PRO.

Between 2009 and 2011, a follow-up health examination was conducted at four ADDITION-DK study centers to establish a cohort baseline. Eligible participants were those still alive, residing near the research centers (Steno Diabetes Center Copenhagen, Aarhus University Hospital, Holstebro Hospital, and the Hospital of South West Jutland, Esbjerg), and who had not withdrawn consent. Eligibility criteria included individuals aged 40–70 years who had previously undergone diabetes screening in ADDITION-DK. Exclusion criteria included pregnancy, psychological or psychiatric disorders preventing informed consent, and life-limiting conditions. One key feature of the data collection was the precise measurement of physical activity and energy expenditure using a combined chest worn accelerometer/heart rate monitor (ActiHeart), which recorded acceleration and heart rate over a week. In study II, participants with at least a 48-hour recording was included for the primary analysis, and then participants with hourly measures of physical acceleration during the

5. Materials and methods

hourly HRV recording was included in the second analysis. Participant with prior CVD ten years before inclusion was also excluded.

Disease history and follow-up data for the population were obtained from Denmark's national registry system, which allows linkage of health records using the personal Civil Registration Number assigned to all citizens. The following national registries were accessed to collect information on incident CVD and mortality, medication use, and healthcare utilization: the National Patient Registry (hospital admissions and outpatient contacts), the National Health Service Registry (general practice visits), the Medical Prescription Registry, the Diabetes Registry, and the Cause of Death Registry.

5.1.1.3. Study III - CANCAN

The CANCAN Study is an observational study conducted at two hospital outpatient clinics in Viborg Regional Hospital and Regional Hospital Gødstrup. It aims to implement a screening protocol for identifying high-risk individuals using CAN assessments, continuous glucose monitoring, and heart failure indicators. All measures were part of routine clinical care for T2D in Central Denmark. We included 200 adults (>18 years) with T2D with a duration of over one year. Exclusion criteria were recent laser-treated eye disease (3 months), pregnancy, lactation, life-threatening illness, or cognitive impairment preventing consent. Participants were identified via electronic records and informed about the study by their doctor during a telephone call. Those interested attended a dedicated meeting before their annual diabetes exam, where study details were discussed. Recruitment took place from 2021 to 2024. In study III, participants without a valid NT-proBNP measurement were excluded.

5.2. Study variables

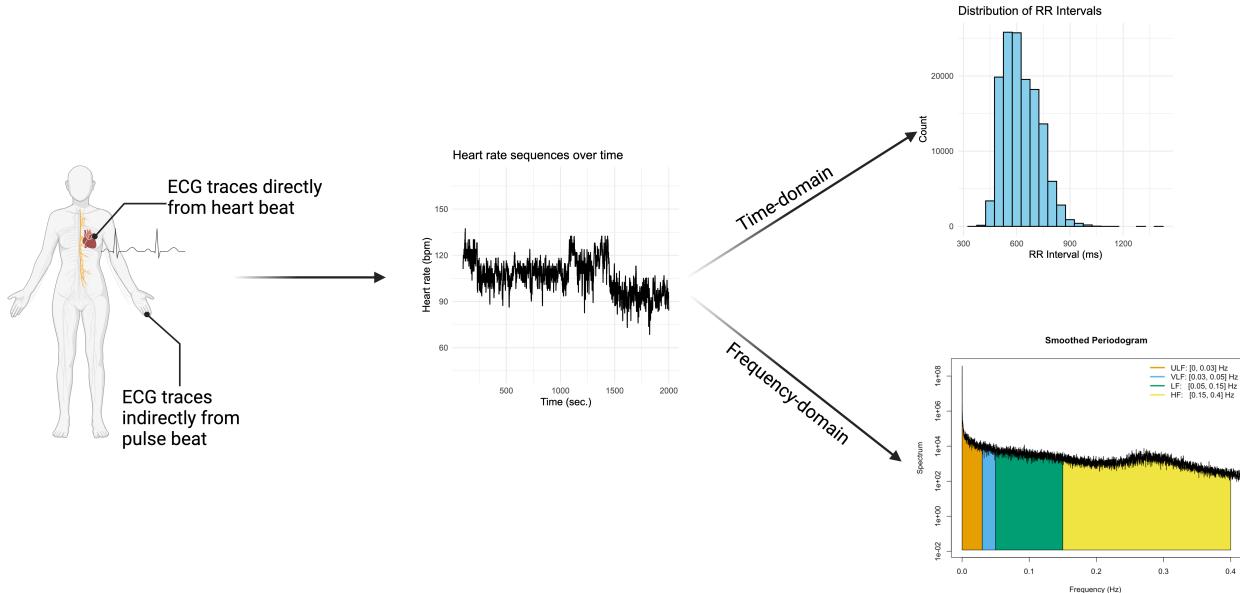
5.2.1. Measures for autonomic dysfunction/ neuropathy



Left: Holter monitor; Middle: Actiheart; Right: Vagus™ device

Heart rate variability

In study I-III different devices were used to capture the distance between each heartbeat defined as RR intervals from electrocardiogram traces either directly from heart-beat traces or indirectly from pulse traces. From this a sequence of successive heart beat intervals is extracted to calculate time- and frequency domain HRV.



Time- and Frequency domain HRV indices (Source: Author)

Time-domain indices

Time-domain measures of HRV are based on the statistical distribution of normal-to-normal (NN) heartbeat intervals. Description of time-domain indices are summarized in [?@tbl-td](#).

Frequency-domain indices

Frequency-domain HRV indices are derived from sequences of NN intervals transformed into the spectral domain using Fourier transformation. These indices quantify heart rate oscillations over different timescales. Short-term variations, such as respiratory sinus arrhythmia, reflect rapid autonomic changes, while longer oscillations capture autonomic responses to posture changes, circadian rhythms, or other physiological processes. Description of frequency-domain indices are summarized in [?@tbl-fq](#).

Holter recordings in study I

All ECG recordings were obtained using a 12-lead Holter system (Fysiologic ECG Services, Amsterdam, the Netherlands) over 24 hours, as previously described.⁷⁴ Participants were instructed to follow their regular daily activities but avoid showering during the recording. The ECG data were processed using proprietary Holter Analysis Software (Fysiologic ECG Services), where artefacts and ectopic beats were excluded through automated processing and manual validation. A

Table 5.2.: **Box 1** Time-domain indices reflections of autonomic function

Time-domain HRV	Description
Standard deviation of NN heart beat intervals (SDNN, in ms)	Measures the total variation in interbeat intervals and reflects both sympathetic and parasympathetic activity ⁶ .
SD of the averages of NN intervals in 5-minute segments throughout the recording (SDANN, in ms)	Measures variations in 5-minute mean interbeat intervals, primarily reflecting autonomic fluctuations associated with the circadian rhythm ⁶
Mean of the SDs of all NN intervals for all 5-minute segments (SDNN index, in ms)	Measures the average short-term variability in interbeat intervals across successive 5-minute periods, reflecting both sympathetic and parasympathetic modulation of heart rate ⁶
NN50 count divided by the total number of all NN intervals (pNN50, percentage)	Measures the proportion of successive interbeat intervals differing by more than 50 ms, primarily reflecting parasympathetic (vagal) activity ⁷⁷ .
Square root of the mean of the sum of squares of differences between adjacent NN intervals (RMSSD, in ms)	Measures variation in successive interbeat intervals during inhalation and exhalation, primarily reflecting parasympathetic (vagal) activity ⁷⁷

Table 5.4.: **Box 2** Frequency-domain indices reflections of autonomic function

Frequency domain	
HRV	Description
Variance of all NN intervals 0.4 Hz, total power (TP, in ms²)	Measures the total variation in interbeat intervals, reflecting both short- and long-term autonomic regulation by the sympathetic and parasympathetic nervous system. ⁶
Ultra low-frequency range (ULF, in ms²; 0.003 Hz)	Measures very long-term oscillations in interbeat intervals, influenced by autonomic responses to circadian rhythms, physical activity, metabolic processes, and thermoregulation. ^{78,79}
Very-low-frequency range (VLF, in ms²; 0.003–0.04 Hz)	Measures oscillations in interbeat intervals over 5-minute periods, reflecting the activity of the renin–angiotensin system and peaks in sympathetic nervous system activity, while also depending on parasympathetic modulation. ^{80,81}
Low-frequency range (LF, in ms²; 0.04–0.15 Hz)	Measures intermediate oscillations in interbeat intervals, reflecting a combination of sympathetic and parasympathetic nervous system activity, particularly associated with baroreflex function and blood pressure regulation. ⁸²
High-frequency range (HF, in ms²; 0.15–0.4 Hz)	Measures short-term oscillations during inspiration and expiration, reflecting parasympathetic modulation of heart rate via the vagus nerve, and closely associated with respiratory sinus arrhythmia. ⁸³

minimum recording duration of 18 hours was required for further analysis.^{[75]8} Inter-beat intervals between consecutive sinus beats were provided in milliseconds (ms). Time-domain HRV indices were calculated, including SDNN, SDANN, RMSSD, SDNN index, and pNN50. Frequency-domain measures were derived using Fast Fourier Transform, including TP, ULF, VLF, LF, and HF.⁷⁵ Outliers were removed. HRV indices were standardised by their mean and SD, and composite Z-scores were computed for time and frequency-domain measures, respectively. This selection of indices covers the main sources of HRV variance.⁷⁵

ActiHeart heart rate and physical activity in study II

Heart rate was measured using a combined accelerometer and heart rate monitor (ActiHeart, Cam-NTech, Cambridge, UK), recording uniaxial acceleration and heart rate. The data collection and processing methods have been described previously. Mean heart rates were recorded in 30-second epochs. Based on a algorithm, distributions of interbeat-intervals was calculated in each 30-second epochs.⁸⁴ HRV calculations were performed using the RHRV package (version 4.2.7) in R.^{[84]85} The algorithm was tested on a dataset with full access to all interbeat intervals.⁸⁴ HRV indices based on global distribution in 24-hour recordings shown high validity.⁸⁵ HRV indices including SDNN, SDANN, SDNN index, TINN, and mean heart rate (HR) were calculated by week, 24-hour cycle, and hour of the day, with hourly values averaged across recording days.⁸⁵

Vagus device for cardiovascular autonomic reflex test in study III

CAN was diagnosed using cardiovascular autonomic reflex tests (CARTs), the gold standard for CAN assessment. R-R intervals were derived from an ECG signal using the Vagus™ device (Medicus Engineering, Aarhus, Denmark.)^{[68]86} We used pulse rate ratios measured under different conditions.⁸⁶ Three standardized cardiovascular autonomic reflex tests (CARTs) were performed: (1) lying-to-standing, (2) deep breathing, and the (3) Valsalva manoeuvre, following a standardized protocol conducted between 8:00 a.m. and 2:00 p.m., after 10 minutes of supine rest.⁸⁶ Smoking and caffeine intake were prohibited two hours before testing. Each test was conducted once by trained examiners.⁸⁶

Manifest CAN was defined as two or more abnormal CARTs using age-specific cut-off values.⁶⁸ The Vagus™ device's accuracy has been validated against FDA standards and stationary devices, showing moderate to high reproducibility⁸⁷. Orthostatic hypertension was defined as a sustained drop in systolic blood pressure of 20 mmHg or diastolic blood pressure of 10 mmHg within three minutes of standing.⁸⁶

Cardiovascular autonomic reflex test

Figure 5.2.: CART

5.2.2. Confounders and variables for instrumental bias

Across Studies I, II, and III, a comprehensive set of covariates and potential confounders were assessed, including lifestyle factors, clinical measurements, biochemical markers, and socioeconomic indicators.^{75,85,86}

Smoking status was self-reported in all studies, categorized as never, former, or current (Study I), current/ex/never (Study II), and smoker/non-smoker (Study III). Alcohol consumption was recorded as average weekly units in all three studies.^{75,85,86} Physical activity was assessed via self-report in Studies I, II, III, with Study I capturing total and moderate-to-vigorous activity (hours/week), Study II used the Recent Physical Activity Questionnaire (RPAQ) to calculate physical activity energy expenditure (PAEE), and Study III classifying activity as sedentary or non-sedentary.^{75,85,86} In Study II also used combined accelerometry and heart rate monitoring (ActiHeart) to estimate PAEE.⁸⁵ Study II included register-based data on socioeconomic status at baseline, including education length, income, and employment status.⁸⁵ All studies included measurements of body mass index (BMI), waist circumference, and systolic and diastolic blood pressure, obtained during clinical examinations.^{75,85,86}

Blood samples were analyzed in all studies for HbA1c, fasting plasma glucose (FPG), triglycerides, total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol.^{75,85,86} Study I also included a 2-hour oral glucose tolerance test (OGTT) to classify glucose metabolism status based on FPG and OGTT (normal, prediabetes, T2D) using WHO 2006 criteria, excluding HbA1c as a diagnostic criterion.⁷⁵ Study III additionally measured creatinine, estimated glomerular filtration rate (eGFR), and urine albumin-to-creatinine ratio.⁸⁶

Self-reported history of CVD and use of anti-hypertensive, glucose-lowering, and lipid-lowering medications were collected in all studies.^{75,85,86} In Study II, history of CVD events in the 10 years prior to baseline were retrieved from national registers.⁸⁵ In Study III, history of CVD was collected from electronic patient records.⁸⁶

5.3. Outcomes

5.3.1. Arterial stiffness

Arterial stiffness is characterized by arteriosclerosis and atherosclerosis properties of the arteries. The stiffness of different segments of the vascular musculature can be assessed both locally and dynamically. Aortic and carotid stiffness were assessed as markers of arterial stiffness, following previously described procedures.⁸⁸

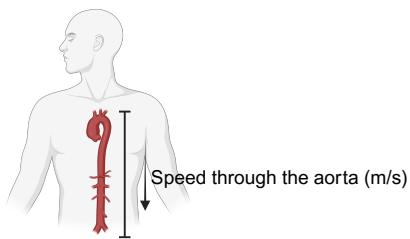
Pulse wave velocity

Aortic stiffness was measured by carotid-femoral pulse wave velocity (cf-PWV) using applanation tonometry (SphygmoCor, Atcor Medical, Sydney, Australia), with the median of at least three consecutive recordings included in the analysis. cf-PWV is calculated based on the time between the ECG systole and the arrival of the pressure wave at the femoral and carotid measurement sites along with the distance between these two measurement sites. cf-PWV is measured with participants in a supine position following a 10-minute rest period. The aortic path length was determined using a tape measure by subtracting the carotid-to-sternal notch distance from the femoral-to-sternal notch distance.⁸⁸

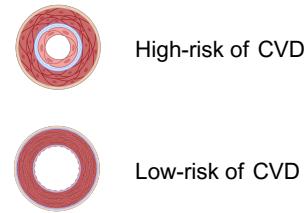
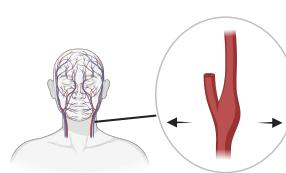
Carotid artery distensibility

Carotid stiffness was assessed by the carotid artery distensibility coefficient (CD), based on ultrasound imaging of the left common carotid artery using a 7.5 MHz linear probe (MyLab 70, Esaote Europe, Maastricht, the Netherlands). CD was calculated as $\Delta D/\text{braPP}$, where ΔD represents carotid distension and braPP is brachial pulse pressure. Mean heart rate and mean arterial pressure (MAP) were recorded every five minutes using an oscillometer device (Accutorr Plus, Datascope, Montvale, NJ, USA).⁸⁸

Pulse wave velocity (PWV)



Carotid artery distensibility (CD)



Measures of arterial stiffness, measured dynamically through the descending aorta and locally at carotid sites. (Source: Author)

5.3.2. Indicators of heart failure

N-terminal prohormone of brain natriuretic peptide (NT-proBNP) is a neuretic peptide that can be used to detect patients with heart failure and the progression of heart failure. It derives from

5.3. Outcomes

Table 5.6.: ?(caption)

Outcome	Diagnosis codes
<i>Heart failure</i>	ICD: I50
<i>Three-point MACE</i>	
• Stroke	ICD: I61 - I64
• Myocardial infarction	ICD: I21-I24
• Cardiovascular death	ICD: I20-I28, I42, I46
• Cardiovascular revascularization	SKA: KPAE10, KPAE25, KPAF10, KPAF20, KPAF21, KPAF22, KPAH10, KPAH20, KPAH21, KPEE, KPEF, KPEH, KPEP, KPEQ, KPFE,, KPFH, KPFP, KPFQ

B-type natriuretic peptid (BNP) which is a cardial neurohormone, that is synthesized and secreted as response to stretched cardiomyocytes and cardiac volume overload. After secretion, proBNP is cleaved, releasing the active hormone BNP along with the remaining N-terminal fragment, known as NT-proBNP. In Study III, a blood sample was taken at the study site. Description of the NT-proBNP analysis of plasma samples is described in supplementary material in Appendix Study III.⁸⁶

A modified version of the validated The WATCH-DM heart failure risk score was used. The risk score is based on 9 variables: two binary (history of myocardial infarction and coronary artery bypass grafting) and seven continuous (age, BMI, systolic/diastolic BP, serum creatinine, HDL cholesterol, and HbA1c). Scores range from 0–39, categorized as very low (11), low (12–13), moderate (14–15), high (16–18), and very high (19) risk.^{[86]8990}

NYHA class stage I–IV was included. Heart failure symptoms were defined as NYHA class II–IV, assessed by a physician.⁸⁶

5.3.3. Cardiovascular events

Information on CVD events and mortality was obtained from the Danish National Patient Registers until 2021 by utilizing the ICD-10 codes for stroke, myocardial infarction, cardiovascular death, cardiovascular revascularization, and heart failure. We defined three-point major adverse cardiovascular events (MACE) as myocardial infarction, stroke, cardiovascular revascularization, and cardiovascular death.⁸⁵

5.4. Statistical Methods

5.4.1. Cross-sectional analysis

Study I

In Study I, we used multiple linear regression to investigate associations between 24-hour HRV and arterial stiffness. Model 1 adjusted for age, sex, education, glucose metabolism status, and mean arterial pressure (MAP) to account for the oversampling of individuals with T2D and potential instrumental bias of arterial pressure flow. Model 2 included additional adjustments for smoking behavior, alcohol consumption, physical activity, body mass index, HbA1c, triglycerides, total-to-HDL cholesterol ratio, and medication use. Arterial stiffness measures were log-transformed to ensure normally distributed residuals and back-transformed into percentage change estimates. A sex interaction term was added to assess if the association differed between sex. We performed sensitivity analyses excluding individuals on antihypertensive treatment or glucose-lowering medication.⁷⁵

Study III

In Study III, we applied logistic regression models to investigate the association between CAN and heart failure, using NT-proBNP as the primary outcome. We adjusted for age, sex, and diabetes duration, smoking behavior, alcohol consumption, body mass index, HbA1c, triglycerides, total cholesterol, and antihypertensive medication, eGFR and prior CVD. We performed sensitivity analyses and excluded participants with beta-blocker treatment or prior CVD. We applied logistic regression to assess the odds of CAN association with heart failure symptoms, defined by a NYHA class II or higher, adjusting for covariates in primary analysis. Linear regression was employed to evaluate differences in the WATCH-DM risk score between individuals with and without CAN.⁸⁶

5.4.2. Time-to-event analysis

In Study II, we used Poisson regression models to quantify the associations between multiday HRV and cardiovascular events, as follow-up data were undisturbed over time and to avoid assumptions of proportional hazards⁹¹. Multiday HRV was modelled using splines with knots at predefined percentiles to assess non-linear associations. Hourly HRV was analysed separately for each hour to observe if the association of HRV had diurnal variation. Both HRV and mHR were standardized by their mean and standard deviation to ensure comparability. Based on assumptions about potential confounding pathways summarized in directed acyclic graphs (DAG), we fitted two models: Model 1 adjusted for age and sex, while Model 2 further adjusted for education, smoking, alcohol consumption, physical activity (physical activity energy expenditure (PAEE) calculated from Recent Physical Activity Questionnaire RPAQ), body mass index, total cholesterol, and HbA1c. Additional analyses were performed with HRV pre-adjusted for concurrent heart rate and physical acceleration to account the influence of these factors. Missing covariates were handled using multiple imputation. Each individual's follow-up period began at the time of their inclusion in the baseline examination.⁸⁵ To calculate age-specific incidence rate (IR) we did the following. Follow-up ended at the earliest occurrence of CVD, heart failure, all-cause mortality, death, or the end of the study period . The

follow-up time was divided into one-year intervals based on the individual's age. Using this age-split data, incidence rates of CVD, heart failure, and all-cause mortality were analyzed in relation to the HRV, with age treated as a time-varying covariate in a Poisson regression model.

5.4.3. Effect modification

Effect modification is used to assess whether the association between an exposure and an outcome varies depending on the level of a third variable, known as the effect modifier⁹².

In Study I, it was hypothesized that the association between 24-hour and arterial stiffness was stronger in strata of progression of diabetes (normal glucose metabolism, prediabetes, T2D). Therefore, interaction term between HRV and diabetes status was included to observe the size of the association across strata.⁷⁵ We did subsidiary analysis in a subpopulation without T2D to check if the effect was modified by HbA1c. In Study II, the association between multiday HRV and CVD endpoints varied by sex was quantified, to explore potential biological dimorphism.⁸⁵ In Study III, we aimed to determine whether the association between CAN and elevated NT-proBNP is present in the subgroup without symptoms, defined as NYHA class < II. Hence, the hypothesis was no significant effect modification between groups with and without symptoms. Similarly, whether the association remains present in the group classified as low to moderate risk of heart failure was explored, based on the WATCH-DM risk score.⁸⁶

A significant effect modification between the exposure and the effect modifier in all analyses was defined as an interaction term with a p-value < 0.05.

5.4.4. Multiple imputed by chained equations

Multiple Imputation by Chained Equations (MICE) is a method for handling missing data in datasets. This procedure imputes missing values through an iterative series of predictive models, generating plausible estimates while preserving the relationships within the data. To avoid one imputation for missing value could give the value the same confidence as the a non-missing value, Rubins Rule was followed. Rubin's rules in MICE combine results from multiple imputed datasets by pooling estimates of interest (e.g., means or regression coefficients) using their within- and between-imputation variances⁹³. Thus, we ensure valid statistical inferences by accounting for the uncertainty introduced by missing data.

In Study II, confounders was imputed to include as many participants and avoid excluding population with our without cardiovascular or mortality events. Dataset was imputed 10 times. In Study III, missing CART was imputed, as a proportion of participants had non-valid test due to insufficient air in the valsalva manuevre, unstable heart beats or data error. These variables was used as auxiliary variables in imputation to reduce bias⁹⁴. All available variables of biochemical measures, diagnosis, medication and cause of non-valid CART was used to impute each missing CART using predictive mean matching.⁸⁶

5.4.5. Instrumental bias

In Study I-III we are investigating the physiological properties by dynamic measures and biomarkers to quantify autonomic function, arterial stiffness, and cardiac function. Other conditions may affect the properties we are attempting to measure, and thus are causing instrumental bias.

Vascular Stiffness

In Study I, we used measurements of arterial stiffness using cf-PWV and carotid distensibility. Both measures are influenced by arterial pressure at the time of examination. Arterial pressure affects the propagation of the pressure wave through the aorta (cf-PWV) and the expansion and contraction of the carotid artery (carotid distensibility).⁹⁵ To account for this, we adjusted for mean arterial pressure in our models.⁷⁵

Cardiovascular autonomic function

In Study II, we assessed autonomic function using multiday HRV recordings and hourly HRV measurements. Studies have highlighted that HRV is dependent on heart rate, and low HRV may simply reflect a higher resting heart rate (rHR). To adjust for this without overcorrecting for a collinear variable, we pre-adjusted HRV by regressing rHR on HRV, extracting the residuals, and using these as the pre-adjusted determinant. For hourly HRV, variability in heart rate may be influenced by changes in physical activity, creating a risk that HRV serves as a proxy for movement rather than autonomic function. To address this, we applied a similar pre-adjustment approach by regressing concurrent heart rate and physical acceleration to account for physical activity.⁸⁵

Biomarker of Heart Failure

In Study III, kidney function and overweight are known to influence NT-proBNP levels independently of heart failure⁵⁶. We adjusted the model to account for the blurred effect of eGFR on NT-proBNP levels in the analysis.⁸⁶

6. Results

In this section, study population characteristics and findings from analysis will be presented.

6.1. Study I

6.1.1. Descriptive

In The Maastricht Study, 7,449 participants were included between November 2010 and December 2020, of whom 1,316 reported a history of CVD.⁷⁵ Participants who had valid 24-hour HRV measured was 4379 and of those 3673 had a valid measurement of either CD or cf-PWV. Study population included 3673 participants. Further characteristic are described in the study in manuscript Table 1 in Appendix Study I.⁷⁵ The study population represented diabetes risk of NGM (65%), prediabetes (15%), and T2D (20%). The median (IQR) cf-PWV (aortic stiffness) became higher with diabetes status: NGM: 8.08 m/s (7.28, 9.16), prediabetes: 8.96 m/s (7.84, 10.32), and T2D: 9.36 m/s (8.16, 10.80). CD (carotid stiffness) decreased: NGM: 15.0 (11.8, 18.8), prediabetes: 13.5 (10.4, 16.9), and T2D: 12.5 (9.9, 16.0) $\times 10^3/\text{kPa}$. SDNN (ms) was highest in NGM and lowered with worsening glucose metabolism: NGM: 138ms (117, 164), prediabetes: 127ms (106, 152), and T2D: 116ms (96, 139). Further description of charaterisitcs by diabetes are described in Table 6.1.

Table 6.1.: Study characteristics in The Maastricht Study (Study I) by diabetes status

Characteristic	Normal glucose metabolism N = 2,389	Prediabetes N = 538	Type 2 Diabetes N = 746
Women	1,361 (57%)	258 (48%)	265 (36%)
Age (years)	58 (51, 64)	62 (57, 68)	63 (57, 68)
Moderate to vigorous physical activity (hours/week)	5.3 (3.0, 8.3)	4.5 (2.3, 7.5)	3.8 (1.5, 6.8)
BMI (kg/m ²)	25.0 (22.9, 27.4)	27.2 (24.9, 30.1)	28.8 (26.0, 31.7)
HbA1c (%)	5.35 (5.17, 5.63)	5.63 (5.35, 5.90)	6.54 (6.08, 7.09)
HDL (mmol/L)	1.60 (1.30, 2.00)	1.40 (1.20, 1.80)	1.30 (1.00, 1.50)
Total cholesterol (mmol/L)	5.50 (4.80, 6.20)	5.50 (4.80, 6.30)	4.50 (3.90, 5.20)
Triglycerides (mmol/L)	1.05 (0.80, 1.45)	1.39 (1.03, 1.90)	1.51 (1.08, 2.14)
Duration of type-2 diabetes (only for diagnosed participants)			3 (0, 9)
Mean IBI (ms)	838 (775, 907)	815 (760, 897)	806 (744, 889)
SDNN (ms)	138 (117, 164)	127 (106, 152)	116 (96, 139)
RMSSD (ms)	26 (21, 34)	24 (19, 33)	22 (17, 31)
TP (ms ²)	12,596 (8,880, 17,498)	10,615 (7,134, 15,374)	8,880 (6,064, 12,722)
ULF (ms ²)	10,771 (7,392, 15,142)	8,948 (5,852, 13,374)	7,524 (5,036, 11,001)
VLF (ms ²)	1,198 (833, 1,692)	1,015 (685, 1,478)	816 (541, 1,267)
HF (ms ²)	94 (57, 158)	78 (47, 138)	63 (36, 117)
Systolic blood pressure (mmHg)	123 (114, 133)	129 (122, 140)	130 (122, 139)
Diastolic blood pressure (mmHg)	75 (71, 80)	78 (73, 83)	76 (72, 81)
Mean arterial pressure (mmHg)	95 (88, 102)	99 (93, 107)	98 (92, 105)
Carotid artery distensibility (10-3/kPa)	15.0 (11.8, 18.8)	13.5 (10.4, 16.9)	12.5 (9.9, 16.0)
Carotid-femoral pulse wave velocity (m/s)	8.08 (7.28, 9.16)	8.96 (7.84, 10.32)	9.36 (8.16, 10.80)
Glucose-lowering medication	0 (0%)	0 (0%)	519 (70%)
Antihypertensive medication	431 (18%)	199 (37%)	478 (64%)
Beta blockers	149 (6.2%)	77 (14%)	195 (26%)
Lipid-lowering medication	280 (12%)	141 (26%)	484 (65%)

Note:

makecell[l]Categorical variables: N (%); Continous variables: Median (IQT:25th-75th) Table are adapted from supplementary material in Appendix Study I

6.1.2. 24-hour HRV and arterial stiffness

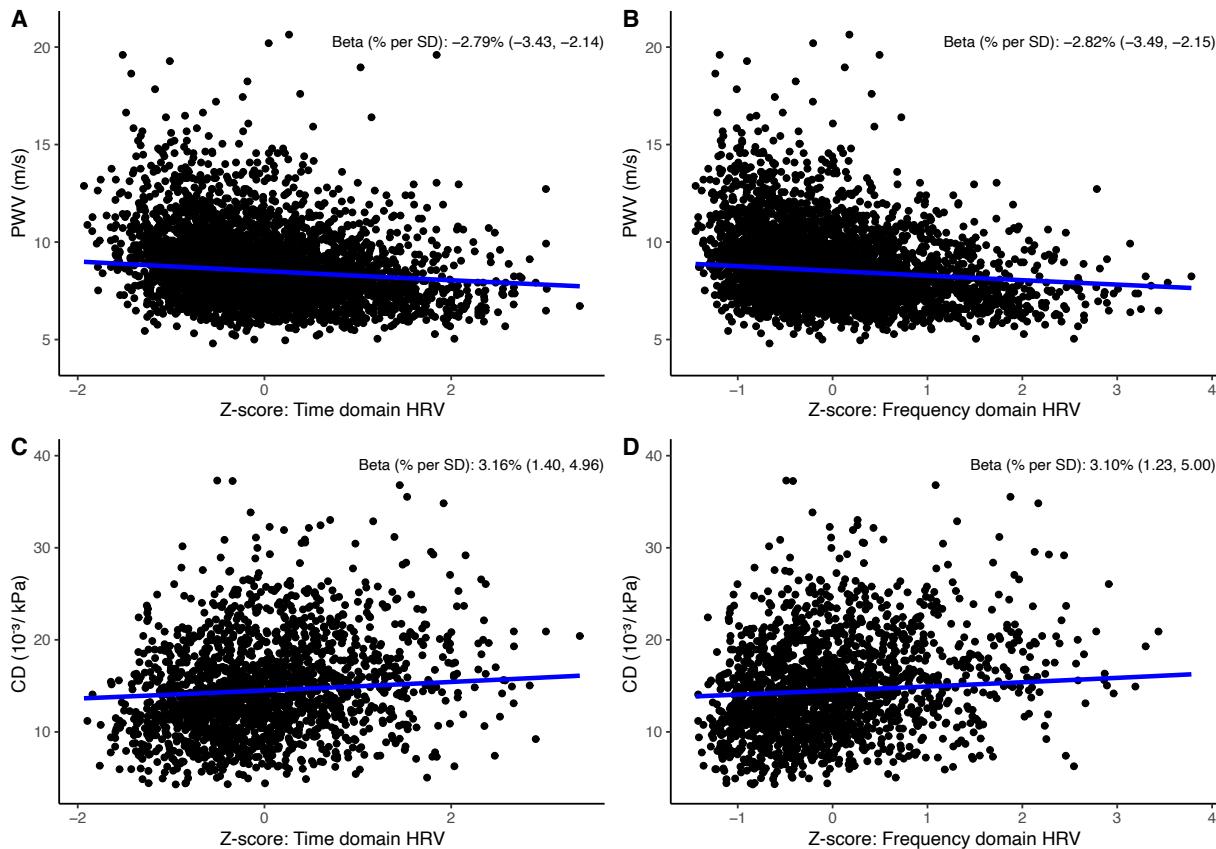
Time-domain HRV

In the fully adjusted model 2, cf-PWV was 2.8% (CI: 2.1; 3.4) lower, while CD was 3.3% (CI: 1.5; 5.1) higher per SD higher in HRV time-domain Z-score (see see Figure 6.2 A and B). Among the time-domain indices, SDNN, SDNNi, and SDANN showed the strongest associations, with cf-PWV being lower by 2.5% (CI: 2.0; 3.1), 2.5% (CI: 1.9; 3.4), and 2.2% (CI: 1.7; 2.7), respectively.⁷⁵ Conversely, CD was higher by 3.2% (CI: 1.7; 4.7), 3.0 % (CI: 1.4; 4.6), and 2.8% (CI: 1.3; 4.3), respectively. RMSSD and pNN50 showed a weaker association with cf-PWV (-1.1% [CI: -1.4; -0.4], and -1.1 [-1.7; -0.6]), while no evidence for an association was found with CD.⁷⁵

Frequency-domain HRV

In the fully adjusted model 2, cf-PWV was 2.8% (CI: 2.1; 3.5) lower, while CD was 3.2% (CI: 1.3; 5.1) higher per SD higher in HRV frequency-domain Z-score (see see Figure 6.2 C and D). Among the frequency-domain indices, total power, VLF, and ULF showed the strongest associations, with cf-PWV being lower by 2.2% (CI: 1.7; 2.8), 2.4% (CI: 1.9; 4.0), and 2.1% (CI: 1.5; 2.6), respectively.⁷⁵ Conversely, CD was higher by 2.7% (CI: 1.2; 4.2), 2.4% (CI: 0.9; 4.1), and 2.6% (CI: 1.1; 4.1), respectively. HF showed a weaker association with cf-PWV (-0.9% [CI: -1.4; -0.4]), while no evidence for an association was found with CD. Mean interbeat interval was associated with 2.4 % (CI: 1.8; 2.9) lower cf-PWV and 4.5% (3.1; 6.1) higher CD.⁷⁵

6.1. Study I



A: Percentage PWV per SD in time-domain composite z-score B: Percentage PWV per SD in frequency-domain composite z-score C: Percentage higher CD per SD in time-domain composite z-score D: Percentage CD per SD in frequency-domain composite z-score. All regression lines were adjusted for being a male, 60 years old, low educational level, without prediabetes or type-2 diabetes, and with 96mmHg mean arterial pressure. From Figure 1 in Appendix Study I⁷⁵

Figure 6.1.: Association between HRV and arterial stiffness

6.1.3. Effect modification of diabetes status

The association between HRV time-domain Z-scores and cf-PWV and CD was significantly modified by prediabetes (cf-PWV: -4.9 [CI: -6.523; -3.243] $^{interaction(*p-value<0.01)}$ CD: 8.0 [CI:3.8; 12.5] $^{*p-value<0.01}$) but not by T2D (cf-PWV: -3.5 % [CI: -4.8; -2.1]) $^{*p-value<0.1}$ CD: 4.8 % [CI:1.3; 8.4] $^{*p-value<0.1}$)⁷⁵. For the indices SDNN and SDANN, the association with both cf-PWV and CD was significantly modified by both prediabetes and T2D⁷⁵.

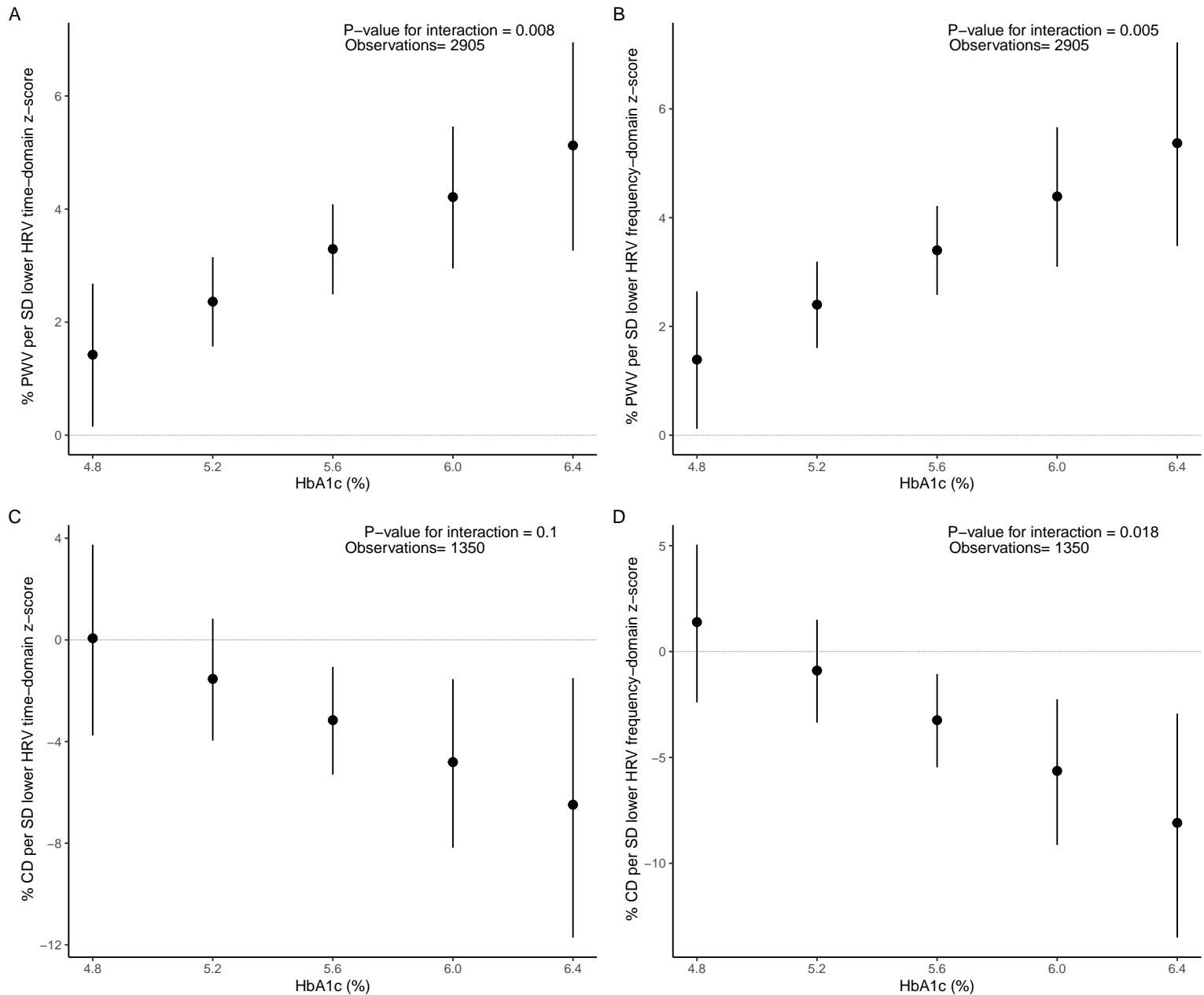
The association between HRV frequency-domain Z-score and cf-PWV was significantly modified from NGM by prediabetes (-5.7 %[CI:-7.4; -3.9] $^{*p-value<0.01}$) and T2D (-3.9 %[CI:-5.4; -2.3] $^{*p-value<0.05}$) while CD was only modified by prediabetes (8.3 %[CI:3.6; 13.2] $^{*p-value<0.01}$) but not by T2D (5.3

6. Results

%[CI:1.4; 9.4]^{**p-value*<0.1})⁷⁵. For the indices total power and ULF, the association with both cf-PWV and CD was significantly modified by both prediabetes and T2D. Mean inter beat interval association with cf-PWV or CD was not significantly modified by diabetes status⁷⁵.

As no stepwise increase was observed in the modification of glucose metabolism status from prediabetes to T2D, subgroup with T2D was excluded to test whether the association was gradually modified by dysglycemia. In this subgroup, the association between HRV time and frequency domain Z-scores and measures of arterial stiffness was modified by HbA1c (range of interaction p-values: 0.1 to 0.005) (see Figure Figure 6.2). For example, per SD lower HRV frequency domain Z-score at HbA1c 6.4% was associated with a 5.4% higher (CI: 3.5; 7.2) cf-PWV, which was 2.0% to 4.0% higher compared to the magnitude of association at HbA1c levels of 5.6% and 4.8% (see see Figure 6.2 B). In CD, per SD lower HRV frequency domain Z-score at HbA1c 6.4% was associated with an 8.1% lower (CI: -13.5; -2.9) CD, which was 4.8% to 9.5% lower compared to the magnitude of association at HbA1c levels of 5.6% and 4.8% (see Figure 6.2 D). No association between HRV frequency domain Z-score and CD was observed at HbA1c levels between 4.8% and 5.2%.

6.1. Study I



A: Percentage PWV per SD in time-domain composite z-score B: Percentage PWV per SD in frequency-domain composite z-score C: Percentage higher CD per SD in time-domain composite z-score D: Percentage CD per SD in frequency-domain composite z-score. Model adjusted for sex, age, educational status, diabetes status, and mean arterial pressure, physical activity, smoking behaviour, alcohol use, body mass index, hba1c, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication. Figure was based on data in Study I⁷⁵

Figure 6.2.: Association between HRV and arterial stiffness modified by HbA1c in subpopulation without T2D

6.2. Study II

6.2.1. Descriptive

The ADDITION-PRO population consisted of 1,627 participants with at least 48-hour HRV measures, while 1,432 had all hour represented with hourly HRV and physical acceleration. The study population included different tiers of diabetes risk: 154 individuals at low risk (9%), 889 at high risk (51%), 314 with impaired fasting glucose (IFG) (18%), 226 with impaired glucose tolerance (IGT) (13%), and 161 with both IFG and IGT (9%). SDNN was split into categories by very-low (SDNN < 100 ms), low (SDNN 100-120 ms), middle (SDNN 121-140 ms), high (SDNN 141-160 ms) and very-high (SDNN > 160 ms).

Characteristics are described in Table 6.2. Participants in the lowest SDNN group (<100 ms) were older (67.4 ± 6.9 years), had higher BMI (28.1 ± 5.4), HbA1c (5.9 ± 0.9 mmol/L), triglycerides (1.5 ± 0.9 mmol/L), and resting heart rate (67.8 ± 5.7 bpm), were more likely to use anti-hypertensive medication (61%), and had lower physical activity energy expenditure (46.8 ± 24.0 kJ/day) compared to those with higher SDNN levels.

