

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

Jonas Rosborg Schaarup

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# **Acknowledgements**

Thanks for all the fish.

# **Supervisors and assessment committee**

## **PhD student:**

**Jonas Rosborg Schaarup, Msc in Public Health**  
Department of Public Health, Aarhus University, Denmark

## **Supervisors:**

**Professor Daniel R. Witte, PhD (main supervisor)**  
Department of Public Health, Aarhus University, Denmark  
Steno Diabetes Center Aarhus, Denmark

**Lasse Bjerg, MD PhD**  
Department of Public Health, Aarhus University, Denmark  
Steno Diabetes Center Aarhus, Denmark

**Signe Toft Andersen, MD PhD**  
Department of Public Health, Aarhus University, Denmark Steno Diabetes Center  
Aarhus, Denmark

**Christian Stevens Hansen, MD PhD**  
Steno Diabetes Center Copenhagen, Denmark

## **Assessment committee:**

**Professor Morten Schmidt (chair and moderator of the defence)** Department  
of Clinical Medicin - Department of Clinical Epidemiology, Aarhus University

**Associate Professor Ilonca Vaartjes** University Medical Center Utrecht, Julius Cen-  
ter, Department of Epidemiology and Health Economics

**Cardiologist Peter Godsk Jørgensen** Herlev and Gentofte Hospital, Capital Region  
of Denmark

# **Abstract**

This is a thesis skeleton written with quarto. Make a copy of this thesis repo and start to write!

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# **1. Introduction [under construction]**

This is a book created from markdown and executable code.

## **2. Background [needs to be fine-tuned]**

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

The body regulates glucose and insulin to maintain glucose homeostasis. During fasting, pancreatic alpha cells secrete glucagon, which stimulates hepatic glucose production via glycogenolysis and gluconeogenesis. Meanwhile, glucose is endogenously produced by the liver and kidneys and utilized by body tissues. 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. Glucose homeostasis is dynamically regulated by inhibitory factors (insulin, hyperglycemia, parasympathetic activity, and substrate availability) and stimulatory factors (glucagon, counterregulatory hormones, hypoglycemia, sympathetic activity, and gluconeogenic substrates).

The progression from normal glucose metabolism to type 2 diabetes is characterized by sustained elevations in blood glucose levels, primarily driven by insulin resistance and followed by a gradual decline in beta-cell function. Insulin resistance occurs when certain cells, such as muscle and liver cells, lose their sensitivity to insulin. As a result, glucose is not effectively taken up by these tissues and remains in blood circulation. Meanwhile, beta-cell function deteriorates, leading to a diminished insulin response to glucose intake. Years before diagnosis, these changes contribute to rising fasting and postprandial glucose levels. [note: read Omar introduction!!!]

Diabetes progression is a continuum, with type 2 diabetes defined based on glucose thresholds associated with an increased risk of diabetes-specific microvascular complications, particularly retinopathy. The WHO (2011) and ADA (2015) diagnostic criteria for type 2 diabetes include fasting plasma glucose 7.0 mmol/L, 2-hour plasma glucose 11.1 mmol/L during an oral glucose tolerance test, or hemoglobin A1c (HbA1c) 6.5% (48 mmol/mol). However, many complications of diabetes, such as macrovascular disease, neuropathy, cancer, and cognitive impairment, can begin to develop at earlier stages of dysglycemia. 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

## *2. Background [needs to be fine-tuned]*

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levels between 7.8–11.0 mmol/L (WHO criteria), and HbA1c levels between 5.7–6.4% (39–47 mmol/mol) (ADA criteria) [ref.].

Risk factors for progression to type 2 diabetes and its complications range from genetic predisposition to lifestyle and socio-environmental factors. The most common precursor to diabetes is central obesity, characterized by excess body fat. The accumulation of diabetes risk factors is linked with a combination of adverse changes in cardiometabolic markers, including increases in low-density lipoprotein (LDL) cholesterol, triglycerides, body mass index (BMI), and systolic blood pressure, along with decreases in high-density lipoprotein (HDL) cholesterol. [Lancet Diabetes Endocrinol 2013; 1: 43–51].

Diabetes increases the risk of both microvascular and macrovascular complications, which are major determinants of the disease's associated morbidity and mortality. Diabetes and cardiovascular disease share common risk factors, including lifestyle factors and conventional clinical markers. As individuals progress toward diabetes, their cardiovascular risk increases, making them more susceptible to developing cardiovascular disease (CVD). However, the identification of preclinical stages of CVD and how CVD risk differs among individuals at high risk of diabetes and individuals with type 2 diabetes needs further definition.

## **2.2. Cardiovascular disease**

Globally, CVD remains the leading cause of death. However, the main cause of mortality varies by country income levels, with cancer becoming the leading cause in some high-income countries. CVD risk is primarily attributable to modifiable lifestyle behaviour such as stress, sedentary behaviour, unhealthy diet, alcohol consumption, and smoking, as well as socio-environmental factors like socio-economic status and air pollution<sup>1</sup>. Along the causal pathway towards CVD, these risk factors contribute to comorbidities such as clinical obesity, diabetes, hypertension, and hypercholesterolemia, further accelerating overall CVD risk. While risk factors are largely shared across CVD types, the mechanisms differ, involving structural, signalling, inflammatory, and dynamic changes in the cardiovascular system.

### **2.2.1. Arteriosclerosis**

Hard CVD end-points are the primary focus of CVD prevention measures. However earlier markers of arterial stiffness assess the gradual vascular pathology and reflect arteriosclerosis, atherosclerosis, vascular calcification. With aging, elastin fibers are gradually replaced by collagen fibers, leading to increased vascular stiffness<sup>2</sup>. Arterial stiffness

is characterized by changes between collagen, elastin, and smooth muscle cells. Stiffening of the arterial vasculature increases systolic pressure, reduces coronary perfusion, and raises the pulsatile load on the micro- and macrocirculation. This process can be accelerated by modifiable CVD risk factors. The progression of arterial stiffness is an early marker in the trajectory of CVD development, as increased stiffness precedes rises in blood pressure and CVD.

### **2.2.2. Atherosclerosis**

Atherosclerosis is characterized by plaque build-up in the arteries, which can develop into a thrombus, leading to artery blockage or haemorrhage. The underlying process of atherosclerosis is where cholesterol, fat, and other substances accumulate in the arterial walls, causing narrowing and reducing blood flow to the heart. When the oxygen supply to the heart is insufficient, it can result in chest pain (angina) or, in severe cases, myocardial infarction. Over time, chronic ischemia can lead to structural and functional changes in the heart, increasing the risk of heart failure and arrhythmia.

#### **Myocardial infarction**

Myocardial infarction (MI) 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 rate of MI has decreased in high-income countries, while improvements in early detection and treatment (both intravenous and therapeutic) have led to a steep decline in MI-related mortality. These advancements have also contributed to a lower prevalence of individuals living with prior MI. In type 2 diabetes, the risk of MI is elevated by 72%, with an approximately threefold risk among patients under 60 years compared to age under 60 without type 2 diabetes<sup>3</sup>. Similar to the general population, its incidence and fatality have declined in diabetes.

#### **Stroke**

Stroke is a cardiovascular disease that can be either ischemic or hemorrhagic. The majority of strokes are ischemic, caused by an obstruction in a cerebral artery, often due to an atherosclerotic plaque or embolism. 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 and increased intracranial pressure<sup>4</sup>. Ischemic stroke remains one of the global leading contributor to mortality and disability<sup>5</sup>. The incidence, prevalence, and cause-specific mortality of stroke remain high but have stagnated, although some declines have been observed in

## *2. Background [needs to be fine-tuned]*

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high-income countries<sup>6</sup>. Stroke risk is already elevated at high levels of glucose (fasting plasma, OGTT, and HbA1c) among people in a pre-diabetic range where the risk exceed of 26% higher risk compared to population without diabetes [7]<sup>8</sup>. In type 2 diabetes, the ischemic stroke risk is elevated almost two-fold<sup>3</sup>.

### **2.2.3. Heart failure**

Heart failure develops gradual with age and often accelerates with the progression of type 2 diabetes. As treatment of ischemic CVD events has improved survival over the last years, the incidence and prevalence of heart failure has increased. Therefore, early detection are important as heart failure leads to lower life-expectancy and quality of life.

Heart failure is a clinical condition characterized by symptoms of breathlessness, fatigue, and fluid retention, often accompanied by clinical signs such as pulmonary crepitations, jugular venous elevation, and peripheral edema. Heart failure can also 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. Therefore, 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 characterized by an LVEF <40%, while HFpEF is identified using various clinical criteria. HFrEF is often followed by ischemic caused heart failure.

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>9</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 abnormal echocardiographic measures of, e.g., left ventricular hypertrophy, left atrial enlargement, or elevated filling pressure<sup>10</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 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>10</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.

[insert figure of brain heart and sympathetic nerves]

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 autonomic dysfunction. Autonomic dysfunction reflects a stressed cardiometabolic environment, as both dysfunction in lipid and glucose metabolism are associated with increased sympathetic activity<sup>11</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>11</sup>. Therefore, the relationship between autonomic dysfunction and cardiometabolic factors is likely a vicious cycle<sup>12</sup>. The consequences can lead to cardiovascular autonomic dysfunction/neuropathy (CAN), resulting dysregulation in heart rate and vascular dynamics.

Cardiovascular 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 be observed in its natural 24-hour (diurnal) pattern<sup>13</sup>. Most studies have examined

## *2. Background [needs to be fine-tuned]*

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cardiovascular 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). Heart rate variability [from exercises physiology to psychology, to cardiovascular research, to diabetes research]. Type 2 diabetes has shown to significantly modify the expression of sympathetic bursts, measured by resting muscle sympathetic nerve activity, and even higher when co-existing with hypertension compared to normotensive without diabetes<sup>14</sup>. Parasympathetic activity is as well deteriorated among high cardiometabolic risk, and type 2 diabetes, shown by impaired baroreflex sensitivity<sup>15</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 [<sup>12</sup>]<sup>16</sup>. Cardiovascular autonomic reflex tests (CARTs) are considered the gold standard for assessing CAN. 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. Both HRV and CARTs have been associated with cardiovascular disease, heart failure, and all-cause mortality, primarily in populations with type 2 diabetes 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 type 2 diabetes. The 2022 ADA/EASD guidelines for the management of hyperglycemia in type 2 diabetes recommend, cardioprotective medication GLP-1 receptor agonists 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 [<sup>17</sup>]<sup>18</sup>. During the progression and after the onset of type 2 diabetes, preclinical stages may manifest with markers of higher cardiovascular risk, underscoring the potential for risk stratification. Risk stratification is the process of classifying individuals based on risk scores, biomarker levels, omic data (metabolomic, proteomics, and genomic) or pre-clinical conditions. This approach aids in subtyping patients for prognostic or diagnostic

purposes, identifying subgroups that require further evaluation, intensified treatment, or lifestyle modifications.

