

# **Cardiovascular autonomic dysfunction impact on cardiovascular complications across glucose metabolism**

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# **Acknowledgements**

Thanks for all the fish.

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## **Study III**

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

## **Additional publications**

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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  
**DKD:** Diabetic kidney 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

## *Abbreviations*

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**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)

**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

# 1. Introduction

Diabetes mellitus is a growing global health concern, posing pressing challenges for public health systems<sup>1</sup>. As prevalence rises, more individuals are exposed to an increased risk of premature mortality and cardiovascular disease (CVD)<sup>1</sup>. At the same time, people live longer with diabetes, and therefore endure extended periods under the burden of diabetes-related complications<sup>2</sup>. 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<sup>[3]4</sup>. Early detection of CVD risk and asymptomatic heart failure, is desired.

Over the last decades, cardiovascular autonomic dysfunction has repeatedly gained attention as a risk factor for CVD<sup>5</sup>. 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<sup>6</sup>. Despite its recognition as a CVD risk factor, 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)<sup>7</sup>. Signs of autonomic dysfunction, may already be present in individuals with prediabetes<sup>8</sup>. Despite rising prevalence and increased CVD risk, people with prediabetes often remain outside structured treatment pathways [9]<sup>10</sup>. 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<sup>[11]12</sup>. This increased accessibility allows for continuous monitoring and a better understanding of HRV over extended periods and under various free-living conditions<sup>13</sup>. However, long-term HRV patterns and specific diurnal responses in relation to 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

## *1. Introduction*

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and carotid artery distensibility in populations covering the whole glycemic continuum, from healthy glucose metabolism to type 2 diabetes.

## **2. Background**

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.

### **2.1. Type 2 diabetes and prediabetes**

The progression from normal glucose metabolism 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 [<sup>14</sup>]<sup>15</sup>. Insulin resistance occurs when certain tissues, such as muscle and liver tissues, lose their sensitivity to insulin. As a result, glucose is not effectively taken up by these tissues and remains in the circulation. Meanwhile, beta-cell function deteriorates, leading to a diminished insulin response to glucose levels. Years before diagnosis, these changes contribute to rising fasting and postprandial glucose levels<sup>14</sup>.

The body regulates glucose through various mechanisms. During fasting, pancreatic alpha cells secrete glucagon, which stimulates hepatic glucose production via glycogenolysis and gluconeogenesis. 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. Insulin and incretins work together to suppress hepatic glucose production, while insulin promotes glucose uptake in muscle and adipose tissue. 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. 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<sup>15</sup>.

Progression towards diabetes is a continuous process, with T2D defined based on glucose thresholds associated with an increased risk of diabetes-specific microvascular complica-

## *2. Background*

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tions, particularly retinopathy. The World Health Organization (WHO)<sup>16</sup> and American Diabetes Association (ADA)<sup>17</sup> 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). The OGTT measures glucose levels two hours after the ingestion of a standard 75-gram glucose load in the fasting state. Many complications of diabetes, such as macrovascular disease, neuropathy, cancer, and cognitive impairment, may start to develop at earlier stages of dysglycemia<sup>[18]1920</sup>. 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)<sup>17</sup>. In parallel with the growing prevalence of T2D, the prevalence of prediabetes is also on the rise<sup>9</sup>.

Risk factors for progression to T2D and its complications range from genetic predisposition to lifestyle and socio-environmental factors. The most common risk factors to diabetes is obesity, and in particular central obesity<sup>21</sup>. 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<sup>22</sup>.

Diabetes increases the risk of both microvascular and macrovascular complications, which are major contributors to the morbidity and mortality associated with the disease<sup>15</sup>. 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 inflammation<sup>23</sup>. These processes contribute to endothelial dysfunction and vascular damage<sup>23</sup>. 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<sup>10</sup>.

## **2.2. 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<sup>24</sup>. At the individual level, these exposures often manifest through more proximal biological risk factors, including hypertension, hypercholesterolemia, diabetes, and obesity. 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. Risk factors contribute to distinct pathophysiological mechanisms across different types of CVD, involving structural, signaling, inflammatory, and hemodynamic changes within the cardiovascular system. 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.

### **2.2.1. Arteriosclerosis**

Emerging evidence emphasizes the role of vascular aging in early disease development, extending beyond the traditional focus on cardiovascular endpoints<sup>25</sup>. 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<sup>26</sup>. Arterial stiffness arises from progressive remodeling of the arterial wall [<sup>25</sup>]<sup>27</sup>. 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<sup>26</sup>. Remodeling of the arterial wall increases systolic blood pressure and reduces coronary perfusion, thereby contributing to the development of hypertension and, eventually, cardiovascular disease<sup>28</sup>. Additionally, arterial stiffness elevate the pulsatile load on the microcirculation, promoting the progression of chronic kidney disease, vascular dementia, and Alzheimer's disease<sup>25</sup>.

### **2.2.2. 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 (ref.) often at specific sites such as the coronary and carotid arteries. 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. 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,

## *2. Background*

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often contain a large lipid-rich necrotic core, a thin fibrous cap, and infiltration by inflammatory cells such as macrophages. 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<sup>29</sup>. 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<sup>[30][31]</sup>.

### **Myocardial infarction**

Myocardial infarction occurs due to the rupture of an atherosclerotic plaque in the coronary arteries, triggering thrombus formation that blocks blood flow. This leads to oxygen deprivation (ischemia) and subsequent myocardial injury or necrosis. If untreated, this process can cause extensive cardiac damage and fatal arrhythmias. Over the past decades, the incidence of myocardial infarction has declined in high-income countries(ref.) with a marked reduction in MI-related mortality(ref.). 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 [32]<sup>33</sup>. Medically, the improved preventive management of hypertension and hyperlipidemia has reduced the burden of atherosclerotic disease. 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. 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<sup>34</sup>. Similar to the general population, its incidence and fatality have declined among people with diabetes.

**Stroke** x 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 emboli. These emboli then travel through the bloodstream and eventually lodge in the cerebral arterial tree, causing ischemic stroke. 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<sup>35</sup>. Ischemic stroke remains one of the global leading contributors to mortality and disability<sup>36</sup>. The incidence, prevalence, and cause-specific mortality of stroke remain high but have stagnated, although some declines have been observed in high-income countries<sup>37</sup>. 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 [38]<sup>39</sup>. In T2D,

the ischemic stroke risk is elevated almost two-fold compared with individuals without diabetes<sup>34</sup>.

### **2.2.3. 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<sup>40</sup>.

It may arise as a consequence of atherosclerosis, arteriosclerosis, or both, contributing to myocardial ischemia, pressure overload, and structural cardiac changes. 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. It is a complex cardiovascular disease caused by structural and functional changes in the heart musculature, affecting systolic and/or diastolic pumping function. Heart failure is generally classified into two subtypes: heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF). Both subtypes involve cardiac remodeling but are defined by left ventricular ejection fraction (LVEF). 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. HFrEF is often a consequence of repeated, non-fatal myocardial infarctions. These events can leave behind scar tissue in the myocardium, impairing the heart's ability to contract effectively and leading to progressive systolic dysfunction.

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<sup>41</sup>. 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. 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<sup>42</sup>. 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). As a result, HFpEF is commonly underdiagnosed and consequently detected at more severe stages, leading to hospitalization<sup>42</sup>.

### **2.3. Cardiovascular autonomic dysfunction**

The cardiovascular system is regulated by autonomic nervous system which influences heart rate and vasoconstriction through neurotransmitter release by the sympathetic and parasympathetic nerves. The primary neurotransmitter of the sympathetic nervous system is noradrenaline, while the parasympathetic nervous system primarily releases acetylcholine by stimulation through the Vagus nerve. 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. It also slows AV nodal conduction, predominantly via the left vagus nerve, thereby prolonging atrioventricular conduction time. 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 and physical activity.

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 parasympathetic function and an increase in sympathetic activity. Additionally, metabolic-related conditions such as obesity and diabetes have been shown to further contribute to cardiovascular autonomic dysfunction (autonomic dysfunction). Autonomic dysfunction reflects a stressed cardiometabolic environment, as both dysfunction in lipid and glucose metabolism are associated with increased sympathetic activity<sup>43</sup>. 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<sup>43</sup>. Therefore, the relationship between autonomic dysfunction and cardiometabolic factors is likely a vicious cycle<sup>44</sup>. The consequences can lead to autonomic dysfunction/neuropathy (CAN), resulting dysregulation in heart rate and vascular dynamics. 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. 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

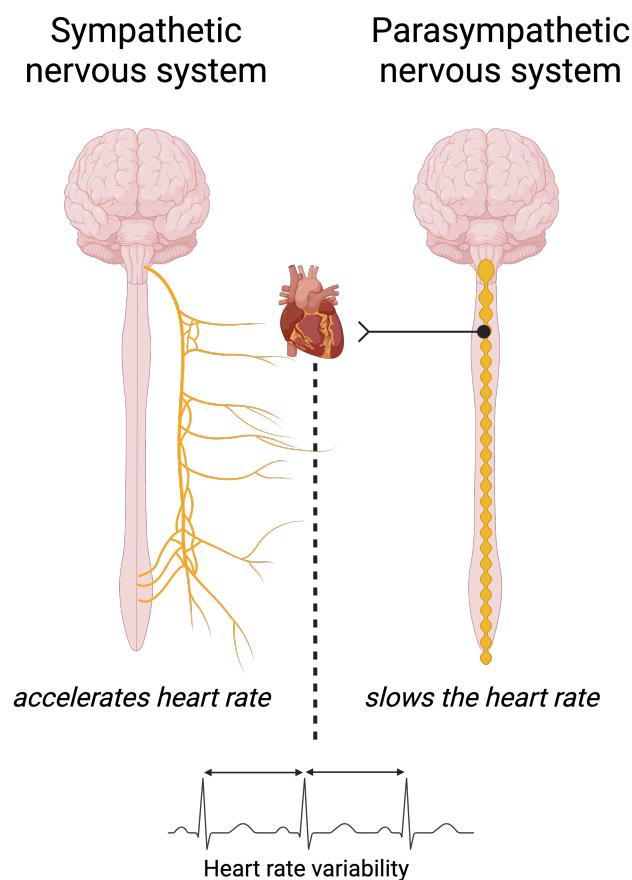


Figure 2.1.: Autonomic nervous system and heart rate variability. (Source: Author)

## *2. Background*

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be observed in its natural 24-hour (diurnal) pattern<sup>13</sup>. Most studies have examined autonomic function using short-term ECG recordings at rest. 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)[reflecting what]. 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(ref.,ref.ref.,ref.). 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<sup>45</sup>. Parasympathetic activity is also impaired in individuals with high cardiometabolic risk and T2D, as reflected by reduced baroreflex sensitivity<sup>46</sup> and lower HF and RMSSD short-term HRV. Before onset of diabetes and during progression of diabetes long-term (24-hour) HRV has shown to be lower compare to those with normal glucose metabolism [44]<sup>8</sup>. Cardiovascular autonomic reflex tests (CARTs) and orthostatic hypotension are considered the gold standard for assessing CAN<sup>47</sup>. 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<sup>47</sup>. 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. 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.

## **2.4. Risk-stratification**

Current cardiopreventive guidelines place strong emphasis on prevent and treat T2D. The 2022 ADA/EASD guidelines for the management of hyperglycemia in T2D recommend, cardioprotective medication (GLP-1 receptor analogues and SGLT2-inhibitors) as first-line options for individuals at high cardiovascular risk. 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 albuminuria. However, no additional preclinical markers are recommended to identify individuals at higher CVD or HF risk. 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

## 2.4. Risk-stratification

[<sup>48</sup>]<sup>49</sup>. During the progression and following the onset of T2D, preclinical stages may be characterized by markers of elevated cardiovascular risk, highlighting the potential 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 preclinical conditions. This approach aids in identifying patients for prognostic or diagnostic purposes, identifying subgroups that require further evaluation, intensified treatment, or lifestyle modifications.

