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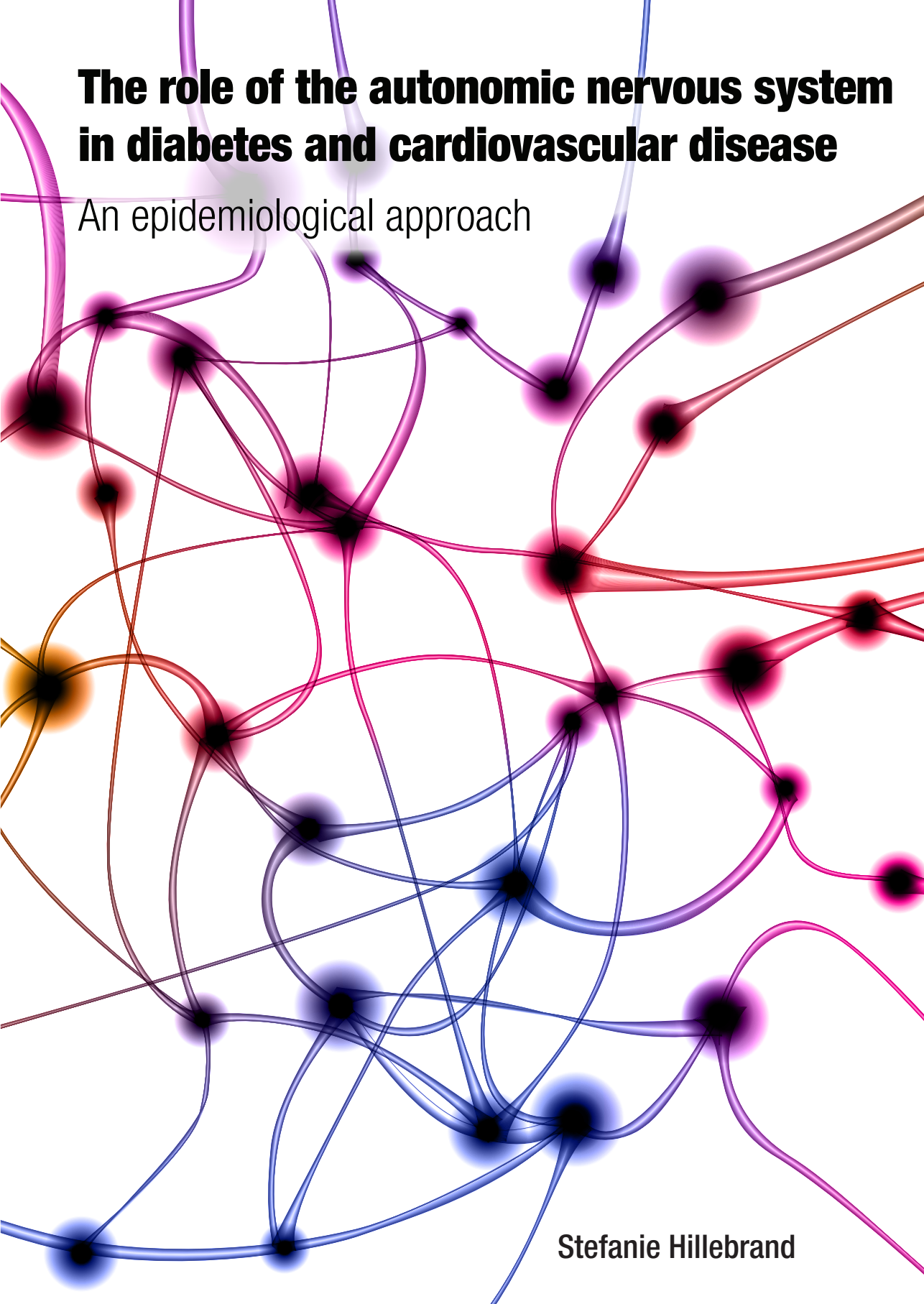
The role of the autonomic nervous system in diabetes and cardiovascular disease

An epidemiological approach



Stefanie Hillebrand

The role of the autonomic nervous system in diabetes and cardiovascular disease

An epidemiological approach

Stefanie Hillebrand

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An epidemiological approach

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Voor mijn ouders

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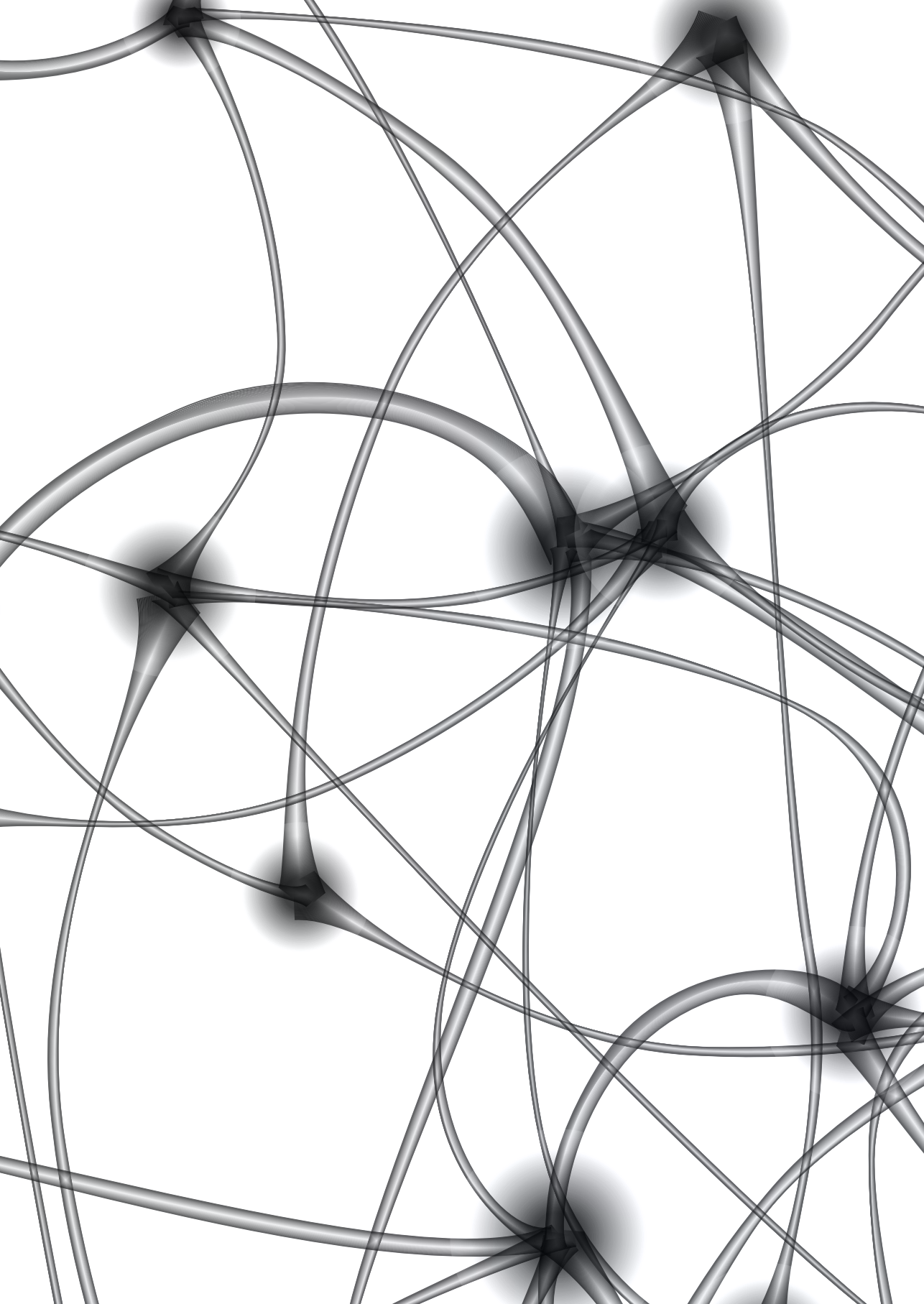

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General Introduction and Outline

The autonomic nervous system (ANS) controls homeostasis and regulates visceral functions. ANS function is impaired in obesity, diabetes and cardiovascular disease. However, it is yet unclear whether this impairment is merely a consequence of underlying disease, or may also be involved in the development of disease. The main objective of this thesis was to study the role of ANS function in the development of diabetes and cardiovascular disease. This general introduction describes the current state of knowledge about ANS function and its role in health and disease. It furthermore describes the outline of this thesis and summarizes the studies and data that were used.

THE AUTONOMIC NERVOUS SYSTEM

Most functions of the ANS are not in conscious control, and are based on feedback loops and reflexes. The central part of the ANS is located in the medulla oblongata of the brainstem and the hypothalamus.[1] Afferent nerves convey information from organs, muscles, the limbic system, the circulatory system and sensory organs to the central part of the ANS. The efferent pathways consist of the parasympathetic and the sympathetic nervous system, which have mostly opposite effects on organs.[2] The functions of the parasympathetic and the sympathetic nervous system are illustrated in the Figure. The sympathetic nervous system is predominant in active situations, ranging from small actions such as posture changes to "flight-fight-or-fright" actions. The parasympathetic nervous system is predominant during periods of lower activity, in most individuals this is the larger part of the day. The sympathetic output originates from the ventrolateral medulla in the

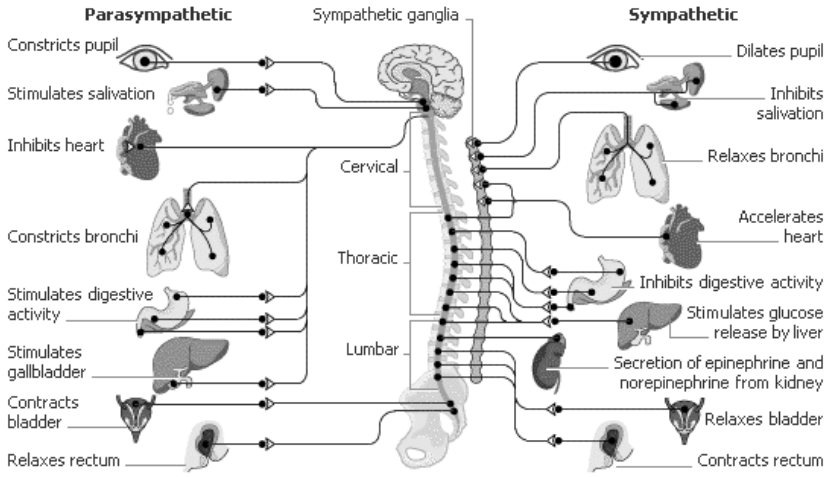

Figure. Functions of the parasympathetic and the sympathetic nervous system
Source: www.medicalterms.info

brain stem and is effectuated through sympathetic preganglionic neurons located in the spinal cord at the T1-L2 segments. The parasympathetic output also originates from the brainstem (nucleus ambiguus) and is effectuated through cranial nerves (CN3, CN7, CN9, CN10) and the spinal cord at the S2-4 segments.[2] The most important neurotransmitters in the efferent part of the ANS are norepinephrine for sympathetic transmission and acetylcholine for parasympathetic transmission.[2] The function of the ANS can be measured in several ways. Often, autonomic control of the cardiovascular system is used to estimate ANS function.