Table 6.2.: Study characteristics in The ADDITION-PRO Study (Study II) by SDNN categories

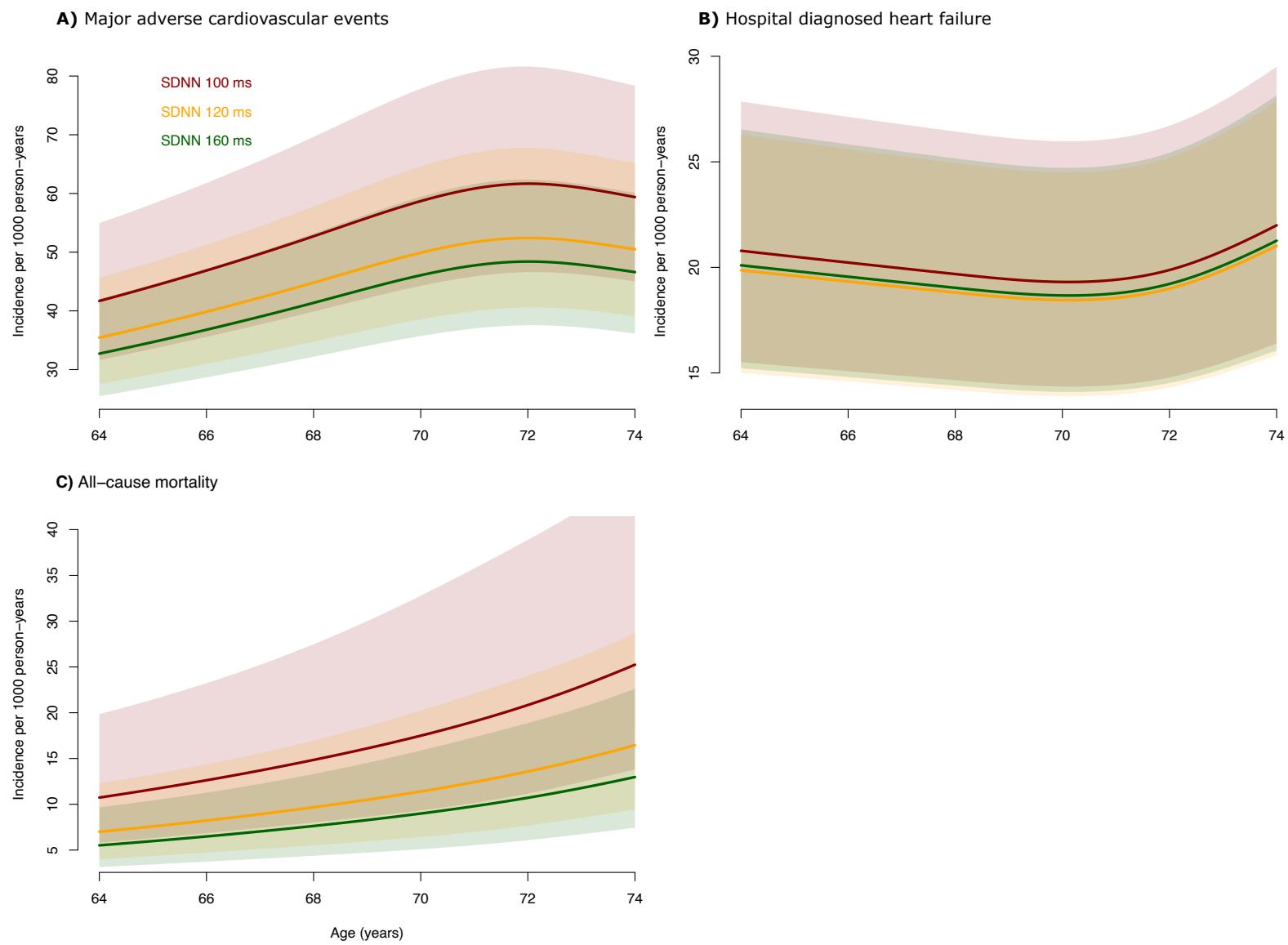
SDNN Categories	Overall, N = 1,625	<100 ms, N = 148	100-120 ms, N = 312	120-140 ms, N = 457	140-160 ms, N = 346	>160 ms, N = 362
Women	759 (47%)	80 (54%)	164 (53%)	251 (55%)	143 (41%)	121 (33%)
Age (years)	65.9 (6.8)	67.4 (6.9)	65.7 (6.9)	66.0 (6.7)	65.5 (6.6)	66.0 (7.0)
Physical activity energy expenditure (KJ / day)	53.1 (25.1)	46.8 (24.0)	49.4 (21.0)	50.7 (21.5)	57.6 (27.2)	57.5 (29.2)
Alcohol consumption (units per week)	9.2 (9.5)	11.3 (10.8)	10.2 (11.3)	8.9 (8.5)	8.5 (9.2)	8.7 (8.2)
Smoking status						
Current	263 (16%)	40 (28%)	70 (23%)	65 (14%)	41 (12%)	47 (13%)
Prior	750 (47%)	58 (40%)	145 (47%)	214 (47%)	162 (47%)	171 (48%)
Never	598 (37%)	47 (32%)	95 (31%)	174 (38%)	140 (41%)	142 (39%)
BMI (kg/m ²)	27.7 (4.7)	28.1 (5.4)	28.2 (4.6)	28.0 (4.7)	27.7 (4.9)	26.9 (4.2)
Waist circumference (cm)	96.7 (13.4)	98.0 (14.9)	98.2 (13.2)	96.7 (13.6)	96.7 (13.1)	94.8 (12.5)
Systolic blood pressure (mmHg)	133.7 (17.3)	134.2 (16.3)	133.7 (17.6)	133.5 (17.8)	133.4 (16.9)	133.8 (17.5)
Diastolic blood pressure (mmHg)	81.9 (10.4)	83.8 (10.1)	82.7 (10.2)	81.7 (10.6)	82.1 (10.2)	80.6 (10.3)
HbA1c (%)	5.8 (0.5)	5.9 (0.9)	5.9 (0.6)	5.8 (0.5)	5.7 (0.4)	5.7 (0.4)
Triglycerides (mmol/L)	1.3 (0.7)	1.5 (0.9)	1.4 (0.7)	1.3 (0.6)	1.2 (0.7)	1.1 (0.6)
Total cholesterol (mmol/L)	5.4 (1.1)	5.2 (1.0)	5.4 (1.2)	5.4 (1.1)	5.4 (1.0)	5.4 (1.0)
HDL cholesterol (mmol/L)	1.6 (0.4)	1.6 (0.4)	1.5 (0.5)	1.6 (0.4)	1.6 (0.4)	1.6 (0.4)
LDL cholesterol (mmol/L)	3.2 (1.0)	3.0 (1.0)	3.2 (1.1)	3.2 (1.0)	3.3 (0.9)	3.3 (0.9)
Urine albumin-creatinine ratio (mg/g)	25.9 (132.8)	36.4 (105.9)	47.9 (275.1)	19.6 (48.2)	19.4 (67.7)	16.4 (36.3)
VO ₂ max (mL/kg/min)	26.6 (7.8)	24.8 (7.5)	24.8 (7.5)	26.1 (6.8)	27.0 (8.0)	28.7 (8.7)
Resting heart rate (bpm)	57.3 (7.3)	67.8 (5.7)	63.3 (5.0)	58.4 (4.5)	55.0 (4.2)	49.8 (4.9)
Anti-hypertensive medication (yes)	753 (47%)	88 (61%)	149 (48%)	216 (47%)	147 (43%)	153 (43%)

Note:

makecell[l]Categorical variables: N (%); Continuous variables: Median (IQT:25th-75th Table are based on data from Study II

6.2.2. Multiday HRV and MACE, heart failure, and all-cause mortality.

The mean multiday SDNN was 139.0 (32.3) ms, and the mean heart rate was 73.5 (9.1) bpm⁸⁵. In the fully adjusted model, SDNN per SD was associated with a lower incidence rate ratio (IRR) for MACE 0.82 (CI: 0.69; 0.97), heart failure 0.76 (CI: 0.58; 0.99), and mortality rate ratio of 0.79 (CI: 0.66; 0.94)⁸⁵. In model with pre-adjustment for resting heart rate, the proportion of the association explained between HRV and MACE, HF, and all-cause mortality was 14%, 25%, and 19%, respectively⁸⁵. When knots were included in the model, the risk higher as SDNN dropped below approximately 120–110 ms (around the 20th percentile), suggesting a potential threshold for elevated risk⁸⁵. Therefore, incidence rate (IR) was calculated at SDNN levels of 100 ms, 120 ms, and 160 ms, respectively, and plotted these as a function of age.



Multiday SDNN levels (100 ms, 120 ms, 160 ms) by age-specific incidence rates for A) major adverse cardiovascular events, B) heart failure hospitalisation, and C) all-cause mortality. Model adjusted for age, sex, education, alcohol consumption, smoking behavior, physical activity, body mass index, total cholesterol, and Hba1c. Figure was based on data in Study II⁸⁵

Figure 6.3.: Multiday SDNN and mean HR association with major adverse cardiovascular events, heart failure, and all-cause mortality

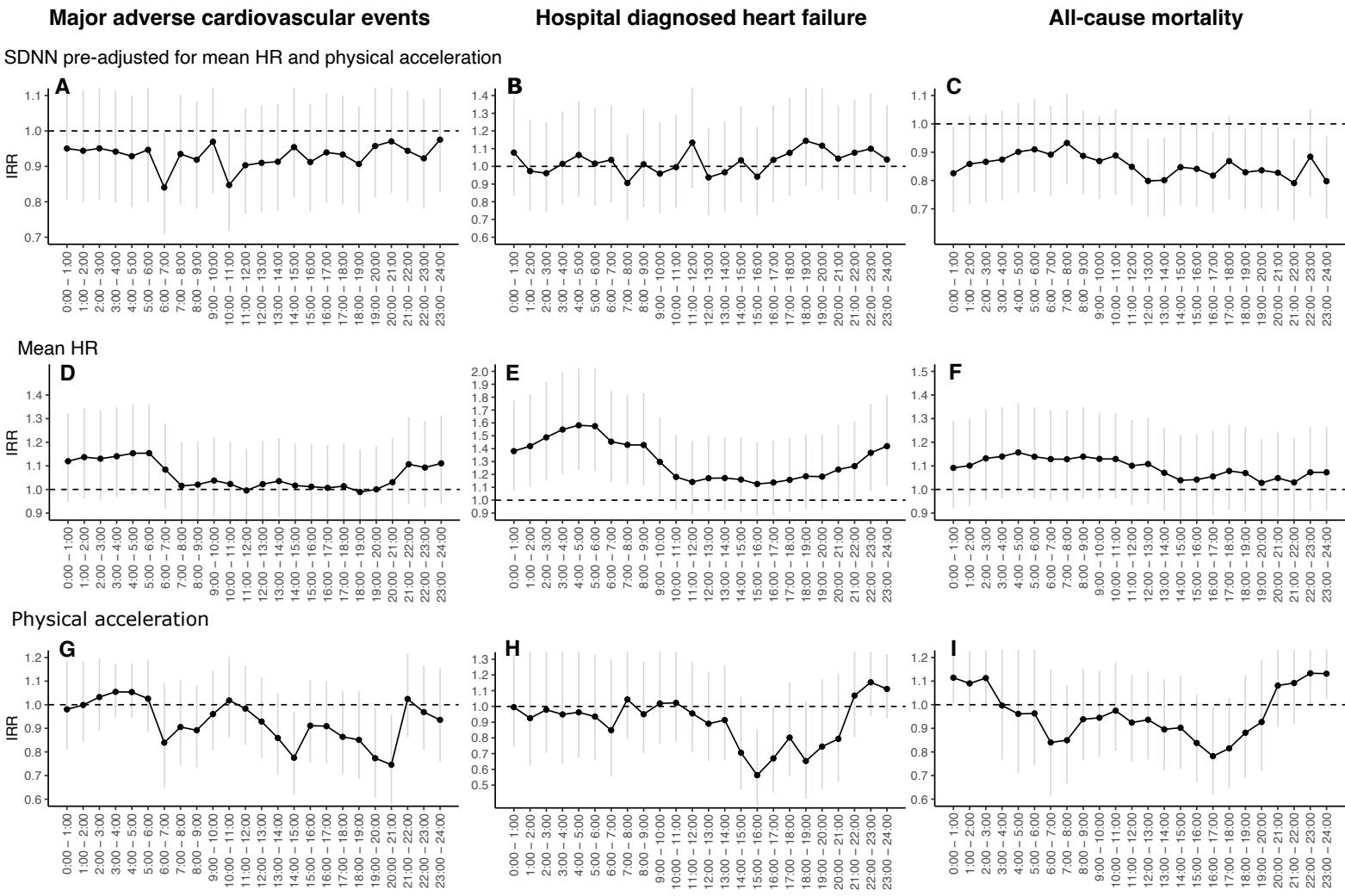
At age 65, the IR per 1000 person-years for MACE was 44.2 (CI: 33.5; 58.3) at SDNN = 100 ms, which was higher than the rates observed at SDNN = 120 ms (IR: 37.6 [CI: 29.2; 48.3]) and SDNN = 160 ms (IR: 34.7 [CI: 27.0; 44.5]) (Figure 6.3 A). The IR became higher with age, reaching its peak at age 72. For heart failure at age 65, the IR was 20.5 (CI: 15.3; 27.5) at SDNN = 100 ms, slightly higher than at SDNN = 120 ms (IR: 19.6 [CI: 14.8; 25.9]) and SDNN = 160 ms (IR: 19.8 [CI: 15.0; 26.2]) (Figure 6.3 B). The IR remained stable until age 70, after which it became higher. For all-cause mortality at age 65, the IR was 11.6 (CI: 6.3; 21.4) at SDNN = 100 ms, higher than at SDNN = 120 ms (IR: 7.6 [CI: 4.3; 13.3]) and SDNN = 160 ms (IR: 6.0 [CI: 3.4; 10.4]) (Figure 6.3 C). The IR for all-cause mortality became higher with age.

6.2.3. Hourly HRV and MACE, heart failure, and all-cause mortality.

From the hourly recordings, I observed a clear periodicity in SDNN, heart rate, sleep patterns, and physical acceleration. Mean (SD) SDNN increased from 5–6 AM (70.2 [28.8] ms), peaking at 8–9 AM (92.1 [29.0] ms), followed by a gradual decline, reaching its lowest point around 2 AM the next day (64.1 [28.1] ms).⁸⁵ A similar circadian pattern was observed in heart rate, although its peak occurred two hours later, starting at 9 AM (76.7 [10.9] bpm).⁸⁵ After peaking, heart rate remained stable throughout the afternoon before gradually decreasing.⁸⁵

In Figure 6.4, I observe hourly SDNN (preadjusted for heart rate and physical acceleration), heart rate, and physical acceleration association. Models was adjusted for age, sex, education, alcohol consumption, smoking behavior, BMI, total cholesterol, and Hba1c. The morning response of SDNN was most indicative of MACE, with the strongest association observed from 6–7 AM (IRR: 0.84; 95% CI: 0.71-1.00 per SD higher SDNN) (see Figure 6.4 A).⁸⁵ Heart rate between 12 AM and 6 AM showed a small trend toward higher risk of MACE (IRR range: 1.11 to 1.15 per SD higher heart rate), although none of the confidence intervals exceeded one (see Figure 6.4 D).⁸⁵ Across all hours, there was a plausible association between SDNN and heart failure. However, this association disappeared after adjusting for physical acceleration and heart rate (see Figure 6.4 B)⁸⁵. In contrast, heart rate between 10 PM and 9 AM was associated with heart failure (IRR range: 1.37 to 1.58 per SD higher heart rate) (see Figure 6.4 E). SDNN was consistently associated with all-cause mortality across all hours, with a stronger inverse association observed between 12 PM and 1 AM (IRR range: 0.79 to 0.88 per SD higher SDNN) (see Figure 6.4 C).⁸⁵ No clear trends of association were observed between heart rate and all-cause mortality.⁸⁵

6.2. Study II



SDNN preadjusted for concurrent physical acceleration and heart rate, as well as mean heart rate (HR) and physical acceleration, were measured hourly from 00:00 to 24:00. The IRR for MACE, heart failure hospitalization, and all-cause mortality are shown by hourly associations of: (A–C) preadjusted SDNN, (D–F) mean HR, and (G–I) physical acceleration. Models were adjusted for age, sex, education, alcohol consumption, smoking behavior, body mass index, total cholesterol, and HbA1c. Figure adapted from Appendix Study II⁸⁵

Figure 6.4.: Diurnal heart rate variability and heart rate association with major adverse cardiovascular events, heart failure, and all-cause mortality risk

6.3. Study III

6.3.1. Descriptive

In study III, 176 participants had measures of NT-proBNP. CAN was present in 31% ($n = 54$) of participants (36% among those with valid CAN measurements (Figure 6.5 A)).⁸⁶ Meanwhile, 23% ($n = 40$) were unable to complete the CART assessment adequately, primarily due to insufficient air pressure during the Valsalva manoeuvre ($n = 21$).⁸⁶ Compare to those without CAN, the participants with CAN were more women (41 % vs 33 %), were more sedentary (45% vs 36%), had a higher proportion with prior major CVD (41% vs 23%) and declined eGFR (< 60ml/min/1.73 m²) (35% vs 22%), higher levels of triglyceride (median 2.05 mmol/L vs 1.95 mmol/ L), were slightly older (median 62 years vs 61 years), had longer duration of T2D (median 19 years vs 15 years), and higher use SGTL2-inhibitors (65% vs 60%) but lower use of GLP-1 RA (63% vs 70%). No other difference in clinical characteristic was observed.⁸⁶

Table 6.3.: Study characteristics in The CANCAN Study (Study IIY) by CAN status

CAN status	Missing	Overall, N = 176	CAN missing, N = 40	No CAN, N = 82	CAN, N = 54
Sex (Women)	0	68 (39%)	19 (48%)	27 (33%)	22 (41%)
Age (years)	0	63 (55; 70)	68 (61; 75)	61 (52; 69)	62 (56; 68)
BMI (kg/m ²)	2	32 (28; 37)	30 (26; 34)	33 (28; 38)	33 (30; 36)
Duration of diabetes (years)	0	17 (11; 24)	20 (13; 30)	15 (9; 21)	19 (13; 24)
HbA1c (mmol/mol)	0	64 (56; 80)	65 (56; 85)	64 (55; 78)	64 (57; 76)
Total cholesterol (mmol/L)	2	3.90 (3.23; 4.78)	3.70 (3.33; 4.18)	4.10 (3.33; 4.88)	3.75 (3.03; 4.98)
HDL (mmol/L)	2	1.00 (0.88; 1.20)	1.00 (0.90; 1.30)	1.00 (0.90; 1.20)	0.97 (0.80; 1.18)
Triglycerides (mmol/L)	3	2.00 (1.30; 2.90)	2.10 (1.10; 2.80)	1.95 (1.30; 2.90)	2.05 (1.40; 2.98)
Systolic blood pressure (mmHg)	1	133 (123; 143)	135 (127; 147)	131 (123; 142)	133 (120; 143)
Diastolic blood pressure (mmHg)	1	76 (68; 82)	73 (66; 79)	78 (72; 83)	74 (66; 82)
Resting heart rate (bpm)	6	77 (66; 84)	68 (62; 80)	78 (69; 84)	80 (67; 89)
CARTS					
Lying to standing (RR ratio)	8	1.02 (1.01; 1.06)	1.03 (1.02; 1.05)	1.05 (1.01; 1.08)	1.01 (1.00; 1.02)
Deep breathing (RR ratio)	4	1.13 (1.07; 1.26)	1.15 (1.10; 1.28)	1.18 (1.11; 1.30)	1.07 (1.03; 1.08)
Valsalva maneuver (RR ratio)	45	1.24 (1.13; 1.36)	1.20 (1.14; 1.25)	1.32 (1.25; 1.45)	1.11 (1.08; 1.16)
NT-proBNP (pg/ml) categories	0				
< 50		72 (41%)	10 (25%)	47 (57%)	15 (28%)
50-124		38 (22%)	11 (28%)	16 (20%)	11 (20%)
125-300		28 (16%)	7 (18%)	10 (12%)	11 (20%)
> 300		38 (22%)	12 (30%)	9 (11%)	17 (31%)
Leisure physical activity	15	68 (42%)	17 (50%)	27 (36%)	24 (45%)
(Sedentary lifestyle vs non-sedentary lifestyle)					
Any antihypertensive medication (yes)	0	140 (80%)	33 (83%)	61 (74%)	46 (85%)
Beta-blockers (yes)	0	52 (30%)	11 (28%)	19 (23%)	22 (41%)
Glucose-lowering medication					
Metformin (yes)	0	123 (70%)	26 (65%)	56 (68%)	41 (76%)
SGLT2-inhibitors (yes)	0	81 (46%)	12 (30%)	40 (49%)	29 (54%)
GLP1 RAs (yes)	0	91 (52%)	15 (38%)	47 (57%)	29 (54%)
Insulin (yes)	0	140 (80%)	33 (83%)	66 (80%)	41 (76%)

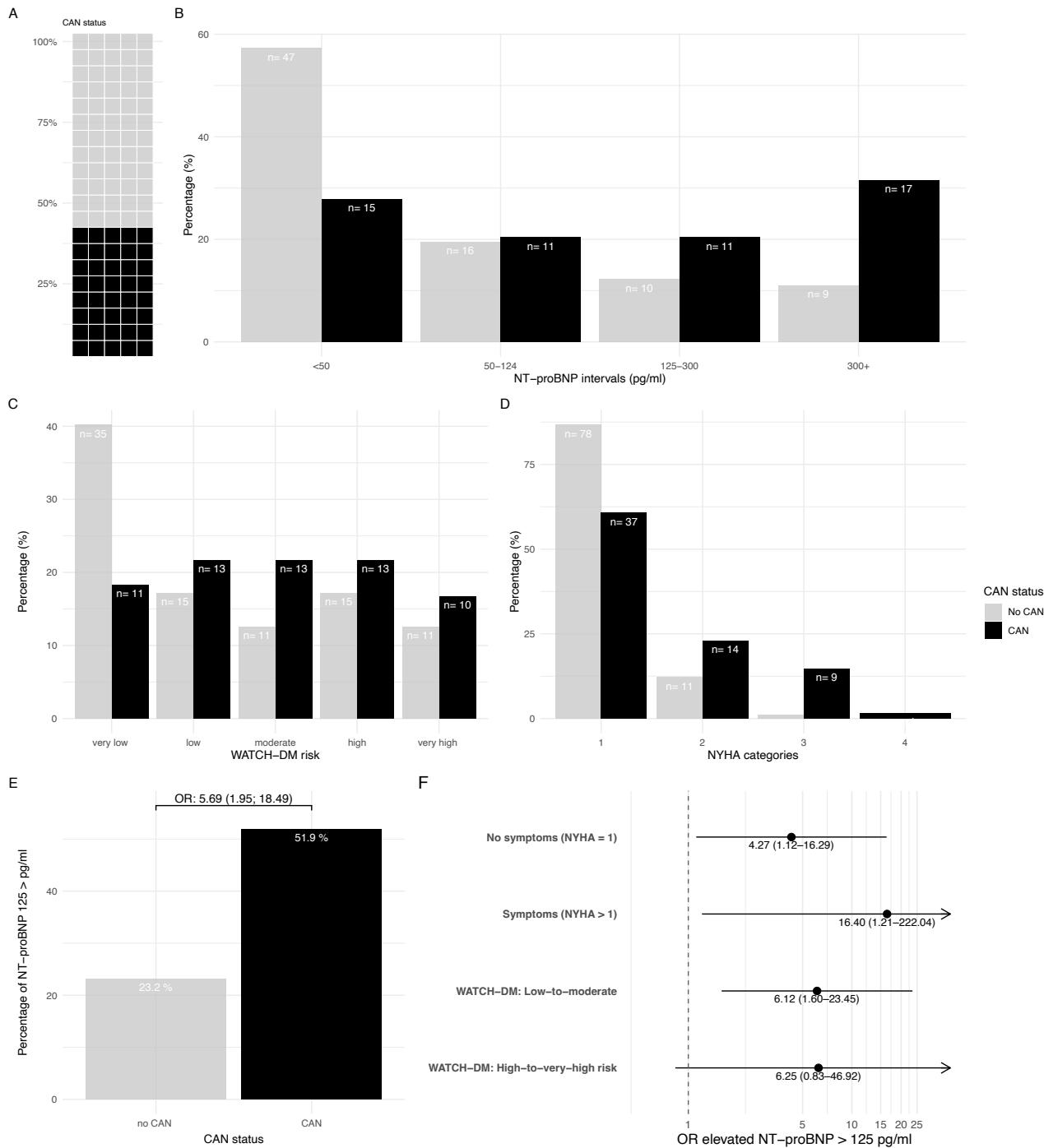
Note:

makecell[1]Categorical variables: N (%); Continous variables: Median (IQT:25th-75th Table are adapted from Table 1 in Appendix Study III

6.3.2. CAN and indicators of heart failure

A greater proportion of individuals with CAN exhibited elevated NT-proBNP levels (>125 pg/ml) (51.9%, n=52/78) compared to those without CAN (23.2%, n=26/112)(Figure 6.5 E)⁸⁶. The fully adjusted odds ratio (OR) for elevated NT-proBNP in individuals with CAN was 5.69 (95% CI: 1.95; 18.49) relative to those without CAN⁸⁶. Among the cardiovascular autonomic reflex tests (CART), the Valsalva maneuver demonstrated the strongest association with NT-proBNP (OR 9.00, 95% CI: 2.88; 33.09; n=51/75), followed by deep breathing (OR 3.30, 95% CI: 1.17; 9.77; n=33/133) and orthostatic hypertension (OR 4.04, 95% CI: 1.27; 13.77; n=24/146).⁸⁶ No significant association was identified for the lying-to-standing test (OR 0.80, 95% CI: 0.32; 1.97; n=54/108).⁸⁶ After imputing missing CART data, the OR for CAN in relation to elevated NT-proBNP declined to 2.94 (95% CI: 1.37; 6.56).⁸⁶ Sensitivity analyses, which excluded participants using beta-blockers or those with a history of CVD, resulted in a smaller sample size and wider confidence intervals, though the overall association remained unchanged.⁸⁶ CAN was associated with elevated NT-proBNP in individuals both without (NYHA I; OR = 4.3, 95% CI: 1.1; 16.3) and with heart failure symptoms (NYHA II; OR = 16.4, 95% CI: 1.2; 222.0), though the interaction was not statistical significant ($p = 0.4$).⁸⁶ Similar associations were seen across WATCH-DM risk groups: very-low-to-moderate (OR = 6.1, 95% CI: 1.6; 23.5) and high-to-very-high (OR = 6.3, 95% CI: 0.83; 46.9).⁸⁶ Participants with CAN had 1.7 (95% CI: 0.3 to 3.0) point higher WATCH-DM risk score compared to those without CAN.⁸⁶ The OR of presenting with NYHA class II or higher was 5.51 (95% CI: 1.9 to 15.97) in the group with CAN.⁸⁶

6.3. Study III



A: Percentage distribution by CAN status (no CAN, CAN). **B:** Percentage distribution of NT-proBNP level categories stratified by individuals with and without CAN. **C:** Percentage distribution of WATCH-DM risk score stratified by individuals with and without CAN. **D:** Percentage distribution of NYHA classification stratified by individuals with and without CAN. **E:** Percentage of individuals with NT-proBNP > 125 pg/ml among those with and without CAN and adjusted odds ratio from Model 4. **F:** Effect modification of the association between CAN and NT-proBNP by symptoms defined by NYHA classification (symptoms: NYHA II vs no symptoms: NYHA = I) and risk score defined by WATCH-DM risk (very-low-to-moderate vs high-to-very-high risk). Figure from Appendix Study III⁸⁶

Figure 6.5.: Distribution of NT-proBNP, NYHA Class, and WATCH-DM Score by CAN Status, and association of CAN with Elevated NT-proBNP

7. Discussion

The aim of this dissertation is to understand how cardiovascular autonomic dysfunction and CAN affect the risk of CVD across stages of glucose metabolism. Given the rising prevalence of prediabetes and T2D, and their association with increased risks of CVD and heart failure, there is a pressing need for earlier indicators to help healthcare providers intervene in a timely manner and prevent progression to more advanced stages of cardiovascular complications. One promising approach involves leveraging data from wearable devices and standardized screening tools. Heart rate dynamics and variability across different circumstances may hold promise as accessible indicators for timely cardiovascular risk stratification.

This chapter presents a summary of the main findings from this dissertation, interpreted in the context of existing evidence in the field, and discusses their clinical relevance across different levels of healthcare. Moreover, the strengths and limitations of the methods and results will be discussed.

7.1. Summary of findings

In this dissertation, autonomic dysfunction, defined by long-term HRV and standardized CARTs, and its relationship with cardiovascular complications were studied across three different cohorts representing populations at varying levels of prevention and care, including public health, primary care, and secondary care. In The Maastricht Study (Study I), I investigated autonomic dysfunction, measured by 24-hour HRV, and arterial stiffness, measured dynamically along the descending aorta and locally at the carotid site among individuals with NGM, prediabetes, and T2D. Lower HRV was associated with higher aortic and carotid stiffness. This association was evident regardless of glucose metabolism status, and was more pronounced in individuals with prediabetes or T2D. The modifying effect of dysglycemia was confirmed by a statistically significant stronger association across higher HbA1c levels. Z-scores of time- and frequency-domain measures showed the strongest associations, primarily driven by HRV indices reflecting total variation in interbeat intervals (SDNN, SDANN, SDNN index, ULF, VLF, TP).

Study II focused on individuals at higher risk of developing diabetes, using data from the ADDITION-PRO cohort. In study II, lower SDNN, measured over a multiple days, was associated with 18%, 24%, and 21% higher risk per SD for ischemic-related CVD, hospitalization of heart failure, and all-cause mortality, respectively. The risk became higher at SDNN levels below 120 ms, supported by a greater difference in incidence rates between individuals with 100 ms and 120 ms than the difference observed between individuals with 120 ms and 160 ms. Hourly measures suggested a specific time point related to ischemic-related CVD, as lower SDNN recorded between 6:00 and 7:00 AM was associated with MACE. Adjustment using the residuals method for concurrent heart rate and physical movement did not explain the observed association. Hourly SDNN was associated with all-cause MRR, although no specific time point showed an exceptionally strong association. While no association between hourly SDNN and heart failure was observed, higher heart rate during the night hours from 02:00 to 06:00 AM was linked to an higher risk of heart failure hospitalization.

These findings suggest that both long-term HRV measures and hourly HRV responses may serve as indicators of CVD risk. However, a key observation is that long-term HRV was assessed under free-living conditions, which restricts the comparability of results to standardized tests of autonomic function, although it provides insights that are more comparable to what may be found with long-term wearable devices. In the CANCAN Study (Study III), standardized CARTs were used to define CAN and to describe indicators of heart failure, including elevated NT-proBNP, WATCH-DM risk, and NYHA classification, among individuals with and without CAN in a population with T2D. In CANCAN, two out of five had CAN. Compared to individuals without CAN, these individuals more often showed signs of heart failure, including elevated NT-proBNP levels, higher WATCH-DM risk scores, and higher classifications on the NYHA. CAN was associated with elevated NT-proBNP levels and these persisted even among individuals without heart failure symptoms based on NYHA classification, as well as among those categorized as having low to moderate heart failure risk according to the WATCH-DM score.

In summary, various aspects of autonomic dysfunction and cardiovascular complications were investigated in populations with NGM, prediabetes, or T2D. The overall findings showed that autonomic

7.2. Cardiovascular autonomic dysfunction and its impact on heart disease across glucose metabolism

function, assessed through heart rate dynamics of long-term HRV and diurnal HRV or heart rate responses to reflex tests, is associated with an increased risk of CVD and heart failure. This relationship appears to be stronger in more severe stages of dysglycemia. Moreover, among individuals with T2D, the presence of CAN may help identify those at higher risk of heart failure, even in the absence of heart failure symptoms.

7.2. Cardiovascular autonomic dysfunction and its impact on heart disease across glucose metabolism

This dissertation shows that autonomic dysfunction, measured by HRV and CARTs, is associated with CVD risk across the spectrum of glucose metabolism dysregulation. This association is evident with measures of arteriosclerosis, atherosclerotic events, all-cause mortality, and heart failure in individuals at high risk of diabetes, as well as with indicators of heart failure in patients with T2D.

7.2.0.1. Arteriosclerosis

In Study I, autonomic dysfunction, measured by 24-hour HRV, showed to be cross-sectionally associated with arterial stiffness, measured both dynamically (cf-PWV) and locally (CD). Adjustments for demographic and lifestyle factors, as well as a range of cardiovascular risk factors, did not explain the association. This suggests that autonomic responses under free-living conditions may contribute to the development of arterial stiffness. The majority of studies in the field have shown an association between autonomic dysfunction, as measured by short-term HRV during rest, and arterial stiffness in populations with either type 1 or T2D.⁹⁶. Study I extended this perspective by examining long-term HRV and focusing on a population without diabetes or prediabetes.

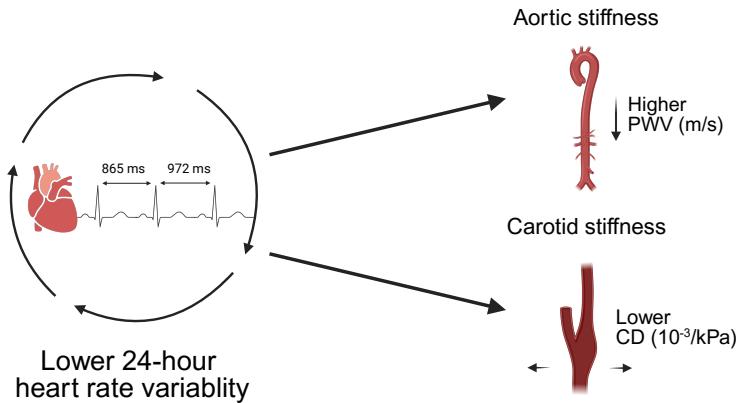


Figure 7.1.: Autonomic dysfunction and arterial stiffness. (Source: Author). —.

Arterial stiffness is not only a structural marker of vascular ageing but is also dynamically modulated by local endothelial signals and autonomic nervous system activity. Several studies have demonstrated a link between elevated sympathetic tone and increased arterial stiffness^{[97]98}. Two possible mechanisms may explain how autonomic dysfunction is related to arterial stiffness. First, autonomic dysfunction may increase the vascular tone of large arteries, thereby impairing arterial elasticity dynamically. This concept is supported by animal studies. In rats, proper autonomic regulation has been shown to be essential for maintaining aortic elasticity. Based on this mechanism, the association between autonomic dysfunction and arterial stiffness might be considered an immediate, and likely transient effect, expected to reverse if autonomic function is restored. Conversely, chronic overstimulation of sympathetic activity can lead to structural remodeling and increased arterial stiffness⁹⁹. While such findings cannot be directly extrapolated to humans, they suggest plausible biological pathways. Although the initial effects of autonomic dysfunction are dynamic and amenable to change by intervention, they may become progressively less reversible over time^{[99]100}. Second, the autonomic nervous system regulates hemodynamic parameters such as heart rate and cardiac contractility⁹⁸. Autonomic dysfunction typically manifests as both reduced HRV and elevated resting heart rate. Arterial shear stress increases as a result of heightened sympathetic activity and parasympathetic withdrawal. A higher resting heart rate may contribute to structurally stiffer arteries by altering blood flow dynamics and by increasing shear stress. Our earlier study using data from the Whitehall II cohort showed that a steeper decrease in short-term (5-minute) HRV over a ten-year period was linked with higher levels of aortic stiffness in the subsequent five years¹⁰¹.

The association between 24-hour HRV and arterial stiffness (Study II) was modified by dysglycemia, suggesting dysglycemia may induce CAN that can affect arterial stiffness, even before the onset of T2D^{[102]103104}. Data from the Whitehall II study showed that aortic stiffness increased more steeply with higher HbA1c values among non-diabetic individuals¹⁰⁵, supporting this notion. In

7.2. Cardiovascular autonomic dysfunction and its impact on heart disease across glucose metabolism

the subpopulation in Study I without diabetes, a modification by HbA1c in both aortic and carotid stiffness was observed. The modifying effect of HbA1c suggests that hyperglycaemia amplifies the consequences of autonomic dysfunction.

7.2.0.2. Atherosclerosis

Study II showed that individuals with a preclinical stage of autonomic dysfunction, measured by multiday HRV, face a higher risk of incident ischemic CVD, heart failure, and all-cause mortality.

Multiday HRV was assessed to capture autonomic activity in real-life settings across several days. Our results are consistent with earlier research linking lower HRV (including measures from 10 seconds to 24 hours) to CVD and mortality^{5,106}. Our findings build on existing evidence by (1) focusing on a population at elevated risk of diabetes, (2) utilizing multiday HRV recordings, and (3) identifying specific periods within the day where HRV patterns were indicative of ischemic-related CVD risk. By using both week-long and hourly data, we identified specific periods that provide a better indication of long-term risk. A strength of using multiday HRV recordings, is to provide more robust insights into individual autonomic patterns by averaging autonomic responses across typical daily conditions. This reduces the influence of random fluctuations caused by factors such as physical activity, emotional states, or sleep on any single day¹⁰⁷.

Multiple mechanisms may explain how autonomic dysfunction contributes to the initiation and progression of ischemic events and stroke. First, as discussed in Study I, autonomic dysfunction may promote arteriosclerosis, leading to arterial stiffness through a dynamic and potentially modifiable process. Arterial stiffness impairs vasodilation, increasing hemodynamic stress and the risk of plaque rupture and thrombus formation.^{108,109} As plaques develop, the associated increase in sympathetic nerve density around the arteries could transiently reduce vascular tone which over time reduce arterial elasticity.¹¹⁰ In a smaller study of people with T2D, lower HRV was linked with increased carotid atherosclerosis.¹¹¹

Second, the autonomic nervous system innervates the adventitia layer of blood vessels, where it modulates vascular tone via sympathetic fibres³². Although atherosclerotic plaques form in the intima layer, recent *in vivo* studies have demonstrated that increased plaque burden is associated with higher local sympathetic nerve density, likely mediated by neuroinflammatory processes.¹¹² Notably, reducing sympathetic innervation has been shown to attenuate plaque formation in animal models.¹¹² These findings suggest that autonomic dysfunction may not only reflect but also actively contribute to atherogenesis.

Third, autonomic nervous dysfunction has been shown to interfere with signalling pathways controlling heart rhythm, potentially leading to arrhythmias that disturb cardiac contraction. Earlier studies have shown lower short-term HRV was associated with incident atrial fibrillation (AF), with a higher risk among participants with T2D [¹⁰⁶]¹¹³. This supports the role of autonomic dysfunction in arrhythmogenesis, which increases the risk of myocardial infarction and stroke. Study II did not include AF as an outcome due to limitations in Danish registries, which often do not distinguish between short- and long-term AF, thereby affecting diagnostic validity.

7. Discussion

A study of individuals with coronary artery disease showed that stress-induced HRV was associated with myocardial infarction, even more than resting HRV, suggesting that lower parasympathetic modulation of heart rate under stress may play a role in ischemia¹¹⁴. Our study focused on long-term HRV under free-living conditions, capturing stress-responsive periods such as morning awakening. These recordings likely reflect underlying autonomic dynamics relevant to cardiovascular risk. A Genome-Wide Association Study (GWAS) in the UK biobank of short-term HRV supports this by identifying mechanisms involving G-protein signaling, pacemaker activity, and mitochondrial function as likely mediators of the genetic contribution to HRV. These pathways influence vagal control, cardiac excitability, and energy metabolism¹¹⁵. Although derived from short-term recordings, these genetic associations may reflect autonomic traits that persist across different time scales, and reinforce the notion of a biological basis for inter-individual differences in HRV, preceding and independent of the onset of dysglycemia. A Mendelian randomization study using data from the Rotterdam Study found that genetically predicted HRV was associated with an higher risk of AF¹¹⁶. However, this association did not extend to all-cause mortality or cardiovascular death in the UK Biobank cohort, where only phenotypically measured HRV showed a significant relationship with these outcomes¹¹⁷. Interestingly, the genetic determinants of HRV exhibited pleiotropic relationships with several autonomic traits, including resting heart rate, heart rate response during exercise, and post-exercise recovery dynamics¹¹⁷. No GWAS has yet been conducted for long-term HRV. Therefore, it is unclear whether the genetic influences identified for short-term HRV are applicable to long-term HRV. Future GWAS efforts targeting long-term HRV could help establish causal relationships to CVD by leveraging methods such as Mendelian randomization, and advancing our understanding of the genetic architecture underlying autonomic regulation under a full day.

7.2.0.3. Heart failure

The relationship between cardiovascular autonomic dysfunction and heart failure is complex¹¹⁸. On one hand, autonomic dysfunction contributes to cardiac remodelling and eventual heart failure. On the other hand, it may reflect compensatory mechanisms of the progression of cardiac remodelling and declining cardiac output. Our findings demonstrated a relationship between autonomic dysfunction and heart failure both cross-sectionally in a population with T2D and prospectively in individuals representing different tiers of diabetes risk. However, our data are limited in determining the extent to which this relationship supports one explanation over the other, as we lack baseline and follow-up measures of both heart failure and HRV. Earlier studies have demonstrated that both short-term and long-term HRV are associated with incident heart failure in populations with and without T2D.^{[119][120][121][122]} Beyond examining a population at elevated risk of diabetes using multiday HRV recordings, we expanded previous research by (1) unmasking the role of resting heart rate in the relationship between HRV and heart failure risk, and (2) identifying specific times of day when heart rate patterns signaled higher risk of heart failure.

Several mechanisms may underlie the role of autonomic dysfunction in advancing heart failure. Findings from Study I confirmed the relationship between autonomic dysfunction and arterial stiffness. It is well established that arterial stiffness is linked to cardiac remodelling, as increased cf-PWV leads to an earlier return of the reflected pulse wave to the aorta, which increases cardiac afterload

7.3. Clinical implications

and reduces coronary perfusion pressure¹²³. Therefore, autonomic dysfunction may have an indirect effect on heart failure, potentially mediated by arterial stiffness. However, structured analyses are needed to confirm these pathways. For example, mediation analysis with repeated measurements could be employed to assess the temporal direct and indirect effects of autonomic dysfunction on measures of cardiac remodeling, with arterial stiffness as the mediator.

Study II showed that multiday HRV was associated with incident heart failure, and approximately one-fourth of the risk was explained by resting heart rate. Data from the Rotterdam Study showed that short-term HRV was longitudinally associated with echocardiographic measures reflecting systolic function, suggesting that autonomic dysfunction contributes to cardiac remodelling.¹²⁴ In contrast to MACE outcomes, findings from Study II showed no specific time point in hourly HRV that was associated with heart failure. Instead, it was the overall daily pattern captured by multiday HRV that was linked to heart failure risk. This suggests that the association is not driven by isolated shifts in autonomic activity in response to circadian stressors, but rather by a consistently impaired autonomic balance under free-living conditions. The effect appears to be driven in part by a failure to show appropriate decreases in heart rate during rest, as individuals with higher hourly heart rates at night had a higher risk of heart failure. In Study III, I observed that individuals with CAN had higher risk of elevated levels of NT-proBNP, a biomarker of myocardial stress and early heart failure. Therefore, CAN is associated with hemodynamic consequences that contribute to both structural and functional cardiac remodeling, which in turn leads to elevated NT-proBNP levels.

We cannot rule out that autonomic dysfunction reflects a compensatory response to progressing heart failure, suggesting potential reverse causation. In addition, it remains unclear to what extent the parasympathetic nervous system can act as a protective mechanism to counterbalance sympathetic dominance, and whether a decline in HRV reflects a breakdown of this balance. The two pathways (autonomic neuropathy and cardiac remodelling) are not mutually exclusive and may interact in a reinforcing cycle. Autonomic dysfunction can lead to increased sympathetic tone and reduced parasympathetic modulation, placing the heart under chronic stress and promoting structural and functional changes.²⁴ In turn, cardiac remodelling may impair autonomic regulation, further exacerbating the imbalance. This interplay may create a self-perpetuating loop that accelerates the progression of heart failure. However, this remains beyond the scope of our current data and analysis.

7.3. Clinical implications

The dissertation investigates autonomic dysfunction in populations ranging from NGM to T2D and yields insights relevant for individuals who engage with the healthcare system at different levels. No specific role has yet been defined for autonomic dysfunction in clinical decision-making within healthcare, as current treatment and intervention options specifically targeting autonomic function remain limited. Despite the fact that the results do not point directly to where and how implementation of autonomic dysfunction in clinical practice may make sense, the included studies broadly represent situations relevant to public health, primary care, and secondary care

settings. In the following section, the clinical implications of using autonomic dysfunction in the prevention of CVD will be discussed. If long-term HRV or CARTs are to be considered for improving risk stratification, it is important to determine at what stage in the progression of diabetes risk, and at which level of care, autonomic dysfunction becomes meaningful for early detection and intervention.

7.3.1. Public health

A central strategy in preventing CVD is the early identification and multifactorial treatment of individuals at high risk.¹²⁵ Public health initiatives support this by promoting healthy lifestyles, facilitating early screening for risk factors, and improving access to essential care and medications. Long-term HRV may enhance these efforts by identifying individuals with elevated cardiovascular risk and by tracking their physiological response to lifestyle changes.

Evidence from Study I showed that lower long-term HRV was associated with increased arterial stiffness, as measured by cf-PWV and CD, even in individuals without T2D. One standard deviation lower HRV corresponded to the effect of 2.7 additional years of ageing on aortic stiffness (cf-PWV) and 1.6 years on carotid stiffness (CD).⁷⁵ These cross-sectional findings suggest that HRV may serve as a marker of early vascular aging and cardiovascular risk. Supporting this, the Whitehall II study demonstrated a longitudinal relationship between short-term HRV and aortic stiffness. Together, these findings highlight the potential of HRV as a indicator of vascular health.

Within the public health setting, individuals with prediabetes represent a particularly vulnerable group at risk for comorbidities.¹²⁶ They often fall between structured care pathways, sometimes encouraged to reassess their cardiovascular risk at more frequent intervals, other times not offered any additional measures or attention beyond general lifestyle advice. Notably, Studies I and II demonstrated that the associations between long-term HRV and CVD risk were especially pronounced in this population. In those at high risk of diabetes, a one standard deviation (33 ms) lower multiday SDNN was equivalent to 4.5 additional years of aging for ischemic-related CVD and 2.2 to 2.4 years for heart failure.⁸⁵ On a population level, lower HRV (SDNN: 100 ms) in individuals with prediabetes was associated with a higher incidence rate of CVD, heart failure, and all-cause mortality compared to individuals with normal-to-higher HRV (SDNN: 120–160 ms). These findings reinforce the role of HRV as an early and sensitive marker of cardiovascular health in populations at cardiometabolic risk.

While these findings highlight HRV's potential, practical implementation faces several challenges. Historically, long-term HRV monitoring has required specialized equipment such as Holter ECG recorders. However, the growing popularity of wearable devices offers a promising alternative. These devices provide a non-invasive, user-friendly way to collect heart rate and HRV data over time, under free-living conditions like those examined in Study II.¹¹

If HRV monitoring proves effective in helping individuals maintain a healthy, age-adjusted HRV range through lifestyle changes and prompts healthcare engagement when HRV deteriorates, it could become a meaningful tool for long-term health tracking. A cross-sectional study of 8 million individuals found that those who took more steps per day had higher HRV, suggesting that HRV

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may also reflect behavioral adaptation.¹³ In addition to the ADDITION-PRO study, the inclusion of data from newer observational cohorts that incorporate wearable devices, along with precise lifestyle measures such as physical activity, diet, and sleep, could enhance the understanding of patterns influencing long-term heart rate variability and diurnal autonomic responses.^{127,128}

A major public health challenge lies in ensuring equitable access to wearable technology. Individuals from lower socioeconomic backgrounds are less likely to own such devices, raising concerns about health disparities. Despite this, there is encouraging evidence that the general population is receptive to digital health innovations. Many are willing to share health data with public institutions and support the use of AI in disease monitoring.^{[11][129]}

Integrating wearable HRV monitoring into public health strategies could represent a transformative step in proactive cardiovascular care. It may support early detection, personalized prevention, and timely referral to primary care when risk levels increase.

7.3.2. Primary care

Cardiovascular risk in primary care is assessed using clinical evaluations and standardized risk prediction tools to identify individuals at elevated risk. Management focuses on lifestyle modification, pharmacological therapy, and regular monitoring to reduce cardiovascular events¹³⁰. In this context, long-term HRV may offer added value by improving the precision of cardiovascular risk stratification and by serving as a marker to monitor the effectiveness of preventive strategies.