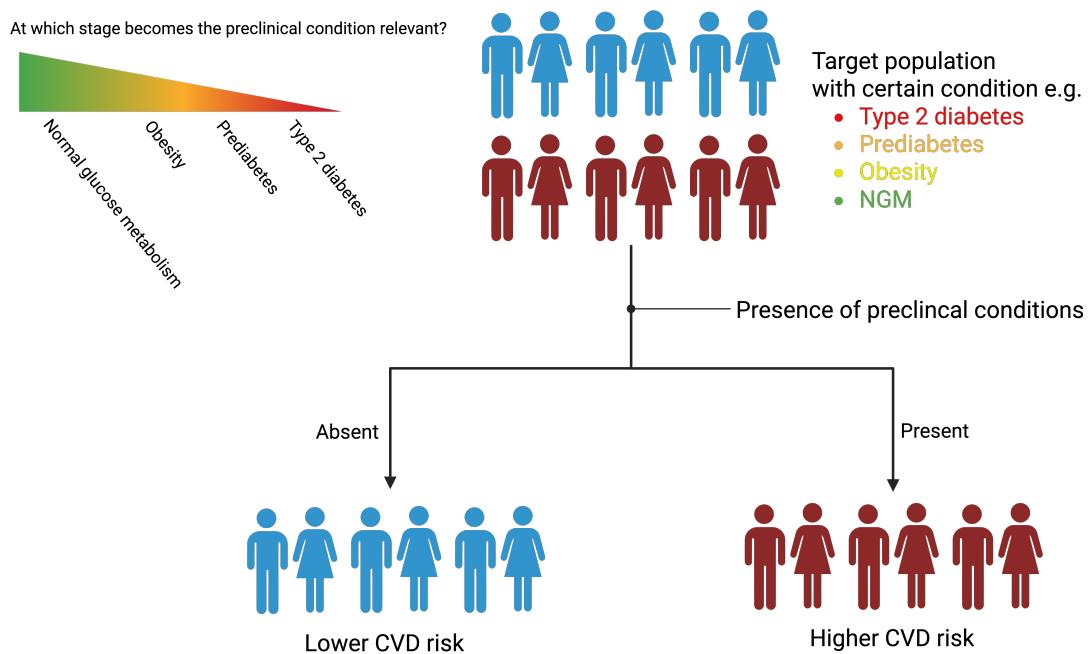


Figure 2.1.: Risk-stratification based on preclinical disease

Cardiovascular autonomic dysfunction despite its relationship with cardiovascular complication has not been defined into clinical practice. Larger epidemiological cohort studies encompassing various stages of diabetes risk, from normal glucose metabolism to prediabetes, onset of type 2 diabetes, and longer term progression of type 2 diabetes, serve as valuable resources for identifying risk-stratification opportunities. Epidemiological studies provide a broad representation of the target population, allowing understand the relationship between cardiovascular 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.

## 2.5. Aetiological research

Aetiology seeks to identify the causes and contributing factors of disease, forming the foundation for understanding its development and underlying mechanisms. Ideally, to

## *2. Background [needs to be fine-tuned]*

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determine causal effects, we would compare outcomes in individuals who were exposed to a risk factor with what would have happened if they had not been exposed. Since this counterfactual scenario is impossible to observe directly, we rely on study designs, such as randomized controlled trials when feasible, and apply statistical methods to data from observational cohort studies to approximate these comparisons. In cardiovascular disease, socio-environmental influences and personal health behaviours play a crucial role in overall health and are considered the outer contributing layer to biological mechanism. The inner layer focuses on biological causal processes, where the connection between these contributing factors and individual predisposition to cardiovascular disease remains a key question in understanding the underlying pathological mechanisms.

Cardiovascular autonomic dysfunction is linked to CVD and all-cause mortality. However, many questions remain regarding the underlying causal mechanisms. Furthermore, as dysglycemia is known to be a primary driver of autonomic dysfunction<sup>19</sup>, the question is to which extent it modulates the relationship between cardiovascular autonomic dysfunction and CVD? This relationship remains unclear, highlighting the need for a deeper understanding of this interplay in target populations representing different stages of glucose metabolism.

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

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

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 normoglycemia, prediabetes or type 2 diabetes.

Study II: Quantify the longitudinal association of week-long and hourly HRV with incidence ischemic-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 type 2 diabetes.

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 type 2 diabetes. In addition autonomic dysfunction is associated with higher levels of carotid-femoral pulse wave velocity and carotid artery distensibility.

## 4. Materials and methods [needs to be fine-tuned]

### 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 type 2 diabetes: The CANCAN study
Design	Aetiological cross-sectional study	Aetiological prospective cohort study	Descriptive cross-sectional study
Cohort	Maastricht study	ADDITION-PRO	CANCAN
Study	3673 people with normal glucose metabolism, prediabetes, and type 2 diabetes	2082 people with high risk of diabetes	173 patients with type 2 diabetes visiting outpatients clinics
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
Determinant	24-hour HRV	Week-long and hourly HRV	Cardiovascular autonomic reflex test NT-proBNP
Primary outcome	Arterial stiffness	Major adverse cardiovascular events, heart failure, and all-cause mortality	

## 4.1. Overview of the studies

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

### 4.1.1. Study population

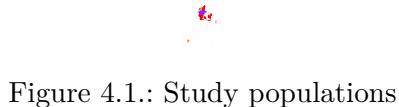


Figure 4.1.: Study populations

#### 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 type 2 diabetes, through the regional Diabetes Patient Registry, to extensively phenotype individuals with type 2 diabetes 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 population included participants with measurements of 24-hour HRV and at least one measure of arterial stiffness (carotid-femoral pulse wave velocity or carotid artery distensibility), both of which were completed within a three-month period between November 2010 and December 2020. The study has been approved by the institutional medical ethics committee (NL31329.068.10) and the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All participants gave written informed consent.

#### *4. Materials and methods [needs to be fine-tuned]*

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##### **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, originally designed as a stepwise screening program for type 2 diabetes in general practice. ADDITION-PRO aims to investigate early markers of cardiovascular disease (CVD) and metabolic dysfunction in individuals in different tiers of diabetes risk.

The ADDITION-Europe screening program identified a large number of individuals with impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and normoglycemia despite having risk factors for diabetes and CVD. Participants for ADDITION-PRO were recruited from the original ADDITION-DK screening cohort, which included individuals from 190 general practices across Denmark. The recruitment strategy focused on individuals at high risk of diabetes without type 2 diabetes, 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 type 2 diabetes by blood measurements including HbA1c, random blood glucose, FPG, and OGTT, were identified patients were invited to the ADDITION-trial. High risk individuals without type 2 diabetes 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 longitudinal cohort. 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 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 the second analysis. We also excluded participant with prior CVD ten years before inclusion.

The population were disease history and follow-up in the unique register system of Denmark, 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 pilot 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 type 2 diabetes in Central Denmark. We included 200 adults (>18 years) with type 2 diabetes 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 cardiovascular autonomic dysfunction/ neuropathy**

#### **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. In study III, we used the ratio in pulse rate in test under different conditions lying-to-standing, in- expiration, and valsalva manoeuvre.

#### *Time-domain indices*

Time-domain measures of HRV are based on the statistical distribution of normal-to-normal (NN) heartbeat intervals. Description of time-domain indices are summarized in box 1.

#### 4. Materials and methods [needs to be fine-tuned]

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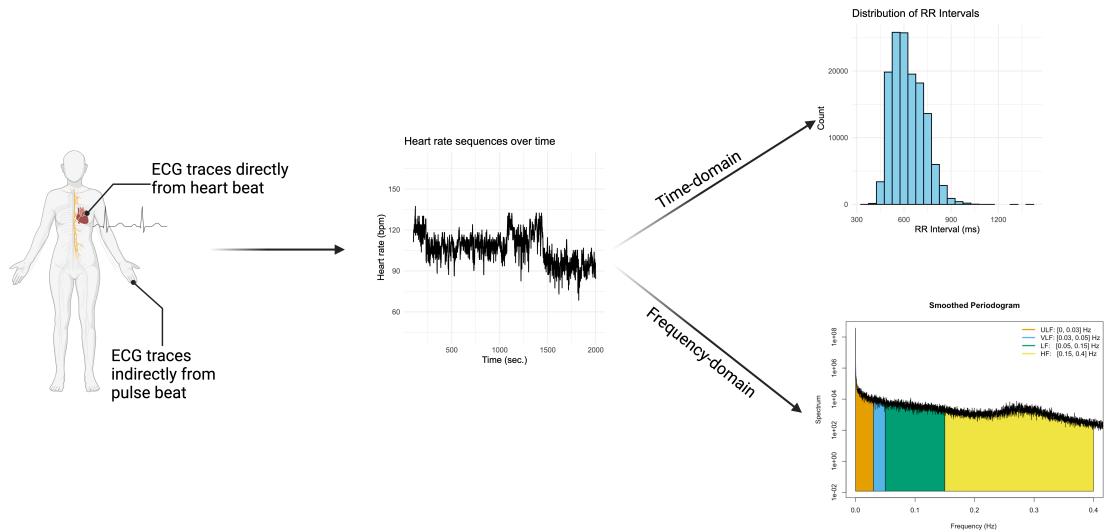


Figure 4.2.: Heart rate variability

Table 4.2.: Box 1: Time-domain HRV indices

Time-domain HRV	Description
<b>Standard deviation of NN heart beat intervals (SDNN, in ms)</b>	Reflects overall HRV and total autonomic nervous system activity over the recording period.
<b>SD of the averages of NN intervals in 5-minute segments throughout the recording (SDANN, in ms)</b>	Measures long-term HRV variations, primarily reflecting circadian and autonomic fluctuations.
<b>Mean of the SDs of all NN intervals for all 5-minute segments (SDNN index, in ms)</b>	Estimates short-term HRV fluctuations and vagal tone by averaging segmental variations.
<b>NN50 count divided by the total number of all NN intervals (pNN50, percentage)</b>	Represents the proportion of successive NN intervals differing by more than 50 ms, indicating vagal activity.
<b>Square root of the mean of the sum of squares of differences between adjacent NN intervals (RMSSD, in ms)</b>	Reflects short-term HRV, mainly parasympathetic (vagal) activity.