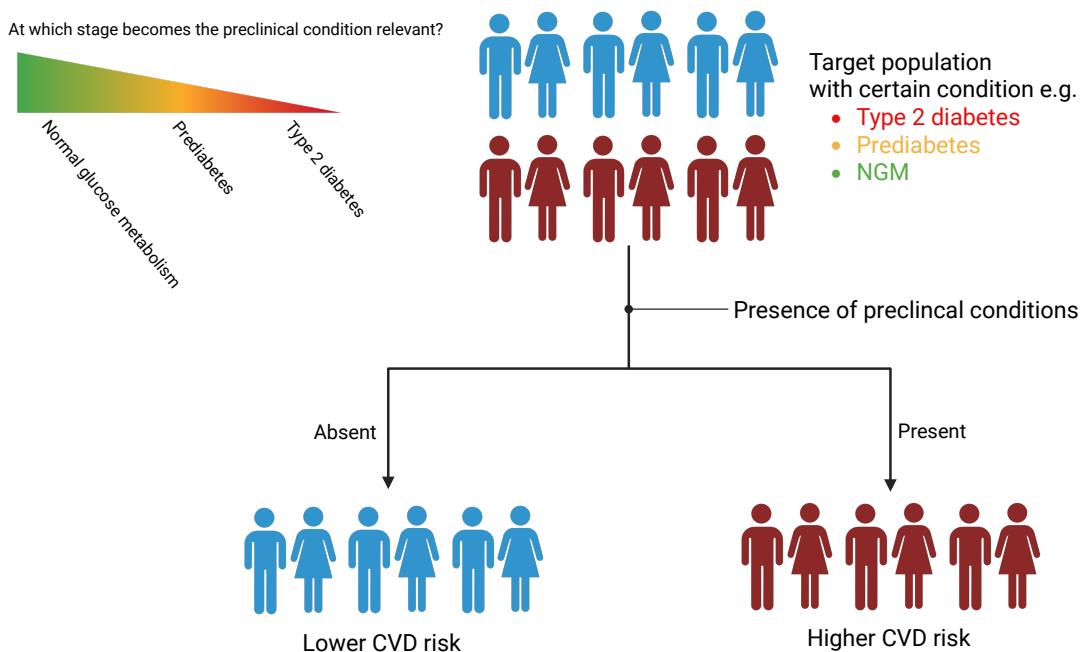


Figure 2.2.: Risk-stratification based on preclinical disease. (Source: Author)

Autonomic dysfunction despite its relationship with cardiovascular complication has not been used in clinical practice. Larger epidemiological cohort studies encompassing various stages of diabetes risk, from normal glucose metabolism to 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, allowing understand the relationship between autonomic dysfunction and cardiovascular complications. They also have potential to determine when, along the trajectory of diabetes progression and duration, autonomic function are meaningful for cardiovascular risk-stratification.

### **3. Aim and hypothesis**

The hypotheses of this dissertation are:

CAN and autonomic dysfunction is associated with CVD and acts as an early risk factor for heart failure and other cardiovascular complications, including stroke, and myocardial infarction in patients with prediabetes and/or T2D. In addition autonomic dysfunction is associated with higher levels of sub-clinical measures 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 normal glucose metabolism, 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 (Pro-BNP), WATCH-DM risk, and New York Heart Association (NYHA) scores among individuals with T2D.

## 4. Materials and methods

### 4.1. Overview of the studies

Table 4.1.: 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   Descriptive cross-sectional study
Design	Aetiological cross-sectional study	Aetiological prospective cohort study	CANCAN study
Cohort	Maastricht study	ADDITION-PRO study	173 patients with T2D visiting outpatients clinics
Study	3673 people with normal glucose metabolism, prediabetes, and T2D	2082 people with high risk of diabetes	
Data	Population-based cohort sources from The Maastricht Study in the Netherlands	Cohort study of selected people based on having high risk of diabetes	Clinical cohort study
Determine	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

#### *4. Materials and methods*

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Study I	Study II	Study III
Statistical analysis Missing data	Poisson regression Multiple imputation of chained equations for confounders	Logistic regression Complete case analysis and multiple imputation of chained equations for CART and confounders
Complete case analysis		

##### **4.1.1. Study population**

###### **4.1.1.1. 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 people 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). In the analysis of Study I, the study among 7449 people 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 i

###### **4.1.1.2. 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.

#### 4.1. Overview of the studies

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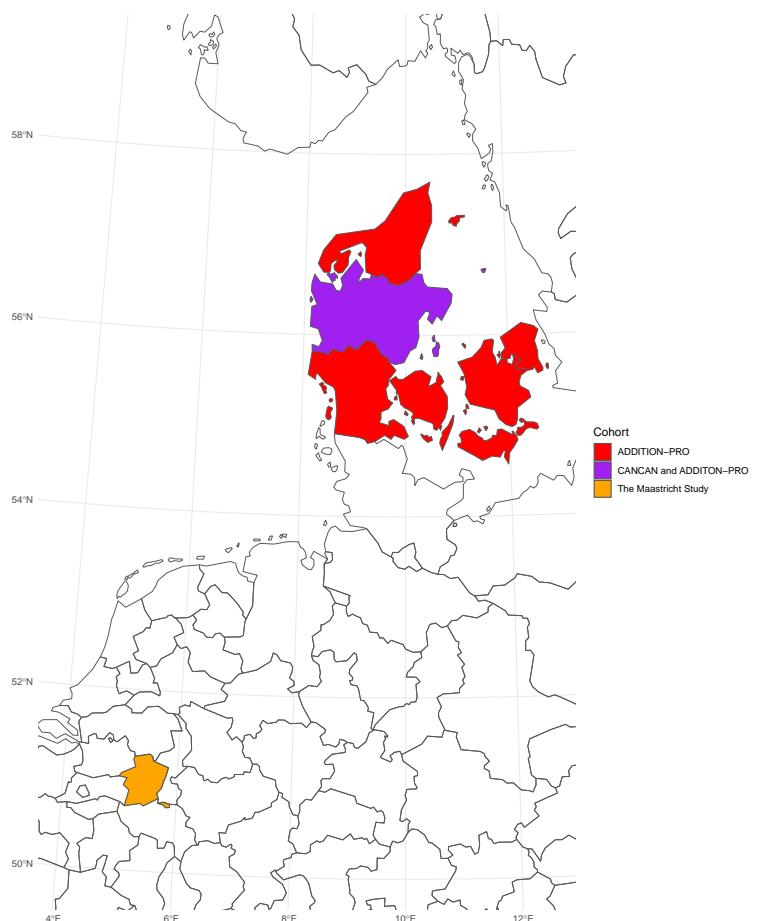


Figure 4.1.: Study populations

#### *4. Materials and methods*

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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. 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 disorder 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, we included participants with a least 48-hour recording for our first analysis, and then include those participants with hourly measures of physical acceleration during the hourly HRV recording for th second analysis. We also excluded participant with prior CVD ten years before inclusion.

Disease history and follow-up data for the population were obtained from Denmark's unique 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.

#### **4.1.1.3. 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 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.

## **4.2. Study variables**

### **4.2.1. Measures for autonomic dysfunction/ neuropathy**



Figure 4.2.: Left: Holter monitor Middle: Actiheart Right: Handheld Vagus™ device

#### **Heart rate variability**

In study I-III a device was 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 HRV. The pool of heartbeat data, we extrapolated time-domain and frequency-domain HRV indices.

#### *Time-domain indices*

#### 4. Materials and methods

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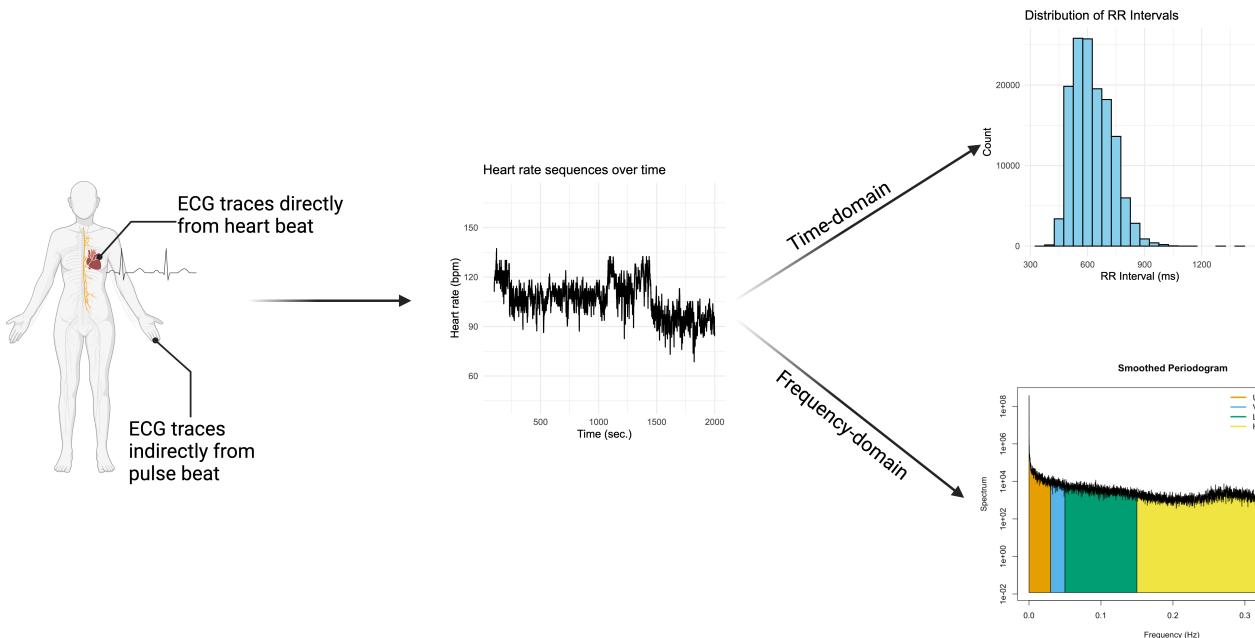


Figure 4.3.: Heart rate variability. (Source: Author)

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. 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 minimum recording duration of 18 hours was required for further analysis. 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. 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.

#### **ActiHeart heart rate and physical activity in study II**

Heart rate was measured using a combined accelerometer and heart rate monitor (ActiHeart, CamNTech, 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, and HRV was derived as the variation between

Table 4.2.: **Box 1** Time-domain indices reflections of autonomic function

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

#### 4. Materials and methods

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Table 4.4.: **Box 2** Frequency-domain indices reflections of autonomic function

Frequency domain HRV	Description
<b>Variance of all NN intervals 0.4 Hz, total power (TP, in ms<sup>2</sup>)</b>	Measures the total variation in interbeat intervals, reflecting both short- and long-term autonomic regulation by the sympathetic and parasympathetic nervous system <sup>6</sup> .
<b>Ultra low-frequency range (ULF, in ms<sup>2</sup> 0.003 Hz)</b>	Measures very long-term oscillations in interbeat intervals, influenced by autonomic responses to circadian rhythms, physical activity, metabolic processes, and thermoregulation [51]52.
<b>Very-low-frequency range (VLF, in ms<sup>2</sup>; 0.003–0.04 Hz)</b>	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[53]54.
<b>Low-frequency range (LF, in ms<sup>2</sup>; 0.04–0.15 Hz)</b>	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 <sup>55</sup> .
<b>High-frequency range (HF, in ms<sup>2</sup>; 0.15–0.4 Hz)</b>	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 <sup>56</sup> .

## 4.2. Study variables

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). We used pulse rate ratios measured under different conditions. Three standardized cardiovascular autonomic reflex tests (CARTs) were performed—lying-to-standing, deep breathing, and the Valsalva manoeuvre, following a standardized protocol conducted between 8:00 a.m. and 2:00 p.m., after 10 minutes of supine rest. Smoking and caffeine intake were prohibited two hours before testing. Each test was conducted once by trained examiners.

Manifest CAN was defined as two or more abnormal CARTs using age-specific cut-off values (ref.). The Vagus™ device's accuracy has been validated against FDA standards and stationary devices, showing moderate to high reproducibility (ref.).

### Cardiovascular autonomic reflex test



Figure 4.4.: CART

HRV was derived from all CARTs using autoregressive spectral analysis. Time domain measures included SDNN and RMSSD, while frequency domain measures included LF, HF, and total power. 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 (ref.).

#### 4.2.2. 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.

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. Physical activity was assessed via self-report in Studies I, II, III, with Study I capturing total and

#### *4. Materials and methods*

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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. In Study II also used combined accelerometry and heart rate monitoring (ActiHeart) to estimate PAEE. Study II included register-based data on socioeconomic status at baseline, including education length, income, and employment status. All studies included measurements of body mass index (BMI), waist circumference, and systolic and diastolic blood pressure, obtained during clinical examinations.

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. 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. Study III additionally measured creatinine, estimated glomerular filtration rate (eGFR), and urine albumin-to-creatinine ratio.

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

### **4.3. Outcomes**

#### **4.3.1. Arterial stiffness**

Arterial stiffness characterized arteriosclerosis and atherosclerosis properties of the arteries. The stiffness of different trees of the vascular musculature can assessed both locally and dynamically. Aortic and carotid stiffness were assessed as markers of arterial stiffness, following previously described procedures<sup>58</sup>.

##### **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 from the time between the ECG systole and the arrival of the pressure wave at the femoral and carotid measurement sites and the distance between these two measurement sites. It 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<sup>58</sup>.