Long-term HRV may improve ranking of individual risk when added to established clinical risk scores. Tools such as SCORE2 and the Framingham Risk Score are widely used in primary care to guide cardiovascular risk assessment^{131,132}. In Study I, models adjusted for conventional CVD risk factors supported the potential added value of 24-hour HRV in relation to arterial stiffness, a surrogate marker of CVD risk. Study II extended this perspective by demonstrating associations between multiday HRV and incident CVD and heart failure. However, these findings are based on associations and do not include formal prediction modeling¹³³, and therefore cannot determine whether incorporating long-term HRV or CARTs into existing risk scores improves predictive performance beyond current guidelines. This study design is not feasible in ADDITION-PRO, as the cohort does not represent high-risk diabetes populations typically identified in primary care (e.g., elevated HbA1c). Likewise, CANCAN is limited by its small sample size and recruitment from secondary care. To assess predictive value, cohorts should reflect individuals with T2D or those at high risk, as defined by current clinical practice.

Most biomarkers have shown limited incremental value beyond established predictors (including age, sex, lipid profiles, diabetes status, and blood pressure). Few studies suggest that 24-hour HRV may improve risk discrimination for CVD and all-cause mortality in individuals with T2D¹³⁴, and for stroke and heart failure in older adults^{[119][135]}. However, these studies often lack calibration or validation in large-scale cohorts and have not been integrated with widely used risk scores such as SCORE2 or the Framingham Risk Score.

Long-term HRV may also help classify preclinical autonomic dysfunction, enabling targeted interventions in a subgroup of patients to prevent CVD. The increasing availability of wearable devices capable of capturing long-term HRV data presents a practical opportunity for continuous monitoring in primary care. These devices may facilitate earlier detection of autonomic dysfunction and support more personalized approaches to cardiovascular risk management. However, the clinical utility of stratifying patients based on preclinical autonomic dysfunction remains uncertain. These considerations are only actionable if interventions in this subgroup can be shown to reduce cardiovascular risk. Emerging evidence suggests that both pharmacological and lifestyle interventions can improve HRV in the short term.^{136,137} For example, high-intensity interval training has been shown to improve autonomic function in obese individuals with and without T2D.¹³⁸ Similarly, lifestyle changes in individuals with prediabetes have been associated with improvements in short-term HRV, which may partly explain a reduction in diabetes risk independently of weight loss.¹³⁹ Nevertheless, it remains unclear whether these effects on HRV are sustainable over time and whether they translate into long-term cardiovascular protection. In many cases, improvements in autonomic function may be mediated indirectly through changes in cardiometabolic markers such as glucose levels, lipid profiles, body weight, maximal oxygen uptake, and blood pressure.

Despite these uncertainties, monitoring autonomic function through long-term HRV may offer a valuable tool for assessing cardiovascular risk and tracking the impact of preventive strategies. In Denmark, prediabetes, defined by HbA1c, is present in 7.1% of adults.¹⁴⁰ One in five of these individuals develops T2D within five years¹⁴⁰, while others either remain in the prediabetic stage or return to normoglycemia. Despite their higher risk of CVD and heart failure¹⁴¹, individuals with prediabetes are not captured by existing preventive strategies. This underscores the need for early and precise risk assessment.¹⁰ Given that the cardiovascular consequences of autonomic dysfunction appear to be more pronounced in individuals with prediabetes compared to those with normoglycemia, HRV has the potential to help identify those at elevated CVD risk within this group. However, evidence demonstrating improved risk prediction and sustained effects leading to better cardiovascular outcomes is needed to establish its relevance for integration into primary care.

7.3.3. Secondary care

In secondary care, endocrinologists assess cardiovascular and heart failure risk by integrating advanced diagnostics, biomarker analysis, and imaging to detect early heart failure, guided by symptoms and risk profiles. The treatment of patients with T2D is guided by evidence-based therapies and multidisciplinary collaboration. The ADA/EASD 2022 consensus on Management of Hyperglycemia in T2D emphasizes that early detection of heart failure in individuals with T2D is crucial, as it enables timely initiation of therapies such as SGLT2 inhibitors, which have demonstrated significant benefits in reducing heart failure-related outcomes⁷⁰. A major challenge in diabetes care is detecting heart failure before symptoms appear, as patients with symptomatic heart failure face a higher risk of mortality and more frequent hospitalizations⁴. The AHA, ACC, and HFSA 2022 guidelines recommend identifying individuals at risk of heart failure based on factors such as diabetes, poor glycaemic control, uncontrolled hypertension, hyperlipidaemia, elevated BMI, albuminuria, renal dysfunction, and a history of CVD⁵⁶. Still, there is a need to identify optimal

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approaches for recognizing and diagnosing heart failure in clinical care, as broad echocardiographic screening in T2D is time-consuming and costly⁴.

Study III demonstrated that CAN may help identify individuals at higher risk of heart failure, beyond what is captured by symptoms or existing risk scores. Our findings support considering CAN as a relevant risk factor for heart failure and suggest it may have value in future risk stratification strategies in T2D. A clinical advantage of using CARTs is that they are standardized tests performed under controlled conditions. CARTs have proven to be reliable and reproducible, with reference values established in large population studies⁶⁸. Beyond these findings and the established evidence of increased heart failure risk, CAN also identifies individuals at high risk for overall CVD, kidney disease, and early mortality in the T2D population^{[61][62]}. In Study III, I observed that two out of five participants had CAN, highlighting it as a complication with considerable prevalence. Therefore, detecting CAN may uncover an often-overlooked condition that is common in individuals with T2D.

Clinical stratification of care includes two key considerations: (1) CAN should be further evaluated for associated cardiovascular complications, such as heart failure; and (2) cardiopreventive strategies should be initiated earlier in this subgroup.

First, patients with CAN may benefit from further cardiovascular assessment, including the use of sensitive biomarkers or echocardiography. NT-proBNP is a strong predictor of heart failure and a validated biomarker for ruling out the condition.⁵⁶ However, its specificity varies across heart failure phenotypes, being less specific for detecting HFpEF compared to HFrEF.⁵⁶ Therefore, additional evaluation using echocardiography may be warranted. Beyond classifying heart failure phenotypes, echocardiography identifies preclinical stages of heart failure through the detection of functional or structural cardiac abnormalities. Including CAN in structured assessments of heart failure could help clarify to which extent CAN overlaps with cardiac abnormalities. Determining the diagnostic and prognostic value of CAN, particularly its sensitivity and specificity in detecting HFrEF and HFpEF, requires further investigation.

Second, the presence of CAN may justify earlier initiation of protective therapies. SGLT2 inhibitors are recommended as second-line treatment in T2D and have demonstrated benefits in reducing the risk of heart failure, CVD, and kidney function decline, complications commonly associated with CAN. Current guidelines recommend initiating these therapies based on a history of CVD, heart failure, or the presence of conventional high-risk cardiovascular factors. However, the specific impact of SGLT2 inhibitors on the progression of cardiorenal outcomes in patients with CAN remains to be fully understood. Furthermore, while antihypertensive treatment is a cornerstone of cardiovascular risk management, whether specific classes of antihypertensive agents offer protective effects in patients with CAN remains to be explored.

The direct clinical implications of our findings in Study III are limited. The generalizability of our results is restricted, as our study population consisted of patients with T2D receiving secondary care. Two out of five patients with CAN showed to have a history of CVD, a group already at higher risk of heart failure due to their prior diagnosis. This overlap may influence the interpretation of CAN as an independent risk factor. Therefore, these findings need to be validated in a broader population with T2D, including individuals without a history of CVD. Doing so would allow for

greater generalizability of our results to the broader T2D population, particularly those visiting primary care.

7.4. Strengths and limitations

7.4.1. Study design

Cross-sectional design

Studies I and III are based on cross-sectional data, with exposure and outcome measured within a three-month period. The main limitation of this design is that it does not allow us to determine whether the exposure led to the outcome or vice versa. As a result, we cannot establish temporality or confirm whether changes in the outcome were caused by the exposure. Based on prior evidence, the direction of the associations in Study I was inferred using physiological knowledge and findings from epidemiological and *in vivo* studies.¹⁰¹

Study III focused on the clinical diagnosis of CAN and the presence of heart failure. The research question was oriented toward the clinical utility of CAN in identifying patients with T2D who may be progressing early toward heart failure. Whether cardiac function progressively worsens due to the underlying mechanisms of CAN remains to be fully established.

Longitudinal design

A major strength of Study II is its longitudinal design, where HRV was measured at baseline and outcomes were captured prospectively through national registries. This temporal structure ensures that the exposure (HRV) preceded the outcome, reducing the risk of reverse causation. The prospective design allows for stronger inference of directionality than cross-sectional studies. Furthermore, the use of high-quality registry data ensures complete outcome ascertainment and minimizes loss to follow-up bias.

Causality cannot be ascertained from the findings in Study I and Study II, and more causally focused methods are needed. Mendelian randomization, which uses genetic instruments for exposure, could help address this question. Additionally, structured mediation analysis involving modifications such as medication or lifestyle changes could clarify whether improving HRV or CART reduces cardiovascular risk, using data from intervention studies.

7.4.2. Internal validity

In this project, I aimed to assess cardiovascular autonomic function both in free-living conditions and in response to standardized test procedures during clinical visits. Additionally, I used dynamic measurements to evaluate arterial stiffness both locally and by velocity, and biomarker assessments to determine the presence of heart failure. In this section, I discuss the validity of 24-hour, multiday, and hourly HRV measurements, as well as the standardized tests of CAN. I also address the validity

7.4. Strengths and limitations

of the included outcomes and discuss the strengths and limitations of using MACE as a time-to-event outcome.

7.4.2.1. Long-term HRV (>24 hours) as measurement for autonomic function

A main consideration in HRV analysis is the reliability of raw inter-beat interval data from ECG recordings. To ensure accurate various HRV measures, the intervals must be captured in a continuous and correctly sequenced manner. Frequency-domain analyses depend on the integrity of the inter-beat interval sequence, while some time-domain measures, such as RMSSD and pNN50, specifically quantify the variability in the differences between successive intervals.

In Study I, data from a 12-lead Holter system was used, which is considered the gold standard for long-term ECG recordings. With detailed and sequential inter-beat intervals, all HRV metrics were calculated.

In Study II, data from the Actiheart device was used for HRV. The device was configured to record continuously over an 8-day period. It captured 30-second epochs of mean heart rate intervals, along with upper and lower prediction intervals. From each epoch, I generated a distribution of inter-beat intervals. An algorithm was applied to estimate HRV from these distributions, and its validation showed strong agreement with established metrics, including SDNN, SDANN, and the SDNN index.^{[6]84} However, a limitation of this dataset is that it did not allow for the calculation of frequency-domain measures or specific time-domain metrics such as RMSSD or pNN50.⁸⁴

In the context of this study, which focuses on long-term HRV in free-living conditions, it is important to acknowledge that the autonomic nervous function we aim to assess may also be influenced by behavioral factors such as physical activity, sleep, meal timing, emotions, smoking, caffeine intake, alcohol consumption, medication use, and prior cardiovascular complications. These factors can potentially mask or mimic underlying physiological dysfunction during recordings, but they can also elicit the HRV responses we are interested in. Therefore, lower long-term HRV cannot be interpreted solely as a marker of autonomic function. HRV is also influenced by lifestyle patterns over time, making it sensitive not only to day-to-day behaviors but also to long-term habits that affect autonomic balance.

In Studies I and II, I accounted for habitual physical activity, and in Study II, I also adjusted hourly HRV for physical movement during recordings to test the influence of concurrent activity. However, further studies are needed to understand how lifestyle patterns affect long-term HRV recordings on subsequent days, in order to separate direct behavioural from physiological components. In both studies, I excluded participants with prior CVD to preserve the etiological order between autonomic dysfunction and cardiovascular outcomes.

Anti-hypertensive medications, especially beta-blockers, are known to increase HRV in randomized controlled trials.¹⁴² However, in cohort studies, participants using anti-hypertensives generally show lower HRV, likely reflecting a higher burden of cardiovascular complications [ref]. Because beta-blockers target the autonomic nervous system, they may introduce bias in HRV measurements by interfering with the function. In sensitivity analyses in Studies I and III, excluding participants on

7. Discussion

anti-hypertensive treatment did not materially change the estimates. Therefore, these participants were kept and adjusted for medication use in the full models.

Beyond the behavioral and pharmacological contribution to HRV, we cannot physiologically distinguish whether autonomic dysfunction is primarily driven by increased sympathetic activity or reduced parasympathetic tone. Much of the current evidence on autonomic contributions to HRV originates from studies involving experimental manipulation of the autonomic nervous system, such as physiological tests (e.g., tilt response, deep breathing, stress response) or pharmacological modulation. Therefore, it remains uncertain whether the mechanisms linking HRV indices to cardiovascular complications are predominantly due to sympathetic overactivity or parasympathetic withdrawal.

HRV levels are influenced by heart rate, as lower resting heart rate allows for greater variability. In Study I, I chose not to adjust for heart rate in our models, as this could introduce multicollinearity. Additionally, elevated heart rate is driven by higher sympathetic activity and may act as a mediator in the pathway leading to arterial stiffness. Our use of full-day recordings captures HRV during both rest and activity, providing a robust representation of autonomic function over a typical day. In contrast, heart rate correction may be more relevant for short-term HRV recordings, where standardized conditions can be affected by random influences such as time of day, smoking, or caffeine intake. These factors would have been relevant in Study III had I included HRV measures. In Study II, I used the residuals method to pre-adjust HRV measures for resting heart rate, which accounted for part of the observed associations, particularly with heart failure and all-cause mortality, and to a lesser extent with ischemic-related CVD events. Similar trends were observed for hourly associations, where heart rate pre-adjustment had comparable effects on the outcomes.

The three studies demonstrate approaches to identifying CVD risk: (1) selecting appropriate HRV indices, (2) segmenting time intervals, and (3) assessing HRV under defined conditions. Our findings reveal varying associations across long-term HRV indices, with RMSSD and HF showing weaker associations. However, previous research has shown that these indices can be informative when analyzed in 5-minute segments.^{136,143} Additionally, SDNN exhibited varying associations with CVD risk depending on the time of day. We also observed that, in CARTs, the Valsalva maneuver and deep breathing test were more indicative of heart failure. These insights highlight the relevance of methodological decisions in HRV research, particularly in aligning index selection, time segmentation, and in response to what with specific research objectives and clinical contexts.

7.4.2.2. Cardiovascular autonomic reflex test

CART provides a practical approach for screening for autonomic dysfunction and has been shown to be a reliable method.⁸⁷ Although certain indices from CARTs may be influenced by factors such as time of day or recent physical activity, these effects are generally minimal. Furthermore, no impact of caffeine intake has been observed on the reference age-based formula⁶⁸. A limitation of the CARTs in this study was the high prevalence of participants who were unable to complete the full battery of tests, primarily due to missing data from the Valsalva manoeuvre.

7.4. Strengths and limitations

7.4.2.3. Measures of cardiovascular risk

In study I, arterial stiffness measures, including pulse wave velocity and carotid artery distensibility, are influenced by mean arterial pressure (MAP), which may confound the assessment of vascular stiffness. In Study I, we adjusted for MAP, which attenuated the observed associations. However, the associations remained statistically significant.

In Study II, outcomes were based on CVD events, heart failure, and causes of death from Danish national registries. Potential misclassification and underreporting, especially of heart failure, may have led to underestimation of associations.¹⁴⁴

In Study III, NT-proBNP was used as a primary indicator of heart failure. While NT-proBNP is a validated biomarker for early-stage heart failure and useful for ruling out the condition, its specificity varies by HF phenotype.⁵⁶ Thus, we cannot determine HFpEF or HFrEF. Its diagnostic accuracy is influenced by factors such as AF, obesity, and kidney function.⁵⁶ Individuals with AF were excluded by design. Analysis was adjusted for BMI, which did not affect the association between CAN and elevated NT-proBNP. After adjusting for eGFR, the association became stronger, suggesting that reduced kidney function may have masked the true link between CAN and heart failure risk.

7.4.3. External validity

7.4.3.1. Selection bias

The Maastricht Study

The target population in Study I was intended to represent individuals at different stages of glucose metabolism. However, our analysis may be affected by selection bias in the representation of individuals with T2D. The Maastricht Study recruited participants who were able and willing to attend multiple research visits and receive personal health feedback, which likely attracted health-conscious individuals with higher education levels. As a result, participants with T2D were relatively healthy, with a median disease duration of three years and a low prevalence of complications. Those who completed both long-term ECG and arterial stiffness assessments may represent an even healthier subgroup. This selection bias may limit the generalizability of the findings to the broader T2D population and could explain why the effect modification did not differ step-wise from that observed in individuals with prediabetes.

ADDITION-PRO

The target population in Study II was intended to represent individuals at high risk of developing T2D. Participants were recruited through a stepwise screening procedure. Initially, individuals were selected based on a risk score derived from a self-administered questionnaire sent by mail. Those with high scores were invited for further testing using HbA1c or random glucose measurements.

This recruitment strategy involved selection by design, as it defined the source population based on specific risk criteria. The questionnaire prioritized risk factors such as older age and hypertension, leading to overrepresentation of these groups.¹⁴⁵ Prediabetes was identified only after biochemical

testing, while the risk score was primarily designed to detect undiagnosed T2D. Although this selection process was intentional and aligned with the ADDITION-PRO objectives, it may limit the generalizability of the findings to the broader population at risk for T2D.

In addition, selection bias may have occurred due to differential participation in the ADDITION screening program. Healthier individuals were more likely to participate, both by completing the risk questionnaire and by attending follow-up testing.¹⁴⁶ As a result, the baseline risk for CVD in ADDITION-PRO participants may have been lower compared to the target population.

CANCAN

The target population in Study III was intended to represent individuals with T2D treated in outpatient clinics. In Denmark, patients with T2D are referred to diabetes specialists at outpatient clinics when their general practitioner is unable to stabilize their condition. A strength of the CANCANC sampling strategy is that patients were already attending endocrinology consultations, and the study examination required only additional time during their visit, without the need for extra transportation or appointments. Assessing selection bias in this study is challenging, as inclusion depended on referral practices by general practitioners.¹⁴⁷ These practices may vary individually, with differing thresholds for referring patients to specialized care based on clinical judgment and patient characteristics.

7.4.3.2. Generalisability

The generalisability of our findings is considered in the context of the targeted recruitment strategies used in each study, which aimed to include individuals across a spectrum of diabetes risk, from NGM to established T2D. As a result, the findings are most applicable to populations with similar clinical profiles and healthcare settings.

Studies I-III include individuals at high risk of diabetes and those with T2D. Therefore, the associations between cardiovascular autonomic dysfunction and cardiovascular outcomes or surrogate biomarkers are relevant to individuals with some degree of diabetes risk and progressed T2D. Study I suggests that the link between autonomic dysfunction and cardiovascular risk, as measured by arterial stiffness, is also present in individuals with NGM, though to a lesser extent. This finding, supported by replication in the Whitehall II cohort indicates that the observed relationship may extend beyond high-risk groups and into the general population.¹⁰¹ In study III, participants represent a higher-risk diabetes group among Danish diabetes patients, while more stable patients remain under general practitioner care. Consequently, the prevalence of heart failure indicators and CAN is likely higher in this selected group, than patients managed in primary care, and thus limits extending our finding to broader T2D populations.

By design, younger individuals with prediabetes or young-adult-onset T2D are underrepresented in our studies. This group may be overlooked in current research and warrants further attention in future studies.^{140,148} The applicability of our findings to other countries may be influenced by differences in demographic composition, risk factor distributions, healthcare systems, and stages of economic development. These factors can affect both the prevalence of diabetes and cardiovascular

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disease and the nature of their associations. While our study populations were primarily of Nordic and Western European descent, differences in ethnic composition are only one of several factors that may influence external validity. These regions also share relatively well-organized, publicly funded healthcare systems, which may differ substantially from those in other parts of the world and further affect the applicability of our findings.

8. Perspective

8.1. Continuous monitoring of cardiovascular health

This dissertation has investigated autonomic function impact on cardiovascular complications across different stages of glucose metabolism. Understanding when and how physiological signals reflect elevated CVD risk is essential for developing early and effective prevention strategies. Incorporating HRV into digital health solutions could support personalized feedback mechanisms, enabling timely lifestyle or therapeutic interventions and contributing to more adaptive and preventive healthcare strategies. Based on our findings and conclusions, we propose further perspectives to define its role in research and healthcare from three aspects: (1) continuous non-invasive health monitoring, (2) risk stratification, and (3) identification as a causal and modifiable marker.

8.1. Continuous monitoring of cardiovascular health

Wearable devices enable comprehensive data collection on behavioral (e.g., sleep and physical activity) and physiological (e.g., heart rate, ECG, temperature) parameters.¹⁴⁹ These devices offer a broader and more feasible approach to long-term heart rate monitoring. Despite growing interest in wearable-based monitoring, the integration of HRV into routine cardiometabolic risk assessment remains limited.

Two key aspects highlight the potential applications of monitoring: (1) identification of risk and (2) assessment of response to intervention.

Identification of risk

Lower long-term HRV is a risk factor for CVD, associated with arterial stiffness and clinical endpoints. Our findings indicate that specific HRV and heart rate patterns under free-living conditions may enhance early risk detection, independent of concurrent physical activity. For improved risk assessment, future predictive models should move beyond adjusting for physical activity as a confounder and instead integrate multiple physiological signals, such as HRV responses to sleep and activity patterns, to better capture dynamic health states. Machine learning offers powerful tools to analyze complex raw time-series data, including interbeat intervals and accelerometer signals, potentially improving risk prediction beyond traditional HRV summary metrics. However, the limited interpretability of these models remains a key barrier to clinical adoption. Nevertheless, HRV can be clinically interpreted from wearable devices and may help identify individuals at elevated cardiovascular risk potentially without the need for blood pressure readings or invasive measures such as blood samples.

Assessment of response to intervention

HRV represents a potential target for intervention, as low HRV may reflect adverse lifestyle patterns. Behaviors such as disrupted sleep, physical inactivity, diet and irregular meal timing can influence circadian fluctuations in HRV.^{136,150–152} HRV has also proven to respond to pharmacological interventions. For example, beta-blockers have been shown to increase HRV, while GLP-1RA may reduce it.^{142,153}

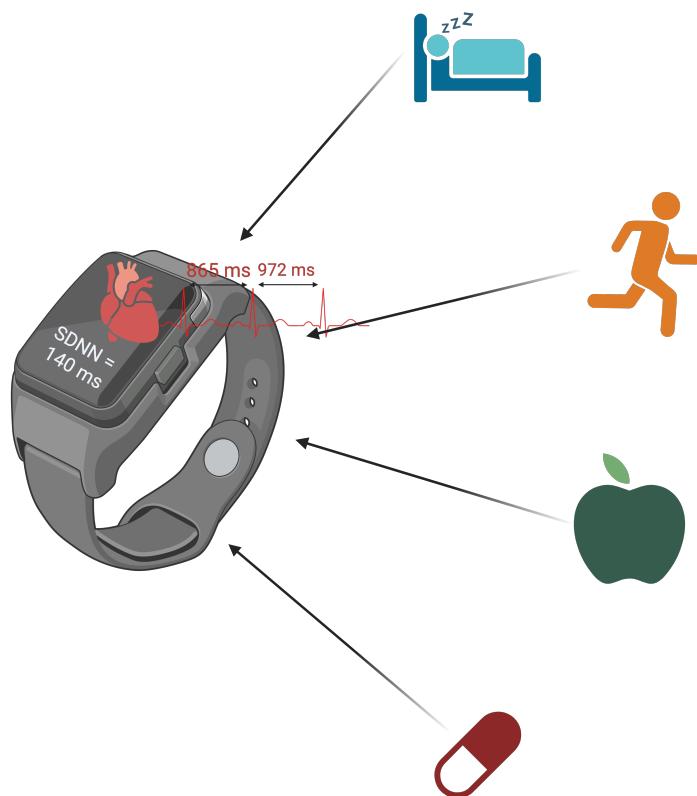


Figure 8.1.: HRV feedback in response to lifestyle and treatment interventions. (Source: Author)

Future research can leverage wearable devices to monitor the effectiveness of behavioral and pharmacological interventions on HRV at the individual level. This approach may support precision real-time monitoring to identify lifestyle patterns or treatments that promote cardiovascular health through HRV modulation or uncover potential side effects.⁷³

However, standardization and transparency across wearable device brands remain a challenge for both research and clinical use. While smartwatches offer convenient heart rate monitoring, their accuracy varies due to reliance on photoplethysmography, which can be affected by motion and other external factors, especially during physical activity.^{154,155} Despite these limitations, ongoing improvements in sensor technology and algorithm calibration are likely to enhance the reliability of wearable-derived HRV and heart rate data.¹⁵⁶ Open data formats are important to ensure that detailed data (e.g. interbeat intervals)^{156,157} from various devices can be used consistently in health prediction algorithms, rather than relying only on summarized outputs.

8.2. Risk-stratification

The distinct roles of long-term HRV and CART in cardiovascular risk stratification remain to be fully established. Building on the concept of continuous monitoring through wearable technology, long-term HRV presents two promising avenues that warrant further investigation:

- **Enhancement of existing risk scores:** HRV may improve the predictive accuracy of established cardiovascular risk models, such as SCORE2 or the Framingham Risk Score.
- **Support for treatment decisions:** Long-term HRV may also help optimize the timing of treatment initiation and guide intermediate clinical decisions.

Cardiovascular risk assessment

Digital CVD risk calculators can optimize the timing of follow-up assessments and treatment initiation. Analyses from Steno Diabetes Center Copenhagen suggest that annual retinopathy screening may not be necessary for all patients. Instead, prediction models using clinical variables can determine optimal re-screening intervals.¹⁵⁸ In prediabetes, a key concern is overmedicalization, since many individuals do not progress to T2D or develop complications.¹⁴ Therefore, efforts to identify subgroups in prediabetes are needed to enable timely prevention of cardiovascular complications for individuals at risk of CVD.¹⁰ In T2D, data-driven methods using clinical characteristics can help identify who will benefit most from intensive treatment¹⁵⁹ It remains to be seen whether wearable technologies, such as those measuring HRV, can help improve individualized screening intervals and identify individuals who require closer clinical attention.

Timing and treatment decisions

In addition to optimizing the timing of follow-up assessments, determining cardiovascular risk can also guide when to initiate treatment. In type 1 diabetes, for example, elevated CVD risk scores are used to inform decisions about starting lipid-lowering therapy.¹⁶⁰ Wearable-derived data may also support intermediate treatment decisions.⁷³ In a UK population with T2D in clinical practice, patient characteristics have been used to predict whether SGLT2 inhibitors or GLP1RA will better improve HbA1c levels.^{[161][162]} A further step is to include long-term HRV as a clinical characteristic to enhance the prediction of treatment response. This could enable more precise stratification of therapy or lifestyle interventions based on the most effective option for each individual. Whether incorporating long-term HRV into predictive models can improve the personalization of treatment, particularly for therapies with cardiovascular effects, remains to be demonstrated. This conceptual framework may also have potential for guiding the selection of first-line antihypertensive medications.

As discussed in the clinical implications of CAN in T2D, it is unclear how well a CAN diagnosis predicts heart failure risk in the broader T2D population seen in primary care. Intermediate clinical decisions are needed for patients diagnosed with CAN to determine whether to proceed with further evaluation for heart failure using echocardiography or to initiate specific cardioprotective therapy.

In summary, future research should explore whether identifying high-risk individuals based on autonomic dysfunction, using HRV or CAN assessed through CART, can support personalized and timely cardiovascular screening or interventions.⁷³

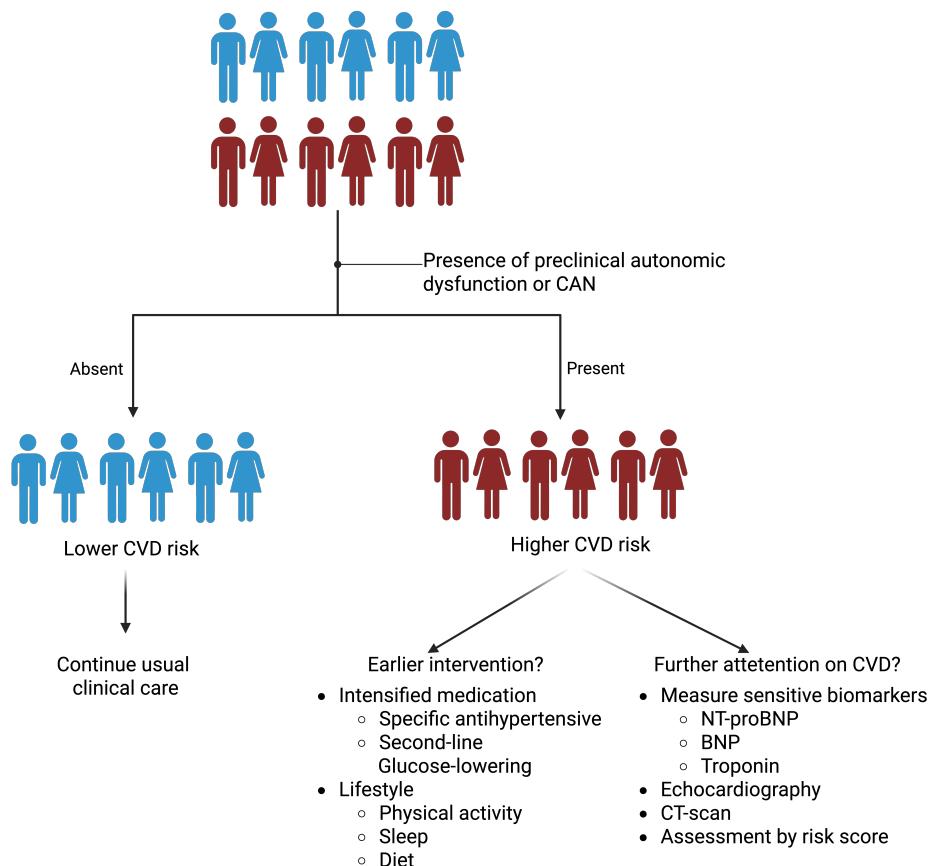


Figure 8.2.: Conceptual model for risk stratification by autonomic dysfunction. (Source: Author)

8.3. Effective causal modifiable marker

Our findings support a potential etiological link between long-term HRV and CVD risk, providing preliminary evidence consistent with a causal relationship. However, the observed association does not confirm causality, and further research is needed to determine whether HRV directly influences CVD outcomes. While randomized controlled trials are the gold standard for establishing causality, isolating the direct effect of HRV is particularly challenging. Interventions that affect HRV often do so indirectly through changes in weight, inflammation, or insulin sensitivity. Similarly, pharmacological treatments may improve HRV as a secondary effect, such as through blood pressure reduction

8.3. Effective causal modifiable marker

from antihypertensive medications. This makes it difficult to determine whether modifying HRV itself leads to improved cardiovascular outcomes.

To address these limitations, modern epidemiological methods such as Mendelian randomization and structured causal mediation analysis offer promising alternatives. These approaches can help infer causality from observational data and estimate indirect effects using trial data. Notably, no GWAS has yet investigated the genetic determinants of long-term HRV. Establishing such associations is essential for understanding its genetic architecture and for using genetic variants as unconfounded proxies to assess HRV's causal role in CVD. However, a challenge arises from findings in short-term HRV, which show considerable pleiotropy. This may complicate the use of Mendelian randomization, as the method relies on the assumption of no horizontal pleiotropy.¹⁶³

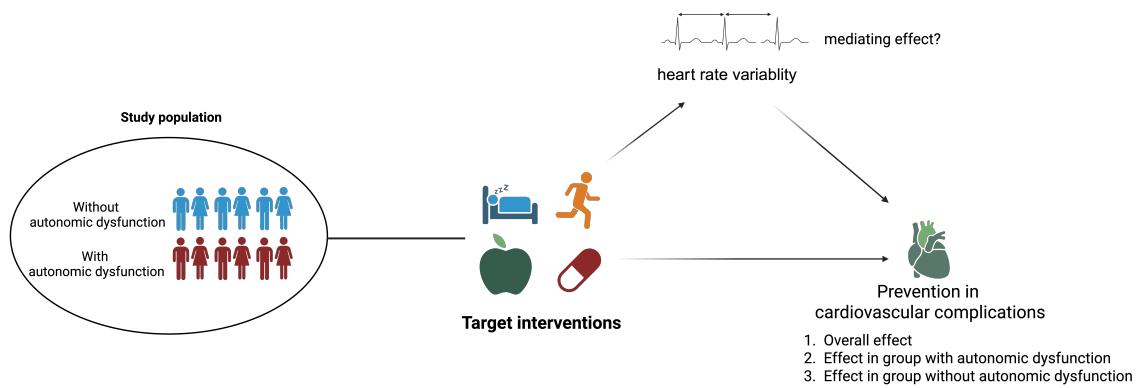


Figure 8.3.: Mediation of HRV by intervention in prevention of CVD. (Source: Author)

Future cardiometabolic intervention trials and longitudinal cohorts, whether focused on lifestyle or pharmacological strategies, should, where feasible, include repeated HRV measurements measurements. This would enable structured mediation analyses and help determine whether modifying autonomic function leads to sustained improvements in cardiovascular outcomes. Such evidence could clarify whether interventions like antihypertensive medications or lifestyle changes in physical activity, diet, and sleep can causally and sustainably improve CVD risk through HRV modulation.

9. Conclusion

This dissertation aimed to investigate how autonomic dysfunction, assessed by HRV and CARTs, is associated with cardiovascular complications across different stages of glucose metabolism. The findings support the hypothesis that autonomic dysfunction is an early and independent marker of cardiovascular risk.

Autonomic dysfunction was associated with higher arterial stiffness not only in individuals with T2D, but also in those with prediabetes and normal glucose metabolism. A particularly pronounced association was observed in individuals with prediabetes, where lower multiday HRV was linked to a higher risk of cardiovascular disease, heart failure, and mortality. These findings suggest that autonomic dysfunction may contribute to cardiovascular complications even before the onset of T2D, potentially through a modifying effect during the early stages of dysglycemia. Among individuals with T2D, standardized CARTs identified those with CAN who had a higher risk of heart failure, even when asymptomatic and not classified as high risk by risk scores.

Early detection is important, as CVD and heart failure are associated with reduced life expectancy and quality of life. This dissertation demonstrates the potential of autonomic dysfunction as a clinically relevant marker of cardiovascular risk across the full spectrum of glucose metabolism, including stages prior to the onset of T2D. However, it remains unclear whether this dysfunction plays a causal role or reflects underlying pathophysiological processes. Further research is needed to determine its clinical utility in risk stratification and its potential as a target for intervention.

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Summary

This dissertation investigated whether cardiovascular autonomic dysfunction is associated with cardiovascular complications in individuals with normal glucose metabolism, prediabetes, and type 2 diabetes. Cardiovascular autonomic dysfunction was assessed using heart rate variability and standardized cardiovascular autonomic reflex tests. Among the measures, 24-hour heart rate variability reflected autonomic balance and circadian shifts in sympathetic and parasympathetic activity. Lower long-term heart rate variability was consistently associated with adverse cardiovascular outcomes.

Across three studies, autonomic dysfunction was linked to higher levels of arterial stiffness, a higher incidence of ischemic cardiovascular events, heart failure, and all-cause mortality. The association with arterial stiffness was observed across the full spectrum of glucose metabolism and was particularly pronounced in individuals with prediabetes, suggesting that autonomic dysfunction may play a role early in the pathophysiological process. Building on this, the subsequent studies focused on individuals at high risk of developing diabetes. In this population, cardiovascular autonomic dysfunction was associated with a higher risk of cardiovascular disease, heart failure, and mortality.

Cardiovascular autonomic neuropathy, as identified through abnormal cardiovascular reflex testing, was associated with a higher risk of heart failure, even in individuals without symptoms. These findings suggest that cardiovascular autonomic dysfunction may serve as an early and independent marker of heart failure risk, particularly in populations at risk of type 2 diabetes.

The dissertation concludes that cardiovascular autonomic dysfunction is a clinically relevant risk factor that warrants further attention in both research and clinical practice. Future studies should explore whether improving autonomic function can reduce cardiovascular risk and whether measures of autonomic dysfunction can be integrated into existing risk models. Additionally, the potential of wearable technologies for continuous monitoring and early detection is a possibility to be evaluated in both clinical and general populations.

Resume

Denne afhandling undersøgte, om kardiovaskulær autonom dysfunktion er forbundet med kardiovaskulære komplikationer hos personer med normal glukosemetabolisme, prædiabetes og type 2-diabetes. Kardiovaskulær autonom dysfunktion blev målt igennme hjeretrymeveriabilitet (HRV) og standardiserede kardiovaskulære autonome refleksundersøgelser (CARTs). Blandt målingerne gav 24-timers HRV indsigt i den autonome balance og døgnrytmens skift mellem sympathisk og parasympatisk aktivitet. Lavere værdier var konsekvent forbundet med ugunstige kardiovaskulære udfald.

På tværs af tre studier blev autonom dysfunktion forbundet med højere niveauer af arteriel stivhed, en højere forekomst af iskæmiske hjertekarsygedom, hjertesvigt og dødelighed. Sammenhængen med arteriel stivhed blev observeret på tværs af hele spektret af glukosemetabolisme og var særligt forhøjet hos personer med prædiabetes, hvilket antyder, at autonom dysfunktion. På baggrund af dette fokuserede det efterfølgende studie på personer med høj risiko for at udvikle diabetes. I denne population var kardiovaskulær autonom dysfunktion forbundet med højere risiko for kardiovaskulær sygdom, hjertesvigt og dødelighed.

Kardiovaskulær autonom neuropati, defineret gennem abnormale CARTs, var forbundet med en højere risiko for hjertesvigt hos individer med type 2 diabetes, selv hos personer uden symptomer. Disse fund tyder på, at kardiovaskulær autonom dysfunktion kan fungere som en tidlig og uafhængig markør for risikoen for hjertesvigt, især i populationer med risiko for type 2-diabetes.

Afhandlingen konkluderer, at kardiovaskulær autonom dysfunktion er en klinisk relevant risikofaktor, som bør tiltrække større opmærksomhed i både forskning og klinisk praksis. Fremtidige studier bør undersøge, om forbedring af autonom funktion kan reducere kardiovaskulær risiko, og om målinger af autonom dysfunktion kan integreres i eksisterende risikomodeller. Derudover er potentialet i smarture til kontinuerlig overvågning og tidlig opsporing en mulighed, der kan vurderes i både kliniske og generelle populationer.

A. Appendix

A.1. Study I

Cardiovascular autonomic dysfunction is linked with arterial stiffness across glucose metabolism: The Maastricht Study

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Abbreviations

CAN: Cardiovascular autonomic neuropathy

CD: Carotid artery distensibility

PWV: carotid-femoral pulse wave velocity

MAP: Mean arterial pressure

CVD: Cardiovascular disease

ECG: Electrocardiogram

HRV: Heart rate variability

rHR: Resting heart rate

SDNN: The standard deviation of normal-to-normal R-R intervals

SDANN: The standard deviation of the averages of NN intervals in 5-minute segments throughout the recording

SDNN index: The mean of the standard deviation of all NN intervals for all 5-minute segments

pNN50: The NN50 count divided by the total number of all NN intervals

RMSSD: The square root of the mean of the sum of squares of differences between adjacent NN intervals

TP: Total frequency

HF: High frequency

LF: Low frequency

VLF: Very-low frequency

ULF: Ultralow frequency

Abstract

Background

Autonomic dysfunction is an established risk factor for cardiovascular disease (CVD). The mechanisms explaining the link between autonomic dysfunction and CVD are less well understood but may involve vascular stiffness. Investigating their interplay across glucose metabolism statuses could provide insights into how vascular changes unfold as people progress towards diabetes.

Objective

To ascertain the cross-sectional association between cardiovascular autonomic function and arterial stiffness across glucose metabolism status.

Methods

We performed a cross-sectional analysis of participants of The Maastricht Study without prior CVD. Cardiovascular autonomic function was based on heart rate variability (HRV) indices from 24-hour electrocardiogram recordings and summarized in Z-scores for time and frequency domains. Aortic and carotid stiffness were assessed by carotid-femoral pulse wave velocity (PWV) and carotid artery distensibility (CD), respectively. We used multiple linear regression to study the associations and adjusted for demographic and lifestyle factors and a range of cardiovascular risk factors. We tested for effect modification of the associations by glucose metabolism status.

Results

PWV and CD measures were available in 3673 and 1802 participants, respectively (median (25th; 75th percentile) age: 60 years (53; 66), 51% women, 20 % type 2 diabetes by design. Participants with lower HRV had higher aortic stiffness, as reflected by 2.8% (CI: 2.1; 3.4) and 2.8% (2.1; 3.5) higher PWV per standard deviation (SD) lower composite HRV time-domain and frequency domain Z-score, respectively. Similar trends were observed for carotid stiffness, reflected by 3.2% (1.4; 5.0) and 3.1% (1.2; 5.0) lower CD per SD lower composite HRV time-domain and frequency domain Z-score, respectively. Associations were stronger among people with prediabetes and type 2 diabetes compared to normal glucose metabolism (p-value for interaction for prediabetes: <0.05; and for type 2 diabetes ranging between: <0.05 - <0.10).

Conclusion

Cardiovascular autonomic dysfunction is associated with higher aortic and carotid stiffness, especially in people with dysglycemia. Thus, autonomic dysfunction may contribute to cardiovascular risk by affecting vascular stiffness.

Short abstract

This study ascertains the association between cardiovascular autonomic dysfunction and arterial stiffness in 3,671 participants of The Maastricht Study without prior cardiovascular disease. Cardiovascular autonomic function was assessed using 24-hour heart rate variability (HRV). Aortic stiffness was measured by pulse wave velocity (PWV), and carotid stiffness by carotid artery distensibility (CD). Lower HRV was associated with 2.8–3.2% higher PWV and 3.1–3.2% lower CD per SD decrease in HRV Z-scores, with stronger associations observed in individuals with prediabetes or type 2 diabetes. These findings suggest that autonomic dysfunction may increase cardiovascular risk through effects on vascular stiffness, particularly in prediabetes and diabetes.

Background

Improvement of targeted cardiovascular disease (CVD) prevention and treatment in people with diabetes and prediabetes requires a deeper understanding of the interplay between early stages of CVD and diabetes complications [1, 2]. Cardiovascular autonomic dysfunction (autonomic dysfunction), expressed by a reduction in heart rate variability (HRV), is an established risk indicator for CVD that can be easily monitored by wearables, such as smartwatches [3, 4]. However, the mechanisms that explain the link between autonomic dysfunction and CVD remain unclear. Arterial stiffness reflects structural changes in the arterial wall as, with ageing, the elastin fibres gradually are substituted with collagen fibres in the media layer of the large arteries [5]. This remodelling is associated with higher left ventricular afterload contributing to the pathogenesis of heart failure [6, 7]. Moreover, arterial stiffness is linked to atherosclerotic CVD events (e.g. myocardial infarction and stroke) and mortality [8].

Cardiovascular autonomic function can be estimated by HRV indices. The variation between the distance of successive normal RR intervals in milliseconds forms the basic observation underlying all HRV indices. It provides a time- or frequency-domain estimate of the balance between the sympathetic and parasympathetic tone influencing the sinoatrial node [9]. Extended recordings of HRV covering the circadian rhythms of sympathetic and parasympathetic activity may give insight into the role of lower-frequency sources of variability i.e. very low frequency and ultra-low frequency [9]. Lower 24-hour HRV reflects poorer adaptation in cardiac and vascular response to internal and external stimuli throughout the circadian rhythm [10]. Autonomic dysfunction may initially be expressed by sympathetic overactivity and reduced vagal activity [11]. Both in type 1 and type 2 diabetes, autonomic dysfunction and its association with arterial stiffness are well established [12-15]. Moreover, the Whitehall II study showed a longitudinal link in the general population, implying that the association can be observed without the presence of diabetes [16]. However, understanding to what degree the link between autonomic dysfunction and arterial stiffness is modified by dysglycemia is needed to highlight at which stage in the progression of diabetes, autonomic dysfunction is important. Most studies have measured arterial stiffness based on aortic stiffness alone [12]. A separate investigation of both aortic stiffness and carotid stiffness reflects different components of the arterial tree structure that are differently associated with types of CVD events [13, 14].

This etiological cross-sectional study aimed to ascertain the association between cardiovascular autonomic function, measured by 24-hour HRV, and arterial stiffness across glucose metabolism status. We hypothesised that autonomic dysfunction, expressed by lower HRV, is associated with higher levels of aortic and carotid stiffness and that the association is more pronounced in people with more advanced dysglycemia.

Methods

Data collection

The exact description of The Maastricht Study is referenced from a previous publications [15]: "We used data from The Maastricht Study, an observational prospective population-based cohort study. The rationale and methodology have been described previously. In brief, the study focuses on the aetiology, pathophysiology, complications and comorbidities of type 2 diabetes mellitus (T2DM) and is characterized by an extensive phenotyping approach. Eligible for participation were all individuals aged between 40 and 75 years and living in the southern part of the Netherlands. Participants were recruited through mass media campaigns and from the municipal registries and the regional Diabetes Patient Registry via mailings. Recruitment was stratified according to known T2DM status, with an oversampling of individuals with T2DM, for reasons of efficiency.

The examinations of each participant were performed within a time window of three months. The study has been approved by the institutional medical ethical committee (NL31329.068.10) and the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All participants gave written informed consent. We examined participants who had both HRV and measurements of aortic- and carotid stiffness within a three-month window around the baseline examination round of The Maastricht Study [15]."