AUTONOMIC CONTROL OF THE CARDIOVASCULAR SYSTEM

The cardiovascular system consists of the heart and peripheral vasculature, and its purpose is to provide an adequate flow of blood and nutrients to the organs.[3] The heart is innervated by the parasympathetic nervous system via the vagal nerve and by the sympathetic nervous system via nerves arising from the upper thoracic region of the spinal cord. Baroreflexes buffer blood pressure, and thereby play a central role in the autonomic regulation of the cardiovascular system. Baroreceptors are not directly sensitive to blood pressure, but rather to the mechanical deformation of the nerve endings during stretching of the vascular wall. Two types of baroreceptors can be distinguished. Cardiopulmonary baroreceptors are localized in the heart, vena cava, and pulmonary vasculature and respond to changes in central venous pressure, whereas arterial baroreceptors in the aortic arch and carotid sinuses respond to changes in arterial pressure. When venous return and arterial blood pressure increase, the sympathetic outflow to the heart and peripheral vasculature is inhibited and the parasympathetic outflow is stimulated, resulting in a decreased peripheral resistance and decreased heart rate.[4] A decrease in venous return and arterial blood pressure results in more sympathetic outflow, decreased parasympathetic outflow, an increase in peripheral resistance and an increase in heart rate. Changes in blood pressure are caused by an adjustment in peripheral resistance rather than by changes in heart rate.

THE AUTONOMIC NERVOUS SYSTEM IN HEALTH AND DISEASE

In healthy individuals, there is modulation in the sympathetic and parasympathetic outflow influenced by the level of activity. This modulation is necessary for an optimal control of homeostasis and visceral functions and is a manifestation of adaptivity. Impaired ANS function is characterized by reduced modulation in sympathetic and parasympathetic outflow, chronic overactivation of the sympathetic nervous system

and chronic decreased activation of the parasympathetic nervous system. Impaired ANS function has been associated with obesity, diabetes and cardiovascular disease.

Obesity

Historically, two hypotheses on the association between ANS function and obesity were postulated. The first hypothesis stated that low activity of the sympathetic nervous system caused weight gain, and the second proposed that activation of the sympathetic nervous system and decreased activity of the parasympathetic nervous system were a result of obesity.[5, 6] The latter hypothesis was adopted by most contemporary researchers and is strongly supported by intervention studies on weight loss, showing that after a decrease in the amount of body fat by a hypocaloric diet or exercise training, activity of the sympathetic nervous system attenuated.[7-13]

Several epidemiological studies have shown inverse associations between body fat and ANS function, and positive associations between body fat and sympathetic nervous system activation.[14-22] However, only few studies were able to investigate the associations between various types of fat, for example visceral fat accumulation within the abdomen, and ANS function. A mechanism underlying of the association between visceral fat and activation of the sympathetic nervous system may be the production of adipocyte derived cytokines (adipokines). Adipocytes, or macrophages invading the adipose tissue, secrete several adipokines.[23] Visceral fat has a higher secretion rate of pro-inflammatory cytokines than subcutaneous fat and inhibits the secretion of anti-inflammatory factors.[24-27] These cytokines can either directly stimulate the central nervous system, by crossing the blood-brain-barrier, or induce low-grade inflammation, resulting in sympathetic activation.[23, 28] Previous studies have shown that visceral fat is stronger associated with cardiovascular risk factors and endpoints than other types of body fat.[29-32] It is, however, unknown whether visceral fat may also stronger influence sympathetic activation than other types of body fat.

Insulin resistance and type 2 diabetes mellitus

Impaired ANS function is a well-known complication of type 2 diabetes mellitus.[33, 34] High blood glucose and insulin levels and an abundance of advanced glycation endproducts can directly damage the nerves or interfere with chemical signalling. Impairment of the ANS worsens with the duration of type 2 diabetes, and is associated with an increased mortality risk.[35] However, several research findings have become known supporting the notion that impaired ANS function may not only be a complication of type 2 diabetes, but may also be involved in the development of the disease. The ANS innervates several organs involved in the glucose metabolism, such as the liver, pancreas, adrenal and skeletal muscles. The parasympathetic branch of the ANS seems responsible for the insulin

sensitivity of several organs.[36] The decreased parasympathetic outflow in impaired ANS function may therefore play a role in the development of type 2 diabetes. Moreover, non-diabetic offspring of type 2 diabetes patients, who are at high risk of developing type 2 diabetes, have a worse ANS function than individuals of the same age without a family history of type 2 diabetes, independent of other risk factors.[37] Together, these arguments suggest that impaired ANS function may not only be a complication of type 2 diabetes, but may also play a role in the development of the disease.

ANS function has been extensively studied in relation with insulin resistance and glucose tolerance. Several cross-sectional studies have shown an inverse association between ANS function and insulin resistance and a positive association between ANS function and glucose tolerance. Insulin resistance, a subclinical stage of type 2 diabetes, is defined as an inadequate response by insulin target tissues to the physiologic effects of circulating insulin. The decreased sensitivity to insulin results in compensatory hyperinsulinaemia. Without this compensation, impaired glucose tolerance or type 2 diabetes mellitus may develop.[44, 45] Therefore, these previously mentioned studies suggest that ANS function is involved in the pathogenesis of type 2 diabetes.[38-43] However, because of the cross-sectional nature of these studies, the direction of the association remains undetermined and it is yet unclear whether impaired ANS function is indeed involved in the development of type 2 diabetes.

Cardiovascular disease

An association between ANS function and cardiovascular disease has frequently been reported. In 1978, an inverse association between variability in heart rate and mortality in patients admitted to the hospital with acute myocardial infarction was first reported.[46] Later studies showed that low baroreflex sensitivity and low heart rate variability were risk factors for cardiovascular events, including myocardial infarction,[47, 48] heart failure,[49] and atrial fibrillation[50] in populations with pre-existing cardiovascular disease. However, in these populations impaired ANS function may have been a result of the pre-existing cardiovascular abnormalities. Therefore, based on existing studies it remains unclear whether impaired ANS function is also a risk factor for the development of cardiovascular disease.

A mechanism underlying the association between impaired ANS function and cardiovascular disease may be an altered lipid metabolism. Experimental studies in rats showed that sympathetic denervation in the liver decreased both hepatic lipogenesis and hepatic secretion of very low density lipoprotein (VLDL).[51, 52] This indicates that sympathetic activation to the liver stimulates lipogenesis and the secretion of VLDL-triglycerides. Furthermore, in adipose tissue, stimulation of the sympathetic nervous

system induces lipolysis, thereby increasing the amount of glycerol and free fatty acids in the circulation.[53] Previous epidemiological studies have reported an association of sympathetic activation with fasting lipid levels in the circulation.[54-56] However, since most individuals are in a postprandial state for the larger part of the day, elevated postprandial serum triglyceride concentrations may be more important for cardiovascular risk.[57, 58] Furthermore, in addition to an increased concentration of lipids in the circulation, activation of the SNS may also result in accumulation of lipids in the liver.[59] The associations of sympathetic activation with postprandial triglyceride concentrations and intrahepatic triglyceride content have not been reported yet.

AIM AND OUTLINE OF THIS THESIS

Although the associations between impaired ANS function, obesity, diabetes and cardiovascular disease have been investigated in previous studies, it remains unclear whether an impaired ANS function is merely a consequence of pre-existing disease, or may also be involved in the development of diabetic and cardiovascular disease. The aim of this thesis was therefore to study the role of ANS function in the development of diabetes and cardiovascular disease.

In **chapter 2** we investigated the association between body fat and ANS function. Since the role of various fat depots has not been unravelled yet, we studied several types of body fat, including abdominal visceral and subcutaneous fat.

In **chapter 3** we investigated whether ANS function is a risk factor for type 2 diabetes. In order to gain more information on the direction of the association we used follow-up data of individuals without diabetes who had been followed until the occurrence of type 2 diabetes. In **chapter 4** we aimed to unravel the interrelationships between body fat, ANS function and insulin resistance.

To investigate whether impaired ANS function is a risk factor for the development of cardiovascular disease, we performed a meta-analysis and meta-regression of prospective studies investigating this association in populations without pre-existing cardiovascular disease in **chapter 5**. We hypothesized that an altered lipid metabolism is a mechanism underlying the association between impaired ANS function and cardiovascular disease and investigated the association between impaired ANS function and alterations in lipid metabolism in **chapter 6**.

STUDY DESIGNS AND DATA USED IN THIS THESIS

The NEO study

The Netherlands Epidemiology of Obesity (NEO) study is a population-based prospective cohort study in 6673 individuals aged 45 to 65 years, with an oversampling of individuals with a BMI ≥ 27 kg/m^2, who were included between September 2008 and October 2012. At baseline, participants completed questionnaires for demographic, lifestyle, and clinical information and underwent an extensive physical examination. Random subsamples of the study population underwent magnetic resonance imaging (MRI) of the abdomen and magnetic resonance spectroscopy of the liver (n=2580), MRI of the heart (n=1207), or were equipped with an Actiheart®, a combined heart rate monitor and accelerometer (CamNtech Ltd, U.K.) for four days (n=955). To answer the research questions in **chapters 2, 4, and 6** of this thesis, we used the baseline measurements of the NEO study.

The Hoorn study

For the research question in **chapter 3** we used data from the Hoorn study, a prospective cohort study including 2484 adults aged 50 to 75 years from the town of Hoorn, the Netherlands. Baseline data were collected between 1989 and 1991 and included data on demographic characteristics, medical history, physical activity and a physical examination. In a subsample of participants (n=631) ANS function was measured. Follow-up examinations were performed in 1996-1998 and 2000-2001.

MEASUREMENT OF AUTONOMIC NERVOUS SYSTEM FUNCTION

Unfortunately, a gold standard for assessing activity of the ANS is lacking. Over the past decennia, several techniques have been developed. The main measures are described below and summarized in the Table.

Microneurography uses fine electrodes that are inserted percutaneously in the nerves to record bursts of activity. Often, the easily accessible common peroneal nerve is used to measure the efferent postganglionic muscle sympathetic nerve activity.[60] Muscle sympathetic nerve activity is a direct measure of sympathetic activity, but it is invasive and can only be used on superficial nerves as nerves to internal organs are not accessible.