#### Frequency-domain indices

## 4.2. Study variables

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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 box 2.

Table 4.3.: Box 2: Frequency-domain HRV indices

Frequency domain HRV	Description
<b>Variance of all NN intervals 0.4 Hz, total power (TP, in ms<sup>2</sup>)</b>	Represents overall HRV, reflecting both short- and long-term autonomic regulation.
<b>Ultralow-frequency range (ULF, in ms<sup>2</sup>; 0.003 Hz)</b>	Captures very long-term oscillations, influenced by circadian rhythms, metabolism, and thermoregulation.
<b>Very-low-frequency range (VLF, in ms<sup>2</sup>; 0.003–0.04 Hz)</b>	Associated with sympathetic activity, inflammation, and hormonal regulation.
<b>Low-frequency range (LF, in ms<sup>2</sup>; 0.04–0.15 Hz)</b>	Reflects a mix of sympathetic and parasympathetic activity, often linked to blood pressure regulation and baroreflex sensitivity.
<b>High-frequency range (HF, in ms<sup>2</sup>; 0.15–0.4 Hz)</b>	Represents parasympathetic (vagal) modulation of heart rate, closely related to respiratory sinus arrhythmia.

### 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.

#### *4. Materials and methods [needs to be fine-tuned]*

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#### **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 consecutive normal heartbeats on the ECG. HRV calculations were performed using the RHRV package (version 4.2.7) in R, including SDNN, SDANN, SDNN index, TINN, and mean HR (mHR). We tested our approach on a dataset with full access to all interbeat intervals to validate our algorithm<sup>20</sup>. These indices have shown high validity for HRV indices based on global distribution (e.g. SDNN, SDANN, SDNNi) in 24-hour recordings. HRV indices were calculated by week, 24-hour cycle, and hour of the day, with hourly values averaged across recording days.

#### **Vagus device for cardiovascular autonomic reflex test in study III**

CAN was diagnosed using cardiovascular autonomic reflex tests (CARTs), the gold standard for CAN assessment. R-R intervals were derived from an ECG signal using the Vagus™ device (Medicus Engineering, Aarhus, Denmark). Three standardized CARTs were performed: lying-to-standing, deep breathing, and the Valsalva manoeuvre, following a standardized protocol 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.



Figure 4.3.: Handheld Vagus™ device

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.).

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

### Cardiovascular autonomic reflex test



Figure 4.4.: CART

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

[Lifestyle] Smoking status was

Clinical markers

Medication]

### 4.3. Outcomes

#### 4.3.1. Arterial stiffness

##### Pulse wave velocity

Arterial stiffness can be characterized by measuring arteriosclerosis and atherosclerosis properties of the arteries. The stiffness of different trees of the vascular musculature can be assessed both locally and dynamically. Aortic and carotid stiffness were assessed as markers of arterial stiffness, following previously described procedures. Aortic stiffness was measured by carotid-femoral pulse wave velocity (PWV) using applanation tonometry (SphygmoCor, Atcor Medical, Sydney, Australia), with the median of at least three consecutive recordings included in the analysis.

##### Carotid artery distensibility

#### *4. Materials and methods [needs to be fine-tuned]*

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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).

[insert figure of PWV and CD]

#### **4.3.2. Biomarker**

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 peptid (BNP) which is a cardial neurohormon, that is syntezied and secreted as response to streched cariomcytes 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 sample were taken at study cite. Description of the NT-proBNP analysis of plasma samples is described in supplementary material [ref.].

#### **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.

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<b>Outcome</b>	<b>Diagnosis codes</b>
<i>Heart failure</i>	ICD: DI50
<i>Three-point MACE</i>	
• Stroke	ICD: I61 - I64
• Myocardial infarction	ICD: I21-I24
• Cardiovascular death	ICD: I20-I28, I42, I46

- SKA: KPAE10, KPAE25, KPAF10, KPAF20, KPAF21, KPAF22,  
Cardiovascular KPAH10, KPAH20, KPAH21, KPEE, KPEF, KPEH, KPEP,  
revasculariza- KPEQ, KPFE,, KPFH, KPFP, KPFQ  
tion
- 

## 4.4. Statistical Methods

### 4.4.0.1. Cross-sectional analysis

In study I, we used multiple linear regression to investigate associations between week-long 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 type 2 diabetes 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.

#### 4.4.0.1.1. Effect modification

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, type 2 diabetes). 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.

#### *4. Materials and methods [needs to be fine-tuned]*

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##### **4.4.0.2. 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>21</sup>. Week-long 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.

##### **4.4.1. 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. All available variables of biochemical measures, diagnosis, medication and cause of non-valid CART was used to impute CART using predictive mean matching.

##### **4.4.2. 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 con-

#### *4.4. Statistical Methods*

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ditions 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 cardiovascular autonomic function using week-long 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 beyond heart failure. We adjusted the model to account for the blurred effect of eGFR on NT-proBNP levels in the analysis.

## **5. Results [needs to be fine-tuned]**

In this section, I will summarize study population characteristics and findings from analysis.

### **5.1. Study I**

#### **5.1.1. Descriptive**

In The Maastricht Study, [10,000 participated by Date], of those 1316 reported prior CVD. Participants who had valid 24-hour HRV measured was 4379 and of those 3673 had a valid measurement of either CD or PWV. Study population included 3673 participants. Further characteristic are described in the study I manuscript [Table 1] [reference to study I].

#### **5.1.2. 24-hour HRV and arterial stiffness**

##### **Time-domain HRV**

In the fully adjusted model 2, PWV was 2.8% (CI: 2.1; 3.4) lower, while CD was 3.3% (CI: 1.5; 5.1) higher per SD increase in HRV time-domain Z-score. 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. 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.

##### **Frequency-domain HRV**

In the fully adjusted model 2, PWV was 2.8% (CI: 2.1; 3.5) lower, while CD was 3.2% (CI: 1.3; 5.1) higher per SD increase in HRV frequency-domain Z-score. Among the frequency-domain indices, total power, VLF, and ULF showed the strongest associations, with cf-PWV being lower by 2.2% (CI: 1.7; 2.8 ), 2.4% (CI: 1.9; 4.0), and 2.1% (CI: 1.5;

## 5.1. Study I

**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)
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 ( $10^{-3}$ /kPa)	15.0 (11.8, 18.8)	13.5 (10.4, 16.9)	12.5 (9.9, 16.0)
Carotid-femoral pulse wave velocity (m/s)	8.08 (7.28, 9.16)	8.96 (7.84, 10.32)	9.36 (8.16, 10.80)
N-HT	833 (35%)	317 (59%)	590 (79%)
Antihypertensive medication	431 (18%)	199 (37%)	478 (64%)
med_HT_beta	149 (6.2%)	77 (14%)	195 (26%)
Lipid-lowering medication	280 (12%)	141 (26%)	484 (65%)

2.6), respectively. 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.

### 5.1.3. Effect modification of diabetes status

The study population represented diabetes risk of normal glucose metabolism (65%), prediabetes (15%), and type 2 diabetes (20%). The median (IQR) cf-PWV (aortic stiffness) increased with diabetes status: NGM: 8.08 m/s (7.28, 9.16), prediabetes: 8.96 m/s (7.84, 10.32), and type 2 diabetes: 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 type 2 diabetes: 12.5 (9.9, 16.0)  $\times 10^{-3}$ /kPa. SDNN (ms) was highest in NGM and decreased with worsening glucose metabolism: NGM: 138ms (117, 164), prediabetes: 127ms (106, 152), and type 2 diabetes: 116ms (96, 139).

The association between HRV time-domain Z-scores and cf-PWV and CD was significantly modified by prediabetes (PWV: -4.9 [CI: -6.523; -3.243]  $interaction(*)^{p-value < 0.01}$  CD: 8.0 [CI: 3.8; 12.5] $^{*p-value < 0.01}$ ) but not by type 2 diabetes (PWV: -3.5 % [CI: -4.8; -2.1])

## 5. Results [needs to be fine-tuned]

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\* $p-value < 0.1$  CD: 4.8 % [CI:1.3; 8.4]\* $p-value < 0.1$ ). For the indices SDNN and SDANN, the association with both cf-PWV and CD was significantly modified by both prediabetes and type 2 diabetes.

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]\* $p-value < 0.01$ ) and type 2 diabetes (-3.9 %[CI:-5.4; -2.3]\* $p-value < 0.05$ ) while CD was only modified by prediabetes (8.3 %[CI:3.6; 13.2]\* $p-value < 0.01$ ) but not by type 2 diabetes (5.3 %[CI:1.4; 9.4]\* $p-value < 0.1$ ). For the indices total power and ULF, the association with both cf-PWV and CD was significantly modified by both prediabetes and type 2 diabetes. Mean inter beat interval association with cf-PWV or CD was not significantly modified by diabetes status.

As we did not observe a stepwise increase in the modification of glucose metabolism status from prediabetes to type 2 diabetes, we excluded the subgroup with type 2 diabetes 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 x). 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 Figure xB). 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 xD). No association between HRV frequency domain Z-score and CD was observed at HbA1c levels between 4.8% and 5.2%.

[figure xx: Maastricht EM HbA1c]

## 5.2. Study II

### 5.2.1. Descriptive

In 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 (9%). We 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).

## 5.2. Study II

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_anti_hypertensive	753 (47%)	88 (61%)	149 (48%)	216 (47%)	147 (43%)	153 (43%)

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. Week-long HRV and MACE, heart failure, and all-cause mortality.

The mean week-long 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 we 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. We included knots in the model, which showed that the risk increased when SDNN fell below 120 to 110 ms (approximately below the 20th percentile), suggesting a potential cut-off point for higher risk. We 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.