### 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  $\Delta D$  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)<sup>58..</sup>

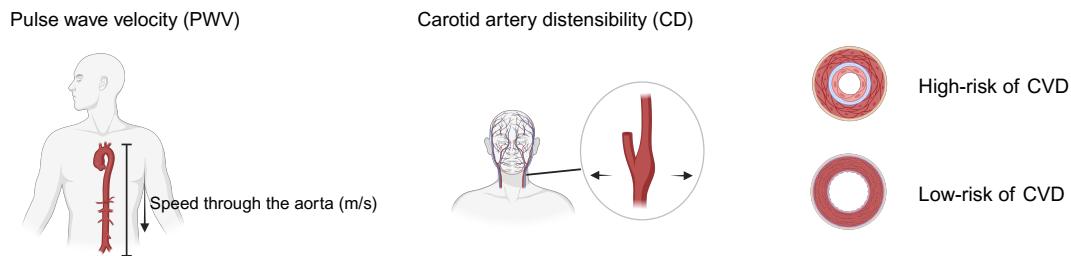


Figure 4.5.: Measures of arterial stiffness, measured dynamically at the aortic and local carotid sites. (Source: Author)

#### 4.3.2. 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. It derives from B-type natriuretic peptide (BNP) which is a cardiac 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, blood samples were taken at Study site. Description of the NT-proBNP analysis of plasma samples is described in supplementary material [ref].

We used a modified version validated The WATCH-DM heart failure risk score. 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.

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

#### 4. Materials and methods

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Table 4.6.: ?(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

#### 4.3.3. Cardiovascular events

Information on CVD events and mortality was obtained from the Danish National Patient Registers until 2021. 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.

### 4.4. Statistical Methods

#### 4.4.1. Cross-sectional analysis

In Study I, we used multiple linear regression to investigate associations between multiday 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. We add interaction sex to observe if the association differed between sex. We performed sensitivity analyses excluding individuals on antihypertensive treatment or glucose-lowering

medication. 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 excluded participants with beta-blocker treatment or prior CVD.

#### 4.4.2. Time-to-event analysis

In Study II, we used Poisson regression models to quantify the associations between HRV and cardiovascular events, as follow-up data were undisturbed over time and to avoid assumptions of proportional hazards<sup>59</sup>. 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. 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.

#### 4.4.3. Effect modification [det kan evt. kortes ned]

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 modifier. This means that the observed relationship between the exposure and the outcome is not uniform across all subgroups. Instead, it differs across strata defined by the effect modifier<sup>60</sup>.

#### *4. Materials and methods*

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In Study I, we hypothesize that the association between 24-hour and arterial stiffness was stronger in strata of progression of diabetes (normal glucose metabolism, prediabetes, T2D). We therefore first stratified by diabetes status to observe the size of the association across strata. We then combine all groups and include an interaction term between HRV and diabetes status. We did subsidiary analysis to check if the effect was modified by dysglycemia by stratifying HbA1c and fasting plasma glucose into deciles. In Study II, we quantified whether the association between multiday HRV and CVD endpoints varied by sex to explore potential biological dimorphism.

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, we hypothesized no significant effect modification between groups with and without symptoms. Similarly, we explored whether the association remains present in the group classified as low to moderate risk of heart failure, based on the WATCH-DM risk score.

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.

##### **4.4.4. 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, we followed Rubins Rule. 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 variances. Thus, we ensure valid statistical inferences by accounting for the uncertainty introduced by missing data.

In Study II, we imputed confounders to include as many participants and avoid excluding population with our without cardiovascular or mortality events. We imputed dataset 10 times. In Study III, we imputed missing CART, 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 bias<sup>61</sup>. 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.

#### 4.4.5. Instrumental bias

In Study I-III we are investigating the body 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) [ref.]. To account for this, we adjusted for mean arterial pressure in our models.

##### *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.

##### *Biomarker of Heart Failure*

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

## 5. Results

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

### 5.1. Study I

#### 5.1.1. Descriptive

In The Maastricht Study, [10,000 participated by Date], of those 1316 reported prior CVD<sup>62</sup>. 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<sup>62</sup>.

#### 5.1.2. 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 5.1 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<sup>62</sup>. 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<sup>62</sup>.

##### 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 5.1 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:

### 5.1. Study I

Table 5.1.: ?(caption)

(a)			
**Characteristic**	**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)
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/m <sup>2</sup> )	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)
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 <sup>2</sup> )	12,596 (8,880, 17,498)	10,615 (7,134, 15,374)	8,880 (6,064, 12,722)
ULF (ms <sup>2</sup> )	10,771 (7,392, 15,142)	8,948 (5,852, 13,374)	7,524 (5,036, 11,001)
VLF (ms <sup>2</sup> )	1,198 (833, 1,692)	1,015 (685, 1,478)	816 (541, 1,267)
LF (ms <sup>2</sup> )	421 (257, 651)	328 (200, 540)	261 (154, 422) 41
HF (ms <sup>2</sup> )	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	15.0 (11.8, 18.8)	13.5 (10.4, 16.9)	12.5 (9.9, 16.0)

## 5. Results

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1.9; 4.0), and 2.1% (CI: 1.5; 2.6), respectively<sup>62</sup>. 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<sup>62</sup>.

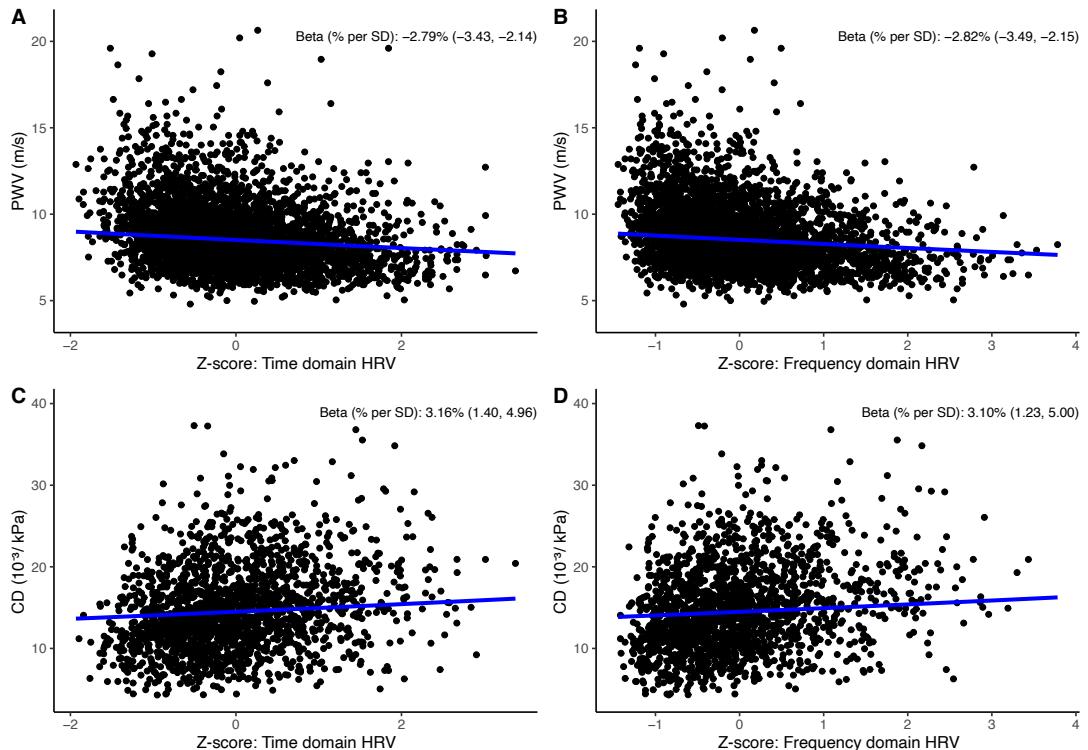


Figure 5.1.: Figure from [authors]. Cardiovascular autonomic neuropathy and indices of heart failure in T2D: The CANCAN Study<sup>62</sup>. (Paper I, appendix)

### 5.1.3. Effect modification of diabetes status

The study population represented diabetes risk of normal glucose metabolism (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 characteristics by diabetes are described in Table 5.1.

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]<sup>interaction(\*)<sup>p-value<0.01</sup></sup> CD: 8.0 [CI:3.8; 12.5]<sup>\*<sup>p-value<0.01</sup></sup>) but not by T2D (cf-PWV: -3.5 % [CI: -4.8; -2.1])<sup>\*<sup>p-value<0.1</sup></sup> CD: 4.8 % [CI:1.3; 8.4]<sup>\*<sup>p-value<0.1</sup></sup><sup>62</sup>. For the indices SDNN and SDANN, the association with both cf-PWV and CD was significantly modified by both prediabetes and T2D<sup>62</sup>.

The association between HRV frequency-domain Z-score and cf-PWV was significantly modified from normal glucose metabolism by prediabetes (-5.7 %[CI:-7.4; -3.9]<sup>\*<sup>p-value<0.01</sup></sup>) and T2D (-3.9 %[CI:-5.4; -2.3]<sup>\*<sup>p-value<0.05</sup></sup>) while CD was only modified by prediabetes (8.3 %[CI:3.6; 13.2]<sup>\*<sup>p-value<0.01</sup></sup>) but not by T2D (5.3 %[CI:1.4; 9.4]<sup>\*<sup>p-value<0.1</sup></sup>)<sup>62</sup>. 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 status<sup>62</sup>.

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 5.2). 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 5.2 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 5.2 D). No association between HRV frequency domain Z-score and CD was observed at HbA1c levels between 4.8% and 5.2%.

## 5.2. Study II

### 5.2.1. Descriptive

The ADDITION-PRO population consisted of 1,627 participant with a 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

## 5. Results

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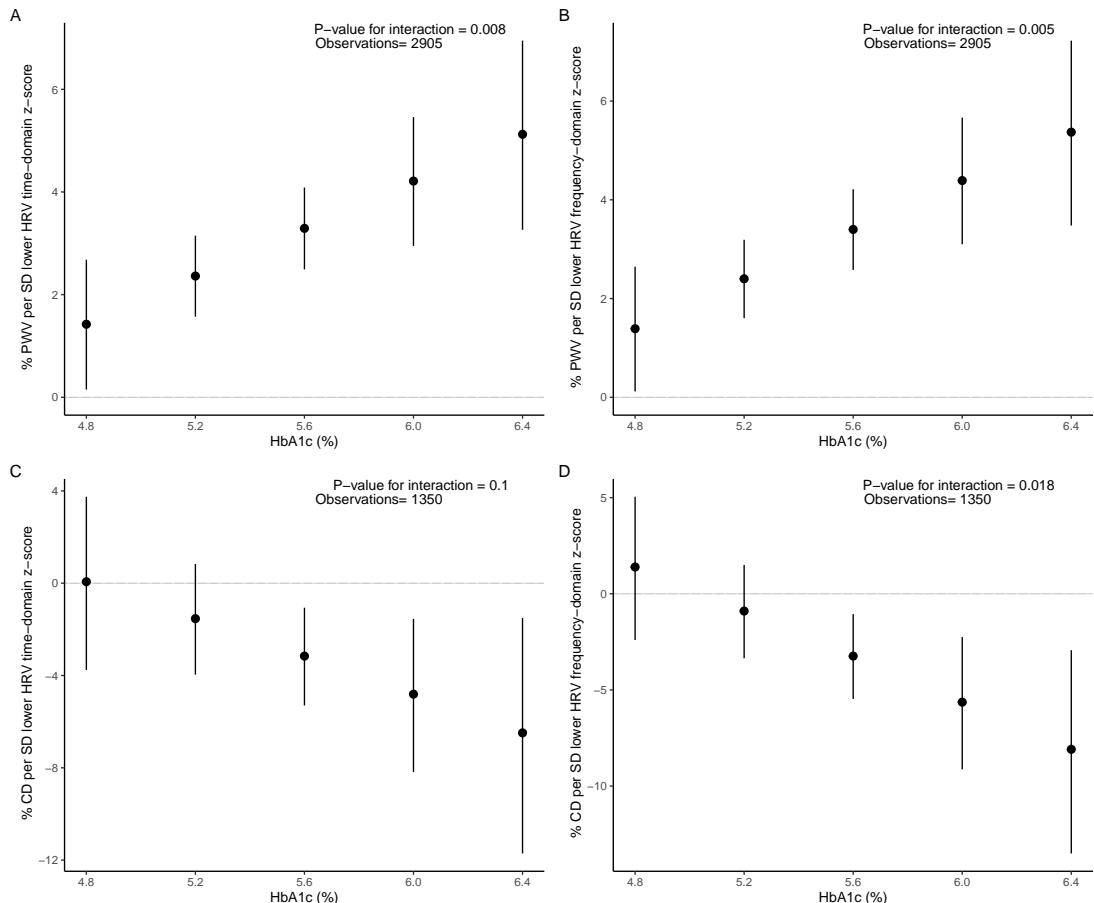


Figure 5.2.: ?(caption)

Table 5.2.: Study participants characteristics

Characteristic	Overall, N = 1,625	<100, N = 148	100-120, N = 312	120-140, N = 457	140-160, N = 346	>160, N = 362
sex						
Men	866 (53%)	68 (46%)	148 (47%)	206 (45%)	203 (59%)	241 (67%)
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						
1	263 (16%)	40 (28%)	70 (23%)	65 (14%)	41 (12%)	47 (13%)
2	750 (47%)	58 (40%)	145 (47%)	214 (47%)	162 (47%)	171 (48%)
3	598 (37%)	47 (32%)	95 (31%)	174 (38%)	140 (41%)	142 (39%)
BMI (kg/m <sup>2</sup> )	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)
Pulse rate (bpm)	67.4 (10.9)	77.7 (11.2)	72.6 (9.3)	67.9 (9.3)	65.3 (9.3)	60.0 (9.8)
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)
vo2max	26.6 (7.8)	24.8 (7.5)	24.8 (7.5)	26.1 (6.8)	27.0 (8.0)	28.7 (8.7)
rest_hr	57.3 (7.3)	67.8 (5.7)	63.3 (5.0)	58.4 (4.5)	55.0 (4.2)	49.8 (4.9)
med_any_antihypertensive	753 (47%)	88 (61%)	149 (48%)	216 (47%)	147 (43%)	153 (43%)

(9%). I splitted SDNN 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).