Catecholamines are sympathetic neurotransmitters which can be measured to estimate sympathetic nervous system function.[61] Dopamine acts mainly in the central nervous system, while epinephrine and norepinephrine mostly have peripheral effects. Epinephrine is exclusively produced by the adrenal medulla, whereas norepinephrine is mainly produced

Table: Commonly used measures of autonomic nervous system function

Measure	Indicative of sympathetic nervous system	Indicative of parasympathetic nervous system	Validity	Advantages	Disadvantages	Used in this thesis (chapters)
Muscle sympathetic nerve activity	Yes	No	High	Direct measurement of nerve activity	Invasive Only peripheral nerves	No
Catecholamines (plasma, urine)	Yes	No	Very high	Direct measurement of neurotransmitter	Expensive	No
Heart rate and mean NN	Yes	Yes	High	Non-invasive Cheap Easy to measure	Influenced by many factors other than the ANS	2, 3, 4, 6
Heart rate variability – time domain (SDNN, RMSSD)	Yes	Yes	Intermediate	Non-invasive Cheap Easy to measure	Influenced by many factors other than the ANS	3, 4, 5, 6
Heart rate variability – frequency domain (HF, LF)	Yes	Yes	Intermediate	Non-invasive Cheap	Difficult to measure Strongly influenced by noise	3, 4, 5, 6
Baroreflex sensitivity	Yes	Yes	Intermediate	Combines RR interval and systolic blood pressure	Influenced by many factors other than the ANS	3
Ewing tests (RR max, RRmax/min, SBP difference, EI-difference)	Yes (EI-difference, RRmax)	Yes (all)	High	Standardized measurements	Time consuming	3
Electrocardiographic measures	Yes	No	Unknown	Non-invasive	No validation studies available	2

NN, normal to normal RR intervals; SDNN, standard deviation of all normal intervals; LF, low frequency; HF, high frequency; EI, expiration-inspiration; SBP, systolic blood pressure; ANS, autonomic nervous system

by sympathetic postganglionic fibres and is therefore more specific to estimate activity of the sympathetic nervous system.[62] Norepinephrine can be measured both in urine or in plasma. All norepinephrine that is formed by the body is eventually excreted in the urine either unchanged or as a metabolite.[63] Therefore, total urinary excretion of norepinephrine or its metabolites can be used to estimate the synthesis rate of norepinephrine.[64, 65] Measurement of plasma norepinephrine is less straightforward, because it depends not only on the release of norepinephrine to plasma, but also on the clearance from the circulation.[66, 67] Spill-over techniques aim to solve this problem. Highly specific radiolabelled norepinephrine is infused into the plasma at a constant rate to reach steady-state conditions.[67] Then, the rate of release of endogenous norepinephrine into plasma and the rate of clearance from the plasma can be estimated using equations.[68] Unfortunately, laboratory measurements of norepinephrine in urine or plasma are expensive.

Because the measurement of muscle sympathetic nerve activity and catecholamines is invasive or expensive, these measurements are not easily employed in large epidemiological studies. In the population studies described in this thesis, we rather used the following measures of ANS function: resting heart rate, heart rate variability, baroreflex sensitivity, Ewing tests and electrocardiographic measures of sympathetic activation. Resting heart rate is a measure of the sympathovagal balance.[69] According to the Rosenblueth-Simeone model, heart rate is determined by the intrinsic heart rate multiplied by a sympathetic factor and a vagal factor.[70] Impaired ANS function with increased sympathetic nervous system activation is reflected in a higher resting heart rate. In 1981, the first findings on spectral analyses of heart rate fluctuations were published.[71] Heart rate variability is defined as the oscillations in the interval between consecutive heart beats.[72] Heart rate variability is a manifestation of activity of the baroreflex, which purpose is to buffer blood pressure by adaptions in sympathetic and parasympathetic outflow.[71, 73] Variability in heart rate can be expressed in several ways. In time domain analyses, a variety of statistical variables are calculated from intervals between adjacent normal R waves (NN intervals). The most commonly used time domain variables are the standard deviation of all NN intervals and the root mean square of successive differences between adjacent RR intervals.[74] The frequency domain analyses quantify cyclic fluctuations of NN intervals by the frequency of the fluctuation using Fourier transformation or autoregressive spectral analysis.[72] High frequency fluctuations, with a frequency range of 0.15-0.40 Hz, are caused by respiration (if breathing is at high frequency[75]) and are effectuated by parasympathetic outflow. Low frequency fluctuations, in the range between 0.04 and 0.15 Hz, are the result of a blood pressure resonance phenomenon mediated by the baroreflexes and are effectuated by both sympathetic and parasympathetic fluctuations in outflow.[73, 76] Impaired function of the ANS is reflected in lower heart rate variability. Heart rate and heart rate variability can be determined from electrocar-

diographic recordings. The total variance in heart rate increases with the duration of the recording, because long measurements (e.g. 24 hours) include more rhythms (e.g. circadian rhythm) than short measurements.[72] In this thesis we used heart rate and heart rate variability from recordings ranging from 10 seconds to 24 hours. In addition to standard electrocardiographic recordings we also used the Actiheart device, an accelerometer combined with a heart rate monitor (CamNtech Ltd, Cambridge, United Kingdom) to calculate heart rate variability. The Actiheart device uses two electrodes at the level of the second intercostal space to measure the electrocardiogram (ECG), after which detection of heart beats occurs and interbeat intervals are recorded. The ECG itself is not stored. After extensive data processing, this recording can be used to estimate heart rate variability over a 24 hour period. The correlation between a Holter ECG and the Actiheart for estimating heart rate (variability) is high (correlation coefficient for mean RR interval 0.998, 95% CI: 0.994-1.000).[77]

The changes in beat-to-beat heart rate are mediated by the baroreflexes. Therefore, also the quantification of baroreflex sensitivity is valuable in the assessment of ANS function.[78] Baroreflex sensitivity is estimated as the change in RR intervals divided by systolic blood pressure (ms/mmHg). Better ANS function is reflected in a higher baroreflex sensitivity.

Ewing tests have been important for many years in the detection of diabetic autonomic neuropathy.[79] In **chapter 3**, we used four measures from the Ewing test battery. Three measures are obtained during an active change in position from lying to standing: the difference between the mean RR interval during one minute of rest prior to standing up and the minimum RR interval within 15 seconds after standing up (RRmax), the maximum RR interval between 15 and 30 seconds after standing up divided by the minimum RR interval 15 seconds after standing up (RRmax/min), and the systolic blood pressure difference (SBP difference) after standing up defined as the mean SBP over 30 seconds during 90-120 seconds after standing up, minus the mean over 30 seconds prior to standing up. Impaired ANS function is reflected in lower RRmax and RRmax/min and a larger (more negative) SBP difference. We also assessed the difference in maximum and minimum RR interval during inspiration and expiration averaged over six breaths (EI-difference) during deep breathing. Lower EI-difference is a reflection of impaired autonomic nervous system function.

Finally, three recent experimental studies showed that activation of the sympathetic nervous system is also reflected in changes on the standard ECG.[80-82] These changes include alterations in conduction times and ventricular repolarization. In **chapter 2** of this thesis, we introduced these novel measures to estimate activity of the sympathetic nervous system.

EPIDEMIOLOGICAL APPROACH

The aim of this thesis was to investigate the role of the ANS in the development of diabetes and cardiovascular disease by using observational population studies. To this end, we used observational data from large cohort studies for causal inference, the process of drawing a conclusion about a causal connection. Causal inferences are most straightforward from randomized controlled trials. However, for the study of ANS function, randomized controlled trials are neither possible nor ethical. Observational studies may be used for causal inferences, after sources of bias have been taken into account, such as confounding and reverse causation.[83]

Confounding may occur in observational studies when the exposure and the outcome variable share a common cause that may obscure the causal association and explain the observed crude association.[84] By adjusting for, or stratifying on, a confounding variable, the effect of the common cause may be eliminated from the association under study. In this thesis, we aimed to adjust our analyses for all known and measured confounding variables of the associations under study.

Reverse causation may occur especially in cross-sectional studies. When the exposure and outcome variables are measured at the same time, the observed exposure may be a consequence of the outcome variable rather than a cause. For example, when glucose tolerance and ANS function are associated in a cross-sectional study, it remains unclear whether glucose tolerances influences ANS function or vice versa. Longitudinal studies are less likely to suffer from reverse causation, as these studies provide information on the temporal relation between the exposure and outcome. However, in longitudinal studies reverse causation may also play a role when the outcome or disease of interest is already (subclinically) prevalent at baseline. For example, when ANS function is studied as a risk factor for the occurrence of cardiovascular disease after a myocardial infarction, we do not know whether impaired ANS function is merely a result of the myocardial infarction, or also a risk factor for cardiovascular disease in general.

In this thesis we used several approaches to overcome an effect of pre-existing diabetes and cardiovascular disease on ANS function in our studies. First, we excluded or adjusted for individuals with prevalent disease in our studies. Second, we restricted our analyses to populations without subclinical cardiovascular disease or diabetes at baseline; and third, we studied associations of ANS function with incident diabetes and cardiovascular disease using prospective follow-up studies. By studying first events of diabetes and cardiovascular disease during follow-up of individuals without pre-existing disease at baseline, we aimed to minimize a potential influence of underlying disease.

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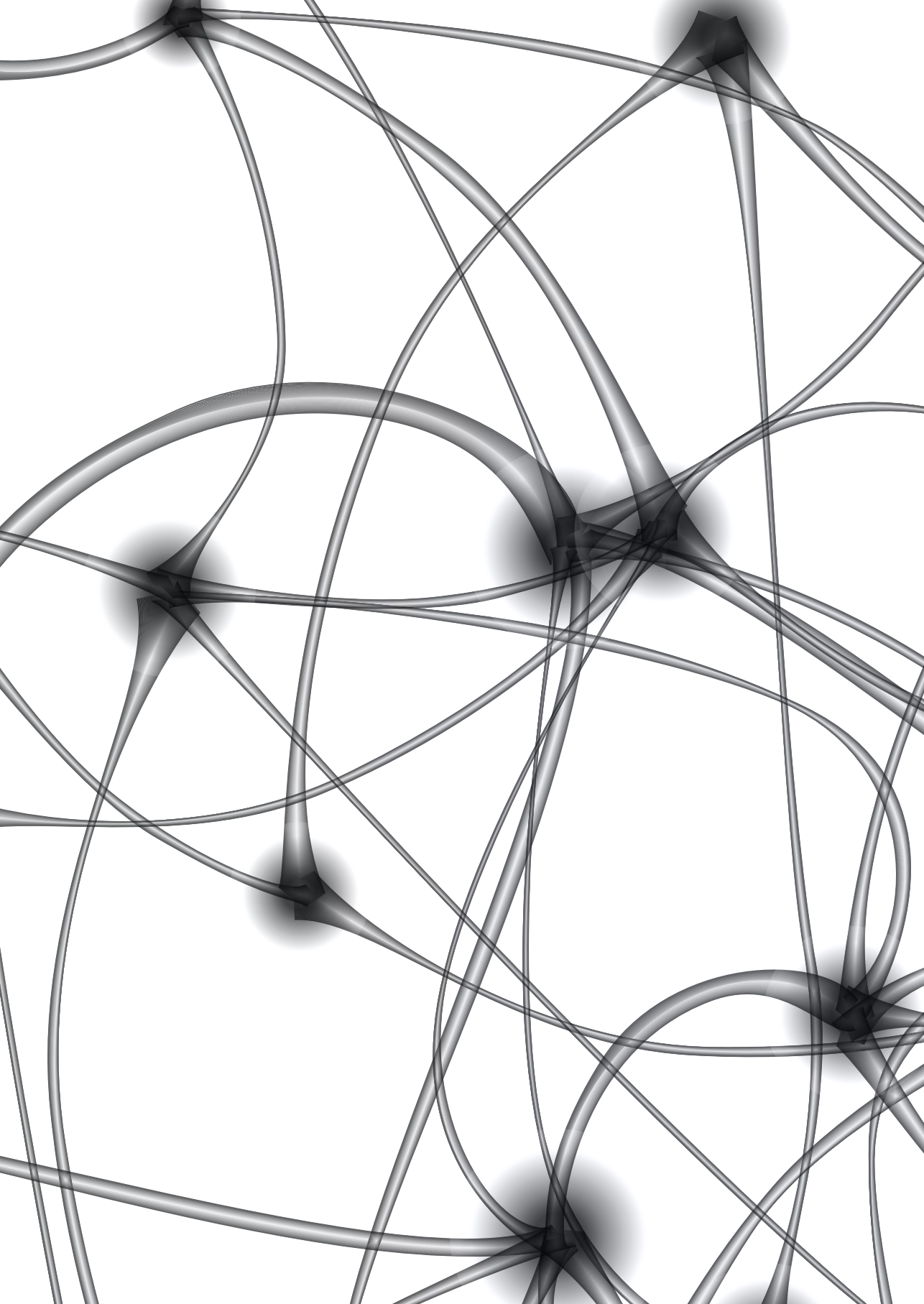

2

Body fat, especially visceral fat, is associated with electrocardiographic measures of sympathetic activation

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ABSTRACT

Objective

Obesity is associated with sympathetic activation, but the role of different fat depots is unclear. The association between body fat, specifically visceral fat, and electrocardiographic measures of sympathetic activation in a population with structurally normal hearts was investigated.