[Incidence rate curves]

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 xA). 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:

## *5. Results [needs to be fine-tuned]*

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19.6 [CI: 14.8; 25.9]) and SDNN = 160 ms (IR: 19.8 [CI: 15.0; 26.2]) (Figure xB). 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 xC). 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, we observed a clear periodicity in SDNN, mean heart rate (HR), sleep patterns, and physical acceleration. SDNN increased from 5–6 AM, peaking at 8–9 AM [mean (SD)], followed by a gradual decline, reaching its lowest point around 1 AM the next day [mean SDNN (SD)]. A similar circadian pattern was observed in heart rate, though its peak occurred two hour later at 10–11 AM. After peaking, heart rate remained stable throughout the afternoon before gradually decreasing.

[include results from hourly results]

## **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). 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 type 2 diabetes (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. Relationship between CAN and elevated NT-proBNP**

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).

### 5.3. Study III

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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.

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## 5. Results [needs to be fine-tuned]

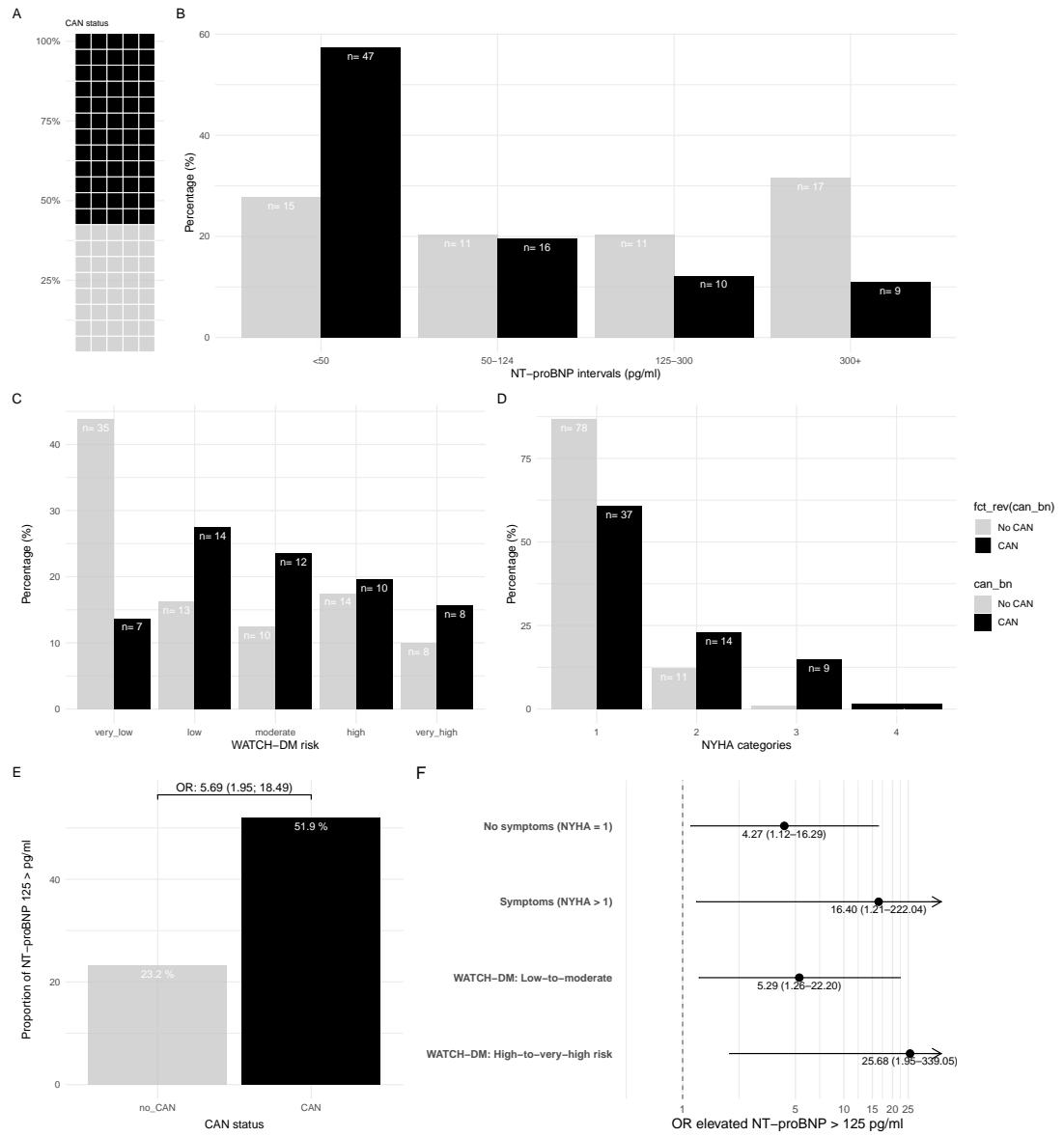


Figure 5.1.: Relationship between CAN and indicators for heart failure

## **6. Discussion [needs to be fine-tuned]**

### **6.1. Strength and limitation**

#### **6.1.1. 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 temporarily or confirm whether changes in the outcome were caused by the exposure. However, based on prior evidence, we assume the direction of the studies based on physiological knowledge and in vivo studies. In Study I, we based our direction of the association based on longitudinal association based on data from Whitehall II study, showing steeper decrease in short-term HRV are associated with subsequent higher levels of cf-PWV<sup>22</sup>. Moreover, [insert in vivo studies]. In Study II, we attempted to mimic temporarily by glucose metabolism, in individuals with type 2 diabetes and without known type 2 diabetes, instead of time, which shows deterioration of glucose metabolism increases the size of the association. The strength of study I, is that sample size is large, making our results more generalisable to wider populations across statuses of glucose metabolism.

In study III, our study design are more focused on the clinical diagnosis of CAN and presence of heart failure. This the question are more focused on clinical utilization of CAN in detecting type 2 diabetes patients who early progress towards heart failure, and thus the aetiological question remain for other study design. Indeed, whether cardiac function progressively worsens due to the etiological mechanisms of CAN remains to be fully established. If confirmed, this would underscore the relevance of CAN as a preclinical marker for early progression to heart failure, one that may be effective to target in efforts to prevent or delay the development of overt heart failure.

##### *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

## *6. Discussion [needs to be fine-tuned]*

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temporal structure ensures that the exposure (HRV) clearly preceded the outcome, reducing the risk of reverse causation. Although repeated measurements of HRV over time would provide more detailed insights into autonomic function dynamics, the prospective design still allows for stronger inference of directionality than cross-sectional studies. Furthermore, the use of high-quality registry data for outcome ascertainment ensures complete follow-up and minimizes loss to follow-up bias.

Based on our studies, we demonstrate first steps in relationship between cardiovascular autonomic function, measured by, heart rate variability or CART, we can only establish an association and cannot conclude with certainty that improvements in HRV measures lead to a reduction in cardiovascular risk. Therefore, we cannot ascertain causal effect based on our findings, and more causal focused methods are needed. Mendelian randomization, which uses genetic instruments for exposure, could help address this causal question. Furthermore, structured mediation analysis involving modification, e.g. by medication or lifestyle, would improve HRV or CART leads to reduction in cardiovascular risk, using data from intervention studies.

### **6.1.2. Intern validity**

In this project, we aimed to assess cardiovascular autonomic function both in free-living conditions and in response to standardized test procedures during clinical visits. Additionally, we used dynamic measurements to evaluate arterial stiffness locally and by velocity and biomarker assessments to determine the presence of heart failure. In this section, I will discuss the validity of 24-hour, week-long, and hourly HRV measurements, as well as the standardized tests of CAN. Furthermore, I will address the validity of the included outcomes. I will as well discuss using the strength and limitation of using MACE as an time to event outcome.

#### **6.1.2.1. Long-term HRV (>24 hours) as measurement for autonomic function**

[Actiheart and Holter monitor]

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 as a consequence to cardiac complications, and in diabetes research as a marker of autonomic neuropathy. In the context of this project, which focuses on long-term HRV in diabetes and cardiovascular research, 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, use of medication, and

## *6.1. Strength and limitation*

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prior cardiovascular complications which can potentially masking or mimicking underlying physiological dysfunction during recordings. Therefore, reduced long-term HRV cannot be interpreted solely as a marker of autonomic function. Moreover, HRV is also influenced by lifestyle patterns over time, making it sensitive not only to acute behaviors but also to long-term habits that affect autonomic balance. In study II, we observed that the lower ranges of HRV had both lower habitual physical activity and lower  $\text{VO}_2 \text{ max}$ , suggesting that lower HRV reflects more sedentary lifestyle and lower cardiorespiratory fitness.

In study I and II, we strived to account for habitual physical activity, while in study II, we also accounted hourly HRV for physical movement during the recording to test the influence of concurrent physical activity. However, further studies are need to understand how lifestyle patterns affect the long-term recording the subsequent day, to understand the behavioral component in long-term HRV. In study I and II, we also excluded participants with prior CVD to ensure that in order to keep etiological order between autonomic dysfunction and the outcome of cardiovascular complication.

Anti-hypertensive medications, especially beta-blockers, are known to increase HRV in randomized controlled trials<sup>23</sup>. However, in cohort studies, participants using anti-hypertensives generally show lower HRV, likely reflecting their higher burden of cardiovascular complications [ref]. Because beta-blockers target the autonomic nervous system, some anti-hypertensive medications may introduce bias in HRV measurements, as they interfere with the autonomic function we aim to assess. In the sensitivity analysis in study I and III, without participants on anti-hypertensive treatment, the estimates did not materially change, thus we included the population and adjusted for medication in the full model

HRV levels are influenced by heart rate as lower heart rate allows higher variability. In study I, we chose not to adjust for heart rate in our models, as doing so could introduce issues of 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 heart rate variability during both rest and activity, providing a robust representation of autonomic function over the course of a typical day. In contrast, heart rate correction may be more relevant for short-term recordings of HRV, where standardized conditions can be affected by random influences such as time of day, smoking, or caffeine intake, which have been relevant for study III if we had included HRV measures. In Study II, we included HRV measures pre-adjusted for heart rate, which accounted for part of the observed associations, particularly with heart failure and all-cause mortality, but to a lesser extent for ischemic-related CVD events. Similar trends were observed in the hourly associations, where heart rate pre-adjustment had comparable effects on the outcomes.