Charteristics are described in Table 5.2. Participants in the lowest SDNN group (<100 ms) were older ( $67.4 \pm 6.9$  years), had higher BMI ( $28.1 \pm 5.4$ ), HbA1c ( $5.9 \pm 0.9$ ), triglycerides ( $1.5 \pm 0.9$  mmol/L), and resting heart rate ( $67.8 \pm 5.7$  bpm), were more likely to use anti-hypertensive medication (61%), and had lower physical activity energy expenditure ( $46.8 \pm 24.0$  kJ/day) compared to those with higher SDNN levels.

### 5.2.2. 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) bpm. 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). When I pre-adjusted for resting heart rate, the proportion of the association explained between HRV and MACE, HF, and all-cause mortality was 14%, 25%, and 19%, respectively. I included knots in the model, which showed that the risk became higher when SDNN fell below 120 to 110 ms (approximately below the 20th percentile), suggesting a potential cut-off point for higher risk. I therefore calculated the incidence rate (IR) at SDNN levels of 100 ms, 120 ms, and 160 ms, respectively, and plotted these as a function of age.

## 5. Results

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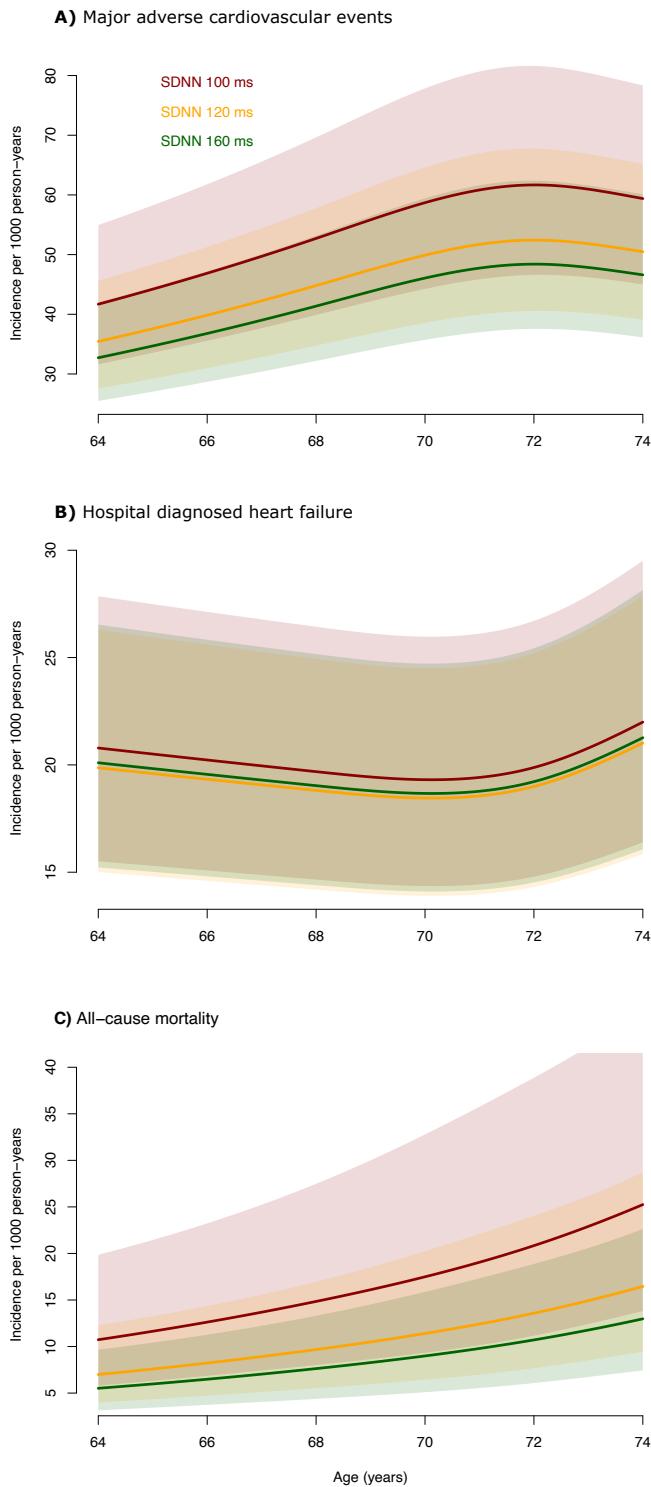


Figure 5.3.: Multiday SDNN levels (100 ms, 120 ms, 160 ms) by age-specific incidence rates for A) major adverse cardiovascular events, B) heart failure, and C) all-cause mortality

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 5.3 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 5.3 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 5.3 C). The IR for all-cause mortality became higher with age.

### 5.2.3. 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). 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). After peaking, heart rate remained stable throughout the afternoon before gradually decreasing.

In Figure 5.4, 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 to 1.00 per SD higher SDNN) (see Figure 5.4 A). 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 5.4 D). 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 5.4 B). 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 5.4 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 5.4 C). No clear trends of association were observed between heart rate and all-cause mortality.

## 5. Results

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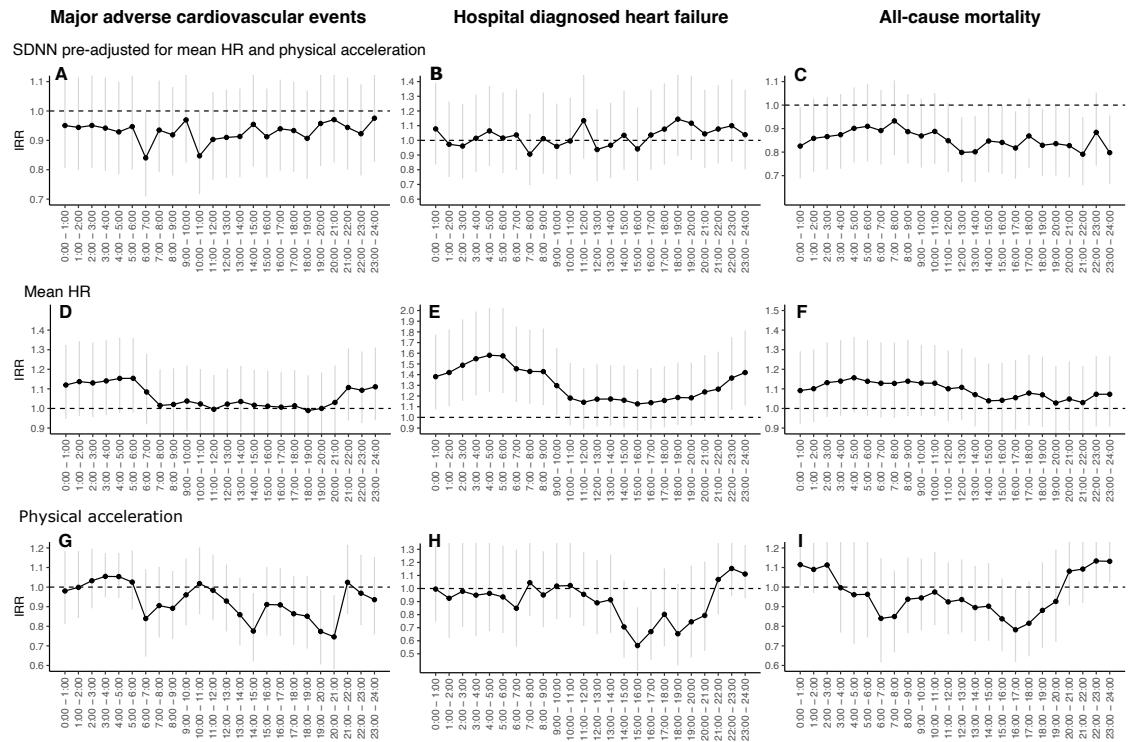


Figure 5.4.: Hourly SDNN levels (100 ms, 120 ms, 160 ms) by age-specific incidence rates for A) major adverse cardiovascular events, B) heart failure, and C) all-cause mortality. Figure adapted from [authors]. —. (Paper II, appendix)

## 5.3. Study III

### 5.3.1. Descriptive

In study III, 179 participants with type 2 had measures of NT-proBNP and performed the CART test. CAN was present in 30% ( $n = 54$ ) of participants (36% among those with valid CAN measurements (Figure 5.5 A)). Meanwhile, 24% ( $n = 43$ ) were unable to complete the CART assessment adequately, primarily due to irregular heart rhythms ( $n = 8$ ) or insufficient air pressure during the Valsalva manoeuvre ( $n = 21$ ). 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 20%) and declined eGFR (< 60) (36% 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.

### 5.3.2. 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 5.5 E). 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 CAN. 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;  $n=24/146$ ). No significant association was identified for the lying-to-standing test (OR 0.80, 95% CI: 0.32; 1.97;  $n=54/108$ ). 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). 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. 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 significant ( $p = 0.4$ ). 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). Participants with CAN had 1.7 (95% CI: 0.3 to 3.0) point higher WATCH-DM risk score compared to those without CAN. 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.

## 5. Results

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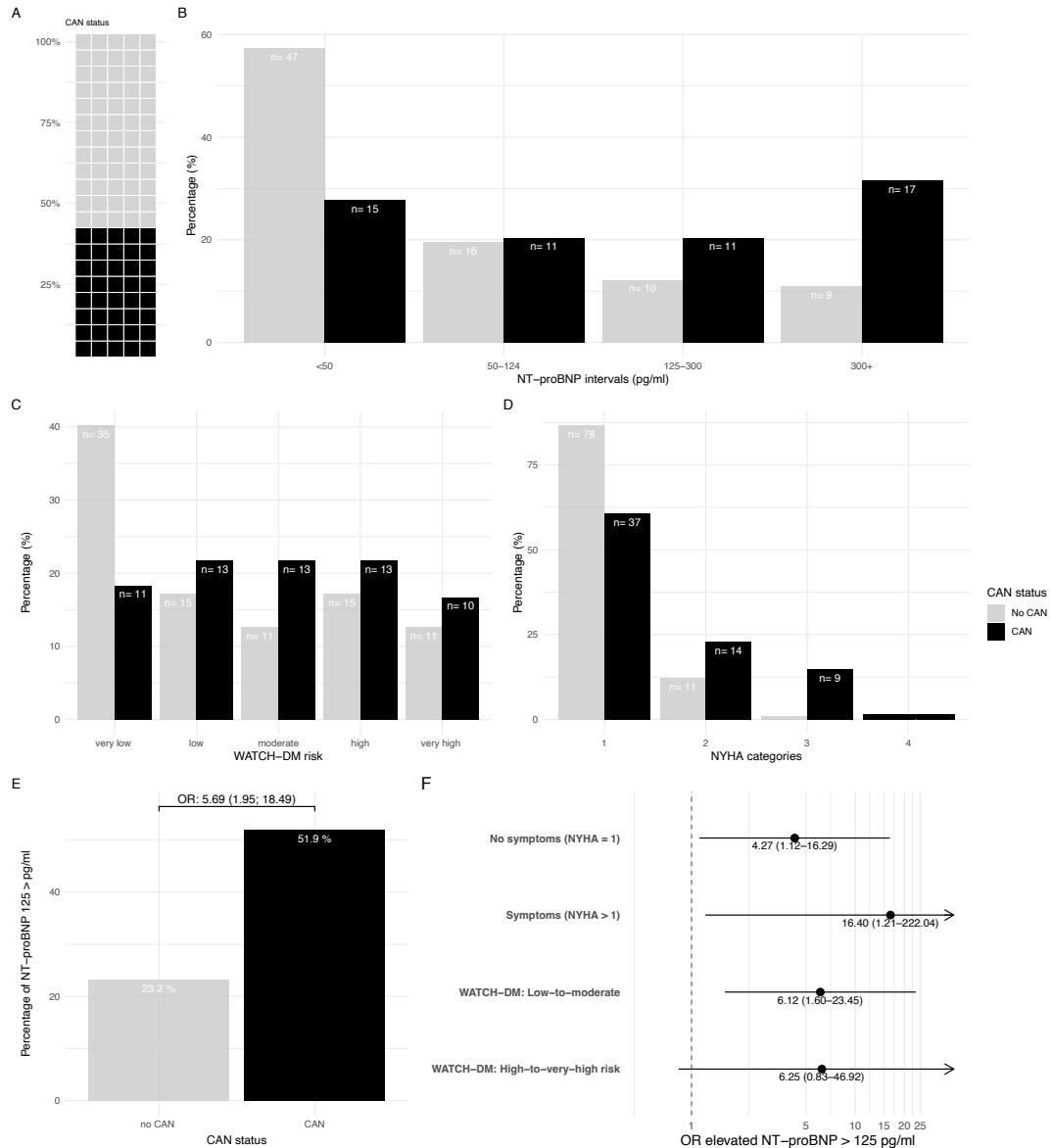