Design and Methods

In this cross-sectional baseline analysis of the Netherlands Epidemiology of Obesity study, body fat percentage was assessed with BIA and abdominal subcutaneous (SAT) and visceral adipose tissue (VAT) with magnetic resonance (MR) imaging. Mean heart rate (HR) and five other electrocardiographic measures of sympathetic activation were calculated. We performed multivariate linear regression analyses.

Results

In 868 participants with a mean age (SD) of 55 (6) years, BMI of 26 (4) kg/m^2, 47% men, body fat was associated with HR and two other measures of sympathetic activation. Per sex-specific SD of total body fat, the difference in HR was 1.9 beats/min (95% CI: 1.0, 2.9; p<0.001) and per SD waist circumference 2.1 beats/min (95% CI: 1.3, 2.9; p<0.001). The difference in heart rate per SD VAT was 2.1 beats/min (95% CI: 1.3, 3.0; p<0.001).

Conclusions

Body fat, especially visceral fat, was associated with electrocardiographic measures of sympathetic activation. Our study implies that already before the onset of cardiovascular disease, excess (visceral) body fat is associated with sympathetic activation.

INTRODUCTION

Obesity is associated with altered function of the autonomic nervous system.[1-6] The autonomic nervous system controls homeostasis and regulates visceral functions by modulations in sympathetic and parasympathetic outflow. Altered autonomic function is characterized by reduced adaptive modulation and sympathetic activation. Neurohumoral activation increases the risk of both first and secondary cardiovascular events and is also associated with subclinical cardiovascular disease (CVD).[7-9]

Body fat may stimulate the sympathetic nervous system and induce autonomic dysfunction by the secretion of adipokines such as leptin, C-reactive protein, TNF-α, plasminogen activator inhitor-1 and interleukin-6. These cytokines either cross the blood-brain-barrier and directly stimulate the central sympathetic nervous system in the hypothalamus, or induce a state of low-grade inflammation that can also stimulate the sympathetic nervous system.[10]

In general, visceral fat seems stronger associated with CVD risk factors than subcutaneous fat. This may be explained by more macrophage infiltration and a higher secretion rate of pro-inflammatory cytokines.[11-14] However, subcutaneous fat is the largest fat depot in the body, accounting for approximately 90% of total body fat. Therefore this depot may still be important in absolute contribution to sympathetic activation. Most previous studies on the association of body fat with sympathetic activation did not distinguish between different abdominal fat depots.[1-4] We hypothesize that visceral fat is stronger associated with sympathetic activation than subcutaneous fat.

Electrocardiographic markers that are commonly used to study sympathetic activation are heart rate and heart rate variability.[15, 16] Three recent experimental studies showed that the sympathetic overdrive is also reflected in other changes on the standard electrocardiogram (ECG).[17-19] These changes include alterations in conduction times and ventricular repolarization. The ECG is an inexpensive, non-invasive and widely available diagnostic tool and may therefore be easy to use in large studies on sympathetic activation.

The aim of our study was to investigate to what extent overall and abdominal fat are associated with both established and novel measures of sympathetic activation in a population without underlying CVD.

METHODS

Study design and population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based prospective cohort study with oversampling of individuals with a BMI ≥ 27 kg/m^2. The design and data collection of the NEO study were described previously.[20] The baseline visit was performed in the NEO study centre in the Leiden University Medical Centre. Prior to this study visit, participants fasted overnight and completed questionnaires. At the NEO study centre an extensive physical examination was performed. In a random subgroup of participants, magnetic resonance (MR) imaging scans of the abdomen and heart were performed.

The present study is a cross-sectional analysis of the baseline measurements of all participants with MR imaging of the heart and abdomen. Exclusion criteria for our study were self-reported CVD (defined as myocardial infarction, angina, congestive heart failure, stroke, or peripheral vascular disease), ejection fraction (EF) <50%, left ventricular hypertrophy (LVH) (defined as >2SD deviation from the mean body surface area corrected left ventricular end-diastolic mass), prevalent diabetes mellitus (DM) (defined as use of glucose lowering drugs, fasting glucose concentration ≥7.0 mmol/L or postprandial glucose concentration ≥11.1 mmol/L), abnormal ECG or missing data on measures of body fat or sympathetic activation. For the analyses including heart rate or heart rate variability, we additionally excluded participants using beta blockers or calcium antagonists. For the analyses including QTc, Tpeak-end, spatial ventricular gradient or spatial QRS-T angle we additionally excluded participants with ventricular conduction defects. The NEO study was approved by the Medical Ethical Committee of the Leiden University Medical Centre. Before inclusion, all participants gave their informed consent.

Data collection

Self-identified ethnicity of participants was grouped into white (reference category) and other. Level of education was grouped in low education (defined as no education, primary education or lower vocational training) versus high education (reference). Tobacco smoking was categorized into current, former or never (reference) smokers. Physical activity was expressed in MET-hours per week. Blood was sampled after an overnight fast of at least 10 hours. Two postprandial blood samples were taken 30 minutes and 2.5 hours after a liquid mixed meal (400 ml, 600 kcal). Fasting and postprandial glucose concentrations were measured with the enzymatic colorimetric method (Roche Modular Analytics P800, Roche Diagnostics, Mannheim, Germany). Participants were asked to bring all their medication. The heart was imaged by MRI (1.5 Tesla MRI, Philips Medical Systems, Best, Netherlands) in short-axis view using an ECG-triggered balanced turbo-field-echo sequence. A 3-dimensional 3-directional velocity-encoded MRI technique was used. End

systolic and end diastolic ventricular masses and volumes were measured. Left and right stroke volumes (SV) were defined as the difference between the end diastolic volume and the end systolic volume. Left ventricular ejection fraction was calculated by dividing left ventricular SV by the end diastolic volume. The left ventricular mass was measured end diastolic and was standardized for body surface area.[21]

Body fat

Height and weight were measured without shoes and one kilogram was subtracted to correct for the weight of clothing. Body Mass Index (BMI) was calculated by dividing the weight in kilograms by the height in meters squared. The circumference of the waist (WC) was measured in the middle of the distance between the lowest rib and the crista iliaca superior with a flexible steal tape. Body fat percentage was measured using a bio-impedance device (TBF-310, Tanita International Division, UK). Abdominal subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) were quantified by MRI (1.5 Tesla MRI, Philips Medical Systems, Best, Netherlands) using a turbo spin echo imaging protocol. At the level of the fifth lumbar vertebra three transverse images with a slice thickness of 10 mm were obtained during a breath-hold. The fat depots were converted from the number of pixels to centimetres squared. The average of the three slices was used in the analyses.

Established and novel measures of sympathetic activation

A 12-lead ECG was obtained using a Mortara Eli-350 electrocardiograph (Mortara Instrument Inc., Best, Netherlands) after a resting period of at least ten minutes. Standard 10-second ECGs were stored in an 8-lead (I, II, II, V1-V6), 5000 sample comma-separated-value file. The Kors matrix was used to calculate vector cardiograms (VCG) from the eight independent ECG leads.[22] ECGs and VCGs were analyzed using the automatic MATLAB-based (The MathWorks, Natick, MA) program BEATS and the semi-automatic program LEADS.[23, 24]

BEATS was used to detect the timings of all QRS complexes and calculated R-R intervals (ms). All ECGs were checked for falsely identified QRS complexes or non-sinus beats, and the timings were manually adjusted. Also the complexes surrounding the non-sinus beat were removed from the mean R-R interval calculations. Mean heart rate (HR) in beats/min was calculated as 60 divided by the mean R-R interval in seconds. The coefficient of variation (CV) was expressed in percentage and calculated as the mean R-R interval divided by the standard deviation of the mean R-R interval x 100.

LEADS was used to calculate QT time (ms), QTc (corrected according to the Bazett formula) and Tpeak-end duration (ms). The QRS and T integral vectors were approximated

by calculating the numerical sum of X-Y-Z deflections (amplitudes of positive deflections are added and those of negative deflections subtracted). The spatial QRS-T angle was defined as the angle (°) between the integral QRS vector and the integral T vector. The spatial ventricular gradient (mV*ms) was calculated as the vectorial sum of these vectors.

Statistical analyses

In the NEO study, individuals with a BMI ≥ 27 kg/m^2 were oversampled. To correctly represent associations in the general population, adjustments for the oversampling of individuals with a BMI ≥ 27 kg/m^2 were made.[25] This was done by weighting individuals towards the BMI distribution of participants from the Leiderdorp municipality, whose BMI distribution was similar to the BMI distribution of the general Dutch population. All results were based on weighted analyses.

Baseline characteristics of the study population were stratified by categories of BMI.[26] Data were expressed as mean (SD), median (25th-75th percentile) or as percentage. The CV was ln-transformed. We performed linear regression analyses to examine the association between body fat and measures of sympathetic activation. As determinants we used BMI (kg/m^2), total body fat (%), WC (cm), VAT (cm^2) and SAT (cm^2). We standardized these values to be able to compare the strength of the association between determinants. Standardization was performed separately for men and women because of the large difference in distribution. In addition, we tested for interaction between measures of body fat and sex by adding an interaction term to all linear regression models. As outcome variables we used HR (beats/min), CV (%), QTc time (ms), Tpeak-end duration (ms), the spatial ventricular gradient (mV*ms) and the spatial QRST angle (°). We calculated the difference and 95% confidence interval per SD of the determinant. For ln-transformed outcome variables, we expressed the regression coefficient as percentage difference. The crude associations were adjusted for age, sex, tobacco smoking, ethnicity, education, physical activity and fasting state (yes/no). The analyses with VAT were additionally adjusted for SAT and vice versa.

RESULTS

Descriptive statistics

In total 6673 participants were included in the NEO study, 1205 with a MRI scan of abdomen and heart. We consecutively excluded participants with pre-existent CVD (n=104), prevalent diabetes (n=106), EF <50% (n=23), LVH (n=81), abnormal ECG (n=2) and missing data (n=21). The total population of the current study consisted of 868 participants. For analyses on heart rate and the coefficient of variation we additionally

excluded participants using beta blockers or calcium antagonists (n=98), resulting in 770 included participants. For the analyses including QTc, Tpeak-end, spatial ventricular gradient and spatial QRS-T angle we additionally excluded participants with ventricular conduction defects (n=73), resulting in 795 included participants. Because of the skewed distribution, the CV was ln-transformed.