## *6. Discussion [needs to be fine-tuned]*

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### **6.1.2.2. Cardiovascular autonomic reflex test**

### **6.1.2.3. Measures of cardiovascular risk**

[ Arterial stiffness - NT-proBNP - MACE limitation in aetiological research - Non-specific heart failure and MI and stroke and death - HRV stronger link with MI or stroke - Perspective: decreasing number of events with prolonging time-to-event - Clinical trial moved to high risk population in lower-income countries (South America) - Challenge for coming observational cohorts (need to increase sample size) [The Problem With Composite End Points in Cardiovascular Studies The Story of Major Adverse Cardiac Events and Percutaneous Coronary Intervention]]

### **6.1.3. External validity**

#### **6.1.3.1. Selection bias**

##### **The Maastricht Study**

In Study I, participants were recruited using different strategies, with a focus on enrolling individuals with type 2 diabetes to ensure sufficient statistical power in this group. Recruitment was conducted through mass media campaigns, municipal registries, and the regional Diabetes Patient Registry via mailings. Thus, participation depended on individuals' awareness of the campaigns and their willingness to attend. Patients with type 2 diabetes were actively targeted with additional mail invitations to encourage participation.

##### **ADDITION-PRO**

In Study II, participants were recruited through a stepwise screening procedure. First, they were selected based on a risk score by self-administrated questionnaire sent by mail , followed by HbA1c or random glucose measurements. These procedures introduce different steps for selection bias as people have both to receive and sent a filled questionnaire, followed by a visit for blood testing if their risk score was high<sup>24</sup>.

First, the population consists of participants who responded to the screening questionnaire and those at higher risk who underwent further blood measurements. Second, the questionnaire itself selects participants based on a risk score developed to identify individuals with type 2 diabetes, while prediabetes identification was based on further measurement on the basis of HbA1c measurements. Certain risk factors, such as age and hypertension, contribute to higher risk by influencing the risk score, and thus may lead to high representation of these groups.

## *6.1. Strength and limitation*

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Hence, selection bias arises from both participation in the risk score assessment and follow-up attendance in ADDITION, as well as from the instruments used to identify risk—namely, the Inter-99 risk score, HbA1c, and random blood glucose measurements. Healthier people are more attentive in screening and cohort studies [ref.].

### **CANCAN**

In Denmark, patients with type 2 diabetes are referred to diabetes specialists at outpatient clinics when their general practitioner is unable to stabilize their diabetes care. As a result, CANCAN participants represent a higher-risk group in Danish diabetes patients, where more stable patients remain under general practitioner care. Consequently, the prevalence of heart failure indicators and CAN is likely higher in this selected group. The strength of the CANCAN sampling strategy in outpatient clinics is that patients were referred to an endocrinologist and attended their consultations. The additional study examination did not require extra transport or appointments but only involved additional time during their visit, with the option of receiving feedback on continuous glucose monitoring.

Overall in this project, the selection bias span across different aspects. In Study I-II, healthier and more health-conscious individuals tend to participate in cohort studies, potentially introducing selection bias. In contrast, attendance in the study III was more successful, as participation was optimized by scheduling study assessments during routine consultations. In epidemiology, we aim to match the source population with our target population. However, limitations due to self-selection in participation arise. Consequently, this can affect the results, as participants may be healthier and better using health care services, leading to less contrast between determinants and outcomes in our etiological analysis. We suspect that one explanation for the lack of a stepwise increase in the association between HRV and arterial stiffness across prediabetes and type 2 diabetes in study II is that the participants with type 2 diabetes represent a well-treated population. Thus, the included participants may be sufficient to demonstrate a relationship, the magnitude of the association to the target population may be limited.

#### **6.1.3.2. Generalisability**

The generalizability of our findings can be discussed on two levels: the extent to which the results apply to the general population within the country and how they translate to populations with different ancestries in other countries.

Studies II–III include individuals at high risk of diabetes and those with type 2 diabetes. Therefore, the associations between cardiovascular autonomic dysfunction and cardiovascular outcomes or surrogate biomarkers extend to individuals with some degree of

## *6. Discussion [needs to be fine-tuned]*

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diabetes risk. However, whether these associations hold in the general populations remains uncertain. Study I suggests that the link between autonomic dysfunction and cardiovascular risk, as measured by arterial stiffness, is also present in individuals with normal glucose metabolism, though to a lesser extent. This finding was further supported by replication in the Whitehall II cohort, strengthening the generalizability of the observed relationship<sup>22</sup>.

The study populations in Studies II–III consist of individuals of Nordic descent, while Study I represents a population of Western European descent. Since the constellation of risk factors for diabetes varies and may manifest differently in population with Asian, South American, African, and other decent, and therefore our findings may not be fully generalizable to these groups. This limitation affects the applicability of the observed associations and their magnitudes to a unknown degree. Further cohort studies including under-represented populations are warranted. As we are studying diabetes risk, all participants in the study were older adults aged 40 years and above. Therefore, our findings are limited to this age group, and whether the results extend to younger adults or children remains to be confirmed. Overall, while our study has the strength of including individuals across different levels of diabetes risk, some limitations in generalizability remain, particularly to more diverse and younger populations.

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

Based on our studies, we have shown that cardiovascular autonomic dysfunction, measured by HRV and CART, is associated with arteriosclerosis across glucose metabolism, atherosclerotic events, mortality, and heart failure in people at high risk of diabetes, as well as indications of heart failure in patients with type 2 diabetes.

### **6.2.1. Arteriosclerosis**

In study II, demonstrated that autonomic dysfunction, as measured by 24-hour HRV, is associated with arterial stiffness measured both dynamically (pulse wave velocity) and locally (carotid distensibility). 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. One potential mechanism of our findings is that autonomic nervous dysfunction may increase the vascular tone of large arteries, thereby impairing arterial elasticity. Animal studies support this notion, in rats, showing proper autonomic regulation is essential for maintaining aortic elasticity, and heightened

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

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sympathetic activity has been shown to damage elastin fibres, resulting in stiffer arteries. While such findings cannot be directly extrapolated to humans, they suggest plausible biological pathways. Additionally, the autonomic nervous system regulates heart rate and cardiac contractility. Autonomic dysfunction typically manifests as both reduced heart rate variability and elevated resting heart rate. A higher resting heart rate may contribute to arterial stiffness 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 HRV over a ten-year period was linked with higher levels of aortic stiffness in the subsequent five years<sup>22</sup>. In study II, we extended this perspective by showing not only short-term HRV during rest but long-term HRV, does link with arterial stiffness, suggesting autonomic response to free-living conditions contributes development of arterial stiffness. In addition, we showed it associated with local measured in carotid distensibility. However, our results are limited by the inability to distinguish between sympathetic and parasympathetic contributions to arterial stiffness, or to determine whether the observed risk is driven by specific responses to living conditions or circadian rhythm variations. The modifying effect of diabetes status, suggest a amplified effect of hyperglycemia of the consequences of autonomic dysfunction. Data from Whitehall II showed how aortic stiffness have a steeper increase by higher HbA1c values among non-diabetic individuals<sup>25</sup>. Our data from Study I extended this perspective by showing that the association between cardiovascular autonomic dysfunction and arterial stiffness is modified by dysglycemia, suggesting that the autonomic nervous system may lie on the pathway from dysglycemia to the development of arterial stiffness, even before the onset of type 2 diabetes. Interestingly, while several time-domain and frequency-domain HRV measures based on the global distribution were modified by diabetes status, the association of mean IBI was not. This suggests that the deterioration of HRV indicators may reflect a different pathogenesis of arterial stiffness in diabetes risk compared to heart rate. In the subpopulation without diabetes, we observed modification by HbA1c at both aortic and carotid stiffness. Hence, our findings support a stronger association within the prediabetes range defined by HbA1c levels of 5.7% to 6.5%<sup>26</sup>, in addition to prediabetes defined by the combination of IGT and IFG.

### **6.2.2. Atherosclerosis**

In study II, we showed that individuals with a preclinical stage of autonomic dysfunction, measured by week-long HRV face a higher risk of incident ischemic-related cardiovascular disease, heart failure, and all-cause mortality.

[ - Discuss what we actual see -> wider range of heart rate lowers risk of cardiovascular complications and mortality.... Will be attempted to discuss 1) the actual mechanism

## *6. Discussion [needs to be fine-tuned]*

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between long-term and hourly HRV and CVD, and 2) potential mechanism of cardiovascular autonomic dysfunctions role in atherosclerosis.]

In the perspective of cardiovascular autonomic dysfunction, we see two possible mechanisms in the formation of ischemic events and stroke. First, autonomic dysfunction drives arteriosclerosis, stiffening the arteries and thereby inhibiting their elasticity<sup>22</sup>. Arterial shear stress increases as a result of heightened sympathetic activity and parasympathetic withdrawal. As a result, the vasodilation of coronary blood vessels may be inhibited, and an increase in vasoconstriction, which can contribute to plaque rupture and thrombus formation<sup>27</sup>. The autonomic nervous system reaches the adventitia layer, innervating the smooth muscles of blood vessels. Despite a separation between the atherosclerotic plaque in the intima layer and the surrounding nerves at the adventitia layer, recent *in vivo* animal studies have shown that higher plaque burden coexists with increased density of sympathetic nerves at local arteries through neuroinflammatory modulation. Furthermore, plaque formation can be lowered by reducing sympathetic nerve density<sup>28</sup>. In this context, findings from Study II suggest that arterial stiffness is not entirely separate from atherosclerosis, which was been confirm by data from the Rotterdam Study<sup>29</sup>. As plaques develop, the associated increase in sympathetic nerve density around the arteries could reduce arterial elasticity. In a smaller study of people with type 2 diabetes, it was shown that lower HRV was linked with increase in carotid atherosclerosis<sup>30</sup>. However, as was asses only nervous response through variation of heart beat, structured studies with precise measures of sympathetic activity are needed to test these hypotheses and confirm them in humans.