The present study includes cross-sectional data from the first 7449 participants, who completed the baseline survey between November 2010 and December 2020 and had measures of arterial stiffness assessed, processed and cleaned. We excluded participants who self-reported prior CVD events, as their pathophysiology and consequent treatment could influence both arterial structural changes and impairment of autonomic balance. We also excluded participants with other types of diabetes than type 2 diabetes, as we investigated the effect modification by glucose metabolism status.

Exposure

All ECG recordings were obtained using a 12-lead Holter system (Fysiologic ECG Services, Amsterdam, the Netherlands) over 24 hours. The procedure for data collection has previously been reported [16]. During the recording period, participants were instructed to keep their regular daily activities but were asked to refrain from showering. The recorded ECG data were then processed using proprietary Holter Analysis Software at Fysiologic ECG Services (Amsterdam, the Netherlands). Non-sinus cardiac cycles i.e. artefacts and premature/ectopic beats were excluded. This process was subsequently validated through manual inspection. Following the exclusion of non-sinus cardiac cycles, the minimum required recording duration for ECG analysis was set at 18 hours. The software from Fysiologic ECG Services provided the inter-beat intervals in milliseconds (ms) between individual R waves of sinus beats. HRV indices were computed using the publicly available GNU Octave software [17], including the time and frequency domain measures established by the Task Force recommendation on HRV [9]. Time domain HRV indices were calculated, including the standard deviation (SD) of all normal-to-normal (NN) intervals (SDNN, in ms), the SD of the averages of NN intervals in 5-minute segments throughout the recording (SDANN, in ms), the square root

of the mean of the sum of squares of differences between adjacent NN intervals (RMSSD, in ms), the mean of the SDs of all NN intervals for all 5-minute segments (SDNN index, in ms), and the NN50 count divided by the total number of all NN intervals (pNN50, percentage). Frequency domain HRV measures were determined using the Fast Fourier Transform based on spectral segment for the whole recording cycle. In the frequency domain HRV, ms² measures the power or energy of the HRV signal within predefined frequency bands. These included the variance of all NN intervals ≤ 0.4 Hz, total power (TP, in ms²), power in the ultralow-frequency range (ULF, in ms² ≤ 0.003 Hz), power in the very-low-frequency range (VLF, in ms²; 0.003–0.04 Hz), power in the low-frequency range (LF, in ms²; 0.04–0.15 Hz), and power in the high-frequency range (HF, in ms²; 0.15–0.4 Hz). We removed outliers in time-domain and frequency domain HRV indices (see description in the supplementary material). We standardised HRV indices by their mean and SD to make indices comparable and calculated composite z-scores for time and frequency domain HRV indices, respectively. The time-domain Z-score included: SDNN, SDANN, RMSSD, SDNN index, and pNN50, and the frequency-domain Z-score included: TP, HF, LF, VLF, and ULF. Prior evidence shows that this selection of indices covers most of the underlying sources of variance determined by calculations of interbeat intervals [9].

Outcome

Aortic and carotid stiffness were included as measures for arterial stiffness. The procedure for arterial measurements has been previously documented [18]. Aortic stiffness was determined by carotid-femoral pulse wave velocity (PWV) and was assessed using applanation tonometry (SphygmoCor, Atcor Medical, Sydney, Australia). We included the median value from at least three consecutive PWV recordings in our analyses.

Carotid stiffness was determined by the carotid artery distensibility coefficient (CD). Ultrasound examinations of the left common carotid artery utilizing a 7.5 MHz linear probe-equipped ultrasound scanner (MyLab 70, Esaote Europe, Maastricht, the Netherlands) were conducted to evaluate local carotid distension. Local carotid stiffness was quantified by computing the CD, using the following equation:

$$CD = \frac{(2 * \Delta D * IAD + \Delta D^2)}{(\text{braPP} * IAD^2) (103 kPa - 1)}$$

, where ΔD represents distension, and braPP signifies brachial pulse pressure. Alongside the vascular assessments, mean heart rate and mean arterial pressure (MAP) were monitored at 5-minute intervals using an oscillometer device (Accutorr Plus, Datascope, Montvale, NJ, USA).

Covariates

Lifestyle factors of smoking (never, former (quit > 6 months ago), former (quit < 6 months ago), current), physical activity: total (hours/week) and moderate to vigorous exercise (hours/week), and alcohol

consumption (average units per week), as well as CVD disease history, and anti-hypertensive, glucose-lowering, and lipid-lowering medication use, were reported through a self-reported questionnaire.

Haemoglobin A1c (HbA1c), fasting plasma glucose (FPG), triglycerides, and total, high-density (HDL) and low-density (LDL) cholesterol levels were measured from blood samples. Anthropometric measures of body mass index (BMI) and waist circumference, as well as systolic and diastolic blood pressure, were measured at the study site [15]. We used World Health Organization 2006 criteria for categorizing glucose metabolism status into normal glucose metabolism, prediabetes (impaired fasting glucose and impaired glucose tolerance) and type 2 diabetes, based on a 2-hour 75 gram oral glucose tolerance test (OGTT) and/or the use of glucose lowering medication [19]. HbA1c was not used as criterion for type 2 diabetes or prediabetes.

Statistical analysis

We describe population characteristics by the distribution (median, 25th and 75th percentile) for continuous variables and frequencies (numbers, percentage) for categorical variables.

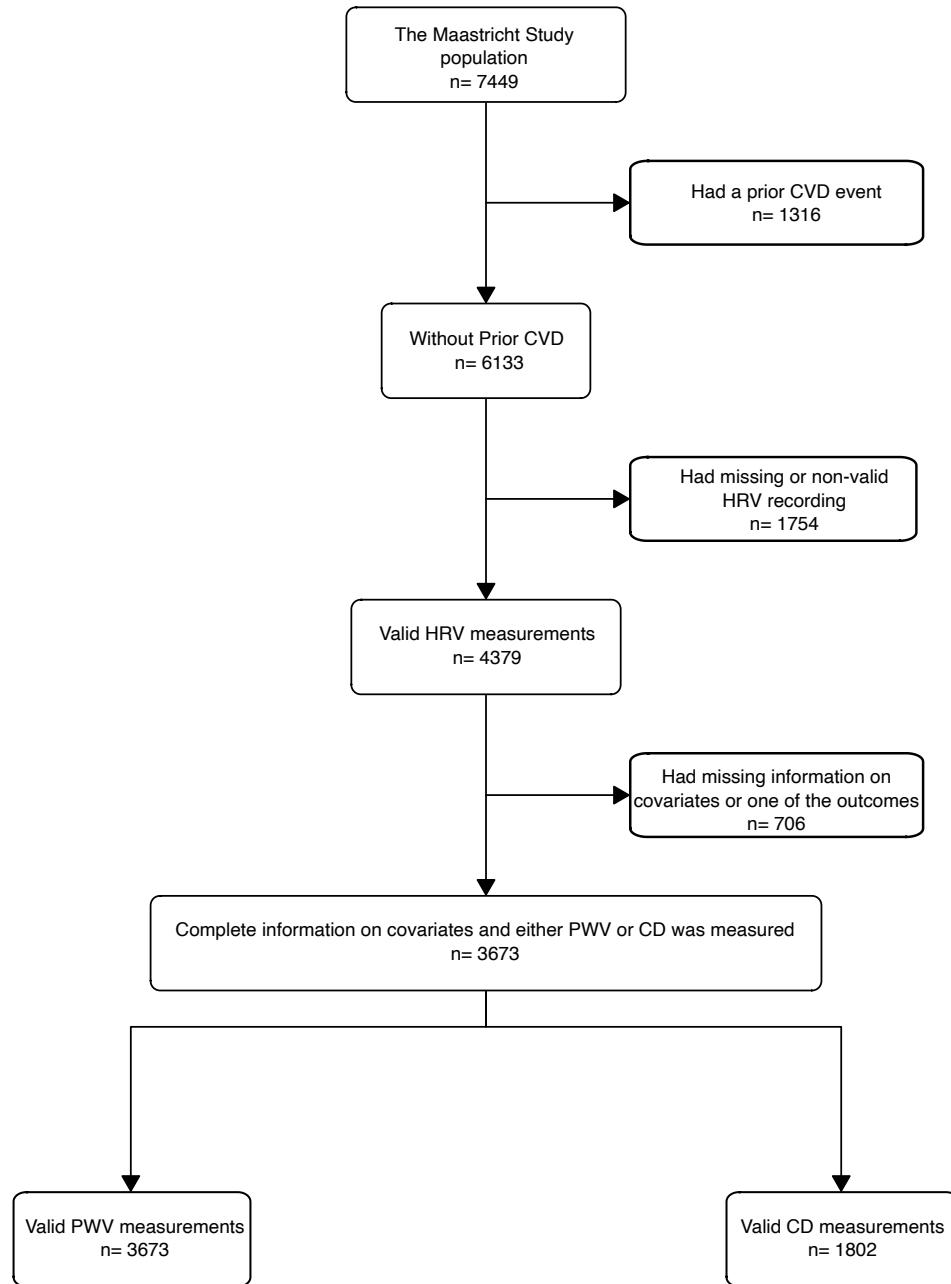
We performed multiple linear regression with heart rate variability indices as exposure for the outcome of arterial stiffness. We included the glucose metabolism status (normal glucose metabolism, prediabetes, and type 2 diabetes) to account for the oversampling of individuals with known type 2 diabetes. We further adjusted for mean arterial pressure (MAP) to account for potential instrumental bias, ensuring that elevated MAP during the measurement of arterial stiffness does not falsely indicate greater stiffness [20]. Model 1 was adjusted for age, sex, education, MAP, and diabetes status. In model 2 we further adjusted for self-reported total physical activity (hours/week), smoking behaviour, alcohol use, body mass index, HbA1c, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication. Blood pressure measures other than MAP are considered a collider as they are affected by autonomic dysfunction and arterial stiffness and thus were not included in the model [21]. To obtain normally distributed residuals, we log-transformed measures for arterial stiffness (PWV and CD) and back-transformed the model estimates into a percentage scale. We further tested for effect modification by sex and diabetes status by including them as multiplicative interaction terms, in separate models. A significant interaction was determined by a p-value<0.05. We also carried out a subsidiary analysis to investigate possible gradual stratified modification by higher glucose levels, using 20th percentiles of either FPG or HbA1c after excluding people using glucose-lowering medication. To test the robustness of our analysis we performed a sensitivity analysis first excluding individuals with antihypertensive treatment and subsequently people with type 2 diabetes. In the effect modification analysis by diabetes status, we performed an additional analysis excluding people using betablockers. We performed a complete case analysis, using the statistical program R (4.3.2) [22].

Results

Descriptive

Of the whole study population with available measures of HRV without prior CVD events and other types of diabetes, 3673 had PWV and 1802 had CD measured. Fifty-one percent were women and participants had a median (25th; 75th percentile) age of 60 (53; 66) years, and 2,387 (65%), 537 (15%), and 747 (20%) had normal glucose metabolism, prediabetes, and type 2 diabetes, respectively. The population with type 2 diabetes more frequently used lipid-lowering and anti-hypertensive medication compared to the populations with prediabetes or normal glucose metabolism (see supplementary table 3S). The median SDNN (HRV) was 133 ms (110; 158). The median PWV (Aortic stiffness) was 8.40 (7.44; 9.76) m/s and CD (Carotid stiffness) 14.2 (11.0; 17.8) 10^{-3} /kPa.

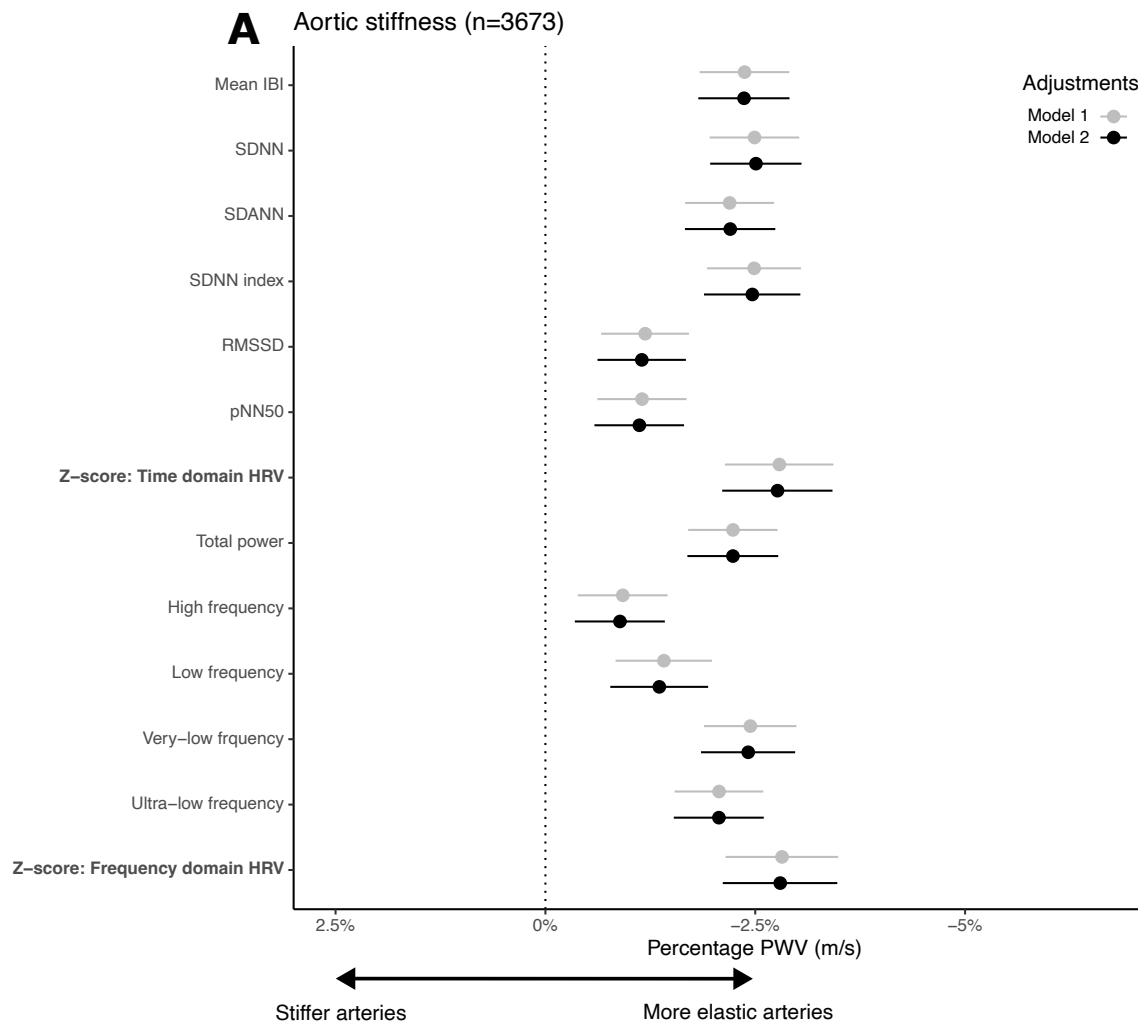
Figure 1: Study flowchart

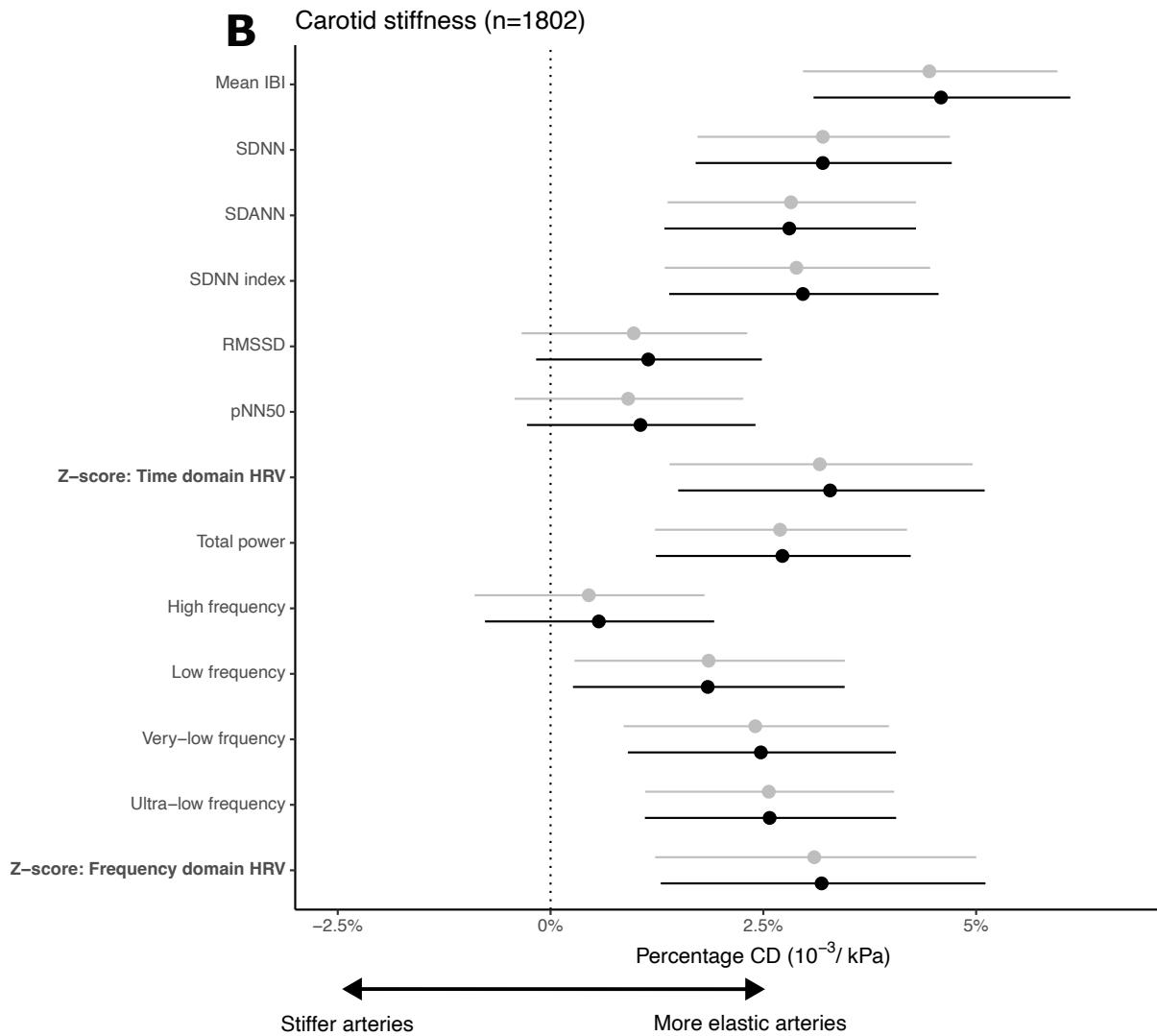


Heart rate variability and aortic stiffness

In model 1, for each SD lower HRV time-domain Z-score, PWV was 2.78% (CI: (2.13; 3.42) higher. For each SD lower HRV frequency-domain Z-score, PWV was 2.82% (CI: 2.14; 3.49) higher (see Fig. 3A and 3B). The strongest associations were seen in SDNN and SDANN for the time domain and in total power, VLF, and ULF for the frequency domain (see Fig. 2A). Associations did not change materially upon adjustment for the confounders in model 2. The sensitivity analyses showed that excluding participants using antihypertensive medication did not materially change the estimates. No interaction was observed by sex (see supplementary material: table 8S).

Figure 2: Association between long-term HRV and arterial stiffness



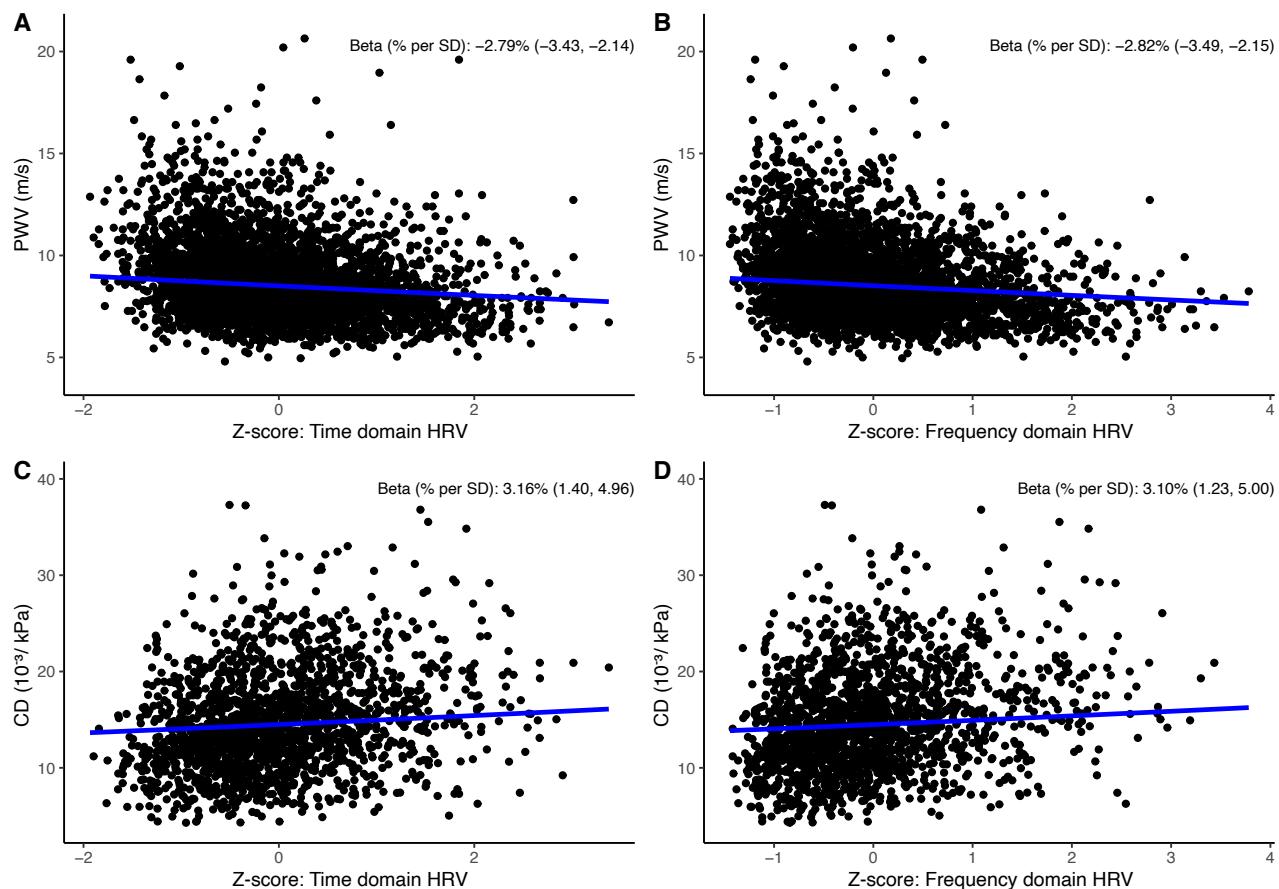


Percentage PWV (A) or CD (B) per SD increase in heart rate variability index and heart period intervals. Model 1: adjusted for sex, age, educational status, diabetes status, and mean arterial pressure. Model 2: Model 1 + physical activity, smoking behaviour, alcohol use, body mass index, hba1c, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication. Z-score: Frequency domain HRV

Heart rate variability and carotid stiffness

In model 1, for each SD lower HRV time-domain Z-score, CD was 3.17% (CI: 1.41; 4.96) lower. For each SD lower HRV frequency-domain Z-score, CD was 3.12% (CI: 1.24; 5.01) lower (see Fig. 3C and 3D). The strongest associations were seen in SDNN and SDANN for time-domain indices and in total power, VLF, and ULF for the frequency domain (see Fig. 2AB). Associations did not change materially upon adjustment for the confounders in model 2. Except for HRV index VLF, the sensitivity analyses showed that excluding participants using antihypertensive medication did not materially change the estimates. No interaction was observed by sex (see supplementary material: table 9S).

Figure 3: Linear relationship between HRV and aortic and carotid stiffness

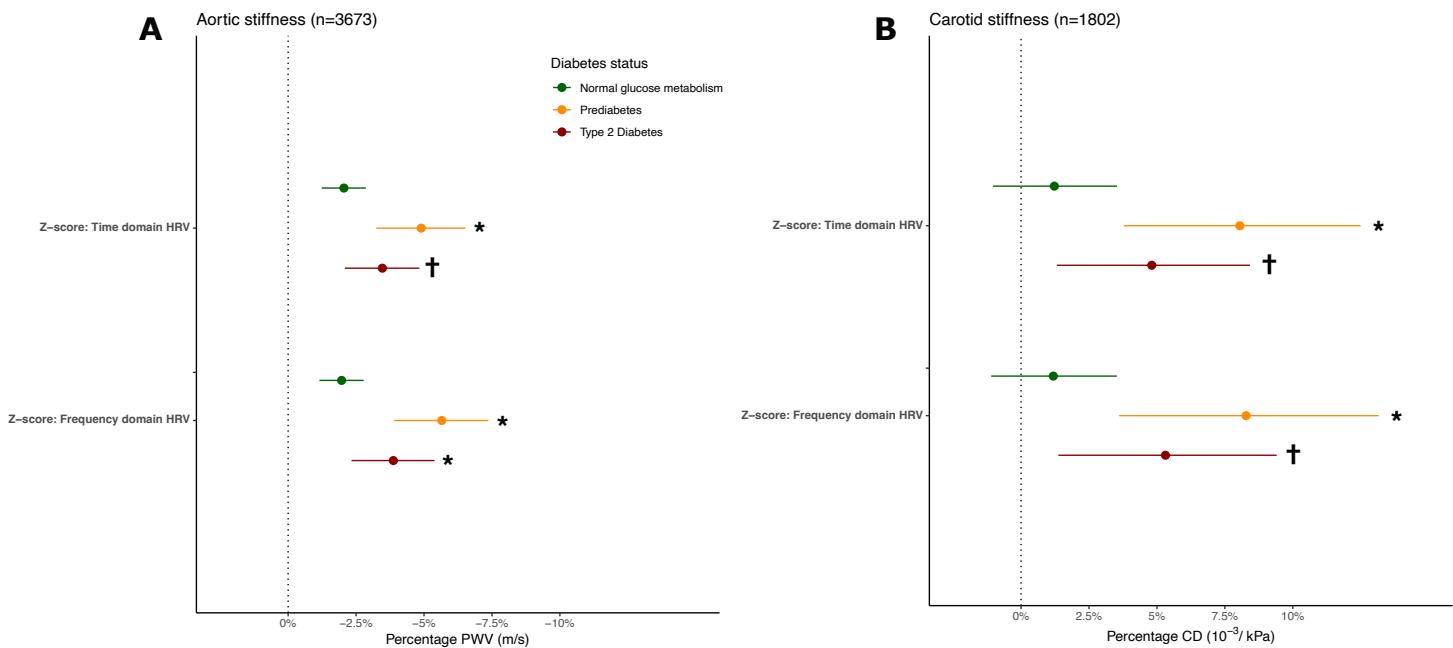


A: Percentage PWV per SD in time-domain composite z-score **B:** Percentage PWV per SD in frequency-domain composite z-score **C:** Percentage higher CD per SD in time-domain composite z-score **D:** Percentage CD per SD in frequency-domain composite z-score. All regression lines were adjusted for being a male, 60 years old, low educational level, without prediabetes or type-2 diabetes, and with 96mmHg mean arterial pressure.

Effect modification by glucose metabolism

The association between HRV and measures of arterial stiffness was stronger in people with prediabetes and type 2 diabetes than in those with normal glucose metabolism (see Fig. 4AB). Indeed, we observed statistically significant interactions when comparing prediabetes and with normal glucose metabolism, whereas the interaction was only significant for type 2 diabetes in the association between HRV frequency-domain Z-score and PWV. Excluding people using betablockers raised the estimates for the type 2 diabetes group in the analysis with PWV as outcome but not in CD (see Fig 3S). Effect modification estimates for each HRV index are presented in the supplementary material (see Table 6S and 7S). When we excluded people using glucose lowering-medication and analysed the stratified modification by quintiles of glycaemia, we found stronger associations between the frequency and time-domain Z-score and PWV and CD in higher percentiles of FPG and HbA1c (see supplementary material Fig1S and Fig2S).

Figure 4: Association between long-term HRV and arterial stiffness modified by diabetes status



A: Percentage PWV per SD in time-domain and frequency-domain composite z-score by diabetes status **B:** Percentage CD per SD in time-domain and frequency-domain composite z-score by diabetes status. Estimates are adjusted for sex, age, educational status, mean arterial pressure, physical activity, smoking behaviour, alcohol use, body mass index, HbA1c, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication. Normal glucose metabolism was defined as reference group.

* Interaction term p-value < 0.05

+ Interaction term p-value < 0.10

CENTRAL ILLUSTRATION



THE
Maastricht
STUDY

Cardiovascular autonomic dysfunction contribution to arterial stiffness across glucose metabolism



Including 3673 participants without prior cardiovascular disease



Without diabetes
n = 2389



Prediabetes
n = 538



Type 2 diabetes
n = 746

A healthy heart responds to challenges by varying heart rate

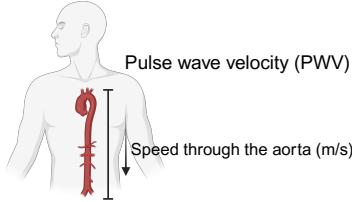


Standard deviation of 24-hour normal interbeat intervals (SDNN)

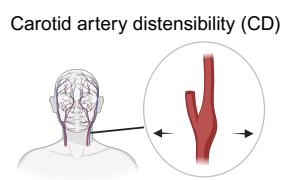
Highest deciles SDNN = 180 ms ~ Good adaptation to changes

Lowest deciles SDNN = 90 ms ~ In range of subclinical cardiovascular autonomic dysfunction

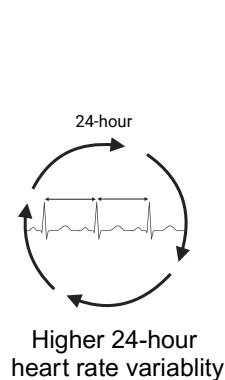
Stiffer arteries are a pathway to cardiovascular disease



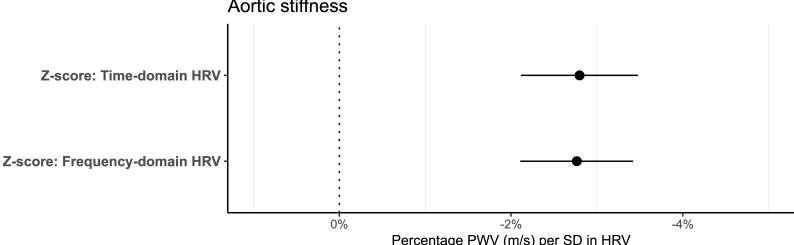
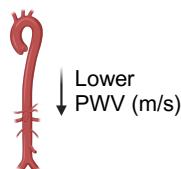
High-risk of CVD



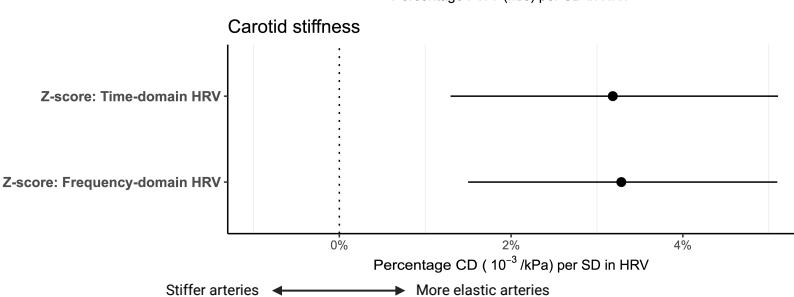
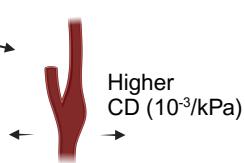
Low-risk of CVD



Aortic stiffness



Carotid stiffness



Discussion

In this study, we showed that cardiovascular autonomic dysfunction, determined by long-term HRV, was associated with both aortic and carotid stiffness among older adults, irrespective of the presence of glucose metabolism status, although the association was stronger in those with prediabetes or type 2 diabetes. Lower HRV was associated with higher stiffness either measured by higher PWV or lower CD.

The association for the time domain Z-score was mainly driven by SDNN and SDANN and that for the frequency domain Z-score was primarily driven by total power, VLF, and ULF. Hence, the associations were mostly determined by HRV indices calculated by global variation of interbeat intervals and lower frequency bands capturing long oscillations in interbeat intervals. Across the HRV indices, SDNN was most strongly associated with measures of arterial stiffness.

The magnitude of the observed associations was modest but relevant when compared to equivalent associations of age with arterial stiffness. One SD lower HRV was equivalent to the effect of 2.7 additional years on PWV and to 1.6 years for CD. A hypothetical individual (male, non-smoker, low alcohol consumption, no diabetes or hypertension, and mean values for all continuous confounders in model 2) with an SDNN at the 10th percentile (90 ms) had 8.77 m/s PWV, and 14.3 10⁻³/kPa CD. A similar hypothetical individual at the 90th percentile of SDNN (180 ms) had a 6.5 % lower PWV and 8.2 % higher CD. Hence, lower 24-hour HRV is associated with arterial stiffness characterized both by local stiffness at the carotid site and by dynamic alterations in the aorta. The link between long-term HRV and these distinctly measured surrogate CVD markers, suggests that long-term HRV is likely also linked to the risk of ischemic and stroke events. Earlier studies have shown that short-term HRV is linked with both coronary heart disease and stroke but are less conclusive with regard to long-term HRV [23, 24].

We accounted for the oversampling of people with type 2 diabetes by adjusting for diabetes status and correcting for the instrument bias in stiffness measures, caused by higher pulse pressure during measurement of PWV and CD, by adjusting for MAP. Adjustment for lifestyle habits and cardiovascular risk factors did not materially change the estimates, suggesting most of the measurable confounding was captured by diabetes status and MAP. In our sensitivity analysis, without participants on anti-hypertensive treatment, the estimates did not materially change, thus we focused on models for the entire study population and adjusted for medication in the full model.

Several studies found lower HRV indices to be associated with aortic stiffness among people with either type 1 or type 2 diabetes [12, 25]. Our study extends these findings by showing that the associations are already present in people without diabetes, albeit to a lesser degree than in people with prediabetes or diabetes.

Both cardiovascular autonomic dysfunction and arterial stiffness are likely to be shared consequences of cardiometabolically disturbed environment including dyslipidaemia, hyperinsulinemia and advanced glycation end-products induced by hyperglycaemia, oxidative stress, and inflammation [16, 26-28] [29, 30]. Our results support the notion that hyperglycaemia modifies the association between HRV and arterial stiffness, as we found stronger associations in the higher quintiles of both FPG and HbA1c in participants without glucose-lowering medication. Early deterioration of glucose metabolism starts a complex cycle of complications, in this case, autonomic dysfunction that along with dysglycemia, likely through neuronal

damage[16], contributes to vascular dysfunction. Although our data is cross-sectional, the effect modification gives a notion that the CVD risks are higher in prediabetes and improvement of glycaemic control could potentially in part modify the contribution of low HRV to arterial stiffness.

Two explanations might clarify why the effect modification did not increase progressively by glycaemic status in the present study. First, because of being diagnosed with type 2 diabetes, participants were more likely to receive cardioprotective care, including glucose-lowering, lipid-lowering, and antihypertensive medication, an effect that cannot be accounted for by adjustment. After exclusion of people using betablockers, the results partly explained why type 2 diabetes showed a smaller modifying effect compared to prediabetes in the outcome of PWV, but not in the outcome of CD. The second explanation could be due to selection bias, as participants with type 2 diabetes, who participated in the Maastricht Study and underwent both long-term ECG recordings and measures of arterial stiffness might be healthier than the background population with type 2 diabetes.

We showed that shorter mean IBI was associated with both aortic and carotid stiffness, emphasising a potential mediating role of higher heart rate in autonomic dysfunction. Sympathetic predominance may result in a higher heart rate and hence lead to higher shear stress on the arterial wall [31]. The association might also be driven by direct sympathetic effects on arteries, caused by increased levels of norepinephrine and reduced clearance [32, 33]. We cannot exclude that the association between HRV and arterial stiffness might be bidirectional, hence arterial remodelling may also cause changes in autonomic balance, which might particularly be expressed in carotid stiffness. The baroreflex receptors located in the carotid artery region become less sensitive as compliance in the carotid region deteriorates, which may result in less adaptive heart rate and blood pressure response [34, 35].

In summary, cardiovascular autonomic function might be a relevant risk indicator of efforts to prevent trajectories towards CVD mediated through arterial stiffness, even before the onset of diabetes. Our findings support the view that lower 24-hour HRV is an indicator of elevated CVD risk.

Hyperglycaemia is rarely an isolated risk factor among people with prediabetes and type 2 diabetes. Therefore, current type 2 diabetes guidelines focus on multifactorial cardiometabolic management, the effect of which on micro- and macrovascular complications has been clearly demonstrated [10, 34]. Closer attention to the mechanisms that mediate these effects offers the prospect of new intervention points. Although it is conceivable that multifactorial risk management slows the progression of arterial stiffening partially by modulating autonomic dysfunction, it remains to be proven whether modification of HRV per se contributes causally to reduction of CVD risk. To ascertain this causality, observational studies using Mendelian randomisation would provide a first line of evidence. Furthermore, cardiometabolic trials, assessing either lifestyle modification or pharmacological intervention should, if possible, measure HRV to enable a structured mediation analysis.

Our findings help us understand that the progression of autonomic dysfunction plays a role in CVD risk and confirm that prediabetes defines a group with a higher risk of complications. Lifestyle and glucose-lowering interventions improve cardiometabolic outcomes in prediabetes but have not yet been shown to effectively prevent CVD or all-cause mortality events [35]. Autonomic dysfunction may serve as a tool for risk stratification among individuals with prediabetes who have high CVD risk. These individuals may particularly benefit from lifestyle interventions to reduce their CVD risk [36, 37]. Lastly, our findings show that autonomic dysfunction plays a smaller, but still meaningful, role in CVD risk among people without diabetes.

The strengths of the study are the large sample size with a large subpopulation with type 2 diabetes and that HRV was determined by long-term 24-hour ECG recordings in free-living conditions. Recordings of 24-hour ECG traces provide a full day measurement of cardiovascular autonomic function during the circadian rhythm, including responses in free living conditions [9]. There are also limitations to consider. First, during ECG recordings, non-stationary activity (including physical activity, meals, consumption of caffeine.) might influence the assessment of cardiovascular autonomic function [9]. Second, the level of HRV may depend on heart rate. We did not include adjustment of heart rate in the model as we believe it violates the principles of multicollinearity. Moreover, as a higher heart rate is determined by increased sympathetic bursts, we consider it to be a mediator on the pathway to arterial stiffness [33]. We have a full-day recording capturing heartbeats in rest and activity. These measures are representative of valid autonomic assessment in a full-day cycle [9]. We believe it is more relevant to consider the correction for heart rate in short-term recordings, as random factors (e.g. time of the day, smoking, caffeine intake) can influence this standardized recording procedure [36]. Therefore, we argue that, in the current study, heart rate should not be included as an adjustment for either confounding or instrumental bias. Third, the generalizability of our findings is limited to populations including middle-aged white people with access to high-quality diabetes care. Finally, our study is based on cross-sectional data and thus, we cannot infer a causal direction. However, we attempted to mimic an aetiological ordering by showing the temporality of glucose metabolism (normal, prediabetes, and type 2 diabetes) in the relationship between autonomic dysfunction and arterial stiffness. Longitudinal data from the Whitehall study showed that a steeper decrease in short-term 5-min HRV over 10 year was associated with subsequent higher levels of aortic stiffness in a five-year trajectory [25]. This suggests that autonomic dysfunction is mainly contributing to arterial stiffness rather than the other way around.

Higher physical activity is longitudinally associated with increased HRV [37]. We included self-reported total physical activity to account for habitual physical activity, but we did not include accelerometer-based physical activity adjustment as this might result in over-adjusting for the concurrent physical movement on the concurrent day of the HRV recordings. Earlier data from The Maastricht Study confirmed that

adjustment for objective physical activity by mean stepping time measured by an accelerometer did not change the estimates of their analysis of HRV compared to self-reported physical activity [16].

Wearable devices have made data collection of physiological measures (e.g. pulse rate, blood oxygen saturation, physical activity etc.) more accessible in general populations e.g. by smartwatches. Global distributed HRV measures as well as lower frequency bands have been shown to be valid [3]. Hence, long-term HRV is becoming more accessible to users and eventually health care providers, however its clinical relevance and role remain to be ascertained before implementation. Our study shows that lower HRV is associated with surrogate markers for CVD risk, even at normal glucose metabolism. Thus, a cycle of 24-hour long-term HRV measured by wearable devices might be an easy and non-invasive tool to detect people who silently have higher CVD risk in all stages of glucose metabolism, beyond conventional CVD markers.

Conclusion

Lower 24-hour HRV was associated with both higher aortic and carotid stiffness. This association was stronger with worse glucose metabolism status. Cardiovascular autonomic dysfunction may contribute to cardiovascular risk by affecting vascular stiffness. The prognostic value of 24-hour HRV in CVD risk, and whether the CVD risk reduction of glucose-lowering intervention is mediated by improved cardiovascular autonomic function remain open for further investigation.

Acknowledgements

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Authors' contributions

JRS, LB, and DW contributed to the conception and design of the study, performed data analysis and interpretation, drafted the manuscript, critically revised it for significant intellectual content, and gave final approval of the version to be published. CS contributed to the conception and design, assisted with data acquisition, interpreted the data, critically revised the manuscript for important intellectual content, and approved the final version for publication. CSH and STA contributed to the conception and design, participated in data analysis and interpretation, critically revised the manuscript for intellectual content, and approved the final version for publication.

MvG, MTS, and BEdG were involved in data acquisition, critically revised the manuscript for significant intellectual content, and approved the final version for publication. JRS is the guarantor of this work, having full access to all study data, and is responsible for the integrity and accuracy of the data analysis.

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Ethics

The study has been approved by the institutional medical ethics committee (NL31329.068.10) and the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All participants gave written informed consent.

Conflicts of interests

All the authors declare that there is no duality of interest associated with their contribution to this manuscript.

Availability of data and materials

The data of this study derive from The Maastricht Study, but restrictions apply to the availability of these data, which were used under license for the current study. Data are, however, available from the authors upon reasonable request and with permission of The Maastricht Study management team.

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Tables and figures

Table 1: Study population characteristics

	N = 3,673
Sex	
Men	1,789 (49%)
Women	1,884 (51%)
Age (years)	60 (53, 66)
Ethnicity	
White	3,633 (99%)
Non-white	40 (1.1%)
Education	
Low (No education, (un)completed primary education, or lower vocational education)	1,094 (30%)
Middle (intermediate vocational education or higher secondary education)	1,050 (29%)
High (Higher vocational education or university education)	1,529 (42%)
Alcohol consumption	
None	609 (17%)
Low (Women: ≤ 7, Men: ≤ 14)	2,147 (58%)
High (Women: > 7, Men: > 14)	917 (25%)
Alcohol total (g/day)	9 (2, 19)
Smoking status	
Never	1,417 (39%)
Former (quit > 6 months ago)	1,733 (47%)
Former (quit < 6 months ago)	62 (1.7%)
Current	461 (13%)
Total physical activity (hours/week)	13 (8, 19)
Moderate to vigorous physical activity (hours/week)	4.5 (2.3, 7.8)
BMI (kg/m ²)	26.0 (23.6, 28.8)
Waist (cm)	93 (85, 102)
HbA1c (mmol/mol)	37 (34, 41)
HbA1c (%)	5.54 (5.26, 5.90)

N = 3,673

Fasting plasma glucose (mmol/L)	5.40 (4.90, 6.00)
LDL (mmol/L)	3.10 (2.40, 3.80)
HDL (mmol/L)	1.50 (1.20, 1.90)
Total cholesterol (mmol/L)	5.30 (4.60, 6.10)
Triglycerides (mmol/L)	1.18 (0.87, 1.65)
Total cholesterol-to-HDL cholesterol ratio	3.40 (2.78, 4.25)
Glucose metabolism status	
Normal glucose metabolism	2,389 (65%)
Prediabetes	538 (15%)
Type 2 Diabetes	746 (20%)
Duration of type-2 diabetes (only for diagnosed participants)	3 (0, 9)
Mean IBI (ms)	828 (765, 904)
SDNN (ms)	133 (110, 158)
RMSSD (ms)	25 (20, 34)
SDANN (ms)	119 (97, 143)
SDNNi (ms)	52 (42, 63)
pNN50 (%)	6 (3, 12)
TP (ms ²)	11,566 (7,991, 16,394)
ULF (ms ²)	9,788 (6,655, 14,183)
VLF (ms ²)	1,105 (736, 1,571)
LF (ms ²)	364 (222, 593)
HF (ms ²)	84 (50, 149)
Systolic blood pressure (mmHg)	126 (116, 136)
Diastolic blood pressure (mmHg)	76 (71, 81)
Mean arterial pressure (mmHg)	96 (89, 103)
Carotid artery distensibility (10-3/kPa)	14.2 (11.0, 17.8)
Carotid-femoral pulse wave velocity (m/s)	8.40 (7.44, 9.76)
Diagnosed hypertension	1,740 (47%)
Glucose-lowering medication	519 (14%)
Antihypertensive medication	1,108 (30%)
Beta blockers	421 (11%)

N = 3,673

Diuretic aldosterone	15 (0.4%)
Diuretics	470 (13%)
Lipid-lowering medication	905 (25%)

Data are shown as n (%) or median (IQR)

Supplemental Material:

Cardiovascular autonomic dysfunction is linked with arterial stiffness across glucose metabolism: The Maastricht Study

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Removal of 24-hour HRV outliers

HRV indices are very sensitive to data error in the time-series collection of interbeat intervals. This can be due to missing heartbeat signals, arrhythmia, ectopic beats and more. These premature, skipped, or non-captured heartbeat recordings might not have been unfiltered from the IBI data and should have been removed, as it does not reflect autonomic activity. The time segment of frequency domain measures in the Maastricht Study was over the whole recording to capture very- and ultra-lower frequency components. Reference values of 24-hour HRV are done with small population size and with either time domain HRV or 5-min segments of frequency domain indices [1]. To determine cutoff for the exclusion of outliers, we visualize each HRV index value across ages and compare it to available reference values by age from studies [1]. Based on visually observing distribution and available reference material, we excluded time-domain indices according to reference material, and the upper 1th percentile in frequency-domain measures.