Table 1: Baseline characteristics stratified by WHO categories of BMI (26)

	BMI category (kg/m^2)		
	< 25 (43%)	**25-30 (45%)**	**> 30 (12%)**
Sex (% men)	35	54	42
Age (years)	56 (6)	55 (6)	55 (6)
Ethnicity (% Caucasian)	98	96	95
Smoking			
Current (%)	9	14	13
Former (%)	40	44	52
Education (% low)	15	22	31
Total body fat (%)			
Men	19 (3)	26 (3)	33 (4)
Women	32 (4)	39 (4)	46 (3)
Waist circumference (cm)			
Men	88 (5)	99 (6)	113 (6)
Women	78 (7)	91 (7)	105 (8)
Mean VAT (cm^2)			
Men	73 (32)	120 (48)	176 (62)
Women	47 (26)	78 (31)	125 (49)
Mean SAT (cm^2)			
Men	150 (42)	209 (49)	327 (80)
Women	194 (54)	295 (54)	431 (94)
Heart rate (beats/min)	64 (10)	65 (10)	68 (10)
CV (%)	2.8 (1.7-4.5)	2.5 (1.6-4.0)	2.6 (1.6-4.1)
QTc (ms)	408 (27)	406 (26)	416 (26)
Tpeak-end (ms)	79 (14)	81 (13)	82 (15)
Ventricular gradient (mV*ms)	66 (24)	67 (25)	58 (20)
QRS-T spatial angle (°)	51 (26)	48 (25)	56 (30)

Data are presented as mean (SD), median (25th-75th percentile) or percentage. Results were based on weighted analyses (n=868)

BMI=body mass index; low education=no education, primary education or lower vocational training; VAT=visceral adipose tissue; SAT=subcutaneous adipose tissue; CV=coefficient of variation; QTc=corrected QT time

Baseline characteristics stratified by BMI categories (< 25 kg/m^2, 25-30 kg/m^2 (overweight), ≥ 30 kg/m^2 (obese)) are shown in Table 1. Participants with overweight were more often men. Obese individuals were on average less educated. Total body fat, waist circumference, visceral fat and subcutaneous fat were higher in de overweight and obese group than in the group with BMI < 25 kg/m^2.

Body fat, heart rate and coefficient of variation

The analyses on heart rate and the coefficient of variation included 770 participants. When stratified by BMI, heart rate was higher and the coefficient of variation in percentage was lower in the overweight and obese group than in the group with BMI < 25 kg/m^2 (Table 1). The interaction terms between body fat measures and sex were non-significant in all linear regression models (*p*-values ranging from 0.41 to 0.84). This suggests that although women have a higher amount of total body fat than men, the association between body fat and sympathetic activation is not essentially different between men and women. BMI,

Table 2: Difference (95% CI) in heart rate and coefficient of variation per SD of body fat

Fat measure (SD)	Model	Mean HR (beats/min)	R^2	P-value	CV (%)	R^2	P-value
BMI (m: 3 kg/m^2; w: 4 kg/m^2)	1	1.3 (0.4, 2.1)	0.01	0.004	−4 (−11, 2)	0.00	0.225
	2	1.4 (0.5, 2.2)	0.05	0.001	−5 (−12, 2)	0.04	0.162
	3	1.4 (0.6, 2.3)	0.07	0.001	−3 (−9, 3)	0.06	0.377
Total body fat (m: 5%; w: 6%)	1	1.9 (1.0, 2.9)	0.04	<0.001	−5 (−12, 2)	0.00	0.204
	2	1.9 (1.0, 2.8)	0.06	<0.001	−4 (−12, 3)	0.04	0.238
	3	1.9 (1.0, 2.9)	0.09	<0.001	−2 (−9, 4)	0.06	0.446
WC (m: 9 cm; w: 12 cm)	1	2.0 (1.1, 2.8)	0.04	<0.001	−6 (−13, 1)	0.01	0.095
	2	2.0 (1.1, 2.8)	0.06	<0.001	−6 (−13, 1)	0.04	0.087
	3	2.1 (1.3, 2.9)	0.09	<0.001	−4 (−11, 2)	0.06	0.149
VAT (m: 54 cm^2; w: 39 cm^2)	1	2.3 (1.4, 3.2)	0.05	<0.001	−8 (−16, −1)	0.01	0.034
	2	2.1 (1.3, 3.0)	0.07	<0.001	−7 (−15, 1)	0.04	0.081
	3	2.1 (1.3, 3.0)	0.09	<0.001	−4 (−12, 3)	0.06	0.216
	4	1.7 (0.6, 2.9)	0.10	0.004	−2 (−12, 7)	0.06	0.642
SAT (m: 71 cm^2; w: 98 cm^2)	1	1.4 (0.5, 2.3)	0.02	0.002	−6 (−13, 1)	0.01	0.095
	2	1.6 (0.8, 2.4)	0.05	<0.001	−7 (−14, 0)	0.04	0.048
	3	1.7 (0.8, 2.5)	0.08	<0.001	−5 (−11, 0)	0.06	0.060
	4	0.8 (−0.3, 1.9)	0.10	0.158	−4 (−12, 3)	0.06	0.248

Results were based on weighted analyses (n=770)
Model 1: Crude; 2: Adjusted for age and sex; 3: Adjusted for age, sex, smoking, ethnicity, education, physical activity and fasting state; 4: Model 3 + SAT (in VAT analyses) or Model 3 + VAT (in SAT analyses)
BMI=body mass index; WC=waist circumference; VAT=visceral adipose tissue; SAT=subcutaneous adipose tissue; HR=heart rate; CV=coefficient of variation

total body fat percentage and waist circumference were positively associated with mean heart rate (Table 2). After adjustment for potential confounders, the association was the strongest for waist circumference. Per SD waist circumference (men: 9 cm; women: 12 cm), the difference in heart rate was 2.1 beats/min (95% CI: 1.3, 2.9; $p<0.001$). There were no associations between body fat and the CV in the adjusted analyses.

As is shown in Table 2, VAT and SAT were both associated with heart rate after adjustments for confounders. After adjustment for SAT, VAT remained independently associated with heart rate: per SD VAT the change in heart rate was 1.7 beats/min (95% CI: 0.6, 2.9; $p=0.004$). However, the association between SAT and heart rate attenuated after adjustment for VAT to a difference of 0.8 beats/min (95% CI: −0.3, 1.9; $p=0.158$).

Body fat, QTc time and Tpeak-end

The analyses on QTc time and Tpeak-end included 795 participants. There was no significant interaction between body fat measures and sex in the linear regression analyses. Waist circumference was stronger associated with QTc than BMI or total body fat percentage. Per SD (men: 9 cm; women: 12 cm) increase in waist circumference, QTc increased with 4.5 ms (95% CI: 2.4, 6.6; $p<0.001$). No associations were observed with Tpeak-end (Table 3).

VAT was independently associated with QTc. After adjustment for all confounders and SAT, the change in QTc per SD VAT was 4.3 ms (95% CI: 1.8, 6.8; $p=0.001$). After adjusting for confounders and VAT, the difference in QTc per SD SAT was 1.0 ms (95% CI: −1.5, 3.4; $p=0.448$).

Body fat, spatial ventricular gradient magnitude and spatial QRS-T angle

Table 3 shows the results of the linear regression analyses between body fat and the spatial ventricular gradient and spatial QRS-T angle. There was no significant interaction between body fat measures and sex. BMI, total body fat and waist circumference were inversely associated with the ventricular gradient magnitude. Waist circumference was most strongly associated with the ventricular gradient magnitude with a difference of −3.4 mV*ms (95% CI: −5.6, −1.2; $p=0.003$) per SD waist circumference (9cm/12cm). After adjustment for potential confounders, BMI, total body fat and waist circumference were not associated with the spatial QRS-T angle.

After adjusting for confounders and SAT, the difference in the ventricular gradient per SD VAT was −4.4 mV*ms (95% CI: −7.0, −1.7; $p=0.001$). SAT was not associated with the ventricular gradient magnitude after adjustment for VAT. After adjustment for confounders and SAT, the difference in QRS-T angle per SD VAT was 3.2° (95% CI: 0.0, 6.3; $p=0.052$).

Table 3: Difference (95% CI) in QTc, Tpeak-end, ventricular gradient and QRS-T angle per SD of body fat

Fat measure (SD)	Model	QTc (ms)	R^2	P-value	Tpeak-end (ms)	R^2	P-value	Ventricular gradient magnitude (mV*ms)	R^2	P-value	QRS-T angle (°)	R^2	P-value
BMI (m: 3 kg/m^2; w: 4 kg/m^2)	1	3.0 (0.7, 5.2)	0.01	0.009	0.4 (−0.8, 1.6)	0.00	0.521	−3.5 (−5.5, −1.4)	0.02	0.001	0.8 (−1.3, 2.9)	0.00	0.453
	2	3.5 (1.4, 5.5)	0.12	0.001	0.5 (−0.8, 1.7)	0.04	0.460	−3.9 (−5.8, −1.9)	0.14	<0.001	0.9 (−1.1, 3.0)	0.04	0.369
	3	3.5 (1.3, 5.7)	0.15	0.002	0.7 (−0.4, 1.8)	0.08	0.197	−3.3 (−5.2, −1.4)	0.16	0.001	0.6 (−1.6, 2.9)	0.05	0.586
Total body fat (m: 5%; w: 6%)	1	4.1 (1.8, 6.5)	0.02	0.001	0.5 (−0.6, 1.6)	0.00	0.392	−3.7 (−6.2, −1.1)	0.02	0.005	−0.2 (−2.4, 1.9)	0.00	0.843
	2	4.1 (1.9, 6.3)	0.13	<0.001	0.4 (−0.7, 1.6)	0.05	0.462	−3.7 (−6.0, −1.4)	0.14	0.002	−0.3 (−2.6, 1.9)	0.04	0.757
	3	4.2 (1.9, 6.5)	0.16	<0.001	0.5 (−0.6, 1.7)	0.08	0.337	−3.1 (−5.4, −0.7)	0.15	0.011	−0.6 (−3.0, 1.8)	0.05	0.614
WC (m: 9 cm; w: 12 cm)	1	4.1 (1.9, 6.4)	0.02	<0.001	0.5 (−0.6, 1.6)	0.00	0.365	−3.7 (−6.1, −1.3)	0.02	0.002	0.0 (−2.2, 2.2)	0.00	0.997
	2	4.3 (2.2, 6.4)	0.13	<0.001	0.5 (−0.6, 1.6)	0.05	0.371	−3.8 (−6.1, −1.6)	0.14	0.001	0.0 (−2.2, 2.2)	0.04	0.983
	3	4.5 (2.4, 6.6)	0.16	<0.001	0.7 (−0.4, 1.7)	0.08	0.198	−3.4 (−5.6, −1.2)	0.16	0.003	−0.2 (−2.6, 2.1)	0.05	0.838
VAT (m: 55 cm^2; w: 40 cm^2)	1	5.4 (3.0, 7.7)	0.04	<0.001	0.9 (−0.3, 2.2)	0.00	0.135	−5.0 (−7.4, −2.7)	0.04	<0.001	1.3 (−0.8, 3.5)	0.00	0.230
	2	4.8 (2.7, 7.0)	0.14	<0.001	0.7 (−0.5, 1.9)	0.04	0.267	−4.7 (−7.1, −2.4)	0.15	<0.001	0.8 (−1.4, 3.0)	0.04	0.485
	3	4.7 (2.6, 6.9)	0.17	<0.001	0.8 (−0.3, 2.0)	0.08	0.159	−4.0 (−6.3, −1.8)	0.16	0.001	1.0 (−1.5, 3.5)	0.05	0.436
	4	4.3 (1.8, 6.8)	0.17	0.001	1.0 (−0.4, 2.3)	0.08	0.167	−4.4 (−7.0, −1.7)	0.16	0.001	3.2 (0.0, 6.3)	0.06	0.052
SAT (m: 72 cm^2; w: 100 cm^2)	1	2.4 (0.1, 4.8)	0.01	0.041	0.1 (−1.0, 1.2)	0.00	0.828	−2.1 (−4.4, 0.1)	0.01	0.060	−1.8 (−3.9, 0.3)	0.00	0.100
	2	3.2 (1.0, 5.3)	0.12	0.004	0.3 (−0.9, 1.4)	0.04	0.657	−2.7 (−4.9, −0.6)	0.13	0.013	−1.5 (−3.6, 0.6)	0.04	0.164
	3	3.3 (1.2, 5.4)	0.15	0.002	0.4 (−0.7, 1.5)	0.08	0.478	−2.2 (−4.3, 0.0)	0.15	0.046	−1.9 (−4.1, 0.3)	0.05	0.086
	4	1.0 (−1.5, 3.4)	0.17	0.448	−0.2 (−1.4, 1.0)	0.08	0.757	0.2 (−2.3, 2.7)	0.17	0.857	−3.5 (−6.2, −0.9)	0.06	0.009