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 a missing modulation of heart rate by parasympathetic response under stress may play a role in ischemia<sup>31</sup>. In our week-long recordings, our data likely included episodes of stress-induced HRV under free-living circumstances, e.g. the first indication observed during the awakening stages in the morning. Hence, capturing autonomic responses to living circumstances and their alignment with the circadian rhythm may provide valuable information about cardiovascular risk. Therefore, understanding autonomic responses to tasks is relevant for comprehending their role in cardiovascular risk, beyond short-term measures taken at rest. Including data to monitor real-time activity, such as physical activity, would bring additional value to capture physiological responses to bodily demands. This could enable the inclusion of heart rate responses (e.g., from rest to standing) and other relevant measures of autonomic function, such as heart rate recovery after physical movement, which is a known risk factor for CVD and all-cause mortality [colechristopherr? .]<sup>32</sup>.

[Secondly, lower HRV may be a result of forming plaques that increase cardiac workload and density of sympathetic nerves. This means the autonomic dysfunction may reflect

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

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a predisposition of establish atheroma. [more meat!!!!]

Moreover the basis of autonomic nervous dysfunction has shown to interfere with signalling pathway controlling the heart rhythm and thus lead to arrhythmias disturbing contraction of the heart. Data from the Atherosclerosis Risk In Communities study of illustrated that lower short-term HRV was associated with incident atrial fibrillation over 20 years, and the risk was higher among participants with type 2 diabetes<sup>33</sup>. This supports autonomic dysfunctions role in the development of arrhythmogenesis which increase the risk of MI and stroke. However, in Study II, we do not have incident atrial fibrillation included as an outcome, therefore it would be needed to be explored to understand whether it could explain the higher risk of MACE.

In a shifting paradigm from a focus on ischemia towards more preventive strategies targeting atheroma, individual risk factors leading to a higher predisposition for plaque formation are receiving greater attention<sup>34</sup>. As increased sympathetic nervous system activity has been linked to greater plaque formation, and may be modifiable by reducing sympathetic drive, the autonomic nervous system could play a role in reducing atherosclerotic thrombus formation. However, more physiological studies are needed to understand the mechanisms of atherosclerosis in the presence of autonomic nervous dysfunction, including the causal direction between the two, and how this interplay may be altered during the progression from normal glucose metabolism to type 2 diabetes. This requires more precise measures of both sympathetic and parasympathetic activity, as well as markers of endothelial dysfunction, beyond what is currently captured by HRV and common indices of arterial stiffness.

### **6.2.3. Heart failure**

Heart failure is commonly classified as either ischemic or non-ischemic in origin. It may arise as a consequence of atherosclerosis, arteriosclerosis, or both, contributing to myocardial ischemia, pressure overload, and structural cardiac changes. The relationship between cardiovascular autonomic dysfunction and heart failure is likely complex<sup>35</sup>. On one hand, autonomic dysfunction may represent complication of that contributes to cardiac stress, sympathetic overactivation, and eventual heart failure. On the other, it may reflect the progression of cardiac remodeling and declining cardiac output. Our findings demonstrated the relationship between autonomic dysfunction and heart failure both cross-sectionally in population with type 2 diabetes and prospectively in people representing different tiers of risk of diabetes. However, our data are limited in determining the extent to which the relationship points toward one explanation or the other, as we lack baseline and follow-up measures of both heart failure and heart rate variability.

## *6. Discussion [needs to be fine-tuned]*

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Findings from Study I confirmed the relationship between autonomic dysfunction and arterial stiffness. It is well known that arterial stiffness is linked to cardiac remodelling, as increased pulse wave velocity leads to an earlier return of the reflected pulse wave to the aorta, which increases cardiac afterload and reduces coronary perfusion pressure<sup>36</sup>. Therefore, arterial stiffness may have an indirect effect on heart failure, potentially driven by autonomic dysfunction. However, structured analyses are needed to confirm these pathways, for example through mediation analysis to assess the direct and indirect effects of autonomic dysfunction. In study II, we observed that week-long HRV was linked with incident heart failure and a 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 autonomic dysfunction contributes to cardiac remodelling<sup>37</sup>. In contrast to MACE outcomes, findings from Study II showed no specific time point in hourly HRV associated with heart failure. Instead, it was the overall daily pattern captured by week-long 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 in 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. 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 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, and thus reverse causation. Hence, elevated sympathetic activity during rest may indicate a greater reliance on compensatory mechanisms to maintain cardiac output. More precise measures are needed to assess sympathetic activity as primary driver of heart failure or secondary compensating mechanism of cardiac dysfunction. In addition, it remains unclear to what extent the parasympathetic nervous system can act as a protective mechanisms to counterbalance sympathetic dominance, and whether a decline in the balance of HRV reflects a breakdown. 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. In turn, cardiac remodeling 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.

In study III, we showed presence of CAN was linked with indicators of heart failure by elevated NT-proBNP, higher WATCH-DM risk score and presence of symptoms defined

### ***6.3. The utility of long-term HRV and CART in cardiovascular disease [under construction]***

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by NYHA. We stratified by the presence of symptoms based on NYHA classification, showing that the association between CAN and elevated NT-proBNP remains. This supports the notion that CAN may help detect asymptomatic cases (ref.). [Discuss the challenge of asymptomatic HF]. Studies have shown that CAN is prognostically linked with incident heart failure and type 2 diabetes<sup>38</sup>. This suggests regardless of the direction between CAN and indication of heart failure, the detection of CAN could be utilized to define individuals with heart failure risk. These individuals could benefit from further cardiovascular assessment e.g. echocardiography or intervention to prevent further progression of CAN. Heart failure risk is above 2 times higher people with type 2 diabetes compared to people without diabetes<sup>39</sup>. In populations with type 2 diabetes, the risk of heart failure varies considerably, making reliable indicators for risk stratification important. Our findings support the potential of CAN as a useful marker to identify people with type 2 diabetes at higher risk of heart failure. However, further studies including measures from echocardiography are needed to validate this approach. If confirmed, this could open the door for targeted interventions based on risk stratification.

### ***6.3. The utility of long-term HRV and CART in cardiovascular disease [under construction]***

When using long-term HRV recordings, careful consideration is needed to determine how best to utilize the data in relation to the specific research objectives. In diabetes and cardiovascular research, 24-hour HRV measures of indices reflecting heart rate responses to in- and expiration such as RMSSD, HF and pNN50 are more sensitive to behavioral influences and therefore have not consistently shown strong associations with cardiovascular or metabolic outcomes, especially when compared to measures based on total variability [12]<sup>16</sup>. However, when HRV is analyzed in shorter segments (e.g., hourly or in 5-minute intervals), measures like RMSSD, pNN50, and HF appear to offer new insights into autonomic function and its relevance in diabetes and cardiovascular disease, e.g. during night-time [40]<sup>41</sup>. SDANN and SDNNi aim to reduce the impact of short-term variability, such as that caused by physical activity, by calculating either the standard deviation of 5-minute segment mean IBI (SDANN) or the mean of standard deviations across 5-minute segments (SDNNi). [include lower-frequency points]. This helps smooth out transient fluctuations and better capture long-term autonomic modulation. Thus, behavioral patterns pose a limitation in physiological research aiming to disentangle the causal pathways between autonomic dysfunction and cardiovascular disease when using long-term HRV measures. These patterns likely introduce high variability between observations that is not attributable to autonomic function itself. However, it simultaneously highlights a potential target for intervention, given that low HRV may be indicative of

## *6. Discussion [needs to be fine-tuned]*

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adverse lifestyle patterns. For instance, behavioral patterns such as disrupted sleep or irregular meal timing may influence circadian fluctuations in HRV. Evidence from studies on night-shift workers suggests that meal timing affects HRV, with daytime meals leading to higher HRV during night hours<sup>41</sup>. Thus, although long-term HRV may lack the precision to disentangle sympathetic and parasympathetic activity due to overlapping behavioral and physiological influences, it may be a valuable tool for assessing autonomic responses in free-living conditions and informing lifestyle-based strategies to improve cardiovascular health. Hence, HRV show potential as a responsive marker to monitor successfullness in CVD risk management.

While GLP-1 receptor agonists are proven to lower cardiovascular risk, they have also been shown to increase heart rate and reduce HRV in short-term trials<sup>42</sup>. This may seem conflicting with the hypothesis that reduced HRV reflects higher cardiovascular risk. However, the beneficial effects of GLP-1 agonists on cardiometabolic risk factors, including improved glycaemic control, weight loss, blood pressure reduction, and anti-inflammatory actions, likely outweigh the mild adverse effects on autonomic markers. Thus, the overall cardiovascular benefit is preserved despite small autonomic changes.

[[HRV is just a proxy for heart rate, controversy?] - HRV is just a proxy for heart rate - direct sympathetic activity at the location, but a proxy from heart rate signals.]

### **6.3.1. Risk-stratification**

We demonstrated that autonomic dysfunction and CAN are linked to cardiovascular risk and complications. Beyond the question of the etiological explanation lies the question of whether the condition can serve as a risk indicator for CVD/heart failure and therefore be used to identify patients at higher risk and thus improve risk stratification of CVD/heart failure. This perspective can be viewed from two angles: (1) long-term HRV may improve precision in predicting individual CVD risk when added to clinical risk scores, and (2) long-term HRV may help identify preclinical manifestations of cardiovascular autonomic dysfunction, which could be targeted to prevent future CVD. If the added value of long-term HRV would change or improve traditional CVD risk score e.g. SCORE-2 and Framingham [<sup>43</sup>]<sup>44</sup>. However, most biomarkers has shown low incremental value, as sex, age, lipids, diabetes status and blood pressure remains most important. Earlier studies support that 24-hour HRV adds value by improving risk discrimination for cardiovascular disease and all-cause mortality in type 2 diabetes<sup>45</sup> and stroke in older adults<sup>46</sup>. However, a limitation of these studies is that the reference model has not been calibrated or validated on a larger scale compared to SCORE2 and Framingham.

Stratifying presence of preclinical manifestation of autonomic dysfunction remains to be understood. These considerations are only relevant if specific interventions demonstrate

### *6.3. The utility of long-term HRV and CART in cardiovascular disease [under construction]*

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a mediating effect of targeting autonomic function in preventing CVD outcomes. In other words, interventions aimed at patients with autonomic dysfunction must show greater benefits in preventing CVD compared to those without autonomic dysfunction. Studies have shown that medication and lifestyle interventions can improve HRV in the short term [41]<sup>47</sup>. High-intensity interval training (HIIT) improves autonomic function in obese individuals with and without type 2 diabetes, with HRV benefits seen only in those without diabetes [@böhnhof2022], while lifestyle changes in prediabetes enhance short-term HRV, which seems to explain the reduction in diabetes risk independently of weight loss [@carnethon2006]. However, it remains to be proven whether these effects are sustainable in maintaining of autonomic function and secondly is effective in preventing CVD. Particularly for lifestyle improvements and intensified diabetes management, a large proportion of the enhancement in autonomic function may be explained by indirect effects through improvements in cardiometabolic markers such as glucose levels, lipid profile, body weight, VO<sub>2max</sub>, and blood pressure.