Reference:

1. Sammito S, Böckelmann I. Reference values for time- and frequency-domain heart rate variability measures. *Heart Rhythm*. 2016;13(6):1309-16. doi: 10.1016/j.hrthm.2016.02.006.

Table S1: Descriptives of included and non-included population

	No, N = 5,514	Yes, N = 3,673
Sex		
Men	2,782 (50%)	1,789 (49%)
Women	2,732 (50%)	1,884 (51%)
Age (years)	61 (53, 67)	60 (53, 66)
Ethnicity		
White	5,418 (98%)	3,633 (99%)
Non-white	93 (1.7%)	40 (1.1%)
Education		
Low (No education, (un)completed primary education, or lower vocational education)	1,911 (35%)	1,094 (30%)
Middle (intermediate vocational education or higher secondary education)	1,449 (27%)	1,050 (29%)
High (Higher vocational education or university education)	2,028 (38%)	1,529 (42%)
Alcohol consumption		
None	1,083 (20%)	609 (17%)
Low (Women: ≤ 7, Men: ≤ 14)	3,237 (60%)	2,147 (58%)
High (Women: > 7, Men: > 14)	1,119 (21%)	917 (25%)
Smoking status		
Never	2,086 (38%)	1,417 (39%)
Former (quit > 6 months ago)	2,511 (46%)	1,733 (47%)
Former (quit < 6 months ago)	106 (1.9%)	62 (1.7%)
Current	740 (14%)	461 (13%)
Total physical activity (hours/week)	13 (8, 18)	13 (8, 19)
Moderate to vigorous physical activity (hours/week)	4.5 (1.8, 7.5)	4.5 (2.3, 7.8)
BMI (kg ² /m)	26.4 (23.9, 29.5)	26.0 (23.6, 28.8)
Waist (cm)	95 (86, 105)	93 (85, 102)
HbA1c (%)	5.54 (5.26, 5.99)	5.54 (5.26, 5.90)
Fasting plasma glucose (mmol/L)	5.40 (5.00, 6.20)	5.40 (4.90, 6.00)
LDL (mmol/L)	2.90 (2.30, 3.70)	3.10 (2.40, 3.80)
HDL (mmol/L)	1.50 (1.20, 1.80)	1.50 (1.20, 1.90)
Total cholesterol (mmol/L)	5.10 (4.30, 5.90)	5.30 (4.60, 6.10)
Triglycerides (mmol/L)	1.19 (0.87, 1.70)	1.18 (0.87, 1.65)
Glucose metabolism status		

	No, N = 5,514	Yes, N = 3,673
Normal glucose metabolism	3,358 (61%)	2,389 (65%)
Prediabetes	844 (15%)	538 (15%)
Type 2 Diabetes	1,259 (23%)	746 (20%)
Duration of type-2 diabetes (only for diagnosed participants)	4 (0, 10)	3 (0, 9)
Mean IBI (ms)	829 (765, 910)	828 (765, 904)
SDNN (ms)	134 (110, 161)	133 (110, 158)
RMSSD (ms)	29 (21, 49)	25 (20, 34)
SDANN (ms)	121 (98, 148)	119 (97, 143)
SDNNi (ms)	54 (43, 71)	52 (42, 63)
pNN50 (%)	8 (3, 20)	6 (3, 12)
TP (ms ²)	11,509 (7,706, 16,540)	11,566 (7,991, 16,394)
ULF (ms ²)	9,665 (6,310, 14,055)	9,788 (6,655, 14,183)
VLF (ms ²)	1,064 (722, 1,606)	1,105 (736, 1,571)
LF (ms ²)	370 (213, 625)	364 (222, 593)
HF (ms ²)	104 (55, 212)	84 (50, 149)
Systolic blood pressure (mmHg)	126 (117, 136)	126 (116, 136)
Diastolic blood pressure (mmHg)	75 (71, 80)	76 (71, 81)
Mean arterial pressure (mmHg)	96 (89, 103)	96 (89, 103)
Carotid artery distensibility (10-3/kPa)	13.8 (10.7, 17.5)	14.2 (11.0, 17.8)
Carotid-femoral pulse wave velocity (m/s)	8.72 (7.60, 10.16)	8.40 (7.44, 9.76)
Prior CVD	1,482 (27%)	0 (0%)
Hypertension (Yes)	3,056 (56%)	1,740 (47%)
Glucose lowering medication	973 (18%)	519 (14%)
Antihypertensive medication	2,192 (40%)	1,108 (30%)
Lipid-lowering medication	1,803 (33%)	905 (25%)

n (%); Median (IQR)

Table S2: Descriptives of participants with both CarDC and cf-PWV measured and participants with only cf-PWV measured

Sex

Men	901 (50%)	888 (47%)
Women	901 (50%)	983 (53%)
Age (years)	60 (54, 66)	59 (52, 66)

Education

Low (No education, (un)completed primary education, or lower vocational education)	506 (28%)	588 (31%)
Middle (intermediate vocational education or higher secondary education)	541 (30%)	509 (27%)
High (Higher vocational education or university education)	755 (42%)	774 (41%)

Smoking status

Never	643 (36%)	774 (41%)
Former (quit > 6 months ago)	892 (50%)	841 (45%)
Former (quit < 6 months ago)	33 (1.8%)	29 (1.5%)
Current	234 (13%)	227 (12%)
BMI (kg ² /m)	26.2 (23.7, 29.2)	25.7 (23.5, 28.6)
Waist (cm)	94 (86, 103)	92 (83, 101)
HbA1c (%)	5.63 (5.35, 5.99)	5.44 (5.17, 5.72)
Fasting plasma glucose (mmol/L)	5.50 (5.00, 6.30)	5.30 (4.90, 5.80)
LDL (mmol/L)	3.10 (2.40, 3.90)	3.10 (2.40, 3.70)
HDL (mmol/L)	1.50 (1.20, 1.80)	1.60 (1.20, 1.90)
Total cholesterol (mmol/L)	5.40 (4.60, 6.10)	5.30 (4.60, 6.00)
Triglycerides (mmol/L)	1.22 (0.89, 1.73)	1.14 (0.84, 1.59)
Hypertension (Yes)	928 (52%)	812 (43%)

Diabetes status

Normal glucose metabolism	1,049 (58%)	1,340 (72%)
Prediabetes	323 (18%)	215 (11%)
Type 2 Diabetes	430 (24%)	316 (17%)

Duration of type-2 diabetes (only for diagnosed participants)	3 (0, 8)	3 (0, 9)
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Mean IBI (ms)	824 (759, 900)	832 (772, 908)
SDNN (ms)	132 (109, 157)	133 (111, 159)
RMSSD (ms)	25 (19, 34)	26 (20, 34)

	CAD measured, N = 1,802	Without CAD measurements, N = 1,871
SDANN (ms)	120 (96, 143)	118 (98, 144)
SDNNi (ms)	51 (42, 62)	53 (43, 64)
pNN50 (%)	6 (3, 12)	6 (3, 12)
TP (ms ²)	11,551 (7,860, 16,410)	11,571 (8,088, 16,366)
ULF (ms ²)	9,850 (6,503, 14,183)	9,673 (6,780, 14,194)
VLF (ms ²)	1,065 (707, 1,520)	1,129 (767, 1,643)
LF (ms ²)	350 (213, 578)	381 (233, 613)
HF (ms ²)	83 (48, 150)	86 (52, 149)
Systolic blood pressure (mmHg)	126 (117, 136)	125 (115, 135)
Diastolic blood pressure (mmHg)	76 (71, 81)	75 (71, 81)
Mean arterial pressure (mmHg)	96 (90, 103)	96 (89, 103)
Carotid-femoral pulse wave velocity (m/s)	8.48 (7.44, 9.84)	8.32 (7.44, 9.60)
Glucose-lowering medication (Yes)	301 (17%)	218 (12%)
Antihypertensive medication (Yes)	601 (33%)	507 (27%)
Using beta-blockers (Yes)	239 (13%)	182 (9.7%)

n (%); Median (IQR)

Table S3: Study population characteristics by diabetes status

	Normal glucose metabolism, N = 2,389	Prediabetes, N = 538	Type 2 Diabetes, N = 746
Sex			
Men	1,028 (43%)	280 (52%)	481 (64%)
Women	1,361 (57%)	258 (48%)	265 (36%)
Age (years)	58 (51, 64)	62 (57, 68)	63 (57, 68)
Ethnicity			
White	2,368 (99%)	533 (99%)	732 (98%)
Non-white	21 (0.9%)	5 (0.9%)	14 (1.9%)
Education			
Low (No education, (un)completed primary education, or lower vocational education)	604 (25%)	192 (36%)	298 (40%)
Middle (intermediate vocational education or higher secondary education)	697 (29%)	145 (27%)	208 (28%)
High (Higher vocational education or university education)	1,088 (46%)	201 (37%)	240 (32%)
Alcohol consumption			
None	338 (14%)	83 (15%)	188 (25%)
Low (Women: ≤ 7, Men: ≤ 14)	1,437 (60%)	298 (55%)	412 (55%)
High (Women: > 7, Men: > 14)	614 (26%)	157 (29%)	146 (20%)
Smoking status			
Never	988 (41%)	185 (34%)	244 (33%)
Former (quit > 6 months ago)	1,070 (45%)	286 (53%)	377 (51%)
Former (quit < 6 months ago)	43 (1.8%)	3 (0.6%)	16 (2.1%)
Current	288 (12%)	64 (12%)	109 (15%)
Total physical activity (hours/week)	13 (9, 19)	13 (9, 19)	12 (7, 17)
Moderate to vigorous physical activity (hours/week)	5.3 (3.0, 8.3)	4.5 (2.3, 7.5)	3.8 (1.5, 6.8)
BMI (kg^2/m^2)	25.0 (22.9, 27.4)	27.2 (24.9, 30.1)	28.8 (26.0, 31.7)
Waist (cm)	89 (81, 97)	98 (90, 105)	103 (96, 112)
HbA1c (%)	5.35 (5.17, 5.63)	5.63 (5.35, 5.90)	6.54 (6.08, 7.09)

	Normal glucose metabolism, N = 2,389	Prediabetes, N = 538	Type 2 Diabetes, N = 746
Fasting plasma glucose (mmol/L)	5.10 (4.80, 5.40)	5.90 (5.40, 6.30)	7.40 (6.60, 8.50)
LDL (mmol/L)	3.20 (2.70, 3.90)	3.30 (2.60, 4.00)	2.40 (1.80, 3.10)
HDL (mmol/L)	1.60 (1.30, 2.00)	1.40 (1.20, 1.80)	1.30 (1.00, 1.50)
Total cholesterol (mmol/L)	5.50 (4.80, 6.20)	5.50 (4.80, 6.30)	4.50 (3.90, 5.20)
Triglycerides (mmol/L)	1.05 (0.80, 1.45)	1.39 (1.03, 1.90)	1.51 (1.08, 2.14)
Duration of type-2 diabetes (only for diagnosed participants)	NA (NA, NA)	NA (NA, NA)	3 (0, 9)
Mean IBI (ms)	838 (775, 907)	815 (760, 897)	806 (744, 889)
SDNN (ms)	138 (117, 164)	127 (106, 152)	116 (96, 139)
RMSSD (ms)	26 (21, 34)	24 (19, 33)	22 (17, 31)
SDANN (ms)	125 (103, 149)	113 (92, 139)	103 (84, 127)
SDNNi (ms)	55 (46, 65)	50 (41, 60)	44 (36, 54)
pNN50 (%)	7 (3, 13)	5 (2, 10)	4 (2, 9)
TP (ms ²)	12,596 (8,880, 17,498)	10,615 (7,134, 15,374)	8,880 (6,064, 12,722)
ULF (ms ²)	10,771 (7,392, 15,142)	8,948 (5,852, 13,374)	7,524 (5,036, 11,001)
VLF (ms ²)	1,198 (833, 1,692)	1,015 (685, 1,478)	816 (541, 1,267)
LF (ms ²)	421 (257, 651)	328 (200, 540)	261 (154, 422)
HF (ms ²)	94 (57, 158)	78 (47, 138)	63 (36, 117)
Systolic blood pressure (mmHg)	123 (114, 133)	129 (122, 140)	130 (122, 139)
Diastolic blood pressure (mmHg)	75 (71, 80)	78 (73, 83)	76 (72, 81)
Mean arterial pressure (mmHg)	95 (88, 102)	99 (93, 107)	98 (92, 105)
Carotid artery distensibility (10-3/kPa)	15.0 (11.8, 18.8)	13.5 (10.4, 16.9)	12.5 (9.9, 16.0)
Carotid-femoral pulse wave velocity (m/s)	8.08 (7.28, 9.16)	8.96 (7.84, 10.32)	9.36 (8.16, 10.80)
Hypertension (Yes)	833 (35%)	317 (59%)	590 (79%)
Glucose-lowering medication (Yes)	0 (0%)	0 (0%)	519 (70%)
Antihypertensive medication (Yes)	431 (18%)	199 (37%)	478 (64%)

	Normal glucose metabolism, N = 2,389	Prediabetes, N = 538	Type 2 Diabetes, N = 746
Using beta-blockers (Yes)	149 (6.2%)	77 (14%)	195 (26%)
Lipid-lowering medication	280 (12%)	141 (26%)	484 (65%)

¹n (%); Median (IQR)

Table S4: Association between 24-hour HRV in the original unit and pulse wave velocity

HRV index	Model 1 PWV % (95% CI)	Model 2 PWV % (95% CI)
Mean IBI (ms)	-0.02255 (-0.02769; -0.017)	-0.02249 (-0.02771; -0.017)
SDNN (ms)	-0.07141 (-0.08689; -0.056)	-0.07189 (-0.08769; -0.056)
SDANN (ms)	-0.06527 (-0.08119; -0.049)	-0.06548 (-0.08166; -0.049)
SDNNi (ms)	-0.16548 (-0.20319; -0.128)	-0.16403 (-0.20266; -0.125)
RMSSD (ms)	-0.09565 (-0.13802; -0.053)	-0.09239 (-0.13495; -0.05)
pNN50 (%)	-0.13984 (-0.20466; -0.075)	-0.13592 (-0.20105; -0.071)
TP (ms ²)	-0.00035 (-0.00043; 0.000)	-0.00035 (-0.00043; 0.000)
HF (ms ²)	-0.00857 (-0.01357; -0.004)	-0.00826 (-0.01326; -0.003)
LF (ms ²)	-0.00445 (-0.00627; -0.003)	-0.00427 (-0.00612; -0.002)
VLF (ms ²)	-0.0034 (-0.00418; -0.003)	-0.00337 (-0.00416; -0.003)
ULF (ms ²)	-0.00035 (-0.00044; 0.000)	-0.00035 (-0.00045; 0.000)

Percentage CD per original unit increase in heart rate variability index and heart period intervals. Model 1: adjusted for sex, age, educational status, diabetes status, and mean arterial pressure. Model 2: Model 1 + physical activity, smoking behaviour, alcohol use, body mass index, hba1c, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication.

Table S5: Association between 24-hour HRV in the original unit and carotid distensibility

	Model 1	Model 2
HRV index	CD % (95% CI)	CD % (95% CI)
Mean IBI (ms)	0.0409 (0.02745; 0.054)	0.04214 (0.02858; 0.056)
SDNN (ms)	0.08919 (0.04849; 0.130)	0.08917 (0.04786; 0.130)
SDANN (ms)	0.08196 (0.04018; 0.124)	0.0814 (0.0391; 0.124)
SDNNi (ms)	0.18749 (0.08769; 0.287)	0.19231 (0.09109; 0.294)
RMSD (ms)	0.07784 (-0.02725; 0.183)	0.09135 (-0.01362; 0.196)
pNN50	0.10975 (-0.05103; 0.271)	0.12704 (-0.03355; 0.288)
TP (ms ²)	0.00041 (0.00019; 0.001)	0.00041 (0.00019; 0.001)
HF (ms ²)	0.00415 (-0.00829; 0.017)	0.00523 (-0.00716; 0.018)
LF (ms ²)	0.00575 (0.00088; 0.011)	0.00572 (0.00082; 0.011)
VLF (ms ²)	0.00327 (0.00118; 0.005)	0.00336 (0.00124; 0.005)
ULF (ms ²)	0.00043 (0.00019; 0.001)	0.00043 (0.00019; 0.001)

Percentage CD per original unit increase in heart rate variability index and heart period intervals. Model 1: adjusted for sex, age, educational status, diabetes status, and mean arterial pressure. Model 2: Model 1 + physical activity, smoking behaviour, alcohol use, body mass index, hba1c, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication.

Table S6: Association between 24-hour standardized HRV and pulse wave velocity by diabetes status

	PWV % (95% CI)	Interaction p-value
Heart period (ms)		
Normal glucose metabolism	-2.542 (-3.213; -1.866)	ref.
Predabetes	-1.85 (-3.163; -0.519)	0.35031
Type 2 Diabetes	-2.225 (-3.313; -1.126)	0.62224
SDNN (ms)		
Normal glucose metabolism	-1.745 (-2.406; -1.08)	ref.
Predabetes	-4.397 (-5.763; -3.012)	0.00065
Type 2 Diabetes	-3.725 (-4.899; -2.537)	0.00402
SDANN (ms)		
Normal glucose metabolism	-1.491 (-2.147; -0.831)	ref.
Predabetes	-4.012 (-5.361; -2.643)	0.00108
Type 2 Diabetes	-3.291 (-4.47; -2.097)	0.00916
SDNN index (ms)		
Normal glucose metabolism	-1.851 (-2.552; -1.146)	ref.
Predabetes	-3.914 (-5.264; -2.544)	0.00692
Type 2 Diabetes	-3.272 (-4.43; -2.1)	0.03746
RMSSD (ms)		
Normal glucose metabolism	-0.967 (-1.651; -0.278)	ref.
Predabetes	-2.011 (-3.269; -0.738)	0.15392
Type 2 Diabetes	-0.995 (-2.042; 0.063)	0.96501
pNN50 (%)		
Normal glucose metabolism	-0.974 (-1.647; -0.297)	ref.
Predabetes	-1.68 (-2.944; -0.4)	0.33419
Type 2 Diabetes	-1.095 (-2.241; 0.063)	0.85863
Time-domain Z-score		
Normal glucose metabolism	-2.053 (-2.863; -1.236)	ref.
Predabetes	-4.897 (-6.523; -3.243)	0.00222
Type 2 Diabetes	-3.467 (-4.83; -2.084)	0.08088
Total power (ms²)		
Normal glucose metabolism	-1.57 (-2.206; -0.93)	ref.
Predabetes	-4.336 (-5.735; -2.916)	0.00046
Type 2 Diabetes	-3.451 (-4.753; -2.132)	0.01121
HF (ms²)		
Normal glucose metabolism	-0.538 (-1.205; 0.133)	ref.
Predabetes	-1.882 (-3.234; -0.511)	0.08129
Type 2 Diabetes	-1.249 (-2.382; -0.103)	0.29055
LF (ms²)		
Normal glucose metabolism	-0.7 (-1.391; -0.004)	ref.
Predabetes	-3.425 (-4.795; -2.035)	4e-04
Type 2 Diabetes	-2.144 (-3.415; -0.855)	0.04795
VLF (ms²)		
Normal glucose metabolism	-1.946 (-2.605; -1.282)	ref.
Predabetes	-3.736 (-5.092; -2.36)	0.01869
Type 2 Diabetes	-3.243 (-4.537; -1.931)	0.07796

	PWV % (95% CI)	Interaction p-value
ULF (ms²)		
Normal glucose metabolism	-1.442 (-2.072; -0.807)	ref.
Prediabetes	-3.975 (-5.364; -2.566)	0.00125
Type 2 Diabetes	-3.273 (-4.582; -1.946)	0.01403
Frequency-domain Z-score		
Normal glucose metabolism	-1.968 (-2.778; -1.153)	ref.
Prediabetes	-5.652 (-7.367; -3.904)	0.00015
Type 2 Diabetes	-3.871 (-5.386; -2.333)	0.02979

Percentage PWV per SD increase in heart rate variability index and heart period intervals Adjustment from model 2 including age, sex, education, mean arterial pressure, physical activity, smoking, alcohol, body mass index, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication.

Table S7: Association between 24-hour standardized HRV and carotid distensibility by diabetes status

	CD % (95% CI)	Interaction p-value
Heart period (ms)		
Normal glucose metabolism	3.755 (1.818; 5.729)	ref.
Prediabetes	5.513 (2.119; 9.021)	0.37357
Type 2 Diabetes	5.663 (2.848; 8.555)	0.26882
SDNN (ms)		
Normal glucose metabolism	1.223 (-0.643; 3.124)	ref.
Prediabetes	7.175 (3.577; 10.898)	0.00349
Type 2 Diabetes	5.358 (2.347; 8.458)	0.0214
SDANN (ms)		
Normal glucose metabolism	1.16 (-0.691; 3.044)	ref.
Prediabetes	5.728 (2.293; 9.277)	0.02074
Type 2 Diabetes	4.76 (1.749; 7.86)	0.04525
SDNN index (ms)		
Normal glucose metabolism	1.338 (-0.632; 3.348)	ref.
Prediabetes	6.916 (3.28; 10.681)	0.00651
Type 2 Diabetes	3.944 (0.981; 6.993)	0.14548
RMSSD (ms)		
Normal glucose metabolism	0.311 (-1.461; 2.115)	ref.
Prediabetes	3.05 (0.15; 6.034)	0.11407
Type 2 Diabetes	1.386 (-1.187; 4.026)	0.50357
pNN50 (%)		
Normal glucose metabolism	0.15 (-1.589; 1.919)	ref.
Prediabetes	2.933 (0.015; 5.936)	0.10757
Type 2 Diabetes	1.64 (-1.131; 4.489)	0.37422
Time-domain Z-score		
Normal glucose metabolism	1.223 (-1.033; 3.53)	ref.
Prediabetes	8.049 (3.778; 12.495)	0.00486
Type 2 Diabetes	4.808 (1.316; 8.421)	0.09046
Total power (ms ²)		
Normal glucose metabolism	1.087 (-0.708; 2.915)	ref.
Prediabetes	6.053 (2.429; 9.807)	0.015
Type 2 Diabetes	5.35 (2.026; 8.782)	0.0261
HF (ms ²)		
Normal glucose metabolism	-0.517 (-2.228; 1.223)	ref.
Prediabetes	3.06 (-0.032; 6.247)	0.04629
Type 2 Diabetes	1.397 (-1.375; 4.247)	0.25123
LF (ms ²)		
Normal glucose metabolism	0.892 (-1.027; 2.849)	ref.
Prediabetes	4.128 (0.516; 7.869)	0.11249
Type 2 Diabetes	2.689 (-0.613; 6.101)	0.34982
VLF (ms ²)		
Normal glucose metabolism	1.258 (-0.641; 3.193)	ref.
Prediabetes	5.66 (2.01; 9.442)	0.03227
Type 2 Diabetes	3.494 (0.206; 6.89)	0.24368

ULF (ms^2)

Normal glucose metabolism	0.996 (-0.778; 2.801)	ref.
Prediabetes	5.535 (1.98; 9.213)	0.0241
Type 2 Diabetes	5.373 (2.027; 8.828)	0.02287
<hr/>		
Frequency-domain Z-score		
Normal glucose metabolism	1.186 (-1.098; 3.523)	ref.
Prediabetes	8.277 (3.61; 13.154)	0.0063
Type 2 Diabetes	5.313 (1.374; 9.405)	0.07353

Percentage CD per SD increase in heart rate variability index and heart period intervals Adjustment from model 2 including age, sex, education, mean arterial pressure, physical activity, smoking, alcohol, body mass index, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication.

Table S8: Association between 24-hour standardized HRV and pulse wave velocity by sex

	PWV % (95% CI)	Interaction p-value
Heart period (ms)		
Men	-1.853 (-2.538; -1.162)	ref.
Women	-3.159 (-3.997; -2.314)	0.01635
SDNN (ms)		
Men	-2.377 (-3.109; -1.639)	ref.
Women	-2.646 (-3.398; -1.889)	0.60266
SDANN (ms)		
Men	-2.095 (-2.829; -1.355)	ref.
Women	-2.312 (-3.055; -1.562)	0.67666
SDNN index (ms)		
Men	-2.041 (-2.769; -1.306)	ref.
Women	-3.012 (-3.823; -2.194)	0.06509
RMSSD (ms)		
Men	-1.012 (-1.711; -0.308)	ref.
Women	-1.316 (-2.093; -0.534)	0.56598
pNN50 (%)		
Men	-1.059 (-1.797; -0.316)	ref.
Women	-1.18 (-1.924; -0.43)	0.81918
Time-domain Z-score		
Men	-2.501 (-3.372; -1.622)	ref.
Women	-3.062 (-3.98; -2.134)	0.37062
Total power (ms²)		
Men	-2.044 (-2.777; -1.305)	ref.
Women	-2.432 (-3.18; -1.678)	0.45651
HF (ms²)		
Men	-0.717 (-1.476; 0.048)	ref.
Women	-1.049 (-1.782; -0.312)	0.532
LF (ms²)		
Men	-0.941 (-1.637; -0.24)	ref.
Women	-2.126 (-3.034; -1.209)	0.03287
VLF (ms²)		
Men	-2.04 (-2.729; -1.345)	ref.
Women	-3.027 (-3.884; -2.164)	0.06757
ULF (ms²)		
Men	-1.903 (-2.639; -1.162)	ref.
Women	-2.232 (-2.971; -1.486)	0.52879
Frequency-domain Z-score		
Men	-2.407 (-3.293; -1.512)	ref.
Women	-3.266 (-4.226; -2.297)	0.17941

Percentage PWV per SD increase in heart rate variability index and heart period intervals. Adjustment from model 2 including age, education, diabetes status, mean arterial pressure, physical activity, smoking, alcohol, body mass index, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication.

Table S9: Association between 24-hour standardized HRV and carotid distensibility by sex

	CD % (95% CI)	Interaction p-value
Heart period (ms)		
Men	5.87 (3.977; 7.797)	ref.
Women	2.489 (0.177; 4.853)	0.02581
SDNN (ms)		
Men	4.006 (2.021; 6.03)	ref.
Women	2.23 (0.136; 4.368)	0.21613
SDANN (ms)		
Men	3.228 (1.262; 5.232)	ref.
Women	2.293 (0.227; 4.402)	0.51292
SDNN index (ms)		
Men	4.253 (2.26; 6.285)	ref.
Women	1.113 (-1.13; 3.407)	0.03247
RMSD (ms)		
Men	2.788 (1.024; 4.582)	ref.
Women	-0.922 (-2.846; 1.04)	0.00546
pNN50 (%)		
Men	2.707 (0.848; 4.6)	ref.
Women	-0.65 (-2.496; 1.232)	0.01171
Time-domain Z-score		
Men	5.021 (2.662; 7.436)	ref.
Women	1.141 (-1.365; 3.711)	0.02393
Total power (ms²)		
Men	3.186 (1.209; 5.2)	ref.
Women	2.147 (0.045; 4.292)	0.47122
HF (ms²)		
Men	2.407 (0.523; 4.325)	ref.
Women	-1.254 (-3.089; 0.616)	0.00618
LF (ms²)		
Men	2.606 (0.698; 4.55)	ref.
Women	0.298 (-2.171; 2.83)	0.13317
VLF (ms²)		
Men	3.274 (1.366; 5.218)	ref.
Women	0.974 (-1.466; 3.473)	0.13568
ULF (ms²)		
Men	2.881 (0.907; 4.893)	ref.
Women	2.198 (0.134; 4.305)	0.6343
Frequency-domain Z-score		
Men	4.491 (2.058; 6.981)	ref.
Women	1.389 (-1.31; 4.162)	0.0849

Percentage CD per SD increase in heart rate variability index and heart period intervals. Adjustment from model 2 including age, education, diabetes status, mean arterial pressure, physical activity, smoking, alcohol, body mass index, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication.

Table S10: Sensitivity analysis: Association between 24-hour HRV and pulse wave velocity

Sub-group	Population size	PWV %	5%	95%
Heart period (ms)				
Main	3673	-0.0225	-0.0277	-0.0173
No beta-blocker medication	3252	-0.0208	-0.0265	-0.0151
No antihypertension medication	2565	-0.0189	-0.0250	-0.0127
No diabetes and antihypertension medication	1958	-0.0199	-0.0266	-0.0133
SDNN (ms)				
Main	3673	-0.0719	-0.0877	-0.0561
No beta-blocker medication	3252	-0.0701	-0.0864	-0.0537
No antihypertension medication	2565	-0.0642	-0.0820	-0.0465
No diabetes and antihypertension medication	1958	-0.0509	-0.0700	-0.0319
SDANN (ms)				
Main	3673	-0.0655	-0.0816	-0.0493
No beta-blocker medication	3252	-0.0641	-0.0808	-0.0474
No antihypertension medication	2565	-0.0586	-0.0767	-0.0404
No diabetes and antihypertension medication	1958	-0.0443	-0.0638	-0.0248
SDNN index (ms)				
Main	3673	-0.1639	-0.2026	-0.1253
No beta-blocker medication	3252	-0.1657	-0.2063	-0.1250
No antihypertension medication	2565	-0.1476	-0.1914	-0.1038
No diabetes and antihypertension medication	1958	-0.1311	-0.1786	-0.0836
RMSSD (ms)				
Main	3673	-0.0923	-0.1349	-0.0498
No beta-blocker medication	3252	-0.1111	-0.1564	-0.0657
No antihypertension medication	2565	-0.1045	-0.1546	-0.0542
No diabetes and antihypertension medication	1958	-0.0932	-0.1486	-0.0377

pNN50 (%)

Main	3673	-0.1359	-0.2010	-0.0707
No beta-blocker medication	3252	-0.1546	-0.2233	-0.0859
No antihypertension medication	2565	-0.1520	-0.2264	-0.0776
No diabetes and antihypertension medication	1958	-0.1510	-0.2330	-0.0689

Total power (ms²)

Main	3673	-0.0003	-0.0004	-0.0003
No beta-blocker medication	3252	-0.0003	-0.0004	-0.0003
No antihypertension medication	2565	-0.0003	-0.0004	-0.0002
No diabetes and antihypertension medication	1958	-0.0002	-0.0003	-0.0002

HF (ms²)

Main	3673	-0.0083	-0.0133	-0.0033
No beta-blocker medication	3252	-0.0096	-0.0148	-0.0043
No antihypertension medication	2565	-0.0088	-0.0145	-0.0032
No diabetes and antihypertension medication	1958	-0.0066	-0.0128	-0.0004

LF (ms²)

Main	3673	-0.0043	-0.0061	-0.0024
No beta-blocker medication	3252	-0.0046	-0.0065	-0.0026
No antihypertension medication	2565	-0.0040	-0.0061	-0.0020
No diabetes and antihypertension medication	1958	-0.0033	-0.0054	-0.0011

VLF (ms²)

Main	3673	-0.0034	-0.0042	-0.0026
No beta-blocker medication	3252	-0.0033	-0.0042	-0.0025
No antihypertension medication	2565	-0.0031	-0.0039	-0.0022
No diabetes and antihypertension medication	1958	-0.0029	-0.0038	-0.0020

ULF (ms²)

Main	3673	-0.0004	-0.0004	-0.0003
No beta-blocker medication	3252	-0.0003	-0.0004	-0.0003
No antihypertension medication	2565	-0.0003	-0.0004	-0.0002

No diabetes and antihypertension medication	1958	-0.0002	-0.0004	-0.0001
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Percentage PWV per original unit increase in heart rate variability index and heart period intervals

Main: Model 2 (adjusted for antihypertensive medication)

No beta-blocker medication: people using betablockers were excluded

No antihypertensive medication: people with antihypertensive medication was excluded

No antihypertensive medication and without diabetes: people with antihypertensive medication and diabetes was excluded

Table S11: Sensitivity analysis: Association between 24-hour HRV (in original unit) and carotid distensibility

Sub-group	Population size	CD %	5%	95%
Heart period (ms)				
Main	1802	0.0420	0.0284	0.0555
No beta-blocker medication	1563	0.0402	0.0252	0.0553
No antihypertension medication	1201	0.0366	0.0201	0.0530
No diabetes and antihypertension medication	846	0.0289	0.0092	0.0485
SDNN (ms)				
Main	1802	0.0887	0.0474	0.1300
No beta-blocker medication	1563	0.0968	0.0529	0.1407
No antihypertension medication	1201	0.0874	0.0388	0.1361
No diabetes and antihypertension medication	846	0.0499	-0.0077	0.1076
SDANN (ms)				
Main	1802	0.0809	0.0387	0.1232
No beta-blocker medication	1563	0.0913	0.0465	0.1361
No antihypertension medication	1201	0.0852	0.0353	0.1352
No diabetes and antihypertension medication	846	0.0572	-0.0017	0.1161
SDNN index (ms)				
Main	1802	0.1902	0.0890	0.2915
No beta-blocker medication	1563	0.1772	0.0670	0.2876
No antihypertension medication	1201	0.1515	0.0317	0.2714
No diabetes and antihypertension medication	846	0.0695	-0.0728	0.2120
RMSD (ms)				
Main	1802	0.0915	-0.0134	0.1965
No beta-blocker medication	1563	0.0712	-0.0444	0.1869
No antihypertension medication	1201	0.1012	-0.0289	0.2315
No diabetes and antihypertension medication	846	0.0436	-0.1123	0.1998
pNN50 (%)				
Main	1802	0.1274	-0.0330	0.2881
No beta-blocker medication	1563	0.1098	-0.0630	0.2829
No antihypertension medication	1201	0.1362	-0.0555	0.3283
No diabetes and antihypertension medication	846	0.0560	-0.1743	0.2868
Total power (ms²)				
Main	1802	0.0004	0.0002	0.0006
No beta-blocker medication	1563	0.0004	0.0002	0.0007
No antihypertension medication	1201	0.0004	0.0002	0.0007
No diabetes and antihypertension medication	846	0.0003	0.0000	0.0006
HF (ms²)				
Main	1802	0.0053	-0.0070	0.0177
No beta-blocker medication	1563	0.0045	-0.0087	0.0177
No antihypertension medication	1201	0.0099	-0.0047	0.0246
No diabetes and antihypertension medication	846	0.0038	-0.0138	0.0214

LF (ms²)				
Main	1802	0.0056	0.0007	0.0105
No beta-blocker medication	1563	0.0047	-0.0006	0.0100
No antihypertension medication	1201	0.0043	-0.0013	0.0099
No diabetes and antihypertension medication	846	0.0017	-0.0048	0.0081
VLF (ms²)				
Main	1802	0.0033	0.0012	0.0054
No beta-blocker medication	1563	0.0031	0.0008	0.0054
No antihypertension medication	1201	0.0026	0.0002	0.0050
No diabetes and antihypertension medication	846	0.0010	-0.0018	0.0038
ULF (ms²)				
Main	1802	0.0004	0.0002	0.0007
No beta-blocker medication	1563	0.0005	0.0002	0.0007
No antihypertension medication	1201	0.0004	0.0002	0.0007
No diabetes and antihypertension medication	846	0.0003	0.0000	0.0006

Percentage CD per original unit increase in heart rate variability index and mean heart period intervals

Main: model 2 (adjusted for antihypertensive medication)

No beta-blocker medication: people using betablockers were excluded

No antihypertensive medication: people with antihypertensive medication were excluded

No antihypertensive medication and without diabetes: people with antihypertensive medication and diabetes were excluded

Table S12: Association between 24-hour standardized HRV and pulse wave velocity

HRV index	Model 1 PWV % (95% CI)	Model 2 PWV % (95% CI)
Mean IBI (ms)	-2.373 (-2.906; -1.838)	-2.366 (-2.908; -1.822)
SDNN (ms)	-2.492 (-3.024; -1.957)	-2.508 (-3.051; -1.962)
SDANN (ms)	-2.195 (-2.724; -1.664)	-2.202 (-2.739; -1.662)
SDNNi (ms)	-2.487 (-3.045; -1.925)	-2.465 (-3.037; -1.890)
RMSSD (ms)	-1.189 (-1.711; -0.664)	-1.148 (-1.673; -0.621)
pNN50	-1.151 (-1.681; -0.619)	-1.119 (-1.652; -0.584)
Time-domain Z-score	-2.787 (-3.431; -2.139)	-2.766 (-3.42; -2.106)
TP (ms ²)	-2.235 (-2.766; -1.701)	-2.234 (-2.773; -1.692)
HF (ms ²)	-0.922 (-1.456; -0.385)	-0.888 (-1.423; -0.35)
LF (ms ²)	-1.412 (-1.984; -0.836)	-1.357 (-1.938; -0.773)
VLF (ms ²)	-2.442 (-2.990; -1.890)	-2.416 (-2.975; -1.854)
ULF (ms ²)	-2.069 (-2.596; -1.539)	-2.067 (-2.601; -1.529)
Frequency-domain Z-score	-2.819 (-3.487; -2.146)	-2.798 (-3.477; -2.113)

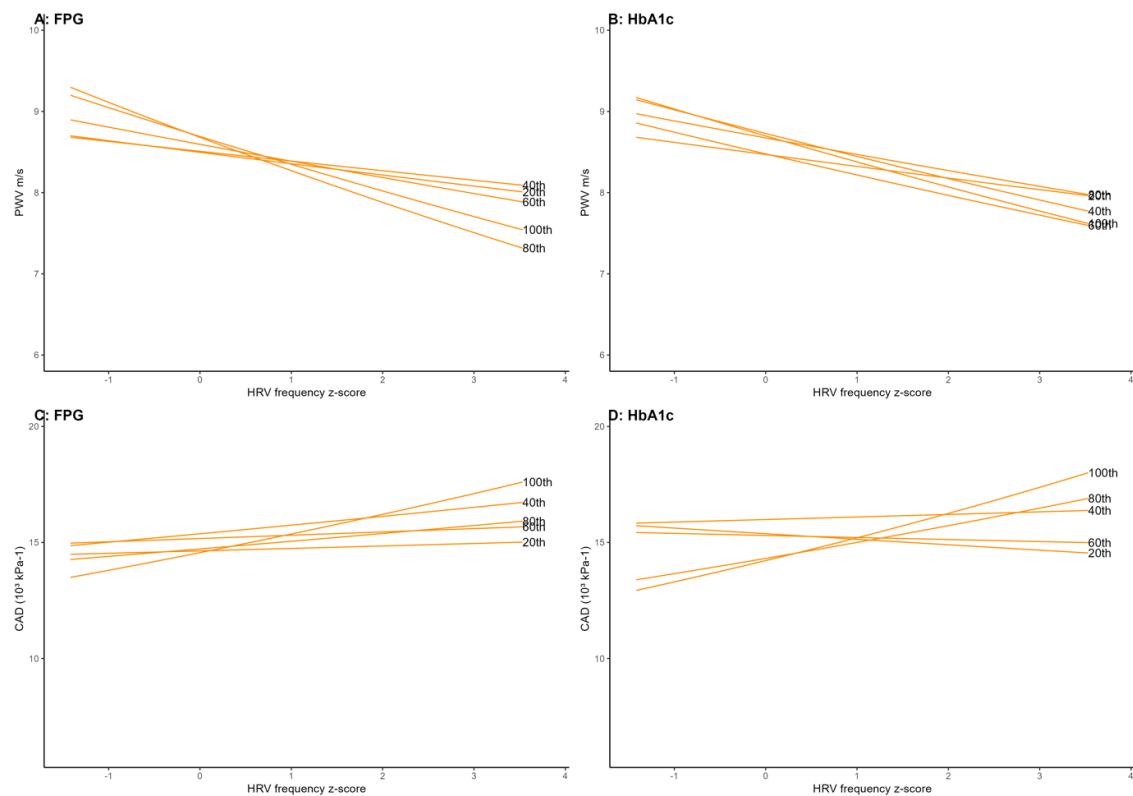
Percentage PWV per SD increase in heart rate variability index and heart period intervals Model 1: adjusted for sex, age, educational status, diabetes status, and mean arterial pressure. Model 2: Model 1 + physical activity, smoking behaviour, alcohol use, body mass index, hba1c, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication.

Table S13: Association between 24-hour standardized HRV and carotid distensibility

HRV index	Model 1	Model 2
	CD % (95% CI)	CD % (95% CI)
Mean IBI (ms)	4.45 (2.966; 5.956)	4.588 (3.09; 6.107)
SDNN (ms)	3.199 (1.727; 4.692)	3.198 (1.705; 4.714)
SDANN (ms)	2.824 (1.375; 4.294)	2.805 (1.338; 4.293)
SDNNi (ms)	2.889 (1.342; 4.46)	2.964 (1.394; 4.559)
RMSSD (ms)	0.977 (-0.34; 2.312)	1.148 (-0.17; 2.483)
pNN50	0.912 (-0.422; 2.263)	1.056 (-0.277; 2.407)
Time-domain Z-score	3.162 (1.397; 4.959)	3.284 (1.500; 5.100)
TP (ms ²)	2.696 (1.226; 4.187)	2.724 (1.237; 4.232)
HF (ms ²)	0.449 (-0.892; 1.809)	0.567 (-0.77; 1.922)
LF (ms ²)	1.857 (0.282; 3.457)	1.847 (0.263; 3.455)
VLF (ms ²)	2.405 (0.858; 3.976)	2.471 (0.908; 4.058)
ULF (ms ²)	2.564 (1.112; 4.037)	2.574 (1.108; 4.061)
Frequency-domain Z-score	3.098 (1.229; 5.001)	3.184 (1.295; 5.109)

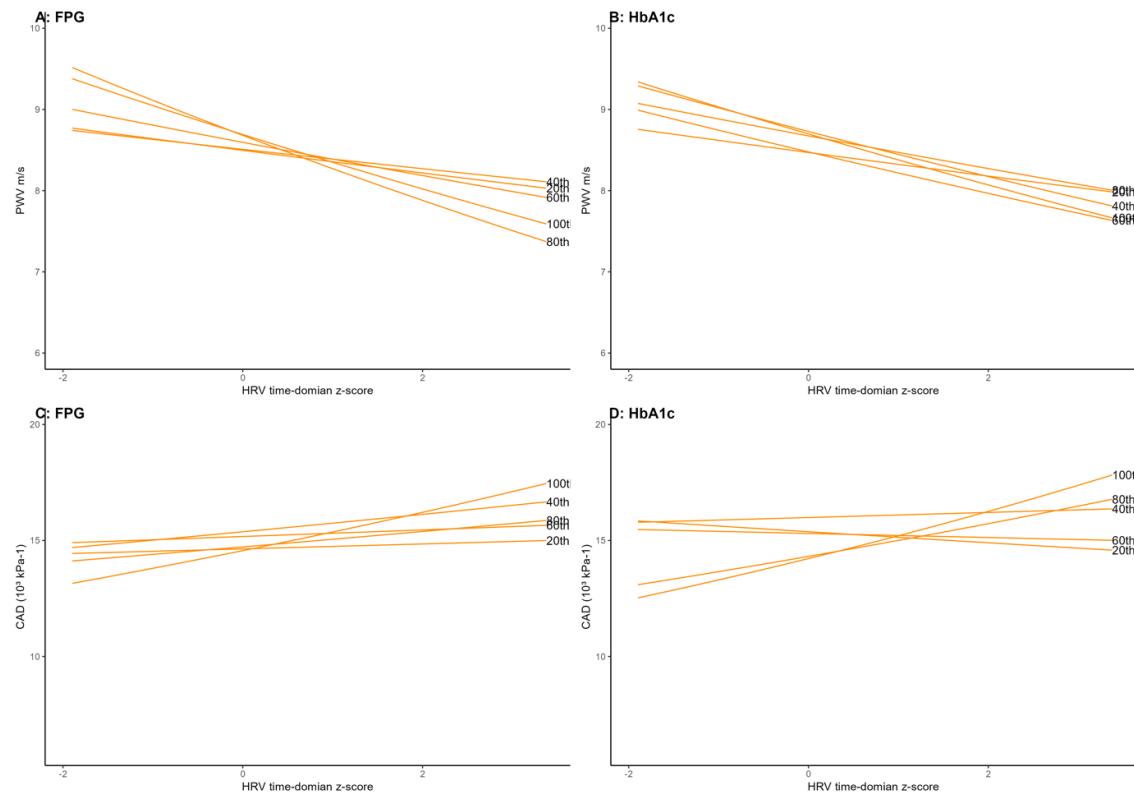
Percentage CD per SD increase in heart rate variability index and heart period intervals. Model 1: adjusted for sex, age, educational status, diabetes status, and mean arterial pressure. Model 2: Model 1 + physical activity, smoking behaviour, alcohol use, body mass index, hba1c, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication.