Results were based on weighted analyses (n=795) Model 1: Crude; 2: Adjusted for age and sex; 3: Adjusted for age, sex, smoking, ethnicity, education, physical activity and fasting state; 4: Model 3 + SAT (in VAT analyses) or Model 3 + VAT (in SAT analyses)

BMI=body mass index; WC=waist circumference; VAT=visceral adipose tissue; SAT=subcutaneous adipose tissue; QTc=correct QT time

Per SD SAT the change in QRS-T angle was −3.5° (95% CI: −6.2, −0.9; *p*=0.009) after all adjustments (Table 3).

DISCUSSION

In the present study, we observed that body fat (BMI, total body fat percentage) and abdominal body fat (waist circumference, VAT, SAT) were associated with mean HR, QTc and the ventricular gradient magnitude. No associations were observed of body fat with the CV and Tpeak-end duration. VAT was positively associated with the spatial QRS-T angle, independent of SAT. There was an inverse association between SAT and the spatial QRS-T angle after adjustment for VAT. The associations between waist circumference and sympathetic activation were stronger than the associations between total body fat and sympathetic activation, suggesting that abdominal fat may be more important for autonomic function than overall fat. All associations were strongest for VAT and associations between SAT and sympathetic activation attenuated after adjustment for VAT. This indicates that different fat depots within the abdomen do not only differ in localization but also in function and that the visceral fat depot is most important with regard to sympathetic activation.

One strength of our study is that we excluded participants not only with pre-existent CVD but also with cardiac abnormalities diagnosed by MR imaging. Abnormalities in cardiovascular structure and function are strongly associated with autonomic function.[7-9] The exclusion of participants with abnormal hearts reduced the possibility of reverse causation because of undetected CVD in our results. Another strength of our study is the availability of visceral and subcutaneous fat measured by MRI, in addition to BMI, waist circumference and total body fat percentage. This enabled us to study the association of the different abdominal fat depots with sympathetic activation.

A limitation of our study is the absence of a direct measure of sympathetic activation. In large epidemiological studies as ours, it is often not feasible to invasively or pharmacologically measure sympathetic activity and we therefore used surrogate markers of sympathetic activation. However, heart rate and heart rate variability are well-established parameters of autonomic function.[15, 16] Heart rate is influenced by sympathetic and parasympathetic outflow to the sinus node, and dominant sympathetic activation over parasympathetic activation results in a higher heart rate and less variability in heart rate. In addition, we investigated four parameters of ventricular repolarization as novel markers of sympathetic activation. These parameters have not been validated as a markers of sympathetic activation. However, the influence of the sympathetic nervous system on

ventricular repolarization has long been recognized.[27] In addition, three recent studies in which the sympathetic nervous system was experimentally stimulated (one in pigs and two in humans) have indicated that our study parameters are strongly influenced by the sympathetic nervous system.[17-19] Because our study is an observational study, the indices of ventricular repolarization in our analyses may be influenced by factors other than sympathetic outflow, such as age, sex, physical activity and smoking. However, by adjusting for these factors, we aimed to minimize their influence on the associations between body fat and sympathetic activation. Another limitation is the use of a single ECG for the measurement of sympathetic activation, because previous studies have indicated that multiple ECGs give a more reliable estimate.[28] However, any random measurement error in the ECG measures would have diluted the true associations, leading to an underestimation in our results. A final limitation is the measurement of confounding variables by questionnaire (smoking, ethnicity, education and physical activity), which may have resulted in some misclassification. Because of this potential misclassification and the fact that this is an observational study, we cannot exclude the presence of residual confounding in our analyses.

The results of our study extend the knowledge from previous papers on body fat and sympathetic activation, by showing that abdominal fat, especially visceral fat, was stronger associated with sympathetic activation than fat mass in general. Previous studies observed an association between BMI and sympathetic activation.[1, 2, 29] However, BMI may not be a valid measure to describe body composition because it does not distinguish between body fat and lean body mass. Total body fat percentage and waist circumference reflect body fat and to some extent fat distribution. In earlier papers, total body fat has been associated with different measurements of the autonomic nervous system, as has waist circumference.[3-6, 30] In our study we measured BMI, total body fat percentage and waist circumference and because we used standardized values for these measures we were able to compare the strength of the associations with sympathetic activation.

Only a few studies were able to differentiate between visceral and subcutaneous fat depots. Three studies reported an association between VAT and sympathetic activation.[31-33] Two of these papers also investigated SAT, and reported that VAT was stronger associated with sympathetic activation than SAT.[32, 33] Another study reported that sympathetic activation in individuals with high subcutaneous fat was not different from non-obese individuals.[34] We were able to extend these observations by showing that VAT was also associated with measures of sympathetic activation on the electrocardiogram (heart rate, QTc and the ventricular gradient) after adjustment for SAT, while the association of SAT with these measures attenuated after adjustment for VAT. Finally, in

line with our results, a recent paper observed an association of BMI with QTc, but not with Tpeak-end.[35] We showed the same results for other body fat measures.

Our results suggest that subcutaneous fat is not associated with sympathetic activation, or that the association between SAT and sympathetic activation is mediated by VAT. Several mechanisms could underlie these associations. Adipocytes, or macrophages invading the adipose tissue, secrete a number of adipokines.[10, 36] Distribution of the adipose tissue is a key determinant of this adipokine secretion. The secretion rate of pro-inflammatory cytokines is higher in visceral fat than in subcutaneous fat. Furthermore, visceral fat inhibits the secretion of anti-inflammatory factors.[11-14] Some pro-inflammatory cytokines, such as leptin, can cross the blood-brain-barrier and directly stimulate the central sympathetic nervous system in the hypothalamus.[10, 37] Other cytokines, for example C-reactive protein, TNF-α, plasminogen activator inhitor-1 and interleukin-6, cause a state of low-grade inflammation that can also stimulate the sympathetic nervous system.[10, 13] Therefore, altered cytokine production may underlie the strong association between visceral fat and sympathetic activation. However, some studies have shown that leptin is more related to total or subcutaneous fat rather than to visceral fat.[32, 34] If this is true, direct stimulation of the central nervous system by leptin could not explain our finding that visceral fat is strongest associated with sympathetic activation.

Because our study is cross-sectional, we cannot make any statements on the direction of the association. Therefore, a pathophysiological mechanism in which sympathetic activation promotes the accumulation of adipose tissue in the visceral compartment, could also explain our findings. Previous studies have shown that chronic activation of the sympathetic nervous system, caused by chronic stress or lack of physical activity could favour the accumulation of visceral fat.[38, 39] This line of reasoning would also explain the inverse association we observed between SAT and the spatial QRS-T angle; less sympathetic activation, reflected in a smaller (more favourable) QRS-T angle, could stimulate the storage of fat in the subcutaneous instead of the visceral compartment.

The association between body fat and sympathetic activation may have clinical implications. A recent study in middle aged and elderly persons without manifest heart disease showed that each increment of 5 beats/min resulted in an 11% higher risk all-cause mortality (95% CI: 1-22; p=0.033) and a 12% higher rate of cardiovascular events (95% CI: 0-26; p=0.044) after 76 months of follow up.[40] In our study, approximately 50 cm^2 of visceral adipose tissue was associated with an increase in resting heart rate of 2.1 beats/min (95% CI: 1.3, 3.0; p<0.001). The explained variance was modest. However, because our population was free of CVD and diabetes, had structurally normal hearts and we adjusted for many factors that increase the risk of cardiovascular disease, our

results still suggest that body fat, especially visceral fat, may be important in the risk of cardiovascular events and mortality.

In conclusion, we observed that body fat, especially abdominal fat, is associated with sympathetic activation. Furthermore, visceral fat seems more important than subcutaneous fat with regard to sympathetic activation. As participants in our study had structurally normal hearts, our results imply that already before the onset of CVD excess (abdominal) body fat is associated with sympathetic activation. Future prospective studies should investigate whether sympathetic activation is a mediator in the association between body fat and CVD.