If long-term HRV or CART is to be considered for improving risk stratification, it remains important to determine at what stage in the progression of diabetes risk, autonomic dysfunction becomes meaningful for early detection and intervention. In Study I, we observed that 24-hour HRV was more strongly associated with arterial stiffness in individuals with prediabetes and type 2 diabetes compared to those with normal glucose metabolism. The modifying association of type 2 diabetes between autonomic dysfunction and cardiovascular complications has been demonstrated in multiple cohorts [33]<sup>40</sup>. In Study II, we observed that long-term measures of HRV were strongly associated with cardiovascular risk, with an association equivalent to 4.5 additional years of aging for MACE risk and 2.2 to 2.4 additional years for heart failure per one standard deviation (33 ms) lower in week-long SDNN. Hence, our findings demonstrate that autonomic dysfunction has a strong link with cardiovascular risk in people with prediabetes or those at high risk of developing diabetes, suggesting that autonomic dysfunction is relevant for cardiovascular risk already in the early stages of diabetes progression. In Study III, we demonstrated promising results supporting the potential of using CART-based CAN screening to identify individuals with type 2 diabetes who may be living with asymptomatic heart failure.

A main limitation of our findings in terms of risk stratification is that we did not investigate, in metabolically at-risk populations, whether incorporating measures from long-term HRV or CART as individual risk factors or as components of a risk score improves risk detection by enhancing sensitivity or specificity compared to existing structured guidelines for screening, prevention, and treatment of CVD and heart failure.

## **7. Perspective [under construction]**

Autonomic dysfunction and cardiovascular risk - pathophysiology

Long-term HRV can serve as a risk indicator and may be divided into segments to identify responses with important implications for disease. Both long-term HRV and its hourly variation can be modulated through interventions targeting lifestyle patterns or medication. However, whether such modulation leads to a reduction in cardiovascular disease risk remains unproven. If subclinical autonomic dysfunction is not addressed, there may be a risk of further progression, including stages such as resting tachycardia with sympathetic overdrive, exercise intolerance, and orthostatic hypotension, where both the sympathetic and parasympathetic nervous systems fail to modulate appropriately (see Figure X).

The availability of wearable devices such as smartwatches is expanding the scale of physiological data collection. Data such as heart rate, temperature, and physical acceleration can now be easily obtained under free-living conditions or through app-guided tests. This opens new opportunities to study heart rate responses in everyday life. Combining heart rate with additional physiological signals may improve understanding of CVD risk. Machine learning methods could further help to integrate heart rate and movement data, identifying early signs and patterns in response in the daily cycle that is linked to CVD risk.

### **7.1. Risk-stratification**

Individuals with elevated glucose levels are at increased risk of developing metabolic complications and cardiovascular disease. However, many remain metabolically stable or even return to normal glucose regulation over time. As a result, structured treatment strategies for this group have not been widely adopted in clinical practice. This is partly due to the high degree of heterogeneity within this population. Therefore, additional indicators beyond glucose levels may be useful to identify those most likely to benefit from early intervention.

In Studies I and II, we demonstrated that autonomic dysfunction, as measured by long-term HRV, was more strongly associated with cardiovascular risk in this population.

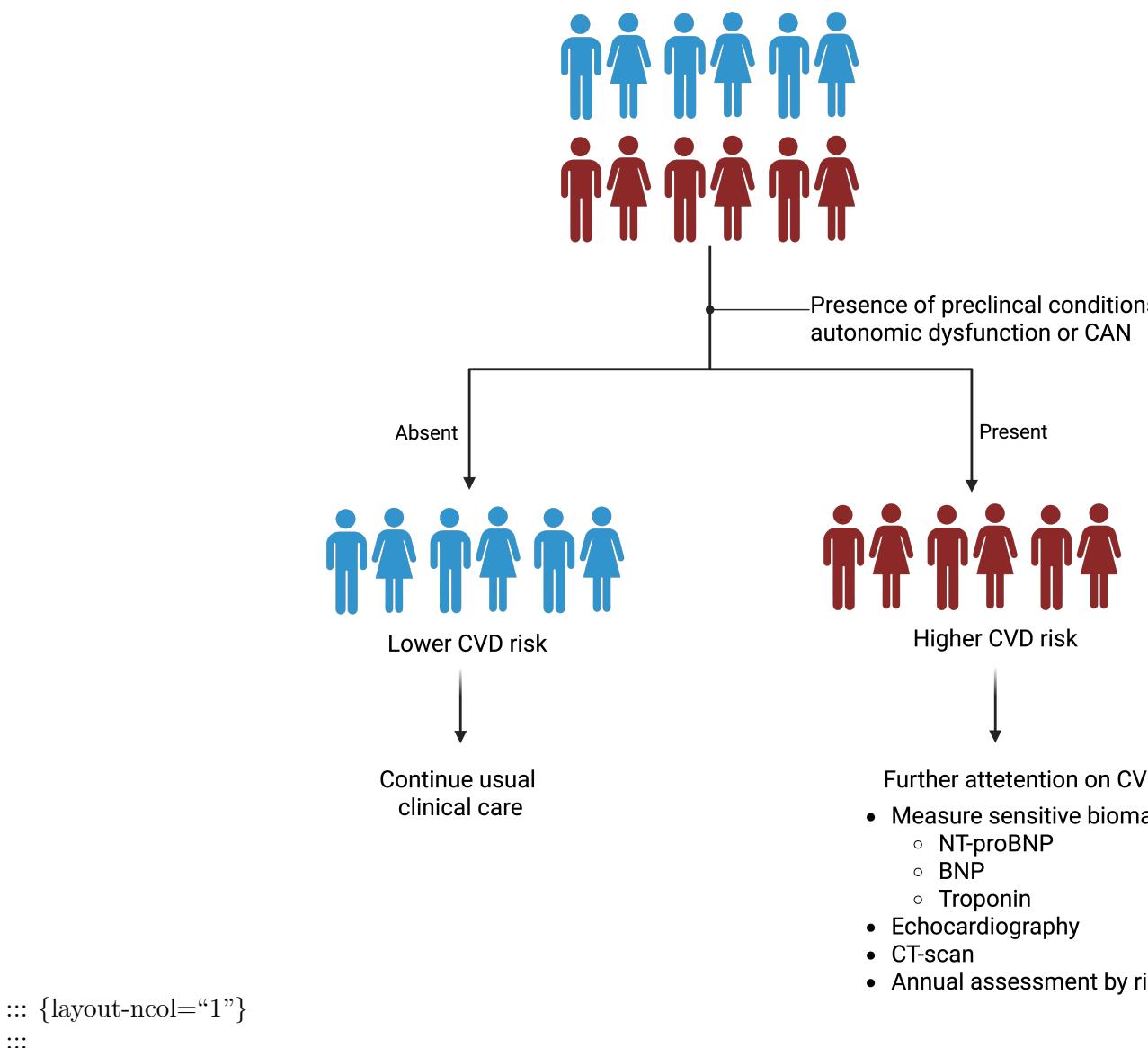
## *7.1. Risk-stratification*

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These findings suggest that HRV may serve as a valuable marker for identifying individuals at elevated risk who could benefit from targeted preventive strategies. Future directions include evaluating whether individuals classified as high-risk based on autonomic dysfunction respond to specific interventions. However, a limitation of long-term HRV measurement is the lack of standardization, as data are collected under free-living conditions and may be influenced by daily behaviors, potentially affecting risk classification.

From a clinical perspective, CART offers a practical approach to screening for autonomic dysfunction, as the test has been shown to be reliable<sup>48</sup>. It is non-invasive and typically takes 8 to 10 minutes to complete. While some CART indices may be influenced by the time of day or recent physical activity, these effects are generally considered minimal. Additionally, no impact of caffeine intake on the reference age-specific values was observed<sup>49</sup>.

In Study III, we demonstrated a relationship between CAN and cardiac dysfunction, as measured by NT-proBNP. Despite . Heart failure is characterized by both structural and functional changes in the heart, such as left ventricular dysfunction, which can be assessed using echocardiography. However, the link between these structural and functional changes and their impact on systolic and diastolic pumping function in relation to CAN remains to be fully understood. Furthermore, the diagnostic and prognostic value of CAN, particularly its sensitivity and specificity in detecting HFrEF and HFpEF, requires further investigation.



## 7.2. Continuous monitoring of cardiovascular health

Over recent years, the use of wearable devices has increased in the general population<sup>50</sup>, enabling comprehensive data collection on behavioral (e.g., sleep and physical activity) and physiological (e.g., heart rate, ECG, temperature) parameters<sup>51</sup>. These devices offer a broader and more feasible approach to long-term heart rate monitoring, requiring less equipment and user burden compared to traditional Holter monitors. Despite growing interest in wearable-based monitoring, the integration of HRV into routine cardiometabolic risk assessment remains limited.

Studies I and II demonstrated a strong association between long-term HRV and CVD risk, with particularly pronounced associations in individuals with prediabetes and type 2 diabetes. These findings suggest that HRV metrics could serve as early indicators for stratifying individuals who may benefit from preventive interventions. In the Whitehall II study, we further showed that a steeper 10-year decline in 5-minute HRV was associated with greater aortic stiffness development over the subsequent five years<sup>22</sup>. Thus, a declining HRV trend detected by smartwatches may help identify individuals who require more intensive interventions.

In Study II, we also observed that specific morning time points were linked to CVD risk, suggesting that physiological responses captured under free-living conditions may provide valuable insights. Rather than adjusting for physical activity as a confounder, future predictive models could integrate multimodal data such as HRV, sleep, and activity patterns to capture dynamic physiological states and improve risk prediction. Incorporating HRV into digital health platforms may support personalized feedback loops, enabling timely lifestyle or therapeutic adjustments.