Figure S1: Association between 24-hour HRV frequency-domain Z-score and aortic (n= 3154) and carotid (n= 1653) stiffness stratified by glucose percentiles in a subpopulation without known type 2 diabetes



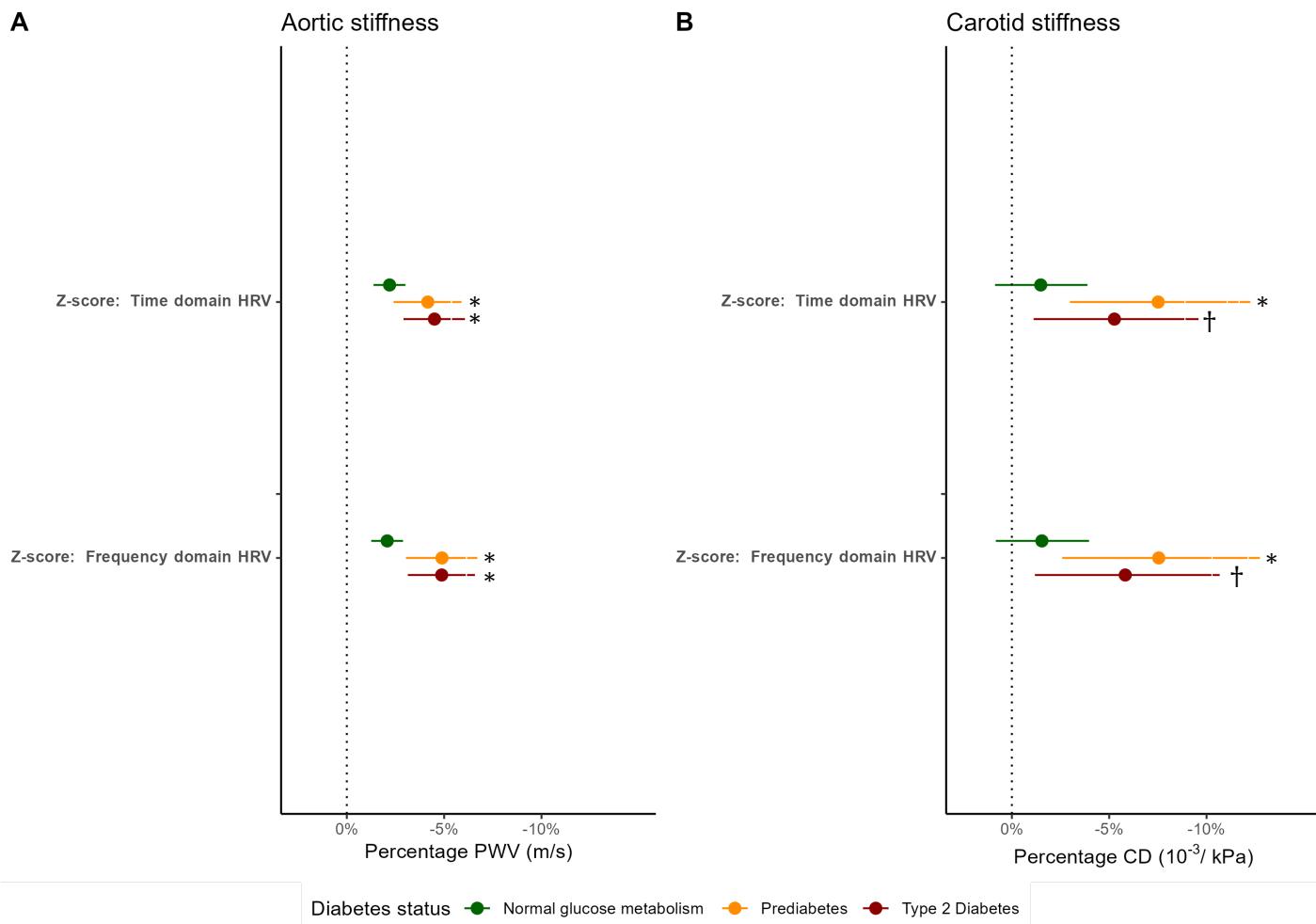
Adjusted for age, sex, and mean arterial pressure

Figure S2: Association between 24-hour time-domain Z-score and aortic (n= 3154 and carotid (n= 1653) stiffness stratified by glucose percentiles in a subpopulation without known type 2 diabetes



Adjusted for age, sex, and mean arterial pressure

Figure S3: Association between 24-hour standardized HRV and arterial stiffness modified by diabetes status without users of beta-blockers



A: Percentage PWV per SD in time-domain and frequency-domain composite z-score by diabetes status **B:** Percentage CD per SD in time-domain and frequency-domain composite z-score by diabetes status. Estimates are adjusted for sex, age, educational status, mean arterial pressure, physical activity, smoking behaviour, alcohol use, body mass index, HbA1c, triglycerides, total-to-high density lipoprotein cholesterol ratio, lipid-modifying- and antihypertensive medication. Normal glucose metabolism was defined as reference group.

*Interaction term p-value < 0.05

+Interaction term p-value < 0.10

A.2. Study II

Cardiovascular autonomic dysfunction precedes cardiovascular disease and all-cause mortality: 11-year follow-up in the ADDITION-PRO study

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Abbreviations

CVD: Cardiovascular disease

MACE: Three-point major adverse cardiovascular events

ECG: Electrocardiogram

HRV: Heart rate variability

rHR: Resting heart rate

SDNN: The standard deviation of normal-to-normal R-R intervals

mHR: Mean heart rate

IRR: Incidence rate ratio

PAEE: Physical activity energy expenditure

Abstract

Aim

We aim to determine the impact of multiday heart rate variability (HRV) on the risk of major adverse cardiovascular events (MACE), heart failure, and mortality in people at high risk of diabetes.

Materials and Methods

Multiday HRV and mean heart rate (mHR) were measured in 1,627 participants from the ADDITION-PRO study between 2009-2011. As measurement for HRV, we calculated a proxy for standard deviation of normal heartbeat (SDNN) both weekly, daily and hourly. Data on MACE and all-cause mortality were obtained from Danish patient registers until 2021. We fitted Poisson regression to determine incidence rate ratios (IRR) for MACE (myocardial infarction, stroke, cardiovascular death), heart failure, and all-cause mortality.

Results

Mean (SD) age was 66 years (7), and 47 % were women. The population had a mean (SD) multiday SDNN of 139.0 (32.3) milliseconds. Multiday HRV index SDNN showed an IRR of 0.82 (CI: 0.69; 0.97), 0.76 (CI: 0.58; 0.99), and 0.79 (CI: 0.66; 0.94) per SD for MACE, heart failure, and all-cause mortality, respectively. SDNN measurements taken from 6:00-7:00 AM showed the strongest association with the risk of MACE. Lower SDNN was associated with all-cause mortality across all hours of the day. Adjustment for physical acceleration and heart rate did not materially change the magnitude of these associations.

Conclusion

Cardiovascular autonomic dysfunction, measured by multiday HRV, is linked with MACE, heart failure, and all-cause mortality. Certain time frames of the day for HRV and heart rate under free-living conditions showed higher risk of cardiovascular disease.

Introduction

Over the past decades, improved treatment and prevention of cardiovascular disease (CVD) have led to lower rates of ischemic events and better post-intervention outcomes in high-income countries [1].

However, the aging population is experiencing more vascular structural changes and cardiac remodeling [2], which can lead to heart failure and subsequent lower quality of life and shorter life expectancy [3]. As acute cardiovascular events, heart failure, and early mortality are still major health concerns, we need to continue to improve early monitoring and identification of individuals with high risk of CVD.

Recent attention has been directed toward individuals at high risk of diabetes, who also face an increased risk of CVD and mortality [4]. Subclinical indicators of diabetes-related microvascular and macrovascular complications can be present in individuals with pre-diabetes or high diabetes risk [5, 6]. As these people do not have a clinical diabetes diagnosis, they often remain outside of structured clinical management.

Cardiovascular autonomic dysfunction (autonomic dysfunction), also known as cardiovascular autonomic neuropathy, can be detected in people with pre-diabetes and is pronounced with diabetes [7]. Autonomic dysfunction increases the risk of both CVD and mortality [8, 9]. Heart rate variability (HRV) is recognized as an indicator of cardiovascular autonomic function, as it quantifies the degree to which the sinoatrial node, which receives input from the autonomic nervous system can modulate the heart rate in response to various circumstances [10]. Studies have demonstrated that autonomic dysfunction (assessed by short period electrocardiograms (ECG)) is linked with CVD [8]. Fewer studies have investigated the association between long-term (> 24-hour) HRV and CVD [8]. Multiple days of HRV recording may capture an average of cardiovascular autonomic responses under regular free-living conditions that are less influenced by a person's random activity during a particular day i.e. physical activity, emotion and sleep [11]. In addition, specific time-frames during circadian variation of HRV may be associated with CVD [12].

We aimed to determine the association between multiday HRV and the risk of incident CVD, heart failure, and all-cause mortality in a population with high risk of diabetes. Secondly, we wanted to identify the hours of the day with the strongest association between HRV and CVD, heart failure and all-cause mortality while accounting for the impact of concurrent physical acceleration and heart rate.

We hypothesized that 1) multiday HRV measures capture HRV patterns associated with risk of CVD events and that 2) the risk of CVD varies between hourly HRV measurements throughout the day.

Materials and Methods

Study population

Participants in the ADDITION-PRO prospective observational study were recruited between 2009 and 2011 from the Danish arm of the ADDITION-Europe study (ADDITION-DK) through a stepwise screening program for type 2 diabetes in primary care [13]. Ethical approval for the ADDITION-PRO study was obtained from the scientific ethics committee of the Central Denmark Region (Reference No. 20000183). The study was conducted in accordance with the Helsinki Declaration, and all participants provided oral and written informed consent for participation and for linkage of their data with national registers. ADDITION-PRO served as the follow-up health examination for individuals at high risk of developing diabetes [14]. The stratification of type 2 diabetes risk in ADDITION-DK was carried out using a Danish diabetes risk score questionnaire [13]. Participants were requested to report information about known risk factors for type 2 diabetes, including age, sex, BMI, known hypertension, family history of type 2 diabetes, gestational diabetes, and leisure time physical activity [15]. Those with a risk score of 5 points or more (out of a maximum of 15 points) were invited to attend a stepwise screening program, which included measures of random blood glucose levels and glycated hemoglobin A1c (HbA1c), a fasting blood glucose test (FPG), and an oral glucose tolerance test (OGTT). The World Health Organization criteria was utilized to diagnose type 2 diabetes [16]. The sampling frame for ADDITION-PRO included participants categorized into groups of increasing type 2 diabetes risk based on their diabetes risk score and glycemic status: low type 2 diabetes risk (less than 5 points on the diabetes risk score); high type 2 diabetes risk (5 or more points on the diabetes risk score) with normoglycemia, isolated impaired fasting glucose (IFG), isolated impaired glucose tolerance (IGT), or both IFG and IGT. A total of 2,082 individuals consented to participate in the ADDITION-PRO health examination, forming the baseline for this study [14]. Individuals with prior CVD events within 10 years before inclusion in ADDITION-PRO were excluded from this analysis. In the present study, we included participants with a valid HRV recording based on at least 48 hours of data and complete information on selected confounders, as described below.

Heart Rate Variability

Heart rate was measured using a combination of an accelerometer and heart rate monitor (ActiHeart, CamNTech, Cambridge, UK). This monitor records uniaxial acceleration and heart rate. The procedure of data collection and processing has been previously described [17]. From the ActiHeart, mean heart rates with prediction intervals were obtained every 30-second epoch. HRV is measured as the beat-to-beat variation between normal heart beat intervals on an electrocardiogram (ECG)[10]. Heart rate processing and calculations of HRV are fully described in supplemental material. Using the RHRV (version 4.2.7) package in R, we calculated HR and HRV indices [18]. We included the standard deviation between normal-to-normal heartbeat intervals (SDNN), the standard deviation of the 5-minute average NN intervals (SDANN), the SDNN index (SDNNi), and the triangular interpolation of NN interval histogram (TINN), and mean HR (mHR). The algorithm for these indices has been tested on a dataset with full 24-hour interbeat intervals (IBI), yielding high validity for global distributed HRV indices [19]. All HRV indices were calculated by up to a week, 24-hour cycle, and for each hour of the day. As our data covered multiple days, we based our 24-hour and hour of the day indices on means across all cycles. As heart rate exhibits cycles longer than two days and up to one week, we defined the measurements as multiday-HRV. To reduce the influence of resting heart rate (rHR) on HRV (a lower rHR allows for greater variability [20]), we pre-adjusted HRV for rHR during the clinical visit using the residual method. Resting pulse rate recordings at study visit were regressed on the logarithm (to obtain normality of residuals) of HRV. We then added the residuals from the model to the intercept and transformed back into the original unit. As autonomic dysfunction may itself elevate rHR, we present both unadjusted and rHR-adjusted HRV results to explore how associations change when accounting for the heart-rate ceiling effect. In order to test the impact of concurrent heart rate and physical acceleration on hourly HRV, we used the same method to pre-adjusted HRV for concurrent mHR and then further included physical acceleration in each particular hour in the pre-adjustment models.

Outcomes

Information on CVD events and mortality, as well as all-cause mortality, was obtained from the Danish National Patient Registers until 2021. ICD-10 diagnosis codes for stroke, myocardial infarction, cardiovascular death, cardiovascular revascularization, and heart failure are described in supplementary material. We defined three outcomes: 1) three-point major adverse cardiovascular events (MACE), including fatal and non-fatal myocardial infarction, fatal and non-fatal stroke, cardiovascular revascularization, and cardiovascular death; 2) hospital-diagnosed heart failure; and 3) all-cause mortality.

Covariates

All covariates were measured at baseline. Lifestyle factors, including smoking (current/ ex-smoker/ never) and alcohol consumption (average units per week), as well as CVD history and use of anti-hypertensive, glucose-lowering, and lipid-lowering medications, were obtained through a self-reported questionnaire. Physical activity energy expenditure kilojoule (KJ) per day (PAEE) was estimated based on combined accelerometry and heart rate data from ActiHeart recordings [17] and by the Recent Physical Activity Questionnaire (RPAQ). The hourly physical acceleration was based on accelerometer (m/s^2) data alone. Blood measurements of HbA1c, OGTT, FPG, triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol were derived from blood samples. Body mass index (BMI), waist circumference, and systolic and diastolic blood pressure were measured during the participant's clinical examination [14]. From the Danish registers, we collected information on CVD events in the 10 years prior to baseline and socioeconomic status at baseline (length of education, income, work status).

Statistical Analysis

Baseline characteristics were described using mean and standard deviation (SD) for continuous variables and numbers (%) for categorical variables. Individual risk time was determined from the time-point of baseline data collection in ADDITION-PRO (2009-2011) until the time-point of CVD, death, or end of follow-up (31 December 2021).

Analysis of multiday HRV

We used Poisson regression models to investigate the association between multiday SDNN and MACE, as well as hospitalized heart failure and all-cause mortality. SDNN and mHR were standardized by their mean and standard deviation. We fitted three models based on assumptions about potential confounding pathways summarized in directed acyclic graphs (DAG) (**Figure S2**). Model 1 included simple adjustments for age and sex. Model 2 accounted for confounding pathways visualized in a DAG including further adjustment for education, alcohol consumption, smoking behavior, physical activity (PAEE calculated from RPAQ), body mass index, total cholesterol, and HbA1c. We used RPAQ to adjust for habitual physical activity and to avoid overadjustment from PAEE estimated from overlapping heart rate data. In model 3, we included systolic blood pressure, anti-hypertensive and glucose-lowering medications to account for use of medication and risk by elevated blood pressure. We performed analyses with HRV pre-adjusted for resting heart rate and included analyses of multiday mHR for comparison. To investigate non-linearity of the associations, we used splines, defining knots-based percentiles in the HRV and mHR distribution. We performed additional analyses including HRV indices SDANN, SDNNi, TINN for the multiday recording and the mean across multiple 24-hour cycles of each HRV. We stratified analyses by sex and tested for interaction effects, considering p-values < 0.05 as statistically significant. These results are shown in the supplementary material (Table 2S and 3S).

Analysis of Hourly HRV

We used Poisson regression models to investigate the association between hourly SDNN and MACE, hospitalization for heart failure, and all-cause mortality. We fitted two models based on assumptions from DAG (**Figure S3**). Each SDNN and mHR per hour was standardized by its mean and standard deviation. Model 1 included adjustments for age and sex. Model 2 was further adjusted for education, alcohol consumption, smoking behavior, PAEE (RPAQ calculated), body mass index, total cholesterol, and HbA1c. To test the influence of concurrent heart rate and physical acceleration, we performed analyses with mHR, physical acceleration, and heart rate and physical acceleration pre-adjusted HRV. All analyses were performed using multiple imputation chained equations to impute missing covariates, in the R statistical computing environment (version 4.2.2).

Results

From the entire cohort, 1,627 (78%) participants had no prior CVD and multiday HRV measured and 1,432 had all hours represented with HRV with concurrent physical acceleration within a full-day (**Figure S1**). The study population included 53% men with a mean (SD) age of 66 (7) years and a mean BMI of 28 (5) kg/m² at baseline. The mean multiday SDNN was 139.0 (32.3) milliseconds (ms) and the heart rate was 73.5 (9.1) bpm. Hourly mean (SD) for SDNN, heart rate, and physical acceleration are presented in supplementary material (**Figure S4**). Forty-six percent had hypertension. Further characteristics of the participants are provided in **Table 1**. In total, the study population was followed for 17,926 person-years (Individual mean follow-up: 11.0 years). There were 172 incident cases of CVD defined as MACE (10.1 per 1000 person-years. Of these, one event was a hemorrhagic stroke, indicating that the majority of MACE events were ischemic in origin. 160 died (8.9 per 1000 person-years), and 71 received a hospital diagnosis of heart failure (4.0 per 1000 person-years).

Multiday HRV index SDNN and mean heart rate association with major adverse cardiovascular events, heart failure and all-cause mortality

In our main analysis (model 2), 1 SD higher multiday SDNN was associated with a 0.83 (CI: 0.70; 0.98) incidence rate ratio (IRR) for MACE, 0.76 (CI: 0.58; 0.99) for heart failure and 0.79 (CI: 0.66; 0.94) for all-cause mortality (**Table 2**). Further adjustments for anti-hypertensive medication, systolic blood pressure, glucose-lowering medications, and pre-adjustment for resting heart rate did not materially change the estimates, except for heart failure where the association was partly attenuated. When examining association by splines, we observed a higher IRR for MACE, heart failure, and all-cause mortality when SDNN was below approximately 120 ms (**Figure 1**). We observed no further reduction in IRR from levels of SDNN above 120 ms. The IRR of the whole week cycle was comparable with the mean of multiple 24-hour cycles of SDNN. Other HRV indices TINN, SDANN and SDNNi showed similar tendencies (**Table S2 and S3**). We found no significant sex-specific differences.

In model 2, 1 SD mHR was associated with a 1.34 (CI: 1.07; 1.68) higher IRR of heart failure. Multiday mHR did not show an association with MACE (1.07 [CI: 0.92; 1.25]) or all-cause mortality (1.12 [CI: 0.96; 1.31]). We observed a nonlinear association i.e. the IRR for heart failure and all-cause mortality were

observed at mHR levels above 80 bpm (**Figure 1**). The threshold for higher risk of MACE was higher (92 bpm).

Hourly SDNN and mean HR association with major adverse cardiovascular events, heart failure and all-cause mortality

When SDNN and mHR were divided into hourly segments during a day, we observed differences in risk with MACE, heart failure, and all-cause mortality across the 24-hours (**Figure 3**).

Major adverse cardiovascular events

Per SD, across hourly time periods, SDNN showed similar lower risk of MACE, except for SDNN measured between 6:00 - 07:00 AM, which showed a lower adjusted IRR (0.80 [CI: 0.67; 0.95]) (**Figure 2B**). Pre-adjusting SDNN for concurrent mHR and physical acceleration slightly attenuated the results. mHR showed the strongest association with MACE between 04:00-06:00 AM e.g. per SD in bpm measured showed an adjusted IRR at 05:00-06:00 AM, 1.19 (CI: 1.01; 1.40).

Hospital-diagnosed heart failure

Measurements taken in the morning (from 7:00 - 10:00 AM) showed inverse associations between higher SDNN and IRR of heart failure. These trends were diminished after SDNN was preadjusted for concurrent physical acceleration and heart rate. Higher mHR measured during the night (from 2:00 - 5:00 AM) was associated with high IRR of heart failure (**Figure 2L**).

All-cause mortality

The association between higher SDNN and lower all-cause mortality rate ratio (MRR) was consistent across the 24 hours with a range of an adjusted MRR between 0.78 (CI: 0.65; 0.94) and 0.89 (CI: 0.74; 1.06). These associations were slightly attenuated after pre-adjustment for mHR and physical acceleration (**Figure 2Y**). mHR showed similar consistent trends association across 24-hours, where higher mHR was associated with higher risk of mortality (**Figure 3S**) but was attenuated after further adjustments.

Discussion

Higher HRV index SDNN, assessed over a full week, is linked with a 17%, 24%, and 21% risk reduction per SD for MACE, heart failure, and all-cause mortality, respectively. The association showed a higher risk when SDNN values were below 120 ms. When the HRV periods were divided into hourly cycles, lower SDNN measured between 6:00-7:00 AM showed an association with a higher risk of MACE, whereas no particular time point had an exceptional association with all-cause MRR. Pre-adjusting hourly SDNN for concurrent physical acceleration and heart rate did not materially change the magnitude of these hourly associations. Also, higher mHR showed a higher risk of MACE and heart failure for measurements taken during the night hours from 02:00-06:00 AM.

We investigated multiday HRV in order to capture autonomic responses in free-living conditions over multiple days. Our findings align with previous studies linking lower HRV to cardiovascular events and mortality but extend this evidence to a high risk of diabetes population using both multiday and hourly recordings, identifying specific time frames of heightened risk [21, 22]. We consider it a strength to use recordings of HRV during multiple days and propose that such HRV measures are likely to contain more robust indications of individual autonomic responses to day-to-day situations. The trends of the association between the mean 24-hour HRV across multiple days compared to the complete multiday HRV were similar. Thus, both multiday and mean 24-hour HRV recordings can be used from the multiday measurement.

The link between autonomic dysfunction and both ischemic events and heart failure, may be attributed to an adverse cardiometabolic environment, and thus these risks are more pronounced in populations with high risk of diabetes and overt diabetes [23, 24]. People with autonomic dysfunction, measured by a low multiday HRV, have a less adaptive autonomic nervous system response during the full day and night. Some of these dysadaptations seem to be more pronounced during specific hours of the day.

Our results underscore the notion that autonomic dysfunction is not only linked with a high risk of CVD, but also a higher risk of all-cause mortality. Autonomic dysfunction might reflect a poor autonomic nervous adaptation, by parasympathetic impairment and sympathetic hyperresponsiveness, that affects adaptability

in certain target organs e.g. the heart [25, 26]. Our results highlight that autonomic dysfunction in the morning, measured by lower HRV, is the strongest hourly indicator for higher risk of acute CVD endpoints and cardiovascular mortality. In the morning hours, the heart needs to make its biggest adaptation with the increase in sympathetic activity as the body experiences a peak in cortisol and changes from a longer rest to rise and movement [27]. Interestingly, lower HRV levels throughout the day (i.e. sleeping, waking, responding to physical movement, stress) are all indicative of mortality risk. The link between autonomic dysfunction with fatal and non-fatal CVD might be attributed to the arrhythmogenesis [26]. Why low HRV is linked with non-cardiovascular related death remains to be explored in future studies.

Lower multiday HRV might be an indicator of autonomic dysfunction driven by more chronic sympathetic dominance that leads to poorer deceleration of heart rate, changes of hemodynamics, and direct arterial constriction, and thus higher cardiac workload [28]. Hence, in the long term, autonomic dysfunction might cause pathological cardiac and arterial remodeling, which in turn increases the risk of ischemic events and heart failure [24, 29]. Our findings show that higher heart rate in the night hours is a strong indicator for both heart failure, CVD, and mortality. These findings may highlight the reduced parasympathetic activity during the sleeping/resting hours leading to minor changes in heart rate during rest. The explanation of the sympathetic overactivity is complex in whether the underlying cause is vagus nerve damage or a compensating sympathetic mechanism to keep sufficient ejection fraction [7, 30]. Therefore, we cannot exclude that higher heart rate might be an early indicator for progression to heart failure, as loss of stroke volume needs compensation by higher sympathetic activity, leading to higher heart rate that is needed throughout the day and night.

Recordings of long-term HRV are both influenced by habitual physical activity and actual physical activity during the measurement of HRV [11, 31]. Participants in ADDITION-PRO generally had low physical activity levels during the multiday recording [32]. When we included preadjusted hourly HRV for both heart rate and physical activity in the concurrent hour, the associations were slightly attenuated, but the trends of the associations were unchanged. Thus, we conclude that the association between higher HRV and lower CVD and mortality risk, is not solely explained by higher physical activity during the measurement time

frame. Data from NHANES suggests that the timing of physical activity influences diabetes risk [33]. Our findings build on this perspective by demonstrating how hourly heart rate and its variability in response to free-living conditions are linked to CVD risk. Further studies on autonomic responses to physical movement at specific times of the day may improve our understanding of CVD risk in free-living conditions.

We prespecified our DAG to close confounding pathways and avoid over-adjustments. The measurable confounding in the associations was mostly attributable to lifestyle factors, BMI, and biochemical markers. No material changes were observed when adding adjustments for systolic blood pressure, anti-hypertensive, and glucose-lowering medication. Pre-adjusting SDNN for baseline heart rate had a clearer impact on the IRR for heart failure, supported by the observed association between multiday mHR and heart failure. Thus, our findings suggest that heart rate serves as a marker of the development of heart failure, potentially reflecting either a predisposition due to a less healthy heart or the subtle progression of heart failure [34, 35]. The proportion explained between HRV and our outcomes showed that 25% of the SDNN association with heart failure was explained by baseline heart rate, compared to 14% for hard CVD events and 19% for mortality. Hence, this might underscore differences in the degree to which heart rate is an indicator of pre-clinical stages of the two outcomes. Therefore, we kept heart failure and the composite MACE as separate outcomes.

SDNN was included as our main determinant because it is the most frequently employed HRV index [10]. Participants in the ADDITION-PRO were invited based on their high risk of diabetes and have been followed up over a decade [14]. Therefore, our results highlight the potential use of measuring multiday heart rate and its variability for assessing incident CVD in populations with high risk of diabetes. Further studies are needed to determine whether these associations are valid in the general population, and in which risk and age groups HRV proves as a potential marker of CVD risk.

Our results demonstrate that in a population with a high risk of diabetes, assessing cardiovascular autonomic function may capture valuable knowledge of clinical relevance. In the current study population, the impact of one SD (33 ms) lower multiday SDNN was of equivalent magnitude to 4.5 additional years of

aging for MACE risk and to 2.2-2.4 years for heart failure and all-cause mortality. Focus on cardiometabolic risk factor management could potentially lead to lower CVD and all-cause mortality risk by improving HRV. Findings support the notion but remain inconclusive due to the lack of trial evidence from drug or exercise interventions demonstrating a mediating role of HRV in the cardiometabolic prevention of CVD [36-38]. People living with high risk of diabetes can effectively modify their cardiometabolic risk profile through increased physical activity, which in addition can lead to improved autonomic function [39, 40]. Therefore, HRV and heart rate are dynamic and responsive modifiable markers which potentially could be used to monitor potential successfulness in CVD risk management [12].

Our findings are based on events and causes of death from Danish national registries. Misclassification of CVD and undercapture of heart failure may have introduced bias, potentially leading to an underestimation of the observed associations [41, 42]. Echocardiographic assessments at baseline and follow-up could have addressed issues of identification and classification of heart failure. A key strength is the comprehensive adjustment for confounders, particularly both habitual and accelerometer-measured physical activity. However, residual confounding remains possible, for example from inflammation, which may affect both HRV and CVD. While multiple testing may introduce type 1 error when testing independent hypotheses, we interpret our hourly and long-term HRV findings as different evaluations of the same overarching hypothesis and find consistent results.

Over the past years, dynamic cardiovascular measures of heart rate and HRV have become more accessible by wearable devices [43]. Therefore, revisiting the use of these dynamic measures as a potential tool in risk management has become more relevant. Wearable devices have potential to improve health monitoring and facilitate targeted and individualized intervention based on physiological data [44]. We showed that both long-term and hourly mHR and HRV contain relevant information about CVD and mortality risk. For example, morning HRV may represent an indicator for morning autonomic response that is stronger linked to MACE compared to the rest of the day response and thus gives a notion for risk assessing time point in free living condition. Hence, monitoring heart rate and HRV could help us take early action when cardiovascular autonomic function deteriorates.

Cardiovascular autonomic dysfunction, expressed by lower multiday HRV, is associated with higher risk of CVD and all-cause mortality in people with high risk of diabetes. There is heterogeneity in the associations across the hours of the day under free-living conditions that are not explained by physical acceleration and heart rate. Thus, long-term HRV and the diurnal autonomic response may capture different risks. Whether long-term HRV has potential as an effective modifiable marker or a prognostic indicator in higher risk populations, and where in the cardiovascular prevention trajectory it may play a role need further definition.

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Conflicts of interests

ELG reports the following general conflicts of interest: ELG has received speaker honoraria or consultancy fees from AstraZeneca, Bayer, Boehringer Ingelheim, Bristol-Myers Squibb, Pfizer, Novo Nordisk, Lundbeck Pharma and Organon. He is an investigator in clinical studies sponsored by AstraZeneca, Idorsia or Bayer and has received unrestricted research grants from Boehringer Ingelheim. DV has received research grants from Bayer A/S, Sanofi Aventis, Novo Nordisk A/S, and Boehringer Ingelheim and holds shares in Novo Nordisk A/S. The remaining authors declare no conflicts of interest related to this manuscript.

Authors' contributions

Study concept and design: JRS, DRW, LB, DV, ELG, CSH. Contributed to the data: DRW, DV, AS. Planning the statistical analysis: JRS, DRW, LB. Conducted the statistical analysis: JRS, LB, DRW. JRS wrote the initial draft of the manuscript. All authors contributed to, critically revised, and approved the final version of the manuscript. JRS is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1: Baseline characteristics

	Without CVD or HF event N = 1,398	With CVD or HF event N = 227
Sex		
Men	722 (52%)	144 (63%)
Women	676 (48%)	83 (37%)
Age (years)	65.6 (6.9)	68.0 (6.3)
Education (years)		
<=10 years	257 (19%)	48 (21%)
>= 15 years	450 (33%)	63 (28%)
10-15 years	672 (49%)	113 (50%)
Smoking status		
Current	225 (16%)	38 (17%)
Prior	639 (46%)	111 (49%)
Never	521 (38%)	77 (34%)
Physical activity energy expenditure (KJ / day)	53.2 (24.8)	52.4 (26.5)
Alcohol consumption (units per week)	9.1 (9.4)	9.7 (9.6)
BMI (kg/m ²)	27.6 (4.7)	28.2 (4.7)
Waist circumference (cm)	96.4 (13.3)	98.8 (13.6)
Systolic blood pressure (mmHg)	133.2 (17.2)	136.3 (17.7)
Diastolic blood pressure (mmHg)	81.8 (10.3)	82.6 (10.7)
Pulse rate (bpm)	67.2 (10.7)	68.2 (12.1)
HbA1c (%)	5.8 (0.5)	5.9 (0.6)
Triglycerides (mmol/L)	1.3 (0.7)	1.4 (0.8)
Total cholesterol (mmol/L)	5.4 (1.1)	5.3 (1.1)
HDL cholesterol (mmol/L)	1.6 (0.4)	1.5 (0.4)
LDL cholesterol (mmol/L)	3.2 (1.0)	3.2 (1.0)
Urine albumin-creatinine ratio (mg/g)	22.6 (117.6)	45.7 (201.6)
Use glucose lowering medication (yes)	197 (14%)	31 (14%)
Use anti-hypertensive medication (yes)	617 (44%)	136 (60%)

	Without CVD or HF event N = 1,398	With CVD or HF event N = 227
Use diuretics medication (yes)	233 (17%)	54 (24%)
Use beta blockers medication (yes)	146 (11%)	42 (19%)
Use calcium channel blockers medication (yes)	183 (13%)	53 (23%)
Use ACE inhibitors medication (yes)	373 (27%)	85 (38%)
Multiday standard deviation of all NN intervals (ms)	139.6 (32.0)	135.2 (33.6)
Multiday standard deviation of the averages of NN intervals in 5-minute segments (ms)	116.5 (34.8)	113.1 (36.0)
Multiday mean of the standard deviation for all 5 minutes segments (ms)	54.8 (29.6)	52.8 (27.7)
Multiday mean HR (bpm)	73.4 (9.0)	73.8 (9.8)

n (%); Mean (SD)

Table 2: Multiday SDNN and mean HR risk with major adverse cardiovascular events, heart failure, and all-cause mortality

	Model 1 IRR (95% CI)	Model 2 IRR (95% CI)	Model 3 IRR (95% CI)
Major adverse cardiovascular events			
SDNN	0.80 (0.68; 0.94)	0.83 (0.70; 0.98)	0.83 (0.71; 0.99)
SDNN pre-adjusted for rHR	0.82 (0.70; 0.97)	0.85 (0.73; 1.00)	0.86 (0.74; 1.01)
Mean HR	1.09 (0.94; 1.26)	1.05 (0.90; 1.23)	1.06 (0.91; 1.24)
Hospital diagnosed heart failure			
SDNN	0.72 (0.56; 0.93)	0.76 (0.58; 0.99)	0.77 (0.59; 1.00)
SDNN pre-adjusted for rHR	0.79 (0.62; 1.01)	0.81 (0.63; 1.04)	0.83 (0.65; 1.05)
Mean HR	1.41 (1.14; 1.74)	1.34 (1.07; 1.68)	1.38 (1.10; 1.72)
All-cause mortality			
SDNN	0.69 (0.58; 0.82)	0.79 (0.66; 0.94)	0.80 (0.67; 0.95)
SDNN pre-adjusted for rHR	0.75 (0.63; 0.88)	0.84 (0.71; 0.99)	0.85 (0.72; 1.00)
Mean HR	1.23 (1.06; 1.42)	1.12 (0.96; 1.31)	1.14 (0.97; 1.32)

Incidence rate ratio for all-cause mortality and cardiovascular disease endpoints per SD in SDNN, HR corrected SDNN, and mHR over a week. Model 1: adjusted for age and sex; Model 2: model 1 + education, alcohol consumption, smoking behavior, physical activity, body mass index, total cholesterol, and Hba1c; Model 3: model 2 + systolic blood pressure, anti-hypertensive, and glucose-lowering medication.

Tables and figures

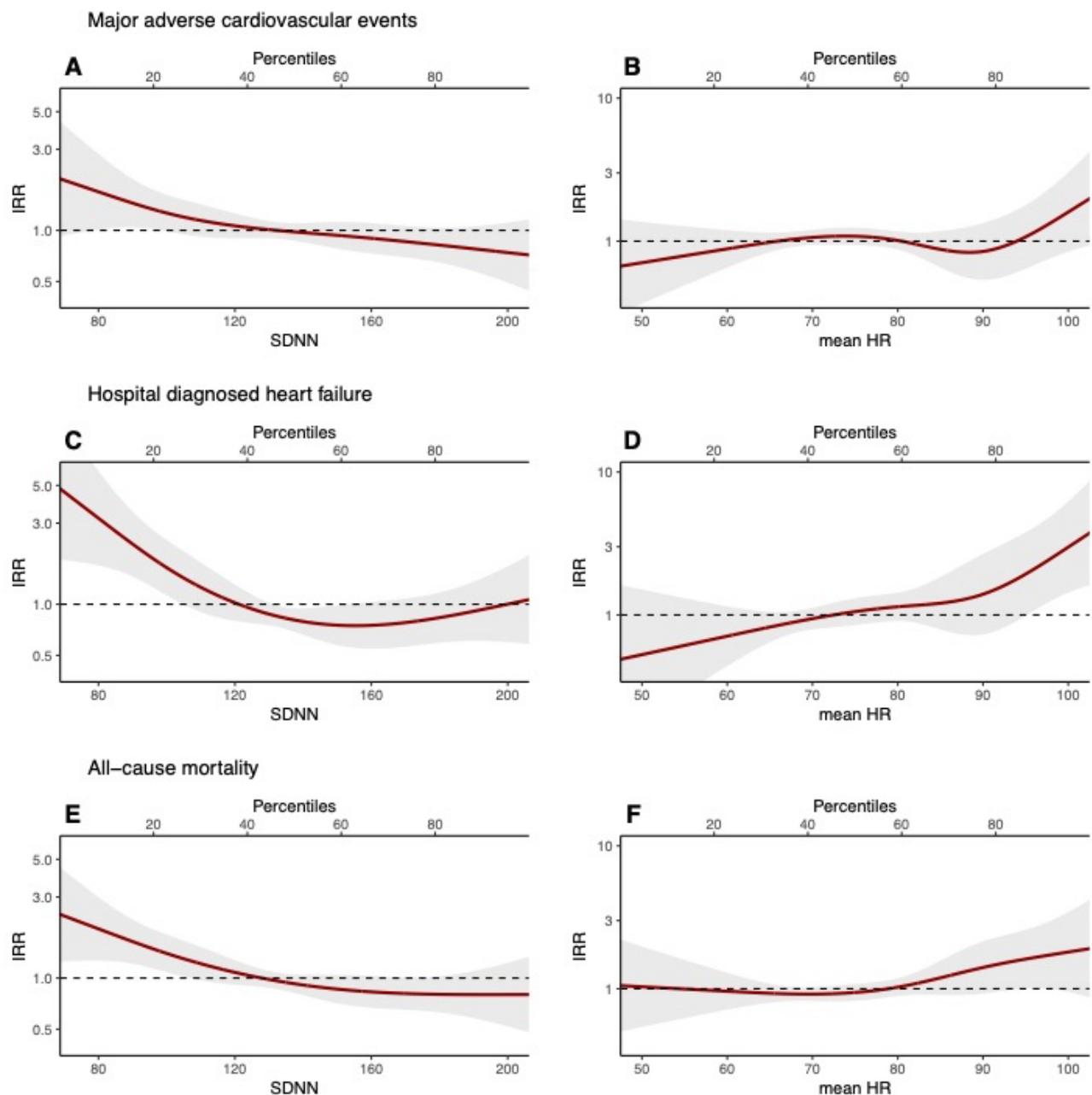
Table 1: Baseline characteristics

Table 2: Multiday SDNN and mean HR risk with Major adverse cardiovascular events, heart failure, and all-cause mortality

Figure 1: Multiday SDNN and mean HR association with major adverse cardiovascular events, heart failure, and all-cause mortality

Figure 2: Diurnal heart rate variability and heart rate association with major adverse cardiovascular events, heart failure, and all-cause mortality risk

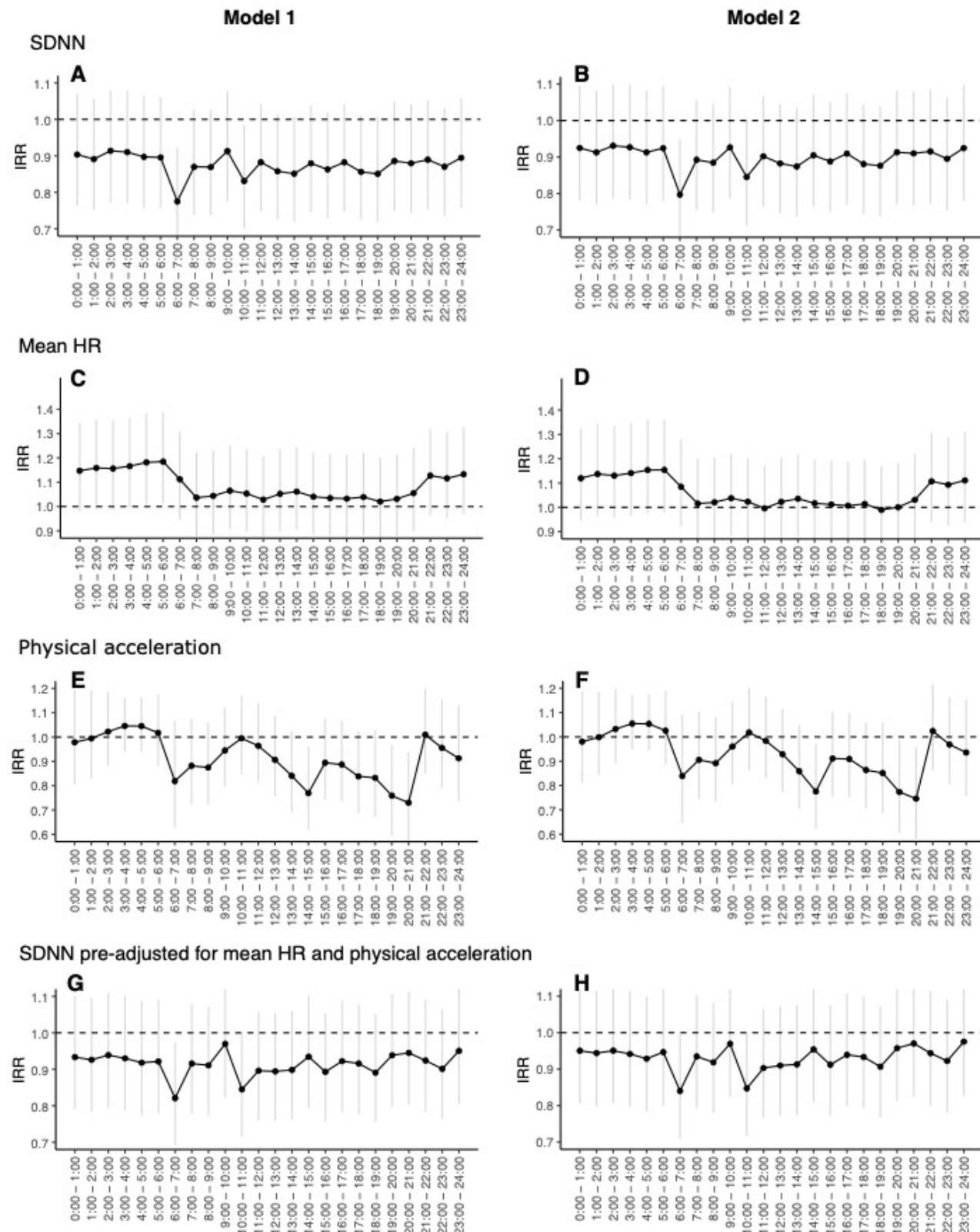
Figure 1: Multiday SDNN and mean HR association with major adverse cardiovascular events, heart failure, and all-cause mortality



Association between multiday SDNN / mHR and MACE, hospital-diagnosed heart failure, and all-cause mortality. IRR are adjusted for age and sex, education, alcohol consumption, smoking behavior, physical activity, body mass index, total cholesterol, and Hba1c.