Figure 5.5.: Relationship between CAN and indicators for heart failure. Figure from [authors]. Cardiovascular autonomic neuropathy and indices of heart failure in T2D: The CANCAN Study. (Paper III, appendix)

## **6. 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 early 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.

## 6.1. 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, assessed dynamically at the aortic site 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. While no significant difference was observed between prediabetes and T2D, the association was modified by HbA1c after excluding individuals with T2D, supporting the modifying effect of dysglycemia. 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).

Following Study I, individuals at higher risk of developing diabetes came into focus, using data from the ADDITION-PRO (Study II) cohort, which represents a high-risk population for diabetes. In Study II, per lower SD in SDNN, measured over a multiple days, was associated with 18%, 24%, and 21% higher risk 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. Residual adjustment of 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.

Our findings suggest that both long-term HRV measures and hourly HRV responses may serve as indicators of CVD risk. However, a limitation is that long-term HRV was assessed under free-living conditions, which restricts the ability to perform standardized tests of autonomic function. In the CANCAN Study (Study III), I used standardized CARTs to define CAN and describe indicators of heart failure, defined by elevated NT-proBNP, WATCH-DM risk, and NYHA classification among those with and without CAN in a population with T2D. In CANCAN, two out five had CAN. Compared to individuals without CAN, these individual more often showed signs of heart failure, in-

## *6.2. Cardiovascular autonomic dysfunction and its impact on heart disease across glucose metabolism*

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cluding elevated NT-proBNP levels, higher WATCH-DM risk score, and higher classifications on the NYHA. CAN was association elevated NT-proBNP levels and the persisted even among individuals without heart failure symptoms based on NYHA classification, as well as 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, and T2D. The overall findings showed that autonomic function, assessed through heart rate dynamics of long-term HRV and diurnal HRV and 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.

## **6.2. Cardiovascular autonomic dysfunction and its impact on heart disease across glucose metabolism**

This dissertation show 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 arteriosclerosis and indications of heart failure in individuals with T2D.

### **6.2.0.1. Arteriosclerosis**

In Study I, autonomic dysfunction, measured by 24-hour HRV, showed to be associated with arterial stiffness, measured both dynamically (cf-PWV) and locally (CD). This suggests that autonomic responses under free-living conditions contribute to the development of arterial stiffness. Majority of studies 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<sup>63</sup>. Study I extended this perspective by long-term HRV and including a population without diabetes or prediabetes.

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<sup>[64][65]</sup>. Two possible mechanisms may explain how autonomic dysfunction is related to arterial stiffness. First, autonomic dysfunction may increase the vascular

## 6. Discussion

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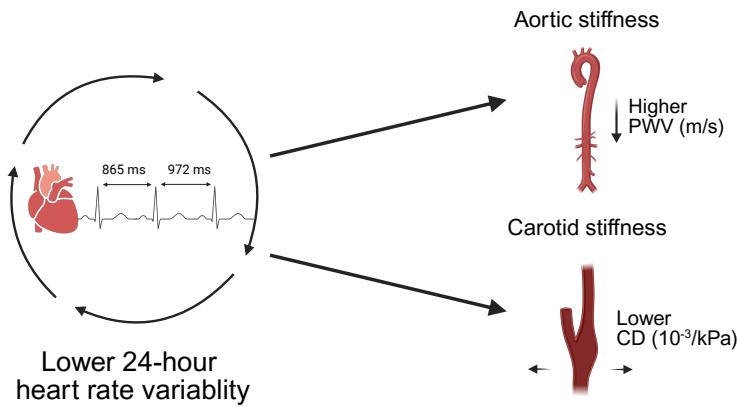


Figure 6.1.: Autonomic dysfunction and arterial stiffness. (Source: Author). —.

tone of large arteries, thereby impairing arterial elasticity. This concept is supported by animal studies. In rats, proper autonomic regulation has been shown to be essential for maintaining aortic elasticity. Chronic overstimulation of sympathetic activity can lead to structural remodeling and increased arterial stiffness<sup>66</sup>. 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<sup>[66]</sup><sup>67</sup>. Second, the autonomic nervous system regulates heart rate and cardiac contractility. 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 structural stiffer arteries by altering blood flow dynamics and 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 years<sup>68</sup>.

The association between 24-hour HRV and arterial stiffness was modified by dysglycemia. Data from the Whitehall II study showed that aortic stiffness increased more steeply with higher HbA1c values among non-diabetic individuals<sup>69</sup>. In 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. One possible explanation for the modification is that dysglycemia may induce early-stage CAN<sup>[70]</sup><sup>71</sup><sup>72</sup>. This, in turn, could affect

## *6.2. Cardiovascular autonomic dysfunction and its impact on heart disease across glucose metabolism*

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arterial stiffness to a greater degree than autonomic dysfunction in NGM, even before the onset of T2D.

### **6.2.0.2. Atherosclerosis**

In Study II, I 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.

We assessed week-long HRV to capture autonomic activity in real-life settings across several days. Our results are consistent with earlier research linking reduced HRV to cardiovascular outcomes and mortality<sup>5</sup>. 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 daily periods where HRV patterns were indicative of ischemic-related CVD risk. By using both week-long and hourly data, we identified specific periods of higher 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 day<sup>73</sup>.

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 [74]<sup>75</sup>. In this context, findings from Study I may not entirely distinguish between arterial stiffness and atherosclerosis, as shown by data from the Rotterdam Study<sup>76</sup>. As plaques develop, the associated increase in sympathetic nerve density around the arteries could transiently reduce vascular tone which over time reduce arterial elasticity<sup>77</sup>. In a smaller study of people with T2D, lower HRV was linked with increased carotid atherosclerosis<sup>78</sup>.

Second, the autonomic nervous system innervates the adventitia layer of blood vessels, where it modulates vascular tone via sympathetic fibres. 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. Notably, reducing sympathetic innervation has been shown to attenuate plaque formation in animal models<sup>79</sup>. These findings suggest that autonomic dysfunction may not only reflect but also actively contribute to atherogenesis.

## 6. Discussion

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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, with a higher risk among participants with T2D [80]81. This supports the role of autonomic dysfunction in arrhythmogenesis, which increases the risk of myocardial infarction and stroke. However, in Study II, I did not include incident atrial fibrillation as an outcome. We did not include atrial fibrillation as an outcome in Study II due to limitations in Danish registries, which often do not distinguish between short- and long-term AF, affecting diagnostic validity. Future research is needed to explore whether it could explain the higher risk of major adverse cardiovascular events.

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 reduced parasympathetic modulation of heart rate under stress may play a role in ischemia<sup>82</sup>. 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 support this by identifying mechanisms involving G-protein signaling, pacemaker activity, and mitochondrial function. These pathways influence vagal control, cardiac excitability, and energy metabolism<sup>83</sup>. The GWAS findings demonstrate that autonomic traits of HRV, are present throughout a person's life. This indicates that there is inherent biological variability in the mechanisms that determine and modulate HRV across individuals. Therefore, HRV observed in adulthood is not solely influenced by recent or concurrent risk factors. A Mendelian randomization study using data from the Rotterdam Study found that genetically predicted HRV was associated with an increased risk of atrial fibrillation<sup>84</sup>. 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 outcomes<sup>85</sup>. 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 dynamics<sup>85</sup>. 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.

### 6.2.0.3. Heart failure

The relationship between cardiovascular autonomic dysfunction and heart failure is complex<sup>86</sup>. 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.<sup>[87][88][89][90]</sup> 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 and reduces coronary perfusion pressure<sup>91</sup>. 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, through mediation analysis to assess the direct and indirect effects of arterial stiffness, with autonomic dysfunction as the main determinant. In Study II, I observed 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<sup>92</sup>. 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, 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 an increased 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 hemody-

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namic consequences that contribute to both structural and functional cardiac remodeling, which in turn leads to elevated NT-proBNP levels.

We cannot exclude the possibility that autonomic dysfunction represents an elevated demand for compensatory mechanisms as heart failure progresses. Studies have shown that patients with heart failure and lower HRV tend to have a worse prognosis in terms of mortality. If low HRV or the presence of CAN were primarily driven by existing cardiac complications, it would suggest that individuals with these conditions exhibit more pronounced sympathetic overactivity as a consequence of heart failure progression, indicating potential reverse causation. Hence, elevated sympathetic activity during rest may reflect a greater reliance on compensatory mechanisms to maintain cardiac output. More precise measures are needed to assess whether sympathetic activity acts as a primary driver of heart failure or as a secondary compensatory response to cardiac dysfunction. 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<sup>23</sup>. 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.

### **6.3. Clinical implications**

The dissertation investigates autonomic dysfunction in populations ranging from NGM to T2D and yields insights relevant for individuals engaged at different levels of the healthcare system. No specific role has yet been defined for autonomic dysfunction in clinical decision-making within healthcare, as current treatment and intervention options remain limited. Despite the fact that the results do not extend to the implementation of autonomic dysfunction in clinical practice, the findings from three included cohorts representing populations under public health, primary care, and secondary care. They mark a notable first step in addressing the impact of autonomic dysfunction on cardiovascular complications. In 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.

### 6.3.1. Public health

A central strategy in preventing CVD is the early identification and treatment of individuals at high risk<sup>93</sup>. 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 on cf-PWV and 1.6 years on CD<sup>62</sup>. 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 dynamic indicator of vascular health.

Within the public health setting, individuals with prediabetes represent a particularly vulnerable group at risk for comorbidities<sup>94</sup>. They often fall between structured care pathways, sometimes receiving regular follow-up and other times remaining outside formal healthcare systems. 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) decrease in multiday SDNN was equivalent to 4.5 additional years of aging for major adverse cardiovascular events and 2.2 to 2.4 years for heart failure<sup>95</sup>. 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<sup>11</sup>.

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<sup>13</sup>, suggesting that HRV may also reflect behavioral adaptation.

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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<sup>[11]96</sup>.

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.

### **6.3.2. 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 events<sup>97</sup>. 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<sup>98,99</sup>. 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 modeling<sup>100</sup>, and therefore cannot determine whether incorporating long-term HRV or CARTs into existing risk scores improves predictive performance beyond current guidelines. While most biomarkers have shown limited incremental value beyond established predictors (including age, sex, lipid profiles, diabetes status, and blood pressure), some studies suggest that 24-hour HRV may improve risk discrimination for CVD and all-cause mortality in individuals with T2D<sup>101</sup>, and for stroke and heart failure in older adults<sup>[87]102</sup>. 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

### *6.3. Clinical implications*

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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<sup>103,104</sup>. For example, high-intensity interval training has been shown to improve autonomic function in obese individuals with and without T2D<sup>105</sup>. 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<sup>106</sup>. 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<sup>107</sup>. One in five of these individuals develops T2D within five years<sup>107</sup>, while others either remain in the prediabetic stage or return to normoglycemia. Despite their increased risk of CVD and heart failure<sup>108</sup>, individuals with prediabetes are not captured by existing preventive strategies. This underscores the need for early and precise risk assessment<sup>10</sup>. 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.