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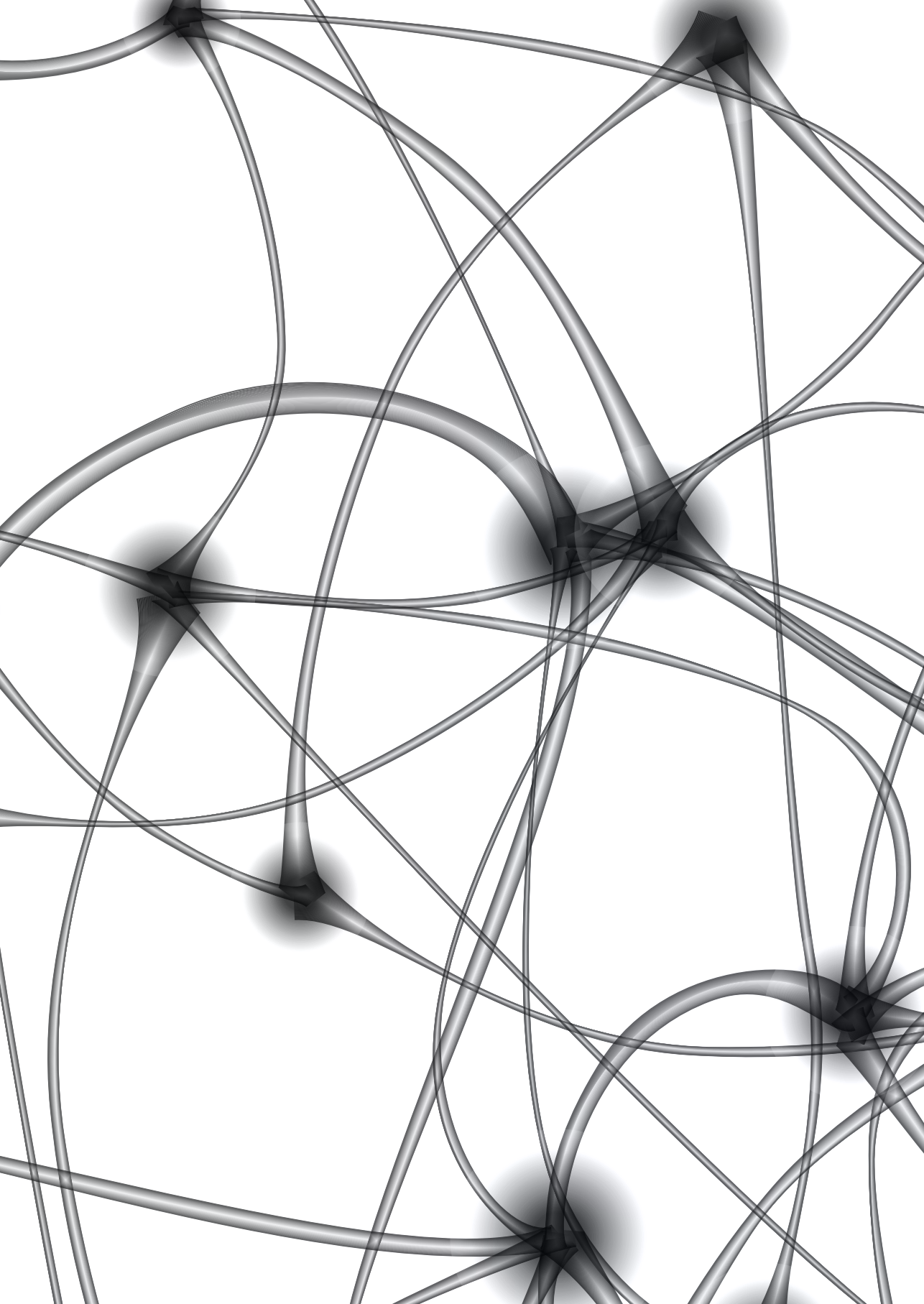
Supplementary table: Correlation matrix between measures of body fat and electrocardiographic measures of sympathetic activation

	Mean HR (beats/min)	P-value	CV (%)	P-value	QTc (ms)	P-value	Tpeak-end (ms)	P-value	Ventricular gradient magnitude (mV*ms)	P-value	QRS-T angle (°)	P-value
BMI (kg/m^2)	0.11	0.002	−0.05	0.120	0.08	0.024	0.05	0.196	−0.10	0.006	0.04	0.211
Total body fat (%)	0.19	<0.001	0.03	0.349	0.28	<0.001	−0.11	0.002	−0.31	<0.001	−0.13	<0.001
WC (cm)	0.13	<0.001	−0.07	0.068	0.03	0.401	0.11	0.002	0.01	0.867	0.07	0.055
VAT (cm^2)	0.17	<0.001	−0.06	0.095	0.09	0.017	0.15	<0.001	−0.07	0.045	0.11	0.002
SAT (cm^2)	0.15	<0.001	−0.03	0.463	0.16	<0.001	−0.05	0.130	−0.16	<0.001	−0.12	0.001

Results were based on weighted analyses

BMI=body mass index; WC=waist circumference; VAT=visceral adipose tissue; SAT=subcutaneous adipose tissue; HR=heart rate; CV=coefficient of variation; QTc=correct QT time



3

Autonomic function is not associated with the incidence of type 2 diabetes mellitus in a population at high risk of diabetes: the Hoorn study

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ABSTRACT

Aim

Impaired autonomic function is a complication of type 2 diabetes mellitus (DM2), but may also be involved in its development. For this reason, this study looked at the association of autonomic function with the incidence of DM2 in a homogeneous Caucasian population.

Methods

The Hoorn study is a prospective population-based study of individuals aged 50–75 years. For the 631 participants, the standard deviation of all normal-to-normal intervals (SDNN) and eight other parameters of autonomic function were calculated at baseline. Fasting and 2-h glucose were measured during follow-up by oral glucose tolerance test (OGTT). DM2 at baseline and follow-up was ascertained by questionnaire and OGTT. After excluding participants with DM2 at baseline, the association of parameters of autonomic function with incident diabetes was examined using logistic-regression analysis while adjusting for possible confounders.

Results

After excluding those with known ($n = 67$) or newly diagnosed ($n = 126$) DM2 at baseline and those missing follow-up data ($n = 140$), 298 participants were eligible for the study (182 with normal glucose tolerance, 19 with impaired fasting glucose and 97 with impaired glucose tolerance). During a median follow-up of 9.2 (range 4.5–11.1) years, 94 incident cases of DM2 were observed. After adjusting for confounding variables, the DM2 odds ratio was 1.12 (95% CI: 0.77, 1.64) per SDNN increase. Results for other parameters of autonomic function were similar.

Conclusion

The present study found no evidence for an association of autonomic function with the incidence of DM2 in a population at high risk of diabetes. This implies that previously observed associations between autonomic function and glucose metabolism in cross-sectional settings may reflect reverse causation.

INTRODUCTION

Type 2 diabetes mellitus (DM2) is an increasing public health problem[1] and autonomic dysfunction is one of its complications.[2] The autonomic nervous system is an involuntary nervous system with a sympathetic and a parasympathetic branch. Its purpose is to control homeostasis and regulate visceral functions. Autonomic dysfunction is characterized by less adaptive changes and relative sympathetic overdrive and is associated with an increased risk of morbidity and mortality.[3, 4] Previous cross-sectional studies showed that impaired autonomic function is associated with increased glucose levels and reduced glucose tolerance in various populations.[5-9]

Although altered autonomic function is considered a consequence of DM2, there are also indications that it may be involved in its development. First, autonomic function has been associated with glucose tolerance in normoglycemic individuals.[10] Second, several organs involved in the glucose metabolism, such as the liver, pancreas, adrenal and skeletal muscles are autonomically innervated. The parasympathetic autonomic nervous system is responsible for the release of insulin from the pancreas and the insulin sensitivity of several organs. The decreased parasympathetic modulation in autonomic dysfunction may therefore play a role in the development of insulin resistance.[11] Third, non-diabetic offspring of DM2 patients, who are at high risk of DM2, have a worse autonomic function than individuals of the same age without a family history of DM2, independent of other risk factors.[12] These findings suggest that autonomic dysfunction may not only be a complication of DM2, but may also play a role in its development. Thus, the aim of the present study was to prospectively investigate the association between autonomic function and the incidence of DM2 in a middle-aged population.

METHODS

Study design and participants

The Hoorn study is a population-based cohort study of glucose tolerance and cardiovascular risk factors in a Caucasian population aged 50 to 75 years. Baseline data were collected from 1989 to 1991. Two follow-up visits were performed to record incident diagnoses of DM2: the first was between 1996 and 1998, and the second was between 2000 and 2001. Details on the Hoorn study have been described elsewhere.[13] In brief, a random sample of men and women aged 50 to 75 years was selected from the municipal registry of the town of Hoorn, the Netherlands. At baseline, 2484 persons participated in a study visit that included a 75-g oral glucose tolerance test (OGTT). Within 3 to 5 weeks of the initial baseline visit, a subset of 631 individuals was invited for additional

baseline measurements. Selection of this subset was stratified by the 2-h glucose values of the first OGTT as well as age and gender. Because of the stratification by 2-h glucose values, the subset included 259 people with normal glucose tolerance (NGT), 28 with impaired fasting glucose (IFG), 151 with impaired glucose tolerance (IGT) and 193 with DM2. The additional baseline visit included measurements of autonomic function. The present study included all participants with available data for at least one parameter of autonomic function. Excluded were those with DM2 at baseline and with missing follow-up data. The Hoorn study had the approval of the ethics committee of the VU University Medical Centre, and all study participants gave their informed consent.

Data collection

During the first baseline visit to the Hoorn study centre, extensive information on demographic characteristics, smoking behaviour, medical history and use of medication was obtained by questionnaire. Physical activity was measured by a sum score of nine equally weighted yes/no questions about the regular performance of the following: sports, bicycling, gardening, walking, doing odd jobs, climbing stairs, household activities, daily food shopping and working.[14] During the physical examination, weight and height were measured with the participants barefoot and wearing light clothing. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m^2). Blood pressure was assessed twice on the right arm while sitting with a random-zero sphygmomano-meter (Hawksley, Lancing, Sussex, UK), and the mean of the two measurements was used for the analyses. Hypertension was defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg.

During both the first and additional baseline visit, blood samples were taken after overnight fasting, and a 75-g OGTT administered to those with no previously diagnosed diabetes. Fasting and 2-h glucose concentrations were measured in plasma (mmol/L) using the glucose dehydrogenase method (Merck, Darmstadt, Germany). Baseline glucose concentrations were defined as the mean of the first and additional baseline measurements. At baseline, participants were classified into categories of glucose tolerance according to World Health Organization (WHO) criteria.[15] NGT was defined as fasting plasma glucose (FPG) < 6.1 mmol/L and 2-h plasma glucose (2-hPG) < 7.8 mmol/L. IFG was defined as FPG 6.1–6.9 mmol/Land 2-hPG < 7.8 mmol/L; IGT with or without IFG was defined as FPG < 7.0 mmol/L and 2-hPG 7.8–11.1 mmol/L; and DM2 was defined as FPG ≥ 7.0 mmol/L or 2-hPG ≥ 11.1 mmol/L.

Fasting serum insulin levels were quantified by insulin-specific double-antibody radioimmunoassay (antibody SP21, EMD Millipore, Billerica, MA, USA).

Autonomic function tests

Participants were asked to refrain from smoking and drinking coffee for 2 hours prior to the additional baseline visit. Tests were performed at a temperature of 19–22°C between 08.30 and 16.00 at least 1 hour after a light meal and with participants in supine position. The tests were preceded by a rest period of at least 10 minutes. During the tests, heart rate and blood pressure were continuously recorded on a PC-based data-acquisition system. RR intervals were obtained by a bipolar electrocardiography (ECG) chest lead and a QRS detector device with an accuracy of 1 ms. Blood pressure was recorded continuously using the Finapres (finger arterial blood pressure) method (model BP2000, GE Datex-Ohmeda, Madison, WI, USA), digitally sampled at 200 Hz, and offline low-pass-filtered and down-sampled to 100 Hz. Systolic blood pressure values were obtained by an automated procedure verified by visual inspection.

Cardiac cycle duration (RR interval) and continuous finger arterial pressure were measured under three conditions: (1) spontaneous breathing for 3 min; (2) six deep breaths over 1 min; and (3) active change in position from supine to standing. The breathing frequency of six breaths/min was dictated by the investigator. When offline spectral analysis showed that the participants failed to breathe at the appropriate frequency, the recording was discarded. After each test, a rest period of 1 min was included to prevent any effects from previous tests.