Figure 2: Diurnal heart rate variability and heart rate association with major adverse cardiovascular events, heart failure, and all-cause mortality risk

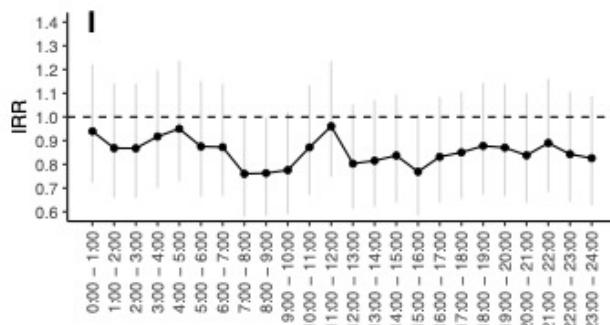
Major adverse cardiovascular events



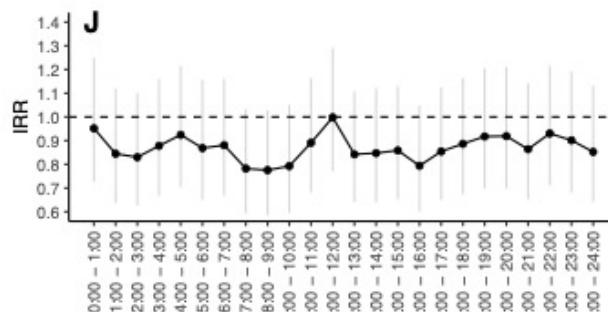
Hospital diagnosed heart failure

Model 1

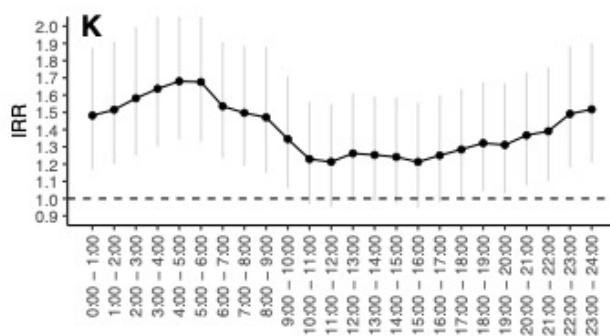
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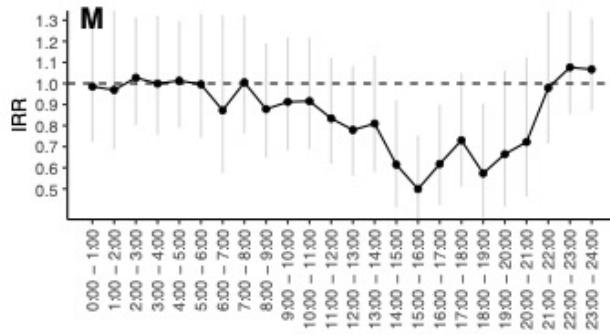
Model 2



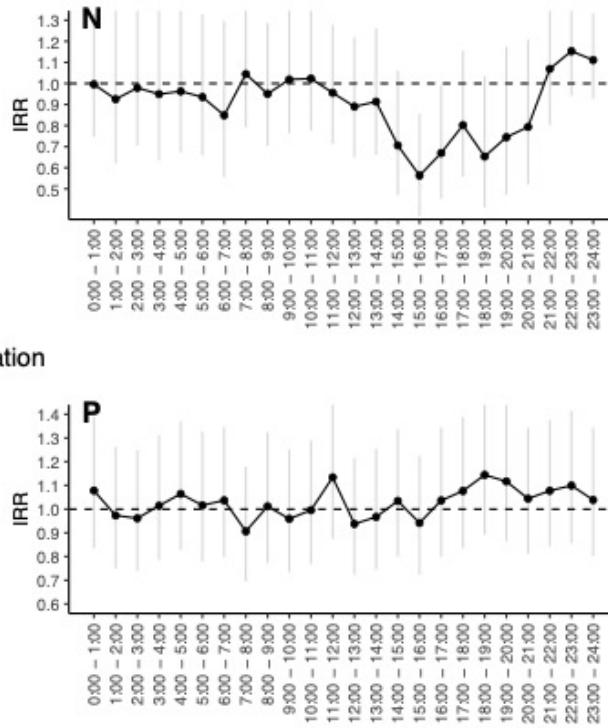
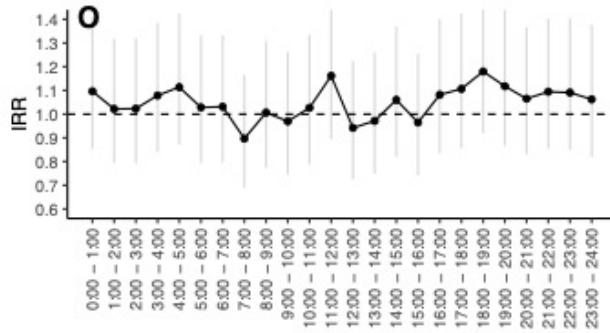
Mean HR



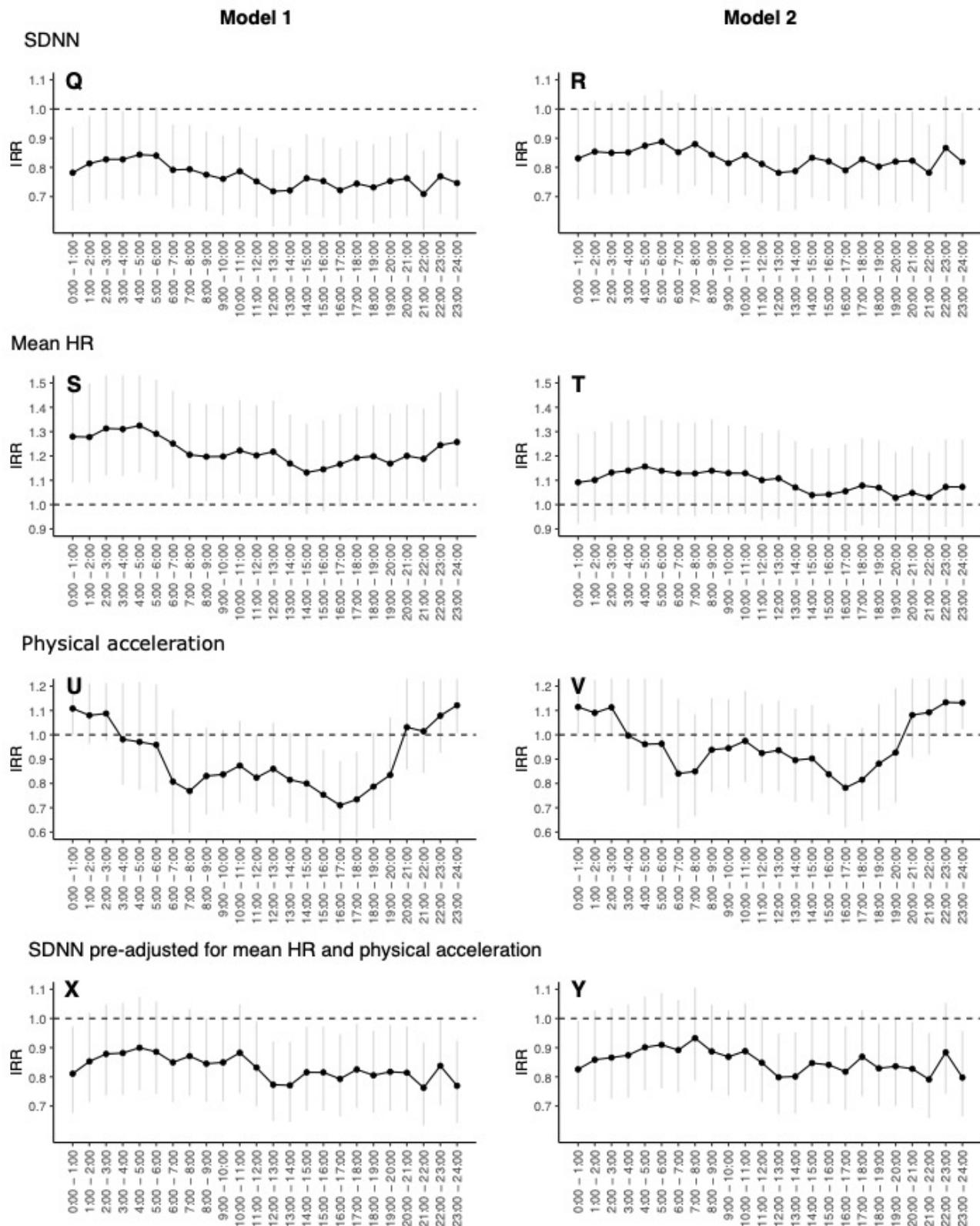
Physical acceleration



SDNN pre-adjusted for mean HR and physical acceleration



All-cause mortality



SDNN, mHR, physical acceleration (PA), and preadjusted SDNN for concurrent physical acceleration and heart rate were measured each hour starting from 00:00 to 24:00. The figures (A-Y) are showing IRR of MACE, heart failure, and all-cause mortality per SD increase by each measurement across hour specific timeframes. Model 1: adjusted for

age and sex; Model 2: model 1 + education, alcohol consumption, smoking behavior, body mass index, total cholesterol, and Hba1c.

Supplementary material:

Cardiovascular autonomic dysfunction precedes cardiovascular disease and all-cause mortality: 11-year follow-up in the ADDITION-PRO study

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Heart rate variability based on 30-seconds mean heart rate and 95% prediction interval

We did not have access to the time series of successive normal to normal inter-beat intervals (IBI), also known as interbeat intervals (IBI), during the measurement period. Therefore, we generated random normal distribution IBIs for every 30-second interval based on the 30-second epoch of mean heart rate and prediction intervals. As earlier studies have shown that IBIs are normally distributed per 30-second epoch, we generated the IBI 30-second distribution using mean heart rate and standard deviation. To calculate SD from prediction intervals, we ensured that the prediction intervals differed symmetrically from the mean by calculating the difference between the upper and lower prediction intervals from the mean heart rate and visually observing their symmetry over time. Using the RHRV (version 4.2.7) package in R, we calculated HRV indices [1]. As we did not have successive time-series measurements, we only used HRV indices based on the distribution of RR intervals, available in time-domain and geometrical HRV indices [2].

Reference:

1. Martínez CAG, Quintana AO, Vila XA, Touriño MJL, Rodríguez-Liñares L, Presedo JMR, et al. Heart rate variability analysis with the R package RHRV. 2017.
2. Schaarup J: Actiheart validation of time-domain heart rate variability.
https://figshare.com/articles/online_resource/Actiheart_validation_of_time-domain_heart_rate_variability/26182361 (2024). Accessed.

Table S1: Diagnosis codes for cardiovascular events

We defined CVD events by including ICD-10 diagnostic codes for stroke (ICD: I61 - I64) (SKA: KAAL10, KAAL11, KPAQ10, KPAQ20, KPAQ21), myocardial infarction (ICD: I21-I24), heart failure (ICD: I50), and cardiovascular death (ICD: I20-I28, I42, I46, I50), and surgical codes for cardiovascular revascularization (SKA: KPAE10, KPAE25, KPAF10, KPAF20, KPAF21, KPAF22, KPAH10, KPAH20, KPAH21, KPEE, KPEF, KPEH, KPEP, KPEQ, KPFE, KPFH, KPFP, KPFQ).

Type of CVD event	Diagnosis codes
Stroke	ICD: I61 - I64
Myocardial infarction	ICD: I21-I24
Heart failure	ICD: I50
Cardiovascular death	ICD: I20-I28, I42, I46
Cardiovascular revascularization	SKA: KPAE10, KPAE25, KPAF10, KPAF20, KPAF21, KPAF22, KPAH10, KPAH20, KPAH21, KPEE, KPEF, KPEH, KPEP, KPEQ, KPFE, KPFH, KPFP, KPFQ

Table S2: Week-long HRV indices and mean HR association with major adverse cardiovascular events, heart failure, and all-cause mortality

	Model 1 IRR (95% CI)	Model 2 IRR (95% CI)	Model 3 IRR (95% CI)
Five-point MACE: AMI + Stroke + HF + All-cause mortality			
SDNN	0.80 (0.71; 0.89)	0.85 (0.76; 0.96)	0.86 (0.77; 0.97)
SDNN pre-adjusted for rHR	0.84 (0.75; 0.94)	0.89 (0.80; 1.00)	0.90 (0.81; 1.01)
SDANN	0.87 (0.78; 0.97)	0.92 (0.82; 1.03)	0.93 (0.83; 1.04)
SDANN pre-adjusted for rHR	0.91 (0.82; 1.01)	0.96 (0.86; 1.07)	0.97 (0.87; 1.07)
SDNNIDX	0.89 (0.80; 0.99)	0.91 (0.82; 1.02)	0.91 (0.81; 1.01)
SDNNIDX pre-adjusted for rHR	0.92 (0.82; 1.02)	0.93 (0.83; 1.03)	0.92 (0.83; 1.03)
TINN	0.81 (0.72; 0.90)	0.87 (0.78; 0.97)	0.87 (0.78; 0.98)
TINN pre-adjusted for rHR	0.85 (0.76; 0.94)	0.91 (0.81; 1.01)	0.91 (0.82; 1.02)
Mean HR	1.17 (1.05; 1.30)	1.11 (1.00; 1.24)	1.12 (1.01; 1.25)
All-cause mortality			
SDNN	0.69 (0.58; 0.82)	0.79 (0.66; 0.94)	0.80 (0.67; 0.95)
SDNN pre-adjusted for rHR	0.75 (0.63; 0.88)	0.84 (0.71; 0.99)	0.85 (0.72; 1.00)
SDANN	0.80 (0.68; 0.94)	0.91 (0.77; 1.07)	0.92 (0.78; 1.08)
SDANN pre-adjusted for rHR	0.85 (0.73; 1.00)	0.94 (0.81; 1.10)	0.95 (0.82; 1.12)
SDNNIDX	0.82 (0.69; 0.97)	0.85 (0.72; 1.00)	0.84 (0.71; 1.00)
SDNNIDX pre-adjusted for rHR	0.87 (0.73; 1.02)	0.88 (0.75; 1.04)	0.87 (0.74; 1.03)
TINN	0.72 (0.61; 0.85)	0.83 (0.70; 0.99)	0.84 (0.71; 0.99)
TINN pre-adjusted for rHR	0.77 (0.66; 0.90)	0.88 (0.75; 1.03)	0.88 (0.75; 1.04)
Mean HR	1.23 (1.06; 1.42)	1.12 (0.96; 1.31)	1.14 (0.97; 1.32)
Four-point MACE: AMI + Stroke + HF + CV Death			
SDNN	0.84 (0.73; 0.96)	0.87 (0.75; 1.00)	0.87 (0.76; 1.01)
SDNN pre-adjusted for rHR	0.87 (0.76; 1.00)	0.90 (0.78; 1.03)	0.91 (0.79; 1.04)
SDANN	0.89 (0.77; 1.01)	0.91 (0.79; 1.05)	0.92 (0.80; 1.06)
SDANN pre-adjusted for rHR	0.92 (0.81; 1.06)	0.95 (0.83; 1.09)	0.95 (0.84; 1.09)
SDNNIDX	0.92 (0.80; 1.05)	0.93 (0.81; 1.06)	0.93 (0.81; 1.06)
SDNNIDX pre-adjusted for rHR	0.93 (0.82; 1.06)	0.94 (0.82; 1.07)	0.93 (0.82; 1.07)
TINN	0.85 (0.74; 0.97)	0.88 (0.76; 1.01)	0.88 (0.77; 1.02)
TINN pre-adjusted for rHR	0.89 (0.78; 1.01)	0.91 (0.80; 1.05)	0.92 (0.80; 1.05)
Mean HR	1.13 (0.99; 1.28)	1.10 (0.96; 1.26)	1.11 (0.97; 1.27)
Three-point MACE: AMI + Stroke + CV Death			
SDNN	0.80 (0.68; 0.94)	0.83 (0.70; 0.98)	0.83 (0.71; 0.99)
SDNN pre-adjusted for rHR	0.82 (0.70; 0.97)	0.85 (0.73; 1.00)	0.86 (0.74; 1.01)
SDANN	0.87 (0.75; 1.02)	0.90 (0.77; 1.06)	0.91 (0.77; 1.06)
SDANN pre-adjusted for rHR	0.90 (0.78; 1.05)	0.93 (0.79; 1.08)	0.93 (0.80; 1.09)
SDNNIDX	0.87 (0.74; 1.02)	0.89 (0.76; 1.04)	0.89 (0.75; 1.04)
SDNNIDX pre-adjusted for rHR	0.88 (0.75; 1.04)	0.90 (0.77; 1.06)	0.90 (0.77; 1.05)

	Model 1	Model 2	Model 3
	IRR (95% CI)	IRR (95% CI)	IRR (95% CI)
TINN	0.83 (0.71; 0.97)	0.86 (0.73; 1.02)	0.87 (0.74; 1.03)
TINN pre-adjusted for rHR	0.86 (0.74; 1.01)	0.90 (0.77; 1.05)	0.90 (0.77; 1.06)
Mean HR	1.09 (0.94; 1.26)	1.05 (0.90; 1.23)	1.06 (0.91; 1.24)
Hospital-diagnosed heart failure			
SDNN	0.72 (0.56; 0.93)	0.76 (0.58; 0.99)	0.77 (0.59; 1.00)
SDNN pre-adjusted for rHR	0.79 (0.62; 1.01)	0.81 (0.63; 1.04)	0.83 (0.65; 1.05)
SDANN	0.75 (0.59; 0.96)	0.81 (0.63; 1.04)	0.83 (0.64; 1.06)
SDANN pre-adjusted for rHR	0.83 (0.65; 1.05)	0.87 (0.68; 1.10)	0.88 (0.70; 1.12)
SDNNIDX	0.94 (0.74; 1.19)	0.93 (0.73; 1.18)	0.92 (0.72; 1.17)
SDNNIDX pre-adjusted for rHR	0.96 (0.76; 1.22)	0.93 (0.73; 1.18)	0.92 (0.72; 1.17)
TINN	0.68 (0.53; 0.87)	0.72 (0.55; 0.93)	0.72 (0.56; 0.93)
TINN pre-adjusted for rHR	0.73 (0.57; 0.93)	0.75 (0.59; 0.96)	0.76 (0.60; 0.97)
Mean HR	1.41 (1.14; 1.74)	1.34 (1.07; 1.68)	1.38 (1.10; 1.72)

Incidence rate ratio for all-cause mortality and cardiovascular disease endpoints per SD in SDNN, HR pre-adjusted SDNN, and mHR over a week. Model 1: adjusted for age and sex; Model 2: model 1 + education, alcohol consumption, smoking behavior, physical activity, body mass index, total cholesterol, and Hba1c; Model 3: model 2 + systolic blood pressure, anti-hypertensive, and glucose-lowering medication.

Table S3: Mean 24-hour HRV indices and mean HR association with major adverse cardiovascular events, heart failure, and all-cause mortality

	Model 1	Model 2	Model 3
	IRR (95% CI)	IRR (95% CI)	IRR (95% CI)
Five-point MACE: AMI + Stroke + HF + All-cause mortality			
SDNN	0.80 (0.71; 0.89)	0.85 (0.75; 0.95)	0.85 (0.76; 0.96)
SDNN pre-adjusted for rHR	0.83 (0.74; 0.92)	0.87 (0.78; 0.97)	0.88 (0.79; 0.98)
SDANN	0.88 (0.79; 0.98)	0.92 (0.83; 1.03)	0.93 (0.83; 1.04)
SDANN pre-adjusted for rHR	0.91 (0.81; 1.01)	0.94 (0.85; 1.05)	0.95 (0.86; 1.06)
SDNNIDX	0.89 (0.80; 1.00)	0.91 (0.82; 1.02)	0.91 (0.81; 1.01)
SDNNIDX pre-adjusted for rHR	0.92 (0.82; 1.02)	0.93 (0.83; 1.03)	0.92 (0.83; 1.03)
TINN	0.79 (0.71; 0.88)	0.84 (0.75; 0.95)	0.85 (0.76; 0.95)
TINN pre-adjusted for rHR	0.82 (0.74; 0.92)	0.87 (0.78; 0.97)	0.88 (0.78; 0.98)
Mean HR	1.16 (1.05; 1.29)	1.10 (0.99; 1.23)	1.11 (1.00; 1.24)
All-cause mortality			
SDNN	0.71 (0.60; 0.84)	0.80 (0.68; 0.96)	0.81 (0.68; 0.96)
SDNN pre-adjusted for rHR	0.75 (0.63; 0.88)	0.83 (0.70; 0.98)	0.84 (0.71; 0.99)
SDANN	0.84 (0.71; 0.98)	0.93 (0.79; 1.10)	0.94 (0.80; 1.11)
SDANN pre-adjusted for rHR	0.87 (0.74; 1.01)	0.95 (0.81; 1.11)	0.96 (0.82; 1.12)
SDNNIDX	0.81 (0.68; 0.96)	0.84 (0.71; 1.00)	0.83 (0.70; 0.99)
SDNNIDX pre-adjusted for rHR	0.85 (0.72; 1.01)	0.87 (0.73; 1.03)	0.86 (0.73; 1.02)
TINN	0.72 (0.61; 0.85)	0.82 (0.69; 0.97)	0.82 (0.69; 0.98)
TINN pre-adjusted for rHR	0.76 (0.65; 0.90)	0.85 (0.72; 1.00)	0.85 (0.72; 1.01)
Mean HR	1.22 (1.06; 1.41)	1.11 (0.95; 1.30)	1.12 (0.96; 1.31)
Four-point MACE: AMI + Stroke + HF + CV Death			
SDNN	0.82 (0.71; 0.94)	0.85 (0.73; 0.98)	0.85 (0.74; 0.98)
SDNN pre-adjusted for rHR	0.85 (0.74; 0.98)	0.87 (0.76; 1.00)	0.88 (0.77; 1.01)
SDANN	0.87 (0.76; 1.00)	0.90 (0.78; 1.03)	0.90 (0.79; 1.04)
SDANN pre-adjusted for rHR	0.91 (0.79; 1.04)	0.93 (0.81; 1.06)	0.93 (0.82; 1.06)
SDNNIDX	0.93 (0.81; 1.06)	0.94 (0.82; 1.07)	0.94 (0.82; 1.07)
SDNNIDX pre-adjusted for rHR	0.94 (0.83; 1.08)	0.95 (0.83; 1.08)	0.94 (0.83; 1.08)
TINN	0.82 (0.71; 0.94)	0.84 (0.73; 0.97)	0.85 (0.73; 0.98)
TINN pre-adjusted for rHR	0.84 (0.74; 0.97)	0.87 (0.75; 1.00)	0.87 (0.76; 1.00)
Mean HR	1.12 (0.98; 1.27)	1.09 (0.95; 1.25)	1.10 (0.96; 1.26)
Three-point MACE: AMI + Stroke + CV Death			
SDNN	0.80 (0.68; 0.94)	0.82 (0.70; 0.97)	0.83 (0.70; 0.98)
SDNN pre-adjusted for rHR	0.83 (0.71; 0.97)	0.85 (0.72; 1.00)	0.86 (0.73; 1.00)
SDANN	0.89 (0.76; 1.04)	0.90 (0.77; 1.06)	0.91 (0.78; 1.07)
SDANN pre-adjusted for rHR	0.92 (0.79; 1.07)	0.93 (0.80; 1.09)	0.93 (0.80; 1.09)
SDNNIDX	0.87 (0.75; 1.03)	0.90 (0.76; 1.05)	0.89 (0.76; 1.05)
SDNNIDX pre-adjusted for rHR	0.89 (0.76; 1.04)	0.91 (0.78; 1.06)	0.90 (0.77; 1.06)
TINN	0.81 (0.69; 0.95)	0.84 (0.71; 0.99)	0.84 (0.71; 0.99)
TINN pre-adjusted for rHR	0.84 (0.71; 0.98)	0.86 (0.73; 1.01)	0.87 (0.74; 1.02)
Mean HR	1.09 (0.94; 1.26)	1.05 (0.90; 1.23)	1.06 (0.91; 1.24)

	Model 1	Model 2	Model 3
	IRR (95% CI)	IRR (95% CI)	IRR (95% CI)
Hospital diagnosed heart failure			
SDNN	0.67 (0.52; 0.86)	0.70 (0.54; 0.92)	0.71 (0.55; 0.92)
SDNN pre-adjusted for rHR	0.72 (0.57; 0.92)	0.74 (0.57; 0.95)	0.75 (0.59; 0.96)
SDANN	0.70 (0.55; 0.90)	0.76 (0.58; 0.98)	0.77 (0.60; 0.99)
SDANN pre-adjusted for rHR	0.76 (0.60; 0.97)	0.80 (0.63; 1.02)	0.81 (0.64; 1.03)
SDNNIDX	0.95 (0.76; 1.20)	0.95 (0.75; 1.20)	0.94 (0.74; 1.19)
SDNNIDX pre-adjusted for rHR	0.98 (0.78; 1.23)	0.95 (0.75; 1.20)	0.94 (0.74; 1.19)
TINN	0.64 (0.49; 0.82)	0.67 (0.51; 0.87)	0.67 (0.51; 0.87)
TINN pre-adjusted for rHR	0.67 (0.52; 0.86)	0.69 (0.54; 0.89)	0.69 (0.54; 0.90)
Mean HR	1.37 (1.10; 1.70)	1.31 (1.04; 1.63)	1.34 (1.07; 1.67)

Incidence rate ratio for all-cause mortality and cardiovascular disease endpoints per SD in SDNN, HR pre-adjusted SDNN, and mHR over a week. Model 1: adjusted for age and sex; Model 2: model 1 + education, alcohol consumption, smoking behavior, physical activity, body mass index, total cholesterol, and Hba1c; Model 3: model 2 + systolic blood pressure, anti-hypertensive, and glucose-lowering medication.

Table S4: Week-long HRV indices and mean HR association with major adverse cardiovascular events, heart failure, and all-cause mortality stratified by sex

Heart rate index	Strata	Model 1		Model 2		Model 3	
		IRR (95% CI)	p-value*	IRR (95% CI)	p-value*	IRR (95% CI)	p-value*
Three-point MACE: AMI + Stroke + CV Death							
SDNN	men	0.82 (0.63; 1.06)	0.95	0.89 (0.68; 1.17)	1.00	0.93 (0.72; 1.21)	0.91
SDNN	women	0.79 (0.64; 0.97)		0.80 (0.64; 0.99)		0.79 (0.64; 0.99)	
SDNN pre-adjusted for rHR	men	0.79 (0.61; 1.03)	0.61	0.85 (0.65; 1.11)	0.64	0.91 (0.70; 1.18)	0.81
SDNN pre-adjusted for rHR	women	0.84 (0.69; 1.03)		0.85 (0.69; 1.04)		0.84 (0.68; 1.04)	
Mean HR	men	1.05 (0.82; 1.34)	0.75	0.96 (0.74; 1.25)	0.70	1.00 (0.77; 1.29)	0.72
Mean HR	women	1.10 (0.91; 1.33)		1.09 (0.89; 1.33)		1.10 (0.90; 1.35)	
Hospital diagnosed Heart failure							
SDNN	men	0.66 (0.38; 1.16)	0.51	0.69 (0.38; 1.24)	0.56	0.67 (0.37; 1.24)	0.59
SDNN	women	0.74 (0.55; 0.98)		0.78 (0.57; 1.06)		0.78 (0.58; 1.06)	
SDNN pre-adjusted for rHR	men	0.66 (0.38; 1.15)	0.35	0.67 (0.38; 1.20)	0.39	0.66 (0.36; 1.20)	0.44
SDNN pre-adjusted for rHR	women	0.83 (0.63; 1.09)		0.84 (0.64; 1.12)		0.86 (0.65; 1.13)	
Mean HR	men	1.87 (1.32; 2.65)	0.05	1.81 (1.25; 2.63)	0.06	1.83 (1.25; 2.68)	0.07
Mean HR	women	1.26 (0.97; 1.63)		1.21 (0.92; 1.58)		1.26 (0.96; 1.65)	
All-cause mortality							
SDNN	men	0.75 (0.57; 0.99)	0.89	0.82 (0.62; 1.09)	0.98	0.83 (0.62; 1.10)	1.00
SDNN	women	0.67 (0.54; 0.84)		0.78 (0.62; 0.97)		0.78 (0.62; 0.98)	
SDNN pre-adjusted for rHR	men	0.79 (0.60; 1.03)	0.91	0.85 (0.64; 1.12)	0.95	0.84 (0.64; 1.11)	0.90
SDNN pre-adjusted for rHR	women	0.73 (0.59; 0.90)		0.83 (0.67; 1.02)		0.84 (0.68; 1.03)	
Mean HR	men	1.18 (0.95; 1.48)	0.80	1.12 (0.88; 1.42)	0.99	1.10 (0.87; 1.40)	0.91
Mean HR	women	1.24 (1.02; 1.50)		1.10 (0.90; 1.36)		1.11 (0.90; 1.36)	

Incidence rate ratio for all-cause mortality and cardiovascular disease endpoints per SD in SDNN, HR pre-adjusted SDNN, and mHR over a week. Model 1: adjusted for age and sex; Model 2: model 1 + education, alcohol consumption, smoking behavior, physical activity, body mass index, total cholesterol, and Hba1c; Model 3: model 2 + systolic blood pressure, anti-hypertensive, and glucose-lowering medication. p-value: p-value for interaction term.*

Figure S1: Study flowchart

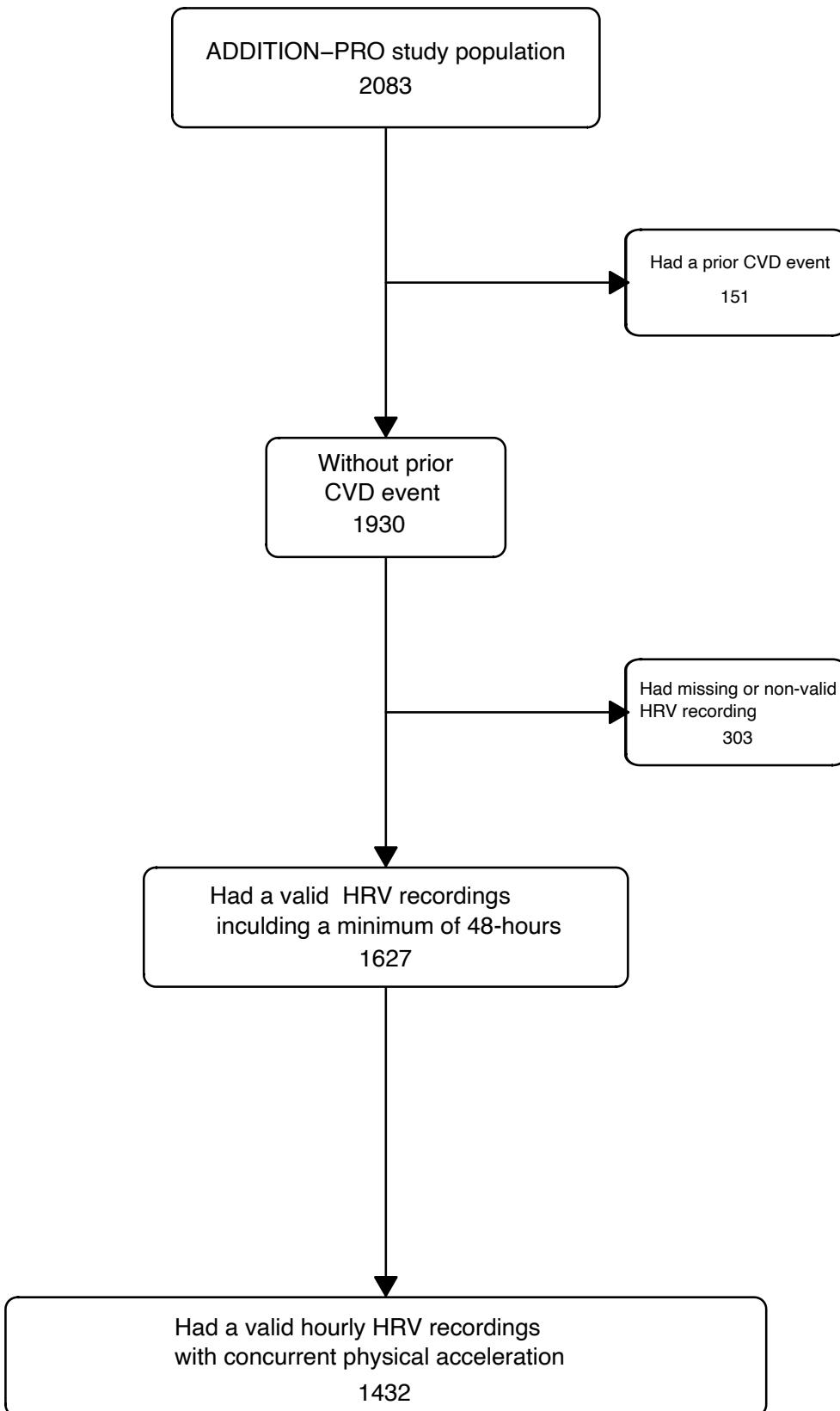
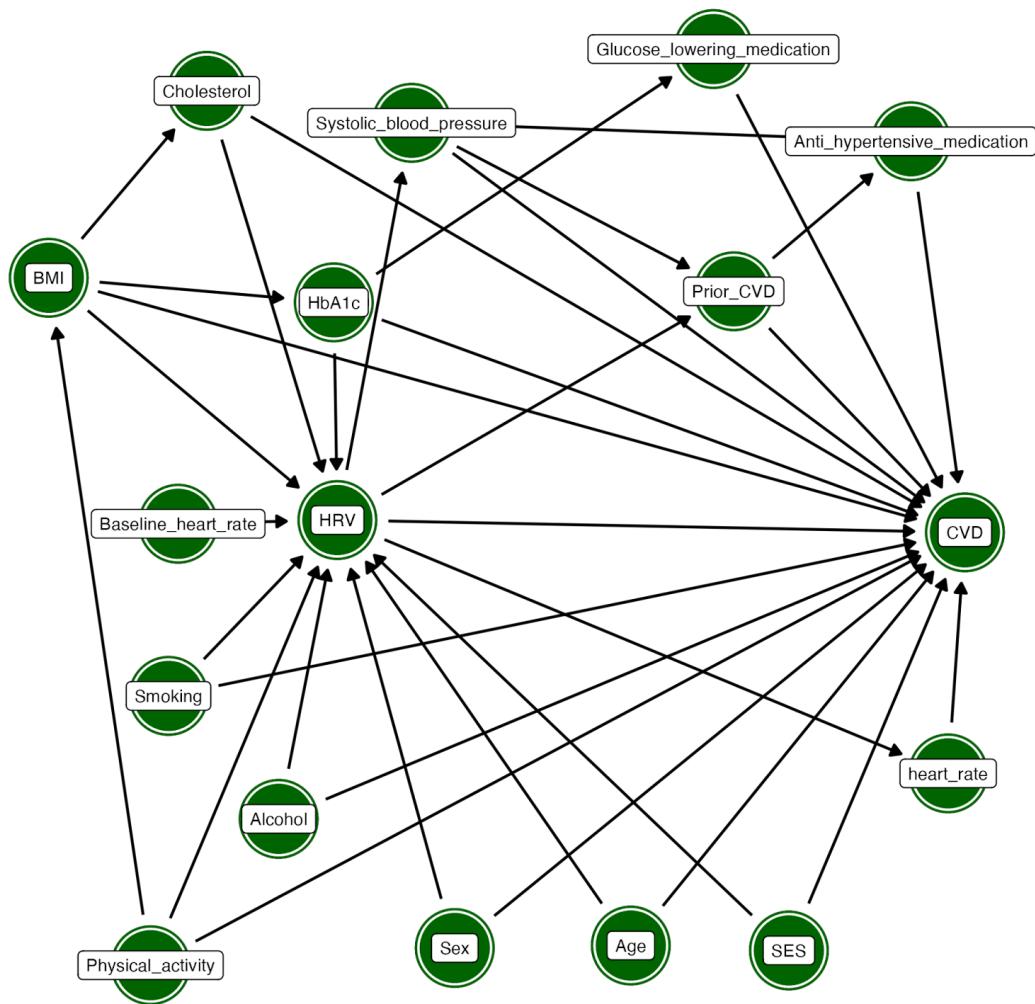
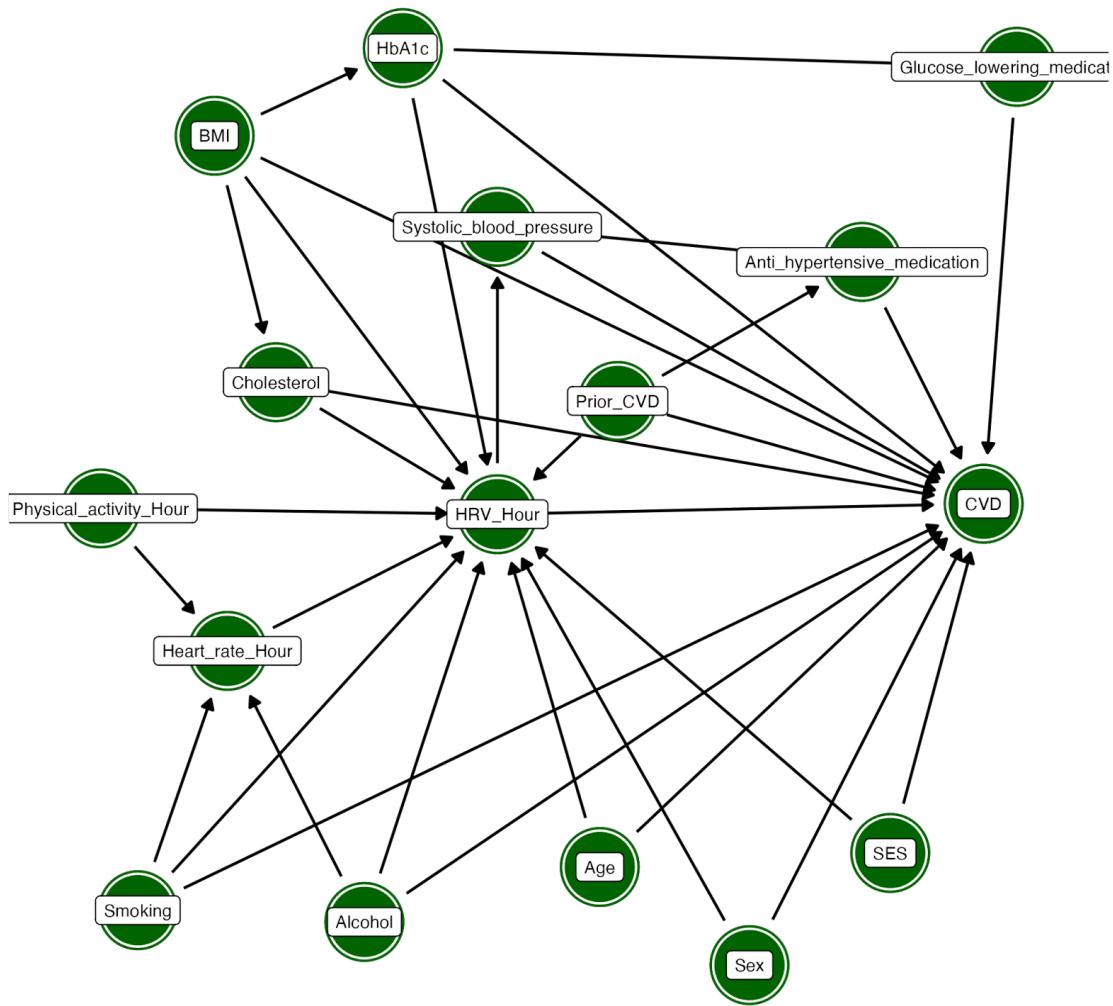


Figure S2: DAG1 – Week-long HRV and CVD, heart failure, and all-cause mortality



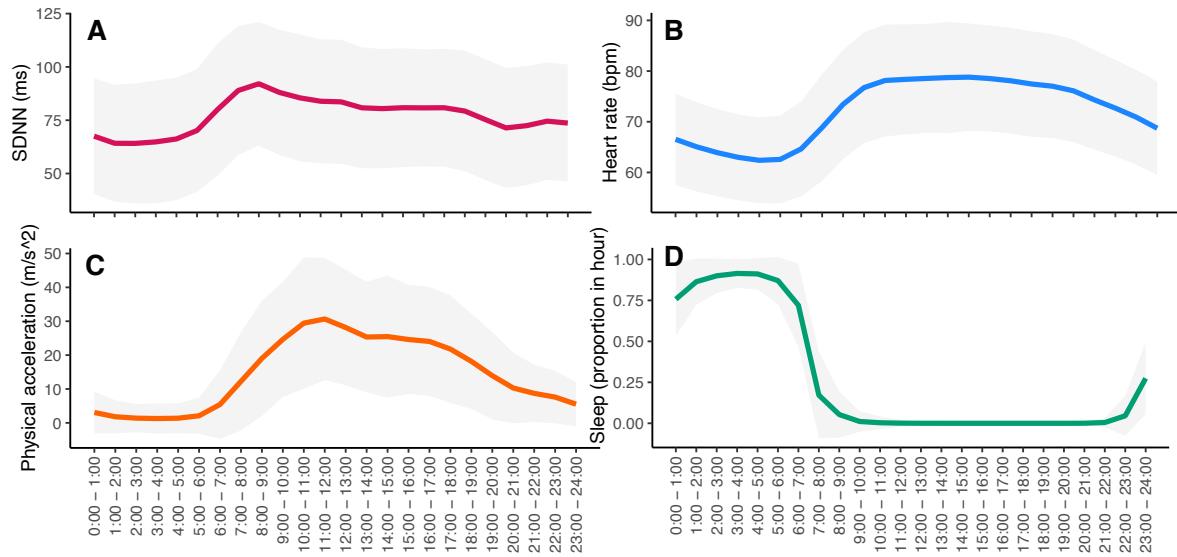
Confounding pathways in the first study aim are visualized by directed acyclic graphs (DAG). Aim: To determine the risk between week-long HRV and CVD, heart failure, and all-cause mortality in a population with high-risk of diabetes.

Figure S3: DAG2 – Hourly HRV and CVD, heart failure, and all-cause mortality



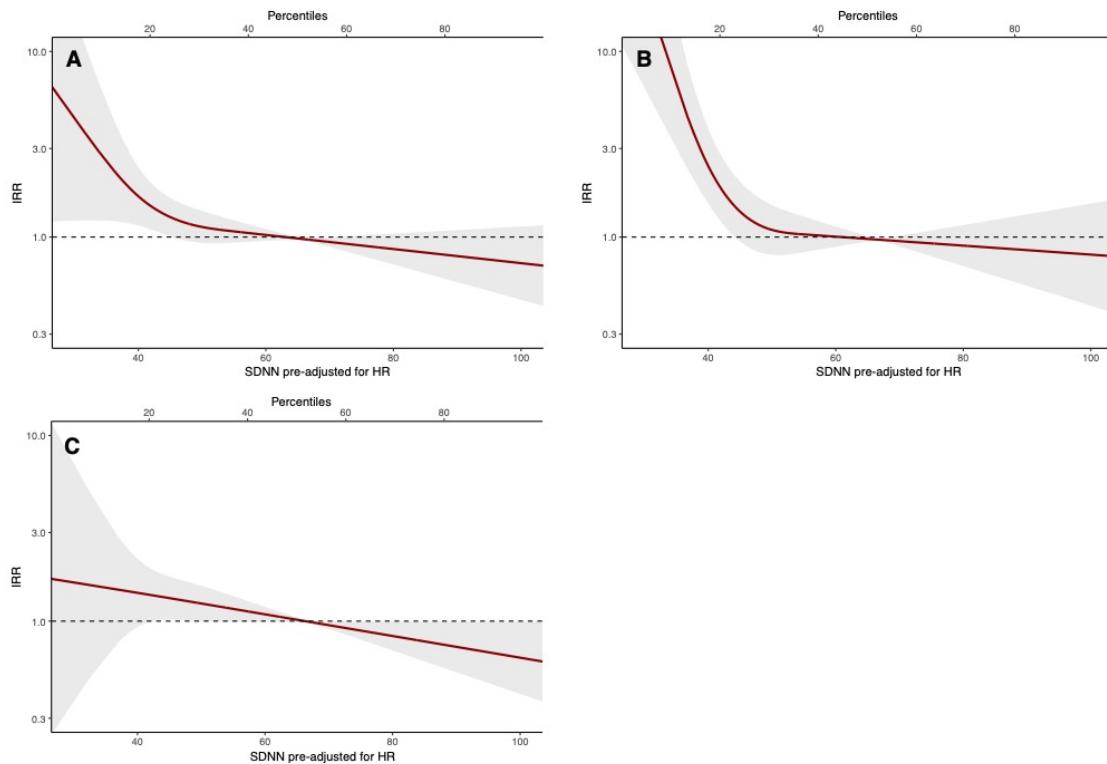
Confounding pathways in the second study aim are visualized by directed acyclic graphs(DAG). Aim: To identify the hours of the day where HRV has the strongest association with CVD, heart failure, and all-cause mortality risk and test the impact of concurrent physical acceleration and heart rate.

Figure S4: Hourly SDNN, heart rate, physical acceleration and sleep over 24 hours



Mean and standard deviation of SDNN (A), heart rate (B), physical acceleration (C), and sleep (D) in each hour time-frame across 24-hours.

Figure S5: Multiday SDNN pre-adjusted for rHR association with MACE, heart failure, and all-cause mortality



Association between week-long SDNN pre-adjusted for rHR and MACE (A), hospital-diagnosed heart failure (B), and all-cause mortality (C). IRR are adjusted for age and sex, education, alcohol consumption, smoking behavior, physical activity, body mass index, total cholesterol, and Hba1c.

A.3. Study III

Cardiovascular autonomic neuropathy and indices of heart failure in type 2 diabetes: The CANCAN Study

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Abbreviations

BMI: Body mass index

CAN: Cardiovascular autonomic neuropathy

CART: Cardiovascular autonomic reflex test

CVD: Cardiovascular disease

ECG: Electrocardiogram

eGFR: Estimated glomerular filtration rate

HbA1C: haemoglobin A1C

HF: Heart failure

HFpEF: Heart failure preserved ejection fraction

HFrEF: Heart failure reduced ejection fraction

HRV: Heart rate variability

NYHA classification: New York Heart Association classification

NT-proBNP: N-terminal pro-brain natriuretic peptide

rHR: Resting heart rate

T2D: Type 2 diabetes

Abstract

Objective

To quantify the association between cardiovascular autonomic neuropathy (CAN) and heart failure (HF) in individuals with type 2 diabetes (T2D).

Research Design and Methods

Two hundred T2D individuals were recruited from two Danish outpatient clinics between 2021-2024. CAN was defined by abnormal cardiovascular autonomic test reflex results. HF outcomes included the primary outcome of elevated N-terminal pro-brain natriuretic peptide (NT-proBNP) levels >125 pg/mL, WATCH-DM risk score. Symptomatic HF was defined by New York Heart Association (NYHA) classification score \geq II. We assessed the association between measures of CAN and HF using logistic and linear regressions, adjusting for confounders and testing for effect modification between CAN and NYHA and WATCH-DM risk scores.

Results

Among 176 individuals with NT-proBNP assessments, the median (interquartile range [IQR]) age was 63 (IQR: 55, 70) years, 61% were men, and median diabetes duration was 17 years (IQR: 11, 24). Among 136 individuals with valid CAN assessment, 40% had CAN and 52% of those had elevated NT-proBNP compared to 23% of individuals without CAN. In fully adjusted model, CAN was associated with 5.7 times higher odds (95% CI: 2.0, 18.5) of elevated NT-proBNP levels compared to individuals without CAN. The association remained statistically significant in asymptomatic individuals, and in individuals with a low-to-moderate WATCH-DM risk score.

Conclusion

CAN is associated with elevated NT-proBNP levels in individuals asymptomatic for HF. This suggest that CAN may complement established biomarkers to identify individuals with earlier HF stages, for timelier treatment initiation.

Article highlights

- **Why did we undertake this study?**

Cardiovascular autonomic neuropathy (CAN) is a common diabetic complication and an independent predictor of major cardiovascular outcomes including heart failure (HF) development and progression. However, its role in the pathophysiology of HF requires further investigation.