#### **6.3.3. Secondary care**

In secondary care, endocrinologists assess cardiovascular and heart failure risk by integrating advanced diagnostics, biomarker analysis, and imaging to detect early dysfunction. 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 outcomes<sup>109</sup>. 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

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and more frequent hospitalizations<sup>4</sup>. 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 CVD<sup>110</sup>. Still, there is a need to identify optimal approaches for recognizing and diagnosing heart failure in clinical care, as broad echocardiographic screening in T2D is time-consuming and costly<sup>4</sup>.

Study III demonstrated that CAN may help identify individuals at increased 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 has proven to be reliable and reproducible, with reference values established in large population studies<sup>47</sup>. Beyond these findings and the established evidence of increased heart failure risk, CAN in the T2D population also identifies individuals at high risk for overall CVD, kidney disease, and early mortality<sup>[111][112]</sup>. In Study III, I observed that two out of five participants had CAN, highlighting it as a prevalent complication. 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 (ref.). However, its specificity varies across heart failure phenotypes, being less specific for detecting HFpEF compared to HFrEF. Therefore, additional evaluation using echocardiography may be warranted. Beyond classify 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 does CAN overlap 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

## *6.4. Strengths and limitations*

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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 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 increased 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.

## **6.4. Strengths and limitations**

### **6.4.1. 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<sup>68</sup>.

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.

## *6. Discussion*

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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.

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

#### **6.4.2.1. 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]57. 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. HF power, RMSSD, and pNN50 typically show earlier diurnal peaks compared to low-frequency power and

#### *6.4. Strengths and limitations*

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SDNN<sup>13</sup>. These measures are more reflective of parasympathetic activity during in- and expiration. It remains to be determined how these hourly measures relate to CVD, heart failure, and all-cause mortality.

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, reduced 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<sup>113</sup>. 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 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 reactivity) or pharmacological interventions. Therefore, it remains uncertain whether the mechanisms linking HRV indices to cardiovascular complications are predominantly due to sympathetic over activity 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

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introduce multicollinearity. Additionally, elevated heart rate is driven by increased 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 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 heart rate variability under defined conditions. Our findings reveal varying associations across 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<sup>103,114</sup>. 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 need for methodological rigor in HRV research, particularly in aligning index selection and time segmentation with specific research objectives and clinical contexts.

### **6.4.2.2. Cardiovascular autonomic reflex test**

CART provides a practical approach for screening for autonomic dysfunction and has been shown to be a reliable method<sup>115</sup>. 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 formula<sup>47</sup>. 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.

### **6.4.2.3. 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.

## *6.4. Strengths and limitations*

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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<sup>116</sup>.

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<sup>110</sup>. Thus, we cannot determine HFpEF or HFrEF. Its diagnostic accuracy is influenced by factors such as atrial fibrillation, obesity, and kidney function<sup>110</sup>. Individuals with atrial fibrillation 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.

### **6.4.3. External validity**

#### **6.4.3.1. 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

## *6. Discussion*

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age and hypertension, leading to overrepresentation of these groups<sup>117</sup>. 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<sup>118</sup>. 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 type 2 diabetes treated in outpatient clinics. In Denmark, patients with type 2 diabetes are referred to diabetes specialists at outpatient clinics when their general practitioner is unable to stabilize their condition. A strength of the CANCAN 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<sup>119</sup>. These practices may vary individually, with differing thresholds for referring patients to specialized care based on clinical judgment and patient characteristics.

#### **6.4.3.2. 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 type 2 diabetes. 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<sup>68</sup>, indicates that the observed relationship may extend beyond high-risk groups and into the general population. 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

#### *6.4. Strengths and limitations*

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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 type 2 diabetes are underrepresented in our studies. This group may be overlooked in current research and warrants further attention in future studies<sup>107,120</sup>. 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 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.

## **7. Conclusion**

This dissertation aimed to investigate how autonomic dysfunction, assessed by minimum 24-hour 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.

## 8. Perspective

We have investigated autonomic function impact on cardiovascular complications across different stages of glucose metabolism. 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

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. Wearable devices enable comprehensive data collection on behavioral (e.g., sleep and physical activity) and physiological (e.g., heart rate, ECG, temperature) parameters<sup>121</sup>. 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.

## 8. Perspective

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### *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, and irregular meal timing can influence circadian fluctuations in HRV (ref. sleep, activity)<sup>103</sup>. Pharmacological interventions also impact HRV: beta-blockers have been shown to increase HRV, while GLP-1 receptor agonists may reduce it<sup>[113][122]</sup>.

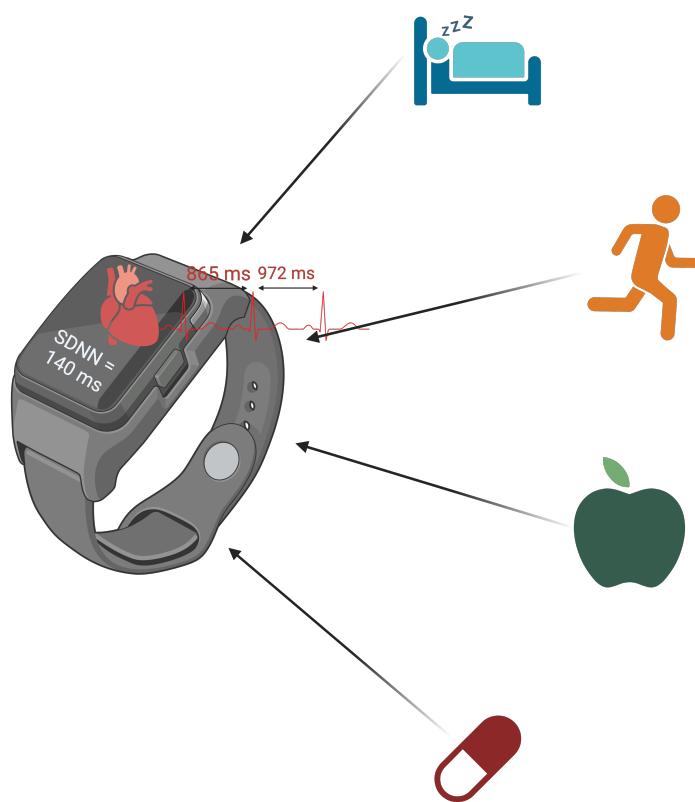


Figure 8.1.: HRV feedback in response to lifestyle and treatment interventions. (Source: Author)

Future research can leverage wearable devices to better understand how behavioral and pharmacological factors influence HRV. This approach may help identify effective lifestyle patterns or treatments that support cardiovascular health through HRV modulation.

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 [123] [124]. 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.

## **8.2. Risk-stratification**

The distinct roles of long-term HRV and CART in cardiovascular risk stratification remain to be fully established. From a wearable technology perspective, long-term HRV offers two promising avenues that warrant further investigation:

1. Non-invasive risk identification: HRV measured via wearable devices may help identify individuals at elevated cardiovascular risk without the need for invasive measures such as blood pressure readings or blood samples.
2. Enhancement of existing risk scores: HRV may improve the predictive accuracy of established cardiovascular risk models, such as SCORE2 or the Framingham Risk Score.

Both applications require further research to determine their clinical utility and integration into routine risk assessment.

A key limitation of long-term HRV measurement is the lack of standardization, as data collected under free-living conditions may be influenced by daily behaviors, potentially affecting risk classification. This highlights the need for standardized protocols. In contrast, CART is a reliable, non-invasive method that typically takes around 10 minutes to perform. A standardized and validated diagnosis of CAN using CART may help identify individuals with T2D who are at elevated risk of complications. However, the extent to which CAN diagnosis predicts heart failure risk and applies to broader populations with T2D or prediabetes remains to be clarified.

Our findings suggest that long-term HRV and CAN may serve as useful markers for identifying individuals at elevated metabolic risk who could benefit from targeted preventive strategies. Future research should explore whether those classified as high-risk based on autonomic dysfunction or CAN would benefit from earlier cardiovascular screening or tailored interventions.

## 8. Perspective

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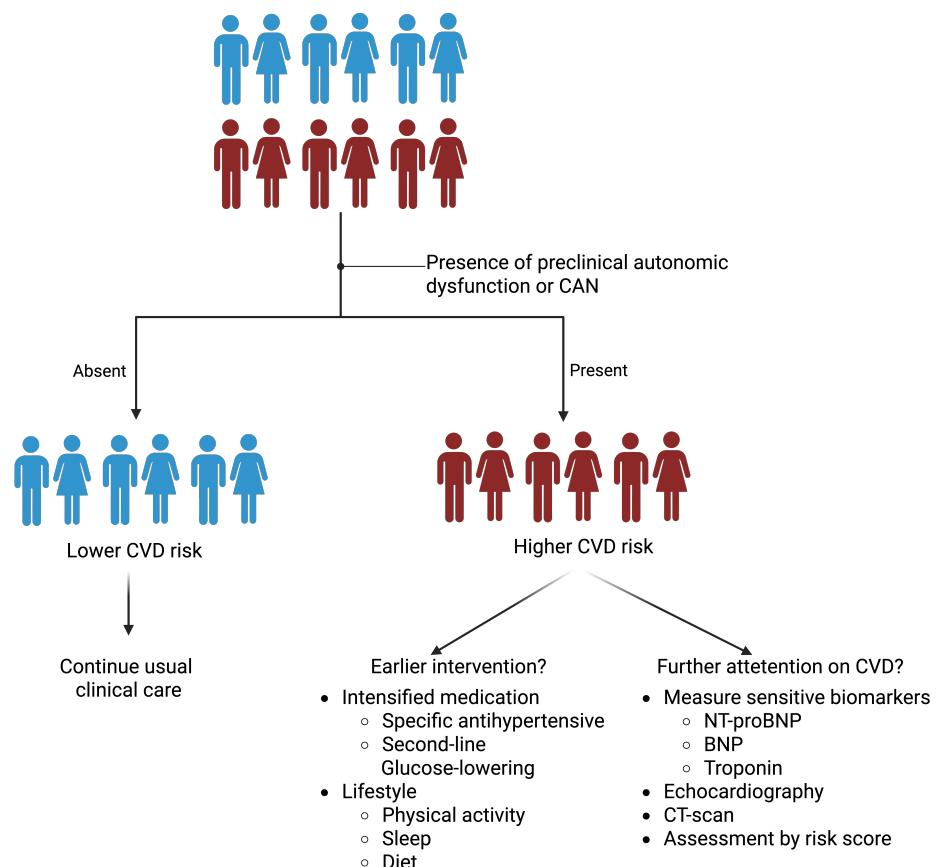


Figure 8.2.: Risk-stratification by autonomic dysfunction. (Source: Author)

### 8.3. Effective causal modifiable marker

Our findings support a potential etiological link between long-term HRV and CVD risk, providing preliminary evidence for 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 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 genome-wide association study 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.

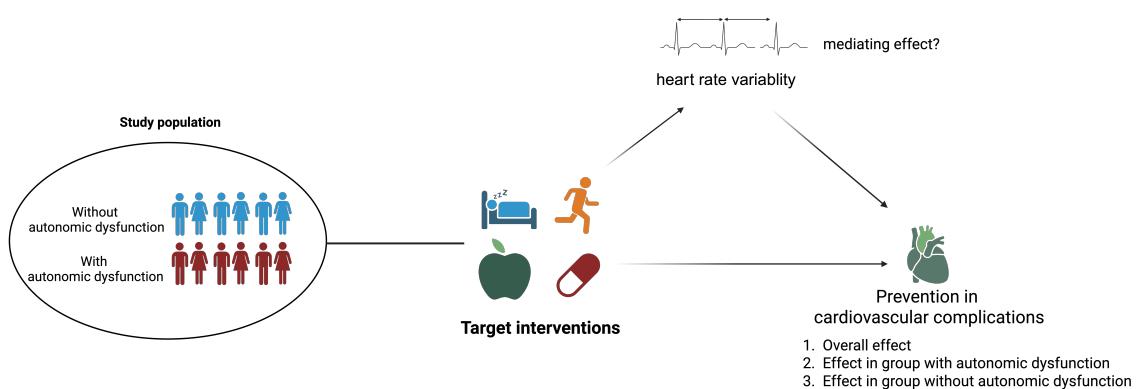


Figure 8.3.: Mediation of HRV by intervention in prevention of CVD. (Source: Author)

## *8. Perspective*

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Future cardiometabolic intervention trials, whether focused on lifestyle or pharmacological strategies, should, where feasible, include HRV 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.