Hence, future studies can leverage wearable devices to continuously monitor risk by HRV and better understand the behavioral factors that contribute to its improvement or deterioration. This approach may help identify effective lifestyle patterns or medications that improve cardiovascular health through modulation of HRV.

However, standardization and transparency across different brands of wearable devices remain a challenge for both research and clinical implementation of heart rate and HRV monitoring. While smartwatches offer a convenient method for heart rate measurement, their accuracy can vary, as they rely on photoplethysmography to detect pulse rate at the wrist. This method can be imprecise under certain conditions, particularly during physical activity, due to motion artifacts and other external factors<sup>52</sup>. Despite these limitations, ongoing improvements in sensor technology and algorithm calibration are likely to enhance the reliability of wearable-derived heart rate and HRV data.

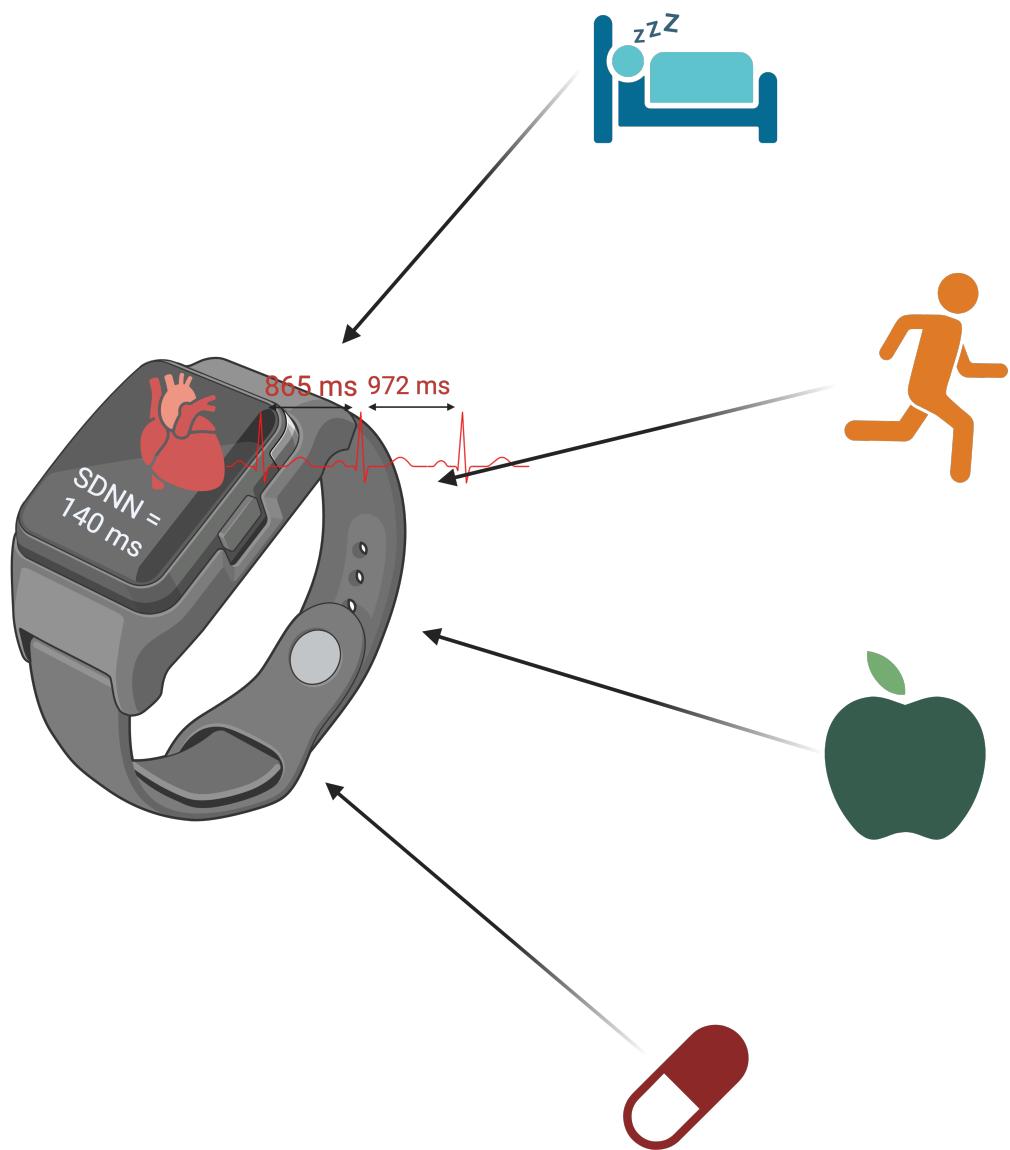


Figure 7.1.: Biofeedback HRV response to lifestyle and treatment solutions

### 7.3. Effective causal modifiable marker

[Closer attention to the mechanisms underlying autonomic dysfunction offers promising opportunities for identifying new intervention targets. While it is plausible that multifactorial risk management may slow the progression of arterial stiffening in part by modulating autonomic function, it remains unproven whether direct modification of heart rate variability (HRV) itself causally reduces cardiovascular disease (CVD) risk. Recent Mendelian randomization studies have emerged as a valuable tool to explore such causal relationships, offering unconfounded estimates by using genetic variants as proxies for exposures.

. These studies can provide a first line of evidence to assess whether HRV is a modifiable causal factor in CVD development. Furthermore, cardiometabolic intervention trials—whether focused on lifestyle modification or pharmacological treatment—should, where feasible, include HRV measurements to enable structured mediation analyses and better understand the role of autonomic function in cardiovascular outcomes.]

- Medelian randomization
- Medation in trails in CVD prevention
- Target trail improves outcome
- Experimental design of mediation of direct target of HRV (medication: beta-blockers, or lifestyle: physical activity, diet and sleep)
- It is responsive.....

The Diabetes Prevention Program (DPP) showed that HRV may modify the effect of lifestyle intervention in preventing type 2 diabetes<sup>53</sup>. However, it remains unclear to what extent this modification applies to cardiovascular outcomes, and whether the intervention was more effective among individuals with lower HRV.

Similar people with type 2 diabetes from young age to a majority of older population. In many cases for prevention effective physical acitivity programs may be out of scope for the more fragile patients, and thus management of medication seems the choice.

## 7. Perspective [under construction]

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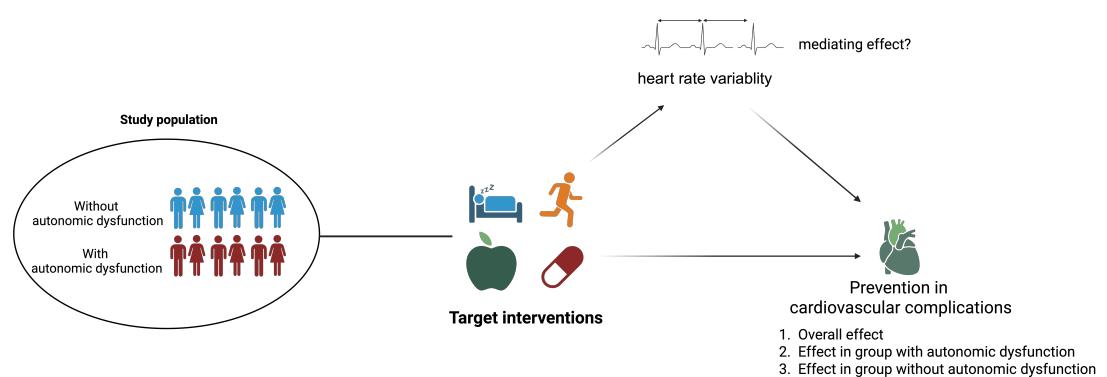


Figure 7.2.: Mediation of HRV by intervention in prevention of CVD

## **8. Conclusion [needs to be fine-tuned]**

Autonomic dysfunction, assessed through 24-hour HRV, is associated with increased arterial stiffness. This relationship is already evident in individuals with normal glucose metabolism and becomes more pronounced in those with prediabetes and type 2 diabetes. In individuals at high risk of type 2 diabetes, lower long-term HRV measured over a week has been linked to ischemic events, heart failure, and all-cause mortality, highlighting HRV's potential as a marker of cardiovascular health. Both HRV and heart rate follow circadian patterns in relation to cardiovascular events. Higher nighttime heart rate is associated with increased risk of heart failure, and specific morning patterns of HRV have been linked to ischemic events. These findings suggest that both long-term and hourly HRV measures provide valuable prognostic information. Structured testing of cardiovascular autonomic function in individuals with type 2 diabetes can detect those with CAN and may help identify individuals at higher risk of heart failure.

We have established an association between HRV and cardiovascular complications. However, the underlying mechanism remains unclear. It is not yet known whether autonomic dysfunction, as indicated by low HRV, is a marker of developing arteriosclerosis, atheroma, or cardiac remodeling, or whether it plays a causal role in their development. While the pathogenic pathways leading to cardiovascular risk appear similar across the spectrum of glucose metabolism, dysglycemia may amplify the impact of autonomic dysfunction. Whether lower long-term HRV in individuals with prediabetes or type 2 diabetes reflects a distinct physiological mechanism involving neuropathy, compared to those with normal glucose metabolism, remains an open question.

Structured studies assessing screening strategies and trial designs, whether focused on lifestyle interventions or targeted pharmacological modulation of HRV, are needed to clarify the clinical role of HRV and CART in cardiovascular prevention. Long-term HRV and its hourly fluctuations provide insight into autonomic responses under free-living conditions. Further research is needed to determine whether modifying these measures can yield sustained preventive effects on cardiovascular disease and mortality. CARTs offer a standardized approach for diagnosing CAN. Clarifying the clinical utility of CARTs in assessing cardiovascular and heart failure risk through the identification of CAN is essential for advancing precision care in individuals with type 2 diabetes. Echocardiographic studies can help establish the link between CAN and the risk of specific heart failure

## *8. Conclusion [needs to be fine-tuned]*

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subtypes. Future research should carefully select HRV measures aligned with specific clinical or research objectives. Long-term HRV and CART have demonstrated potential in cardiovascular risk assessment and should be integrated to evaluate whether autonomic function assessments can monitor treatment or lifestyle effectiveness, or guide stratified cardiovascular risk decisions in individuals with prediabetes or type 2 diabetes.

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## **A. More results**

Some results that wouldn't fit into the main thesis

## **B. Another appendix**

Something else