- **What is the specific question we wanted to answer?**

Whether individuals with type 2 diabetes (T2D) and CAN have higher levels of HF indicators than those without CAN. Additionally, we investigated whether CAN provides clinical value for HF screening beyond established tools such WATCH-DM risk score, or symptoms.

- **What did we find?**

We found that in this cohort of T2D participants, CAN was associated with higher NT-proBNP levels, WATCH-DM and NYHA scores, including in asymptomatic individuals

- **What are the implications of our findings?**

Our findings suggest that CAN detection may help identify individuals with T2D at a higher risk of early-stage, asymptomatic HF that is not captured by conventional tools. This finding supports the potential role of CAN in complementing established biomarkers to identify individuals with earlier HF stages, for timelier treatment initiation.

Introduction

Cardiovascular autonomic neuropathy (CAN) is an important but often overlooked complication of diabetes. CAN is an independent predictor of high cardiovascular disease (CVD) risk, heart failure (HF), diabetic kidney disease, and all-cause mortality[1-6]. Assessment of CAN may identify individuals with diabetes who are at a higher risk of severe complications and premature death. CAN is characterized by impaired autonomic regulation of the cardiovascular system. This condition stems from damage to the autonomic nerve fibres that regulate heart and blood vessel function, leading to disruptions in heart rate regulation and vascular dynamics[7].

HF has also emerged as one of the most prevalent CVD complication in people with type 2 diabetes (T2D) with serious consequences on morbidity, mortality and quality of life[8]. Although most current guidelines recommend proactive screening for HF in T2D [8, 9], most individuals with HF continue to be diagnosed only after experiencing HF symptoms, that unfortunately reflect more advanced HF stages that have a very poor prognosis[8, 10]. Identifying HF in earliest stages, that present with asymptomatic myocardial wall stress is important given that it would enable earlier implementation of guidelines directed therapies that have been shown to substantially reduce the risk of HF hospitalizations and death in T2D [8, 10, 11].

Several scores have been developed to estimate HF risk using demographics and clinical characteristics (e.g. age, sex, blood pressure, biochemical measures, ECG, and CVD history)[12, 13]. The WATCH-DM risk score identifies individuals with T2D at high risk of incident HF, aiding in the detection of asymptomatic cases[12, 14]. This score has been externally validated in several cohorts, demonstrating good discrimination[14]. The New York Heart Association (NYHA) functional classification defines HF severity based on physical activity limitations from score I to IV, but its inability to capture asymptomatic HF reduces its utility for early HF detection [15]. Data from multiple large, well-phenotyped longitudinal cohorts of individuals with and without diabetes have recognized natriuretic peptides, such as N-terminal pro-brain natriuretic peptide (NT-proBNP), as a valid biomarker for HF[16]. NT-proBNP is released in response to increased myocardial wall stress and elevated ventricular filling pressures, which characterize early physiological changes in the development of HF [16]. Elevated NT-proBNP levels (above 125 pg/mL) has demonstrated high sensitivity for identifying HF of both preserved ejection fraction (HFpEF) and reduced ejection fraction (HFrEF)[11].

Simple bedside techniques can be used to determine CAN, including heart rate variability (HRV) from standard ECG recordings [17] and the gold-standard cardiovascular autonomic reflex tests (CARTs), which measure heart rate responses to physiological challenges such as changing position from lying to standing, during deep breathing, and performing the Valsalva maneuver. As CAN is prognostically linked with HF [18], CAN detection may play a role in future strategies for early HF detection. Therefore, screening for CAN shows potential for inclusion in HF detection algorithms. However, the role of CAN in identifying asymptomatic HF and its potential for enhancing risk scores like WATCH-DM was not evaluated yet.

The objective of this study was to describe the distribution of HF indicators including NT-proBNP levels, WATCH-DM risk score, and NYHA classification score among individuals with T2D with and without CAN, and to quantify the association between CAN and these HF indicators. We further evaluated the interactions between CAN, NYHA classification, and WATCH-DM risk scores, to assess the potential role of CAN in addition to established biomarkers and risk scores to identify those with earlier HF stages.

Research Design and Methods

Study design and participants

The Danish cross-sectional study *Cardiovascular Autonomic Neuropathy for risk stratification in type 2 diabetes* (CANCAN study) assessed CAN and indicators of HF and glucometric indices from continuous glucose monitoring (CGM) in individuals with T2D treated in secondary care between 2021 and 2024. The CANCAN study aimed to assess whether identifying CAN detects a high-risk T2D population with higher prevalence of HF indicators and adverse glucose profiles. Results from the CANCAN study on glucometric indices will be analyzed and presented separately. Participants were recruited from two hospital outpatient clinics located in the Central Region of Denmark (Regional Hospital Central Jutland, Viborg and Gødstrup Hospital). We included 200 consecutive individuals (aged >18 years) with T2D for more than one year. Participants were either long-term T2D individuals followed in the outpatient clinic, or they were referred for optimization of their diabetes management. Main exclusions were: arrhythmia precluding CAN assessment, laser treatment for diabetic retinopathy within the past 3 months, pregnancy or breastfeeding, a life-threatening illness with a remaining life expectancy of less than one year, or cognitive impairments that hindered their ability to provide informed consent.

Cardiovascular autonomic neuropathy

CAN was diagnosed using CARTs. Ratios of normal RR-intervals were derived from an ECG using the Vagus™ device (Medicus Engineering, Aarhus, Denmark). Three standardized CARTs were performed to assess R-R intervals in responses to postural changes from lying-to-standing, during deep breathing, and under the Valsalva maneuver [19]. Testing was conducted in a quiet, isolated examination room between 8:00 a.m. and 2:00 p.m. following a standardized protocol. Participants rested supine for 10 minutes before testing. Smoking and caffeine consumption were prohibited for at least two hours before testing. Each CART was performed once per participant by a trained examiner. Manifest CAN was defined by the presence of two or more abnormal CARTs using recently established age-based formulas using normative material for the Vagus™ device [19]. We also tested for orthostatic hypotension, defined as a sustained reduction in systolic blood pressure of at least 20 mmHg or diastolic blood pressure of 10 mmHg within 3 min from changing position from lying to standing [20].

Assessment of indicators of heart failure

NT-proBNP was measured in plasma using a two-step chemiluminescent microparticle immunoassay on the Cobas e601 analyzer (Roche Diagnostics). The analyses were conducted at the Department of Blood Samples

and Biochemistry, Aarhus University Hospital, Denmark. Detection threshold was set at 50 pg/ml. Further description of NT-proBNP analysis is presented in supplementary material. We defined HF as elevated NT-proBNP levels greater than 125 pg/mL, a threshold indicative of structural heart disease, and elevated filling pressures, consistent with previous literature [21].

The WATCH-DM risk score was developed to estimate the 5-year risk of incident HF hospitalization in individuals with T2D based on a previously published risk model incorporating clinical, laboratory, and ECG parameters [12]. For the current study, we adapted the WATCH-DM score to align with available data, using a modified version from a validation study that incorporated haemoglobin A1C (HbA1C) instead of fasting plasma glucose [14]. This version excludes the ECG measurement of QRS duration. The adjusted WATCH-DM risk score includes nine variables: two binary variables (history of myocardial infarction and coronary artery bypass grafting), and six continuous variables (age, body mass index (BMI), systolic- and diastolic blood pressure, levels of serum creatinine, high-density lipoprotein (HDL) cholesterol, and HbA1c). The score ranged from 0 to 39 points, and risk categories were defined as follows: very low (≤ 11 points), low (12–13 points), moderate (14–15 points), high (16–18 points), and very high (≥ 19 points) [14].

New York Heart Association (NYHA) Functional Classification for HF stage I-IV was defined by a physician to determine cardiac functional status (see documentation in supplementary material). We defined NYHA stages II-IV as HF symptoms.

Covariates

We collected data on self-reported lifestyle factors, including smoking status (smoker/ non-smoker), leisure physical activity (sedentary / non-sedentary) (see supplementary material), and average weekly alcohol consumption (number of units). Information regarding CVD history and the use of antihypertensive, glucose-lowering, lipid-lowering, and antithrombotic medications was obtained and verified through electronic health records. Blood and urine samples were collected to measure levels of HbA1C, total cholesterol, HDL cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, creatinine, estimated glomerular filtration rate (eGFR) (based on the CKD-EPI equation [22]), and urine albumin-to-creatinine ratio. Anthropometrics included height, weight, BMI, waist circumference, and clinical assessments of systolic and diastolic blood pressure.

Ethics

The study was approved by the Committee on Health Research Ethics in the Central Denmark Region (#93931 and #1-10-72-221-21). The study was conducted in accordance with the ethical principles for medical research stated in the 1996 Declaration of Helsinki, and all study participants gave oral and written informed consent.

Statistical Analysis

Population characteristics were summarized by CAN status and reported as median and interquartile ranges (IQR) for continuous variables and numbers and percentages for categorical variables. To examine differences in HF risk, we compared the distribution of categorized HF indices (NT-proBNP, WATCH-DM, NYHA) according to CAN status.

HF outcome was defined by an NT-proBNP level >125 pg/mL. We defined symptomatic HF by NYHA score \geq II. The association between CAN and HF was assessed by logistic regression to estimate the odds ratio (OR) of CAN as the determinant for the outcomes of elevated NT-proBNP. Participants without a valid measure of NT-proBNP measurement or CAN assessments were excluded from the complete case analysis. Model 1 was adjusted for age, sex, and duration of diabetes, while Model 2 also included adjustments for smoking status, alcohol consumption, BMI, HbA1c, triglycerides, total cholesterol, and antihypertensive medication. Model 3 was additionally adjusted for history of CVD. Model 4 was further adjusted for eGFR. To assess the impact of beta-blocker treatment and a history of CVD, we conducted a sensitivity analysis. To assess whether the association between CAN and elevated NT-proBNP persisted in the absence of symptoms, defined by NYHA score = I, or among individuals with low-to-moderate WATCH-DM risk, we included interaction terms in separate models for binary NYHA classification (NYHA score = I vs NYHA score \geq II) WATCH-DM risk (very-low-to-moderate [0-15 points] vs. high-to-very-high risk [\geq 16 points]). Logistic regression was used to estimate the OR of CAN as the determinant for the outcome of HF symptoms, defined by NYHA score \geq II, with adjustments in models 1–4. We used linear regression to analyse the difference in WATCH-DM risk score between those with and without CAN.

Analyses were performed using RStudio (4.3.2, RStudio: Integrated Development for R. RStudio, PBC, Boston, MA, USA)[23], with complete case and multiple imputation of chained equation (MICE) approach to handle missing data from CARTs or other covariates. All individuals underwent CARTs, and a technical report was produced when recordings were incomplete. As missingness was related to observed variables, like patient characteristics or report information, we minimized systematic exclusion risk. Therefore, we considered MICE assumptions met.

Results

Participants characteristics

In this study, 176 participants had valid measurement of NT-proBNP of which 108 (61%) were males, median age was 63 (IQT: 55; 70) years, median diabetes duration was 17 (IQT: 11; 24) years, and mean HbA1c was 64 (IQT: 56; 80) mmol/L (Figure S1). Among these individuals, 40 did not complete all CART assessments, with 21 of these unable to perform the Valsalva maneuver. Among individuals with valid CARTs assessment (n=136), the prevalence of CAN was 40% (Figure 1A), 48 (27%) had a history of CVD (myocardial infarction, stroke or peripheral artery disease), and 20 (11%) had been previously diagnosed with HF. Elevated levels of NT-proBNP (>125 pg/ml) were seen in 66 (38%), 45 (26%) had NYHA score \geq II and 56 (33%) had high-to-

very-high risk of HF based on the WATCH-DM risk score (see Figure S2). Among individuals with elevated NT-proBNP, 33% had symptoms indicating HF with NYHA score \geq II and 52% had high risk of HF based on the WATCH-DM score. Complete participants' characteristics are presented in Table 1 contrasting those with CAN with no CAN.

Table 1: Baseline characteristics by CAN status

	Missing	Overall, N = 176	CAN missing,	No CAN,	CAN, N = 54
			N = 40	N = 82	
Sex (Women)	0	68 (39%)	19 (48%)	27 (33%)	22 (41%)
Age (years)	0	63 (55; 70)	68 (61; 75)	61 (52; 69)	62 (56; 68)
BMI (kg/m^2)	2	32 (28; 37)	30 (26; 34)	33 (28; 38)	33 (30; 36)
Duration of diabetes (years)	0	17 (11; 24)	20 (13; 30)	15 (9; 21)	19 (13; 24)
HbA1c (mmol/mol)	0	64 (56; 80)	65 (56; 85)	64 (55; 78)	64 (57; 76)
Total cholesterol (mmol/L)	2	3.90 (3.23; 4.78)	3.70 (3.33; 4.18)	4.10 (3.33; 4.88)	3.75 (3.03; 4.98)
LDL (mmol/L)	3	1.80 (1.40; 2.40)	1.70 (1.30; 1.90)	2.00 (1.50; 2.58)	1.70 (1.10; 2.73)
HDL (mmol/L)	2	1.00 (0.88; 1.20)	1.00 (0.90; 1.30)	1.00 (0.90; 1.20)	0.97 (0.80; 1.18)
Triglycerides (mmol/L)	3	2.00 (1.30; 2.90)	2.10 (1.10; 2.80)	1.95 (1.30; 2.90)	2.05 (1.40; 2.98)
eGFR (ml/min/1.73 m^2) categories	6				
	< 30	16 (9%)	5 (14%)	6 (7.4%)	5 (9%)
	30-59	33 (19%)	7 (19%)	12 (15%)	14 (26%)
	60-89	41 (24%)	12 (33%)	18 (22%)	11 (21%)
	> 90	80 (47%)	12 (33%)	45 (56%)	23 (43%)
Systolic blood pressure (mmHg)	1	133 (123; 143)	135 (127; 147)	131 (123; 142)	133 (120; 143)
Diastolic blood pressure (mmHg)	1	76 (68; 82)	73 (66; 79)	78 (72; 83)	74 (66; 82)
Resting heart rate (bpm)	6	77 (66; 84)	68 (62; 80)	78 (69; 84)	80 (67; 89)
CARTS					
Lying to standing (RR ratio)	8	1.02 (1.01; 1.06)	1.03 (1.02; 1.05)	1.05 (1.01; 1.08)	1.01 (1.00; 1.02)
Deep breathing (RR ratio)	4	1.13 (1.07; 1.26)	1.15 (1.10; 1.28)	1.18 (1.11; 1.30)	1.07 (1.03; 1.08)
Valsalva maneuver (RR ratio)	45	1.24 (1.13; 1.36)	1.20 (1.14; 1.25)	1.32 (1.25; 1.45)	1.11 (1.08; 1.16)
NT-proBNP (pg/ml) categories	0				
	< 50	72 (41%)	10 (25%)	47 (57%)	15 (28%)
	50-124	38 (22%)	11 (28%)	16 (20%)	11 (20%)

	Missing	Overall, N = 176	CAN missing,	No CAN,	CAN, N = 54
			N = 40	N = 82	
WATCH-DM risk score	125-300	28 (16%)	7 (18%)	10 (12%)	11 (20%)
	> 300	38 (22%)	12 (30%)	9 (11%)	17 (31%)
NYHA classification	4	14.0 (11.0; 16.0)	15.0 (12.8; 17.0)	13.0 (10.0; 16.0)	15.0 (12.0; 18.0)
Leisure physical activity (Sedentary lifestyle vs non-sedentary lifestyle)	I	130 (74%)	27 (69%)	72 (88%)	31 (57%)
	II	31 (18%)	9 (23%)	9 (11%)	13 (24%)
	III	13 (7%)	3 (8%)	1 (1%)	9 (17%)
	IV	1 (1%)	0 (0%)	0 (0%)	1 (2%)
Smoking status (smoker vs non-smoker)	1	28 (16%)	6 (15%)	12 (15%)	10 (19%)
Alcohol consumption (units per week)	1	0.0 (0.0; 2.0)	0.0 (0.0; 3.5)	0.0 (0.0; 2.0)	0.0 (0.0; 0.0)
Any antihypertensive medication (yes)	0	140 (80%)	33 (83%)	61 (74%)	46 (85%)
ACE inhibitors (yes)	0	54 (31%)	9 (23%)	25 (30%)	20 (37%)
AT2 antagonist (yes)	0	65 (37%)	16 (40%)	30 (37%)	19 (35%)
Calcium antagonist (yes)	0	71 (40%)	14 (35%)	28 (34%)	29 (54%)
Beta-blockers (yes)	0	52 (30%)	11 (28%)	19 (23%)	22 (41%)
Aldosterone antagonist (yes)	0	18 (10%)	3 (8%)	5 (6%)	10 (19%)
Glucose-lowering medication					
Metformin (yes)	0	123 (70%)	26 (65%)	56 (68%)	41 (76%)
SGLT2-inhibitors (yes)	0	81 (46%)	12 (30%)	40 (49%)	29 (54%)
DPP4 inhibitors (yes)	0	13 (7%)	2 (5%)	8 (10%)	3 (6%)
GLP1 RAs (yes)	0	91 (52%)	15 (38%)	47 (57%)	29 (54%)
Insulin (yes)	0	140 (80%)	33 (83%)	66 (80%)	41 (76%)
Lipid-lowering medication (yes)	0	155 (88%)	34 (85%)	74 (90%)	47 (87%)
Antiplatelet medication (yes)	0	76 (43%)	18 (45%)	29 (35%)	29 (54%)

n (%); Median (IQR)

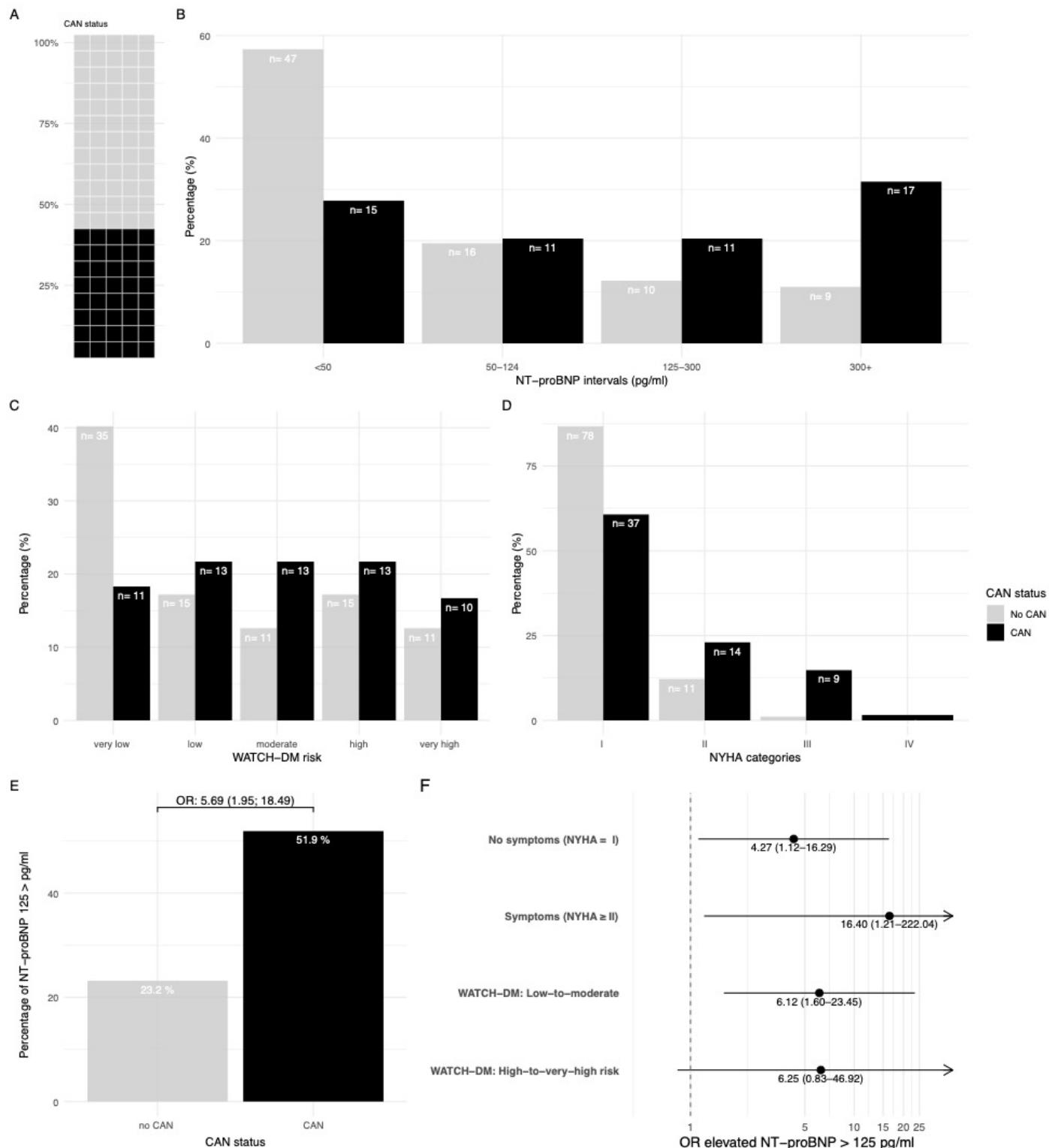
CAN, cardiovascular autonomic neuropathy. CARTs, cardiovascular autonomic reflex test. NT-proBNP, N-terminal pro b-type natriuretic peptide. bpm, beats per minute. BMI, body mass index, LDL, low density lipoprotein. HDL, high density lipoprotein. eGFR, estimated glomerular filtration rate (ml/min/1.73 m²). HbA1c, hemoglobin A1c.

Association between CAN and measures of HF

Individuals with CAN showed a higher prevalence of HF assessed as described in Methods (Figure 1B–D). Specifically, a higher proportion of CAN individuals had elevated NT-proBNP (51.9%) compared to those without CAN (23.2%). In fully adjusted models (model 4) the OR for having elevated NT-proBNP were 5.69 (CI: 1.95; 18.49) compared to those without CAN (Figure 1E). Furthermore, the association between CAN and elevated NT-proBNP was similar in participants with very-low-to-moderate risk in the WATCH-DM score (OR = 6.1 [CI: 1.6; 23.5]) and those with high-to-very-high risk (OR = 6.3 [CI: 0.83; 46.9]).

The association between CAN and elevated NT-proBNP was also present in asymptomatic individuals for HF (OR = 4.3, 95% CI: 1.1; 16.3), although it was stronger in symptomatic HF individuals (OR = 16.4, 95% CI: 1.2; 222.0), although the interaction between groups was not statistically significant ($p = 0.4$).

Figure 1: Figure 1. Distribution of NT-proBNP, NYHA Class, and WATCH-DM Score by CAN Status, and association of CAN with Elevated NT-proBNP



A: Percentage distribution by CAN status (no CAN, CAN). **B:** Percentage distribution of NT-proBNP level categories stratified by individuals with and without CAN. **C:** Percentage distribution of WATCH-DM risk score stratified by individuals with and without CAN. **D:** Percentage distribution of NYHA classification stratified by individuals with and without CAN. **E:** Percentage of individuals with NT-proBNP > 125 pg/ml among those with and without CAN and adjusted odds ratio from Model 4. **F:** Effect modification of the association between CAN and NT-proBNP by symptoms defined by NYHA

classification (symptoms: NYHA $\geq II$ vs no symptoms: NYHA = I) and risk score defined by WATCH-DM risk (very-low-to-moderate vs high-to-very-high risk).

Imputation of missing CARTs showed an OR 2.9 (CI: 1.4; 6.6) between CAN and elevated NT-proBNP. In sensitivity analysis, excluding participants with beta-blocker use or a history of CVD, the direction of the association between CAN and NT-proBNP remained unchanged, although exclusion of these participants reduced sample size and thus to wider confidence intervals compared to the main analysis (see Table S1). CAN was associated with 1.7 (CI: 0.3; 3.0) higher points in WATCH-DM risk, and 5.51 (1.9; 15.97) higher OR for NYHA score ≥ 2 (see Table S2). Among each CART, the Valsalva maneuverer showed the strongest association with elevated NT-proBNP, followed by deep breathing. Orthostatic hypotension was also associated with elevated NT-proBNP (see Table 2). Participants who were not able to perform the Valsalva maneuverer had higher NT-proBNP levels compared to those without CAN (Table S3).

Table 2 CAN diagnosis, orthostatic hypotension, and CARTs association with elevated levels of NT-proBNP (>125 pg/ml)

	Non-cases/ cases	Model 1	Model 2	Model 3	Model 4
CART					
Lying-to-standing	108/54	1.04 (0.5; 2.19)	1.21 (0.56; 2.70)	1.09 (0.49; 2.46)	0.80 (0.32; 1.97)
Deep breathing	133/33	2.76 (1.17; 6.70)	2.95 (1.18; 7.72)	2.93 (1.17; 7.72)	3.30 (1.17; 9.77)
Valsalva maneuverer	75/51	5.65 (2.42; 14.04)	8.13 (3.02; 24.81)	7.14 (2.62; 21.78)	9.00 (2.88; 33.09)
CAN diagnosis	78/52	5.26 (2.28; 12.96)	6.05 (2.41; 16.59)	5.04 (1.97; 13.94)	5.69 (1.95; 18.49)
CAN with imputed CARTs*	127-imputed cases /52+imputed cases	2.61 (1.36; 5.11)	2.98 (1.48; 6.23)	2.88 (1.42; 6.04)	2.94 (1.37; 6.56)
Orthostatic hypertension	146/24	3.31 (1.25; 9.44)	3.20 (1.16; 9.51)	3.11 (1.11; 9.36)	4.04 (1.27; 13.77)

Odds ratio for NT-proBNP > 125 pg/ml comparing orthostatic hypertension, abnormal CARTs or CAN diagnosis to normal CARTs and no CAN. Model 1: Age, sex, diabetes duration. Model 2: Model 1 + HbA1c, smoking status, BMI, anti-hypertensives, total cholesterol, triglycerides, systolic blood pressure. Model 3: Model 2 + history of cardiovascular disease. Model 4: Model 3 + eGFR. CAN, cardiovascular autonomic neuropathy. CARTs, cardiovascular autonomic reflex tests. NT-proBNP, N-terminal pro b-type natriuretic peptide. BMI, body mass index. *Analysis with imputed values for each missing CART.

Conclusions

In this cross-sectional study of contemporary individuals with T2D as followed in outpatient clinics in Denmark, we found that individuals with CAN more frequently showed signs of HF including elevated levels of NT-proBNP, higher WATCH-DM risk score, and higher NYHA classification compared to the individuals without CAN. The association between CAN and elevated NT-proBNP was also present among individuals asymptomatic for HF and in the individuals classified as low-to-moderate risk of HF using the WATCH-DM score.

This study adds to the growing body of evidence that individuals with CAN represent a high-risk T2D population for more severe cardiovascular complications and increased mortality[1-6]. Our findings extend this by demonstrating an increased risk of HF, including early-stage HF, as indicated by elevated NT-proBNP levels. Several lines of evidence showed that individuals with T2D and HF have a poor prognosis including higher rates of hospitalization and a 3- to-5-fold higher mortality compared to individuals with T2D without HF [8, 24]. Furthermore, these data suggest that early identification of CAN in T2D may aid in detecting individuals at particularly high risk of HF, even when asymptomatic. Sympathetic overload in CAN leads to increased cardiac stress through elevated heart rate, stroke volume, and blood pressure [25, 26]. The hemodynamic changes of CAN contribute to cardiac and arterial remodelling [27, 28]. Cardiac and arterial remodelling results in structural and functional alterations in the heart that increase the risk of HF [29, 30]. Hence, CAN may promote the progression of HF before clinical symptoms of HF become apparent.

Although, among those with elevated NT-proBNP, one-third exhibited symptoms consistent with HF, as defined by NYHA score II or higher, the association between CAN and elevated NT-proBNP remained significant in those individuals asymptomatic for HF. Additionally, among individuals with elevated NT-proBNP, half were classified as having a high-to-very-high risk according to the WATCH-DM score, while the other half were categorized as low-to-moderate risk. However, our data showed that the association between CAN and elevated NT-proBNP was similar among those with both high and low-to-moderate WATCH-DM risk. Hence, CAN may provide additional information beyond risk scores such as the WATCH-DM score and symptoms of HF defined by NYHA score and reveal a higher risk of HF than uncovered by these measures alone. These initial data support further studies with follow-up data on HF hospitalizations to confirm a possible additive predictive value of CAN when incorporated into existing risk scores or biomarkers.

Although the American Diabetes Association recommends screening for CAN [9] [8], the implementation of general screening for CAN in routine care remains limited and has not yet been adopted in Denmark. In this study, CAN prevalence was 40%, higher than the 15% reported in the ADDITION study of screen-detected T2D in Denmark and higher than CAN prevalence (19%) in another Danish outpatient clinic study[31, 32]. Thus, the population in our study falls within the higher end of the generally reported prevalence range of 12-73% for CAN in T2D[33]. The reason may be that the cohort in this study comprises individuals with T2D affiliated with a specialist diabetes clinic (secondary care) that usually handle more advanced T2D characterized by multiple complications and suboptimal glycemic control. This contrasts with the broader T2D population in Denmark generally managed in primary care. These factors likely explain the higher prevalence of CAN observed. The individuals with CAN did not differ in conventional cardiovascular risk factors compared to those without CAN yet showed a higher proportion of kidney disease and use of blood pressure lowering medications. The similar levels of blood pressure, triglycerides, HbA1c, lipids, and BMI, may be explained by a more intensive treatment of individuals with CAN who showed a higher cardiovascular risk due to a more prevalent CVD history. Exclusion of individuals with a history of cardiovascular disease, hospitalization for HF, or use of beta-blockers did not alter the association between CAN and elevated NT-proBNP. Of note, even within this high-risk group of individuals with T2D, we identified a subgroup with CAN who may require closer

monitoring for the early detection of HF. Importantly, this subgroup may not have been identified had they not been diagnosed with CAN.

NT-proBNP is recognized as a sensitive marker of early-stage HF that can be used to identify those individuals that will benefit from early interventions before clinical symptoms appear[11, 34, 35]. However, its specificity varies across HF phenotypes, being less specific for detecting HFpEF compared to HFrEF [11, 36]. Therefore, although we cannot link our findings to specific HF phenotypes, NT-proBNP remains a valuable indicator of early HF. The validity of NT-proBNP as a marker for HF may be confounded by atrial fibrillation, obesity, or impaired kidney function[11]. Individuals with concomitant atrial fibrillation were excluded from our analyses, and in this study controlling for BMI did not alter the magnitude of the association between CAN and elevated NT-proBNP. Impaired kidney function, as indicated by decreased eGFR, is linked to greater cardiac dysfunction but may also lead to elevated NT-proBNP levels, independently of HF[11]. After adjusting for eGFR, the association between CAN and NT-proBNP strengthened, suggesting the true link between CAN and cardiac dysfunction may have been underestimated due to the potential elevation of NT-proBNP in individuals with impaired kidney function. Adjustments for smoking status, medication, clinical risk factors, and CVD history did not change the association between CAN and elevated NT-proBNP. Therefore, we consider our findings robust and adequately adjusted for relevant confounding factors.

A relatively large proportion of individuals (23%) did not complete the CARTs assessments, which is consistent with findings from the ADDITION study, where 12% to 33% did not complete the test[31]. The analysis of CAN using imputed CARTs values yielded slightly lower risk estimates and narrower confidence intervals compared to complete case analysis. The direction of the association remained consistent, supporting the validity of our findings after accounting for individuals with missingness of CARTs. Most incomplete CARTs assessments were due to individuals being unable to perform the Valsalva maneuver. These individuals had higher NT-proBNP levels than individuals within the normal Valsalva maneuver values, suggesting that individuals who cannot complete this CART are at higher risk of HF and echocardiogram could be considered in those individuals. Among the CARTs, the Valsalva maneuver followed by deep breathing showed the strongest association with elevated NT-proBNP, while orthostatic hypotension was also strongly associated with elevated NT-proBNP. Our findings show that even a single abnormal CART (excluding the lying-to-standing test) or orthostatic hypotension may indicate increased HF risk and could prompt clinical attention [33].

Strengths and limitations

Strengths of this study are : the large, well-characterized cohort of individuals with T2D, assessed in outpatient clinics and the thoroughly phenotypes for microvascular complications, including autonomic neuropathy, and the use of non-invasive reproducible assessments for CAN and biomarkers [37], making it easy to implement in clinical care. A limitation is that we did not assess echocardiography, which is gold standard for assessing HF and is central to classifying HFrEF and HFpEF[30]. However, previous studies have shown that CAN is associated with both structural and functional cardiac changes in both left atrium and the left ventricle, affecting systolic and diastolic function in individuals with type 1 and T2D[38, 39]. Future studies are warranted

to investigate the association between CAN and defined structural and functional cardiac abnormalities and to determine the prognostic value of CAN, yet we consider the use of validated indicators of HF to support an association between CAN and HF. In HF, compensatory changes in heart rhythm may cause autonomic dysfunction due to increased sympathetic activity, reflecting cardiomyopathy progression rather than diabetic neuropathy[40]. Therefore, due to the cross-sectional design of our study, we cannot exclude the possibility of reverse causality. Longitudinal epidemiological studies support that CAN primarily contributes to the development of HF, rather than being a consequence of it[18, 27]. In addition, since individuals in this study showed low NYHA scores and thus likely possess an early-stage HF less likely to explain CAN in this group of people with long-standing T2D very likely to have developed CAN due to diabetes. The WATCH-DM score is a validated tool for predicting 5-year HF risk in T2D using routine clinical data[13], however the score is not designed for diagnosing HF[18]. The CANCAN study includes secondary care patients with more advanced diabetes compared to the general population with T2D. It remains to be demonstrated whether these findings can be generalized to a broader T2D population without a history of CVD, such as those in primary care.

In conclusion, this study shows an association between CAN and indicators of HF, including elevated NT-proBNP levels, NYHA scores, and WATCH-DM scores in individuals with T2D. Notably, the finding between CAN and elevated NT-proBNP was evident among those individuals who had no symptoms of HF when assessed by NYHA score or had low-to-moderate risk of HF using WATCH-DM score. Our findings, supports a potential role of CAN detection to identify people at overall higher risk of complications and specifically with higher risk of early-stage HF not uncovered using conventional risk scores and HF symptom assessments as WATCH-DM risk score and NYHA score. These findings highlight the role of CAN detection at the point of care to identifying high-risk individuals with T2D who may benefit from a more timely and personalized care.

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Authors' contributions

Study concept and design: JRS, DRW, LB, RPB, AS, STA. Contributed to the data: JRS, LB, HHT, STA, AS. Planning the statistical analysis: JRS, DRW, LB, STA, CSH, RPB. Conducted the statistical analysis: JRS and LB. All authors contributed to, critically revised, and approved the final version of the manuscript. JRS is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflicts of interests

All the authors declare that there is no duality of interest associated with their contribution to this manuscript.

Availability of data and materials

The CANCAN study is managed by a steering committee at Steno Diabetes Center Aarhus, Denmark. The committee encourages interested researchers to use this resource. For further inquiries, please contact STA or JRS.

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Tables and figures

Table 1: Study characteristics

Table 2: CAN and risk of heart failure defined by NT-proBNP

Figure 1: Distribution of NT-proBNP, NYHA, and WATCH-DM by CAN status

Supplementary material:

Cardiovascular autonomic neuropathy and indices of heart failure in type 2 diabetes: The CANCAN Study

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1. Documentation

NT-proBNP analysis

An assay is a two-step immunoassay designed for the quantitative in vitro determination of NT-proBNP in human serum and plasma, utilizing chemiluminescent microparticle immunoassay technology on the Cobas e601 analysis instrument. The sample is first incubated with a biotinylated monoclonal NT-proBNP-specific antibody and a ruthenium-labeled monoclonal NT-proBNP-specific antibody, forming a sandwich complex with the NT-proBNP antigen. In the second incubation, streptavidin-coated microparticles are added, binding the complex to a solid phase via biotin-streptavidin interaction. The reaction mixture is then transferred into the measuring cell, where magnetically captured microparticles are immobilized on an electrode surface. Unbound substances are washed away using ProCell/ProCell M. By applying a voltage to the electrode, a chemiluminescence reaction is induced, and the emitted signal is detected using a photomultiplier. The NT-proBNP concentration is determined using a calibration curve, which is generated instrument-specifically via two-point calibration and a master curve embedded in the reagent barcode or e-barcode.

New York Heart Association classification of heart failure

Classification	Symptoms
NYHA I	No physical limitations. Ordinary physical activity does not cause breathlessness, fatigue, or palpitations.
NYHA II	Mild limitation of physical activity. No symptoms at rest, but ordinary physical activity (e.g., climbing stairs to the second floor, mowing the lawn, vacuuming, carrying heavier groceries) causes some breathlessness, fatigue, or palpitations.
NYHA III	Marked limitation of physical activity. No symptoms at rest, but light physical activity (e.g., walking on flat ground, dressing/undressing, climbing stairs to the first floor) causes pronounced symptoms.
NYHA IV	Symptoms may be present at rest and occur with any physical activity.

Physical activity

Level of physical activity	Description
<i>Sedentary</i>	Reads, watches television, or engages in other sedentary activities
<i>Light activity</i>	Walks, cycles, or performs other light physical activity for at least 4 hours per week (including Sunday walks, light gardening, or walking/cycling to work).
<i>Moderate activity</i>	Participates in recreational sports or performs heavy gardening or similar activities at least 4 hours per week.
<i>High activity</i>	Engages in intense exercise or sports regularly several times per week.

2. Tables and figures

Table S1: Sensitivity analysis excluding individuals without history of CVD or betablockers

Model	OR (CI: 95%)		
	All participants	Without history of CVD or HF ^a	Without betablockers ^b
Model 2	5.81 (2.2; 15.34)	3.74 (0.99; 14.21)	6.25 (1.03; 38.08)
Model 3	5.99 (1.83; 19.61)	9.41 (1.44; 61.33)	9.41 (1.44; 61.33)

Odds ratio for NT-proBNP > 125 pg/ml comparing CAN to no CAN.

^aModel adjustments: Model 2: Age, sex, diabetes duration, HbA1c, smoking status, BMI, anti-hypertensives, total cholesterol, triglycerides, systolic blood pressure. Model 3: Model 2 + eGFR.

^bModel adjustments: Model 2: Age, sex, diabetes duration, HbA1c, smoking status, BMI, anti-hypertensives, total cholesterol, triglycerides, systolic blood pressure. Model 3: Model 2 + history of cardiovascular disease, eGFR.

CAN, cardiovascular autonomic neuropathy. CART, cardiovascular autonomic reflex test. NT-proBNP, N-terminal pro b-type natriuretic peptide. BMI, body mass index.

Table S2: CAN diagnosis association with HF based symptoms defined as NYHA score $\geq II$

	Non-cases/ cases	Model 1	Model 2	Model 3
CAN diagnosis	78/52	5.44 (2.25; 13.15)	6.43 (2.35; 17.62)	5.51 (1.9; 15.97)

Odds ratio for HF based symptoms defined as NYHA score $\geq II$ by CAN diagnosis compared no CAN. Model 1: Age, sex, diabetes duration. Model 2: Model 1 + HbA1c, smoking status, BMI, anti-hypertensives, total cholesterol, triglycerides, systolic blood pressure. Model 3: Model 2 + history of cardiovascular disease and eGFR. CAN, cardiovascular autonomic neuropathy. BMI, body mass index.

Table S3: Study population characteristics by Valsalva maneuver

Characteristic	Missing	Overall N = 155	Normal VM values N = 79	Abnormal VM values N = 52	Low air pressure N = 24
Sex	0				
Men		93 (60%)	54 (68%)	33 (63%)	6 (25%)
Women		62 (40%)	25 (32%)	19 (37%)	18 (75%)
Age (years)	0	63 (55, 70)	61 (52, 69)	63 (57, 69)	73 (63, 78)
Smoking status	0				
Daily		63 (41%)	31 (39%)	20 (38%)	12 (50%)
Weekly/Occasionally		70 (45%)	36 (46%)	23 (44%)	11 (46%)
Quit/Never smoked		22 (14%)	12 (15%)	9 (17%)	1 (4%)
BMI (kg/m ²)	0	33 (28, 37)	32 (28, 38)	34 (30, 38)	30 (27, 34)
Duration of type-2 diabetes (years)	0	16 (11, 24)	15 (9, 20)	19 (14, 24)	23 (13, 33)
eGFR category	4				
<30		15 (10%)	6 (8%)	4 (8%)	5 (22%)
30-59		29 (19%)	10 (13%)	16 (31%)	3 (13%)
60-89		35 (23%)	17 (22%)	10 (20%)	8 (35%)
≥90		72 (48%)	44 (57%)	21 (41%)	7 (30%)
Orthostatic hypertension	0	22 (14%)	9 (11%)	8 (15%)	5 (21%)
NYHA score	0				
I		118 (76%)	69 (87%)	32 (62%)	17 (71%)
II		25 (16%)	8 (10%)	12 (23%)	5 (21%)
III		11 (7%)	2 (3%)	7 (13%)	2 (8%)
IV		1 (1%)	0 (0%)	1 (2%)	0 (0%)
NT-proBNP categories	0				
<50		65 (42%)	46 (58%)	15 (29%)	4 (17%)
50-124		34 (22%)	17 (22%)	9 (17%)	8 (33%)
125-300		22 (14%)	7 (9%)	12 (23%)	3 (13%)
300+		34 (22%)	9 (11%)	16 (31%)	9 (38%)
NT-proBNP > 125 ml/pg	0				
Below		99 (64%)	63 (80%)	24 (46%)	12 (50%)
Above		56 (36%)	16 (20%)	28 (54%)	12 (50%)
WATCH-DM risk score	20	13.0 (10.5, 16.0)	12.0 (10.0, 16.0)	15.0 (12.0, 16.0)	14.5 (13.0, 17.8)
WATCH-DM risk score (high to very-high risk)	20	41 (30%)	19 (26%)	16 (36%)	6 (33%)
History of myocardial infarction	0	18 (12%)	6 (8%)	11 (21%)	1 (4%)
History of stroke	0	18 (12%)	8 (10%)	8 (15%)	2 (8%)
History of major cardiovascular events	0	41 (26%)	17 (22%)	20 (38%)	4 (17%)
History of heart failure	2	15 (10%)	2 (3%)	9 (17%)	4 (17%)

Figure S1: Study and analysis flowchart

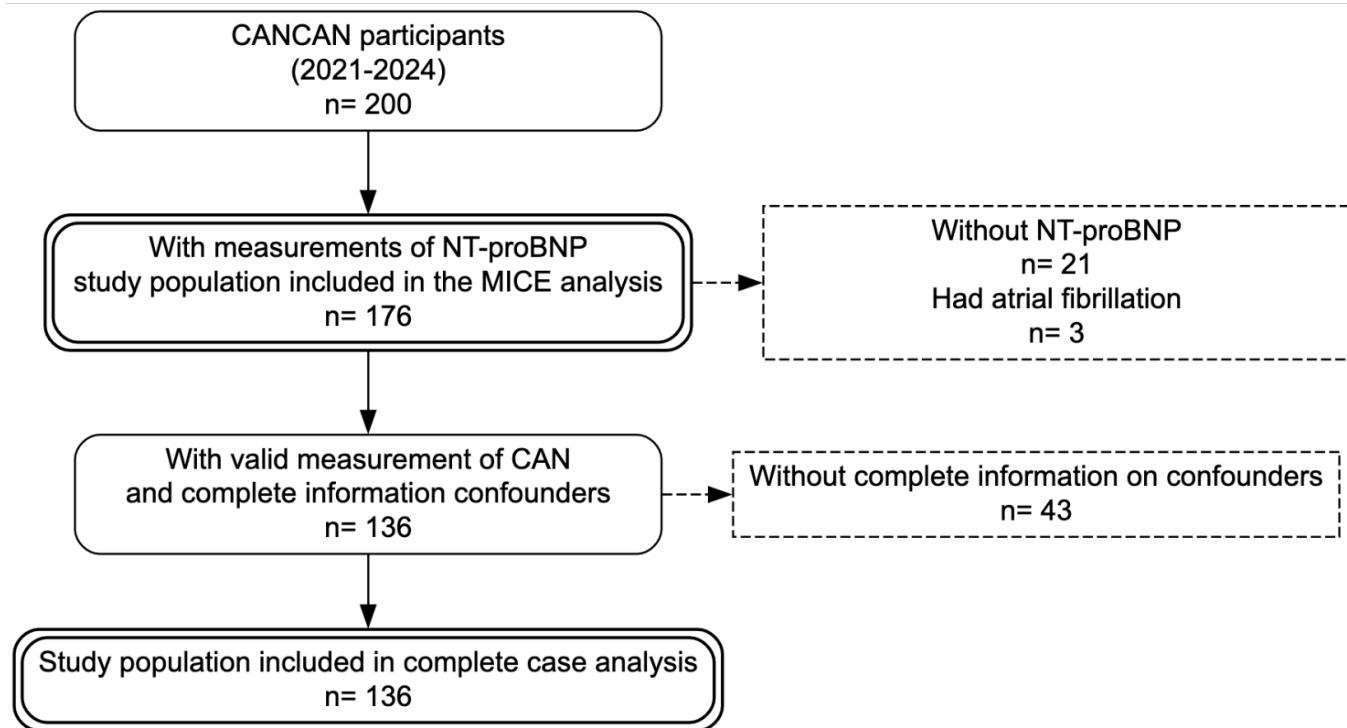


Figure S2: Distribution of NT-proBNP, NYHA, and WATCH-DM