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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 hjeretrymefvariabilitet (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. More results**

Cardiovascular autonomic dysfunction and arterial stiffness\_Maastricht\_study

# **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 (25<sup>th</sup>; 75<sup>th</sup> 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, ms<sup>2</sup> 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 ms<sup>2</sup>), power in the ultralow-frequency range (ULF, in ms<sup>2</sup> ≤ 0.003 Hz), power in the very-low-frequency range (VLF, in ms<sup>2</sup>; 0.003–0.04 Hz), power in the low-frequency range (LF, in ms<sup>2</sup>; 0.04–0.15 Hz), and power in the high-frequency range (HF, in ms<sup>2</sup>; 0.15–0.4 Hz). We removed outliers in time-domain and frequency 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:

$$CD = \frac{(2 * \Delta D * IAD + \Delta D^2)}{(braPP * IAD^2) (10.3 kPa - 1)}$$

, 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, 25<sup>th</sup> and 75<sup>th</sup> percentile) for continuous variables and frequencies (numbers, percentage) for categorical variables.

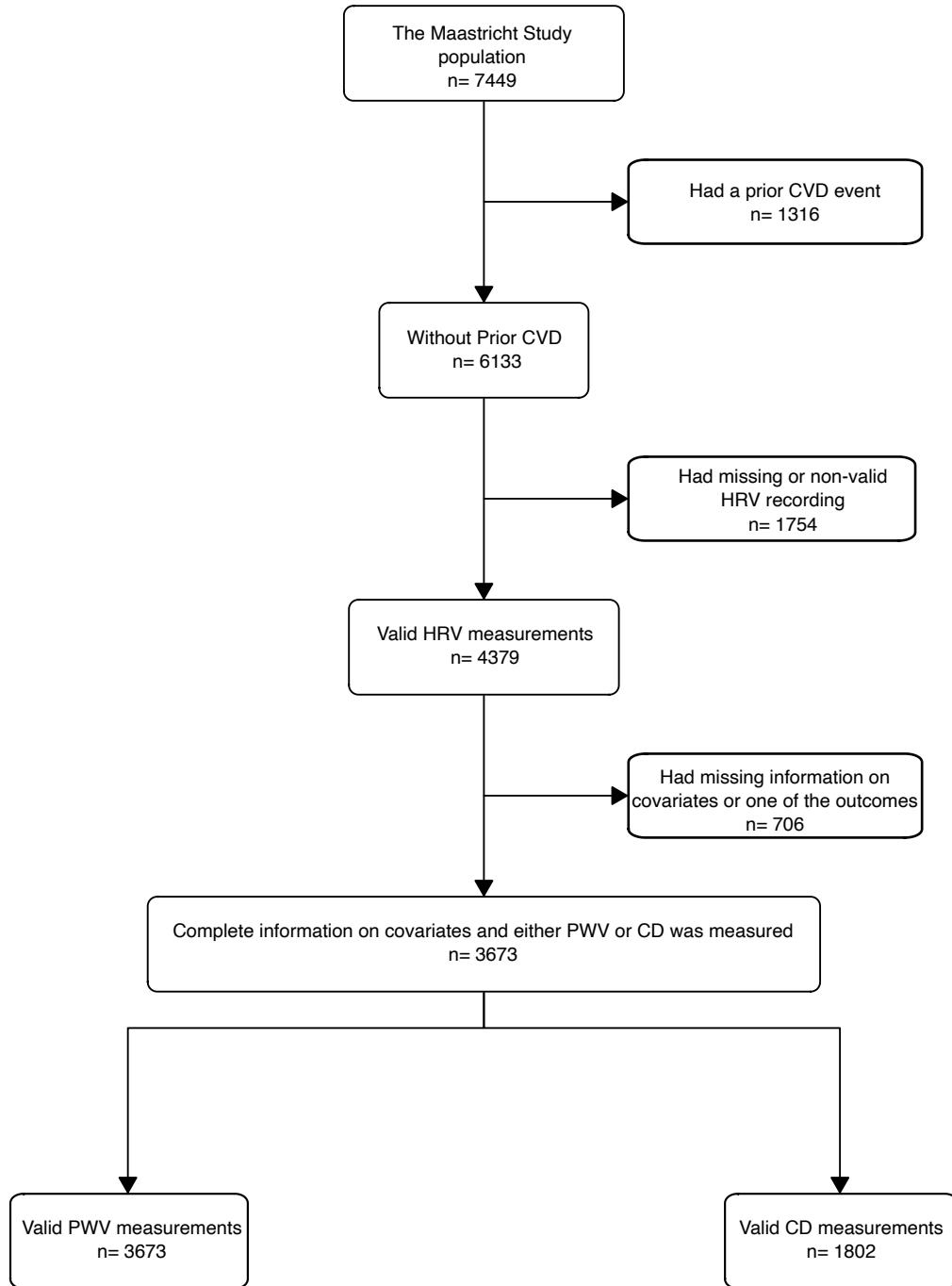
We performed multiple linear regression with heart rate variability indices as exposure for the outcome of arterial stiffness. We included the glucose metabolism status (normal glucose metabolism, prediabetes, and type 2 diabetes) to account for the oversampling of individuals with known type 2 diabetes. 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 (25<sup>th</sup>; 75<sup>th</sup> 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<sup>-3</sup>/kPa.

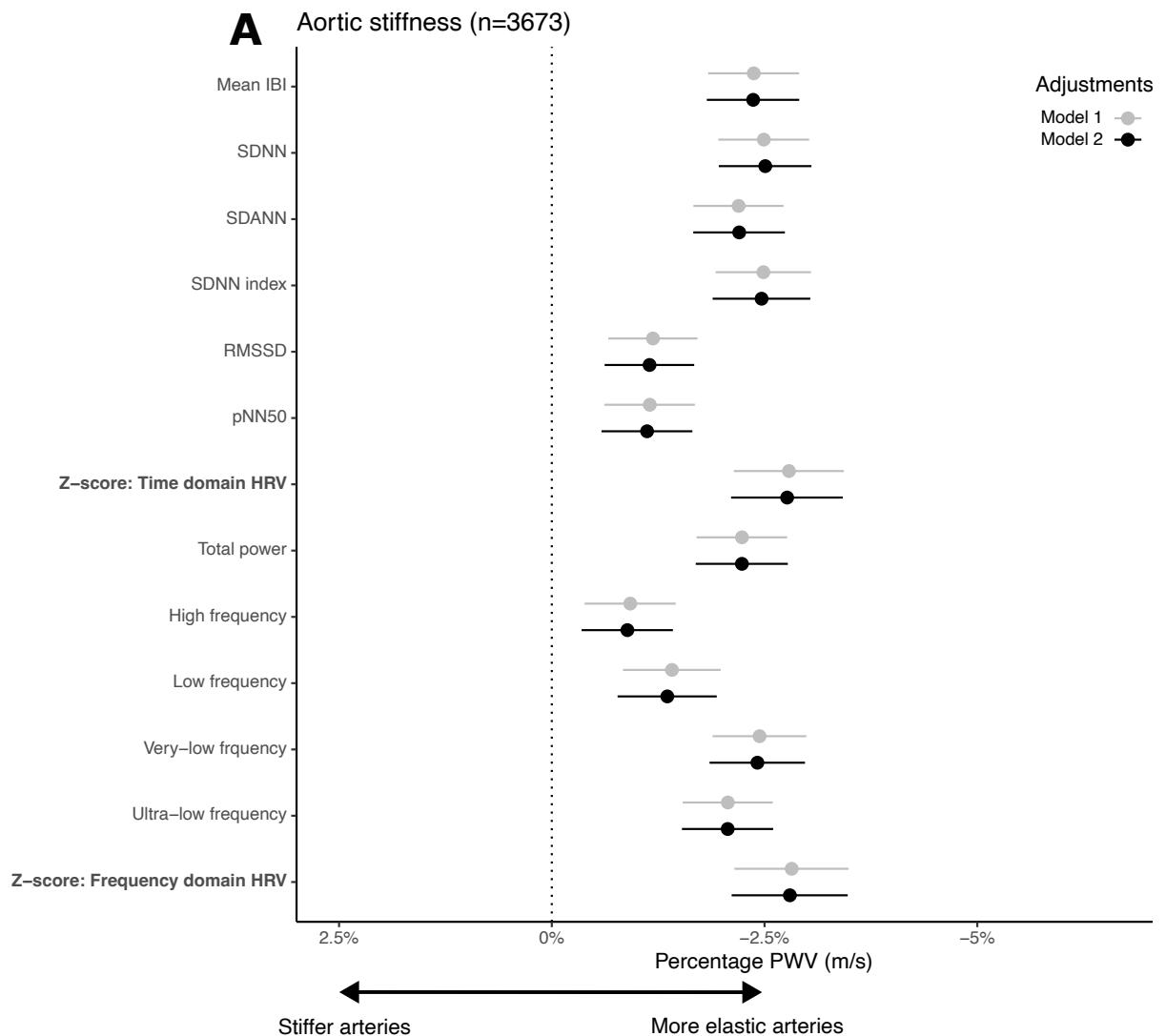
**Figure 1: Study flowchart**

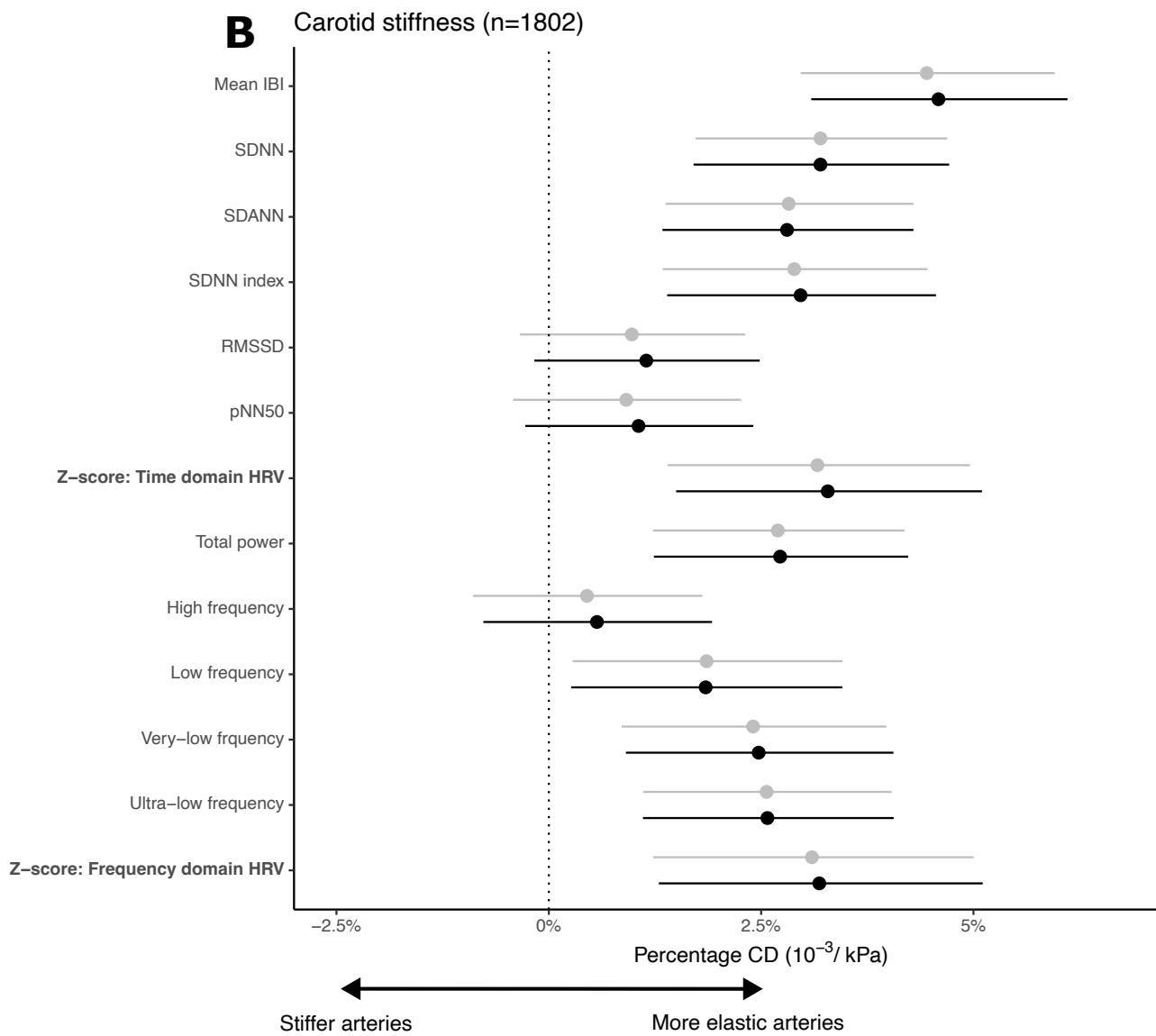


## Heart rate variability and aortic stiffness

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**



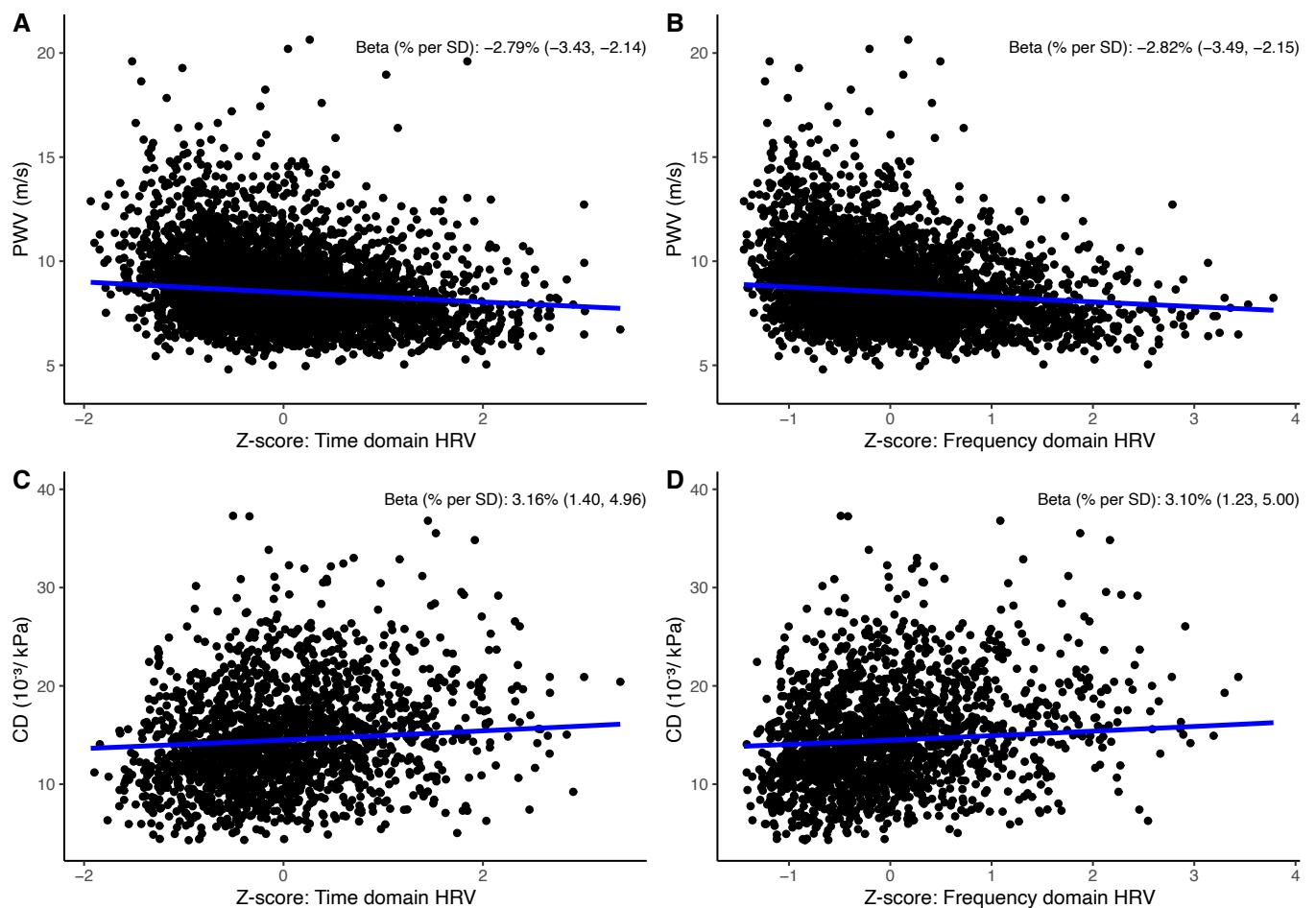


Percentage PWV (A) or CD (B) per SD increase in heart rate variability index and heart period intervals. Model 1: adjusted for sex, age, educational status, diabetes status, and mean arterial pressure. Model 2: Model 1 + physical activity, smoking behaviour, alcohol use, body mass index, hba1c, triglycerides, 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**

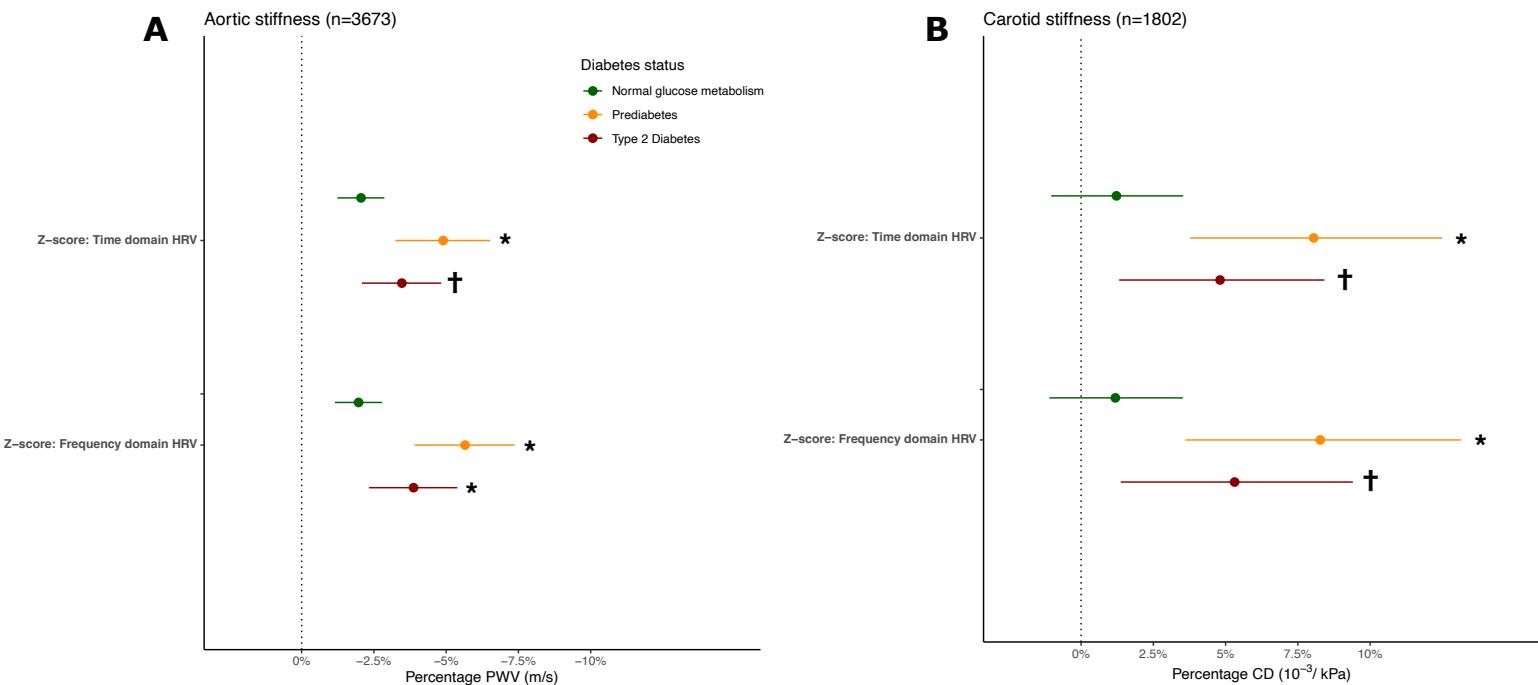


**A:** Percentage PWV per SD in time-domain composite z-score **B:** Percentage PWV per SD in frequency-domain composite z-score **C:** Percentage higher CD per SD in time-domain composite z-score **D:** Percentage CD per SD in frequency-domain composite z-score. All regression lines were adjusted for 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

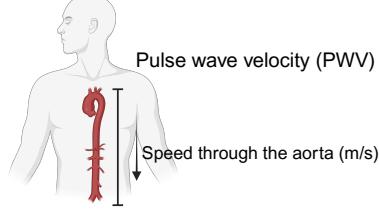


Standard deviation of 24-hour normal interbeat intervals (SDNN)

Highest deciles SDNN = 180 ms ~ Good adaption to changes

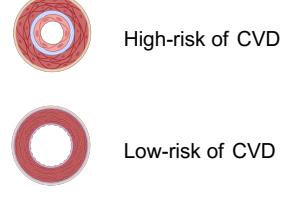
Lowest deciles SDNN = 90 ms ~ In range of subclinical cardiovascular autonomic dysfunction

### Stiffer arteries are a pathway to cardiovascular disease



Pulse wave velocity (PWV)

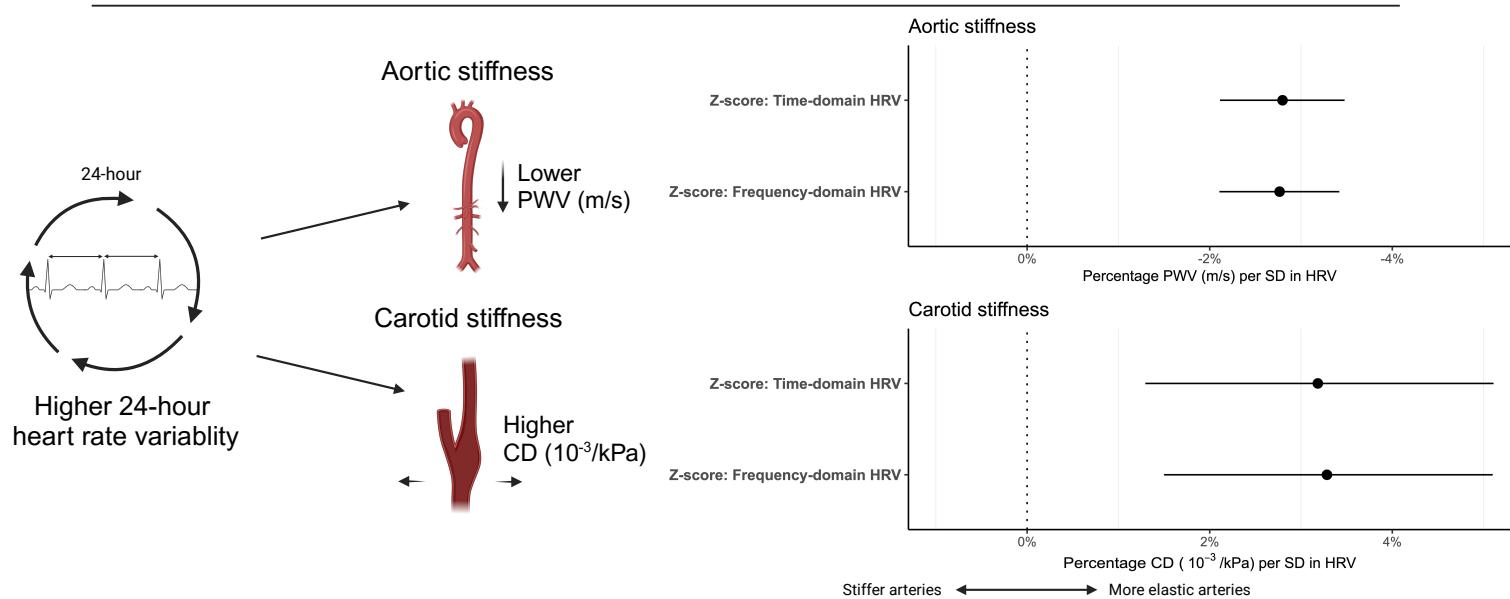
Speed through the aorta (m/s)



Carotid artery distensibility (CD)

High-risk of CVD

Low-risk of CVD



## 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 10<sup>th</sup> percentile (90 ms) had 8.77 m/s PWV, and 14.3 10<sup>-3</sup>/kPa CD. A similar hypothetical individual at the 90<sup>th</sup> 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 <sup>2</sup> )	26.0 (23.6, 28.8)
Waist (cm)	93 (85, 102)
HbA1c (mmol/mol)	37 (34, 41)
HbA1c (%)	5.54 (5.26, 5.90)

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**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 <sup>2</sup> )	11,566 (7,991, 16,394)
ULF (ms <sup>2</sup> )	9,788 (6,655, 14,183)
VLF (ms <sup>2</sup> )	1,105 (736, 1,571)
LF (ms <sup>2</sup> )	364 (222, 593)
HF (ms <sup>2</sup> )	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%)

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**N = 3,673**

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

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

## **B. Another appendix**

Something else