During spontaneous breathing, the mean of the normal-to-normal RR intervals (NN intervals) and standard deviation of all NN intervals (SDNN) were calculated. Furthermore, spectral analysis was used to assess the power of the low-frequency (LF; 0.04–0.12 Hz) and high-frequency (HF; 0.12–0.40 Hz) bands. Impaired autonomic function leads to lower values of these four measurements. From the deep-breathing recordings, it was possible to measure the difference in maximum and minimum RR intervals during expiration and inspiration as averaged over six breaths (EI difference). Baroreflex sensitivity (BRS) was also calculated from the deep-breathing recordings, defined as the change in RR intervals caused by changes in systolic blood pressure (ms/mmHg) and estimated as the gain in transfer function between blood pressure and RR-interval changes. Only spectral components between 0.05 and 0.15 Hz were used, together with a squared coherence (χ^2) of 0.5 or higher. Lower EI difference and BRS sensitivity are a sign of impaired autonomic function. During the active change in position from supine to standing, the difference between the mean RR interval during 1 min of rest prior to standing and the minimum RR interval within 15 s of standing (RRmax) was calculated. In addition, the maximum RR interval 15–30 s after standing divided by the minimum RR interval at around 15 s after standing (RRmax/min), and the systolic blood pressure difference (SBP difference) after standing, defined as the mean SBP over 30 s

within 90–120 s of standing minus the mean over 30 s prior to standing, were calculated. Impaired autonomic function is reflected by lower RRmax and RRmax/min, and a larger (more negative) SBP difference. In some cases, data were missing because the test schedule was not completed, the quality of data was inadequate for processing or non-sinus beats constituted > 10% of the total number of recorded beats.

In addition to individual parameters of autonomic function, a summary score of autonomic function was constructed, as described elsewhere.[16] The results for each parameter of autonomic function were divided into quartiles. Each participant was assigned 0 points if the result was in the most abnormal quartile, 1 point if in the second quartile, 2 points if in the third quartile, and 3 points if in the best quartile of autonomic function. For all parameters except SBP difference, participants in the highest quartile had the best autonomic function. If all nine parameters were available, the scores for each were added together to construct a summary score. If one or two results were missing (41/298), these were replaced by the median value for that score. If three or more results were missing, the summary score was not calculated (59/298). Summary scores ranged from 0 (very poor) to 27 (very good).

Ascertainment of type 2 diabetes during follow-up

At the follow-up visits during 1996–1998 and 2000–2001, examinations including OGTTs were performed. New diagnoses of DM2 were ascertained during a follow-up visit using three methods: (1) by asking 'Have you been diagnosed with DM2 since the last study visit?' and 'Which doctor monitors your DM2?'; (2) by recording the use of glucose-lowering medication; and (3) by OGTTs in participants with no known diabetes. DM2 was defined as FPG ≥ 7.0 mmol/L or 2-hPG ≥ 11.1 mmol/L. At the follow-up visits, both fasting and 2-h glucose concentrations were measured by the hexokinase method (Boehringer Ingelheim Pharma, Ingelheim, Germany) at follow-up.

Statistical analyses

Descriptive statistics were calculated as mean ± standard deviation (SD) or percentage for the total population and stratified by tertiles of SDNN. Also, differences in baseline characteristics between tertiles of autonomic function were tested by analysis of variance (ANOVA) for continuous variables and by chi-square test for categorical variables. As most cases of diabetes were detected at follow-up study visits and the exact dates of onset were unknown, it was not possible to use time-to-event analyses, so logistic regression was performed instead to study the association between the nine parameters of autonomic function at baseline and the incidence of DM2 at follow-up. The nine parameters were standardized to a mean of zero with 1 SD, and crude odds ratios (ORs) were calculated for these standardized parameters with 95% confidence intervals (CI),

while ORs were also adjusted for age (continuous), gender, BMI (continuous), hypertension (yes/no), prevalent cardiovascular disease (CVD; yes/no), cardiac medication (yes/no), smoking (no smoking (reference), current smoking or former smoking), physical activity score (continuous), follow-up duration (continuous) and family history of DM (yes/no). In addition, baseline fasting glucose and insulin concentrations and 2-h glucose concentrations (continuous) were added to the model to correct for the effects of glucose and insulin concentrations on autonomic function. These analyses were also performed in a subgroup of individuals with NGT at baseline. For these participants, a composite endpoint consisting of the development of IFG, IGT or DM2 was calculated, using data from the last available follow-up. The same logistic regression analyses were performed with this composite endpoint as outcome.

Also, linear regression analysis was performed to examine the association of autonomic function with continuous fasting and 2-h glucose concentrations at the follow-up visits, using glucose concentrations from the last available follow-up visit. Statistical analyses were performed with Stata statistical soft-ware, version 12 (StataCorp LP, College Station, TX, USA).

RESULTS

Baseline characteristics

A total of 631 participants had measurements of autonomic function at baseline. Excluded were those with known (n = 67) or newly diagnosed (n = 126) DM2 at baseline. Of the 438 remaining participants, 140 had missing follow-up data. Also, 51 participants died, 30 moved to another region and, for 59 participants, the reason for not attending the follow-up visit was unknown. Those with missing follow-up data were somewhat older (SD) at 66 (7) years compared with 63 (7) years, had higher 2-h glucose levels at 7.1 (2) mmol/L compared with 6.6 (2.0) mmol/L and more often used cardiac medication, with 27% using compared with 19% not. All parameters of autonomic function were slightly lower in those lost to follow-up: their mean (SD) NN interval was 929 (133) ms compared with 974 (152) ms, while BRS was 8 (4) ms/mmHg compared with 9 (6) ms/mmHg.

The present study ultimately included 298 participants: 182 with NGT; 19 with IFG; and 97 with IGT. Their mean age (SD) was 63 (7) years, 51% were male and their mean (SD) BMI was 26 (3) kg/m^2. Baseline characteristics for the entire study population (n = 298) by tertiles of SDNN (n = 272) are shown in Table1.

Table 1: Baseline characteristics for the total study population (n=298) and stratified by tertiles of SDNN (n=272)

	Total	SDNN (ms) in tertiles		
		11-29 ms	30-42 ms	43-118 ms
N	298	91	91	90
Sex (% men)*	51	48	42	62
Age (years)*	63 (7)	64 (7)	63 (7)	61 (6)
BMI (kg/m^2)	26 (3)	27 (3)	26 (3)	26 (3)
Smoking				
- Current (%)	26	23	24	28
- Former (%)	35	32	37	34
Fasting glucose (mmol/L)	5.5 (0.6)	5.6 (0.6)	5.5 (0.5)	5.5 (0.5)
2-hour glucose (mmol/L)	6.7 (2.0)	7.0 (2.0)	6.5 (1.9)	6.5 (2.0)
Activity score (scale 0-9)	6 (2)	5 (2)	6 (1)	6 (2)
Hypertension (%)*	30	42	24	20
Prevalent CVD (%)*	13	18	8	9
Use of cardiac medication (%)*	19	30	13	11
Family history of DM (%)	26	24	25	27
IFG or IGT at baseline (%)	39	42	40	33

Data are presented as mean (SD) or percentage
*p-value<0.05

Those in the highest tertile of SDNN were younger, more often male, less often hypertensive and less often used cardiac medication than those in the lowest SDNN tertile. The prevalence of CVD was highest in the lowest tertile of SDNN. All participants had at least one parameter of autonomic function available, the values of which are presented in Table 2. The first follow-up visit included 286 participants and, of these, 230 attended the second follow-up visit whereas nine participants only attended the second follow-up visit. For one participant the exact date of the follow-up visit was unknown. The median follow-up duration was 9.2 (range 4.5–11.1) years.

Autonomic function and incidence of DM2

Of the 298 participants included in the analyses, 94 developed DM2 at one of the follow-up visits: 24 from the NGT group; 10 from the IFG group; and 60 from the IGT group. At the first follow-up visit (1996–1998), there were 67 cases of DM2 and, at the second follow-up visit (2000–2001), 27 cases of DM2. The ORs (95% CI) for DM2 associated with parameters of autonomic function are shown in Table 3. After adjusting for confounding factors and glucose and insulin levels, ORs ranged from 0.88 (0.59, 1.31) to 1.36 (0.93, 1.98) and were all non-significant. In addition to individual parameters of autonomic

Table 2: Parameters of autonomic function

Parameter of autonomic function	N	Mean (SD)
Mean NN (ms)	272	974 (152)
SDNN (ms)	272	38 (17)
LF power (ms^2)	272	264 (121-547)*
HF power (ms^2)	272	206 (94-451)*
EI-difference (ms)	278	193 (108)
BRS (ms/mmHg)	260	9.4 (5.6)
RR max (ms)	279	260 (98)
RR max/min (ms)	279	1.3 (0.2)
SBP difference (mmHg)	256	–5.5 (14.8)
Summary score	234	14 (6)

*Median (25^{th}-75^{th} percentile)

Table 3: Odds ratio and 95% CI for the incidence of type 2 diabetes per SD of parameters of autonomic function

	N	Events	SD	Model 1	Model 2	Model 3	Model 4
Mean NN (ms)	272	85	152	0.87 (0.67, 1.13)	0.87 (0.67, 1.14)	0.86 (0.65, 1.14)	1.01 (0.69, 1.47)
SDNN (ms)	272	85	17	0.89 (0.68, 1.16)	0.93 (0.71, 1.22)	0.92 (0.69, 1.23)	1.12 (0.77, 1.64)
Ln LF power (ms^2)	272	85	1	1.00 (0.78, 1.30)	1.08 (0.82, 1.41)	1.09 (0.81, 1.47)	1.21 (0.82, 1.77)
Ln HF power (ms^2)	272	85	1	0.95 (0.74, 1.23)	0.99 (0.76, 1.29)	0.93 (0.71, 1.23)	1.17 (0.81, 1.68)
EI-difference (ms)	278	88	108	1.06 (0.82, 1.36)	1.13 (0.87, 1.47)	1.13 (0.85, 1.51)	1.36 (0.93, 1.98)
BRS (ms/mmHg)	260	82	5.6	0.98 (0.75, 1.28)	1.00 (0.76, 1.31)	1.03 (0.77, 1.36)	1.23 (0.83, 1.83)
RR max (ms)	279	90	98	0.79 (0.60, 1.04)	0.82 (0.62, 1.08)	0.82 (0.60, 1.09)	0.92 (0.62, 1.35)
RR max/min (ms)	279	90	0.2	1.03 (0.80, 1.32)	1.11 (0.84, 1.46)	1.17 (0.88, 1.57)	1.35 (0.94, 1.95)
SBP difference (mmHg)	256	82	14.8	0.91 (0.70, 1.18)	0.92 (0.70, 1.20)	0.90 (0.67, 1.20)	0.88 (0.59, 1.31)
Summary score	262	84	6	0.95 (0.73, 1.23)	0.99 (0.75, 1.31)	1.04 (0.78, 1.39)	1.36 (0.92, 2.01)

Model 1: crude
Model 2: adjusted for age and sex
Model 3: adjusted for age, sex, BMI, hypertension, prevalent CVD, cardiac medication, smoking, physical activity, follow-up duration and family history of DM
Model 4: adjusted for age, sex, BMI, hypertension, prevalent CVD, cardiac medication, smoking, physical activity, follow-up duration, family history of DM, and baseline glucose and insulin concentrations

function, a summary score of autonomic function was calculated. For every SD of the summary score, the adjusted OR for DM2 was 1.36 (0.92, 2.01).

Autonomic function and fasting and 2-h glucose concentrations at follow-up

The linear regression analyses used glucose concentrations from the last available follow-up visit (104 from the first follow-up visit and 194 from the second). The Figure shows the crude associations of SDNN (ms) at baseline with fasting (A) and 2-h (B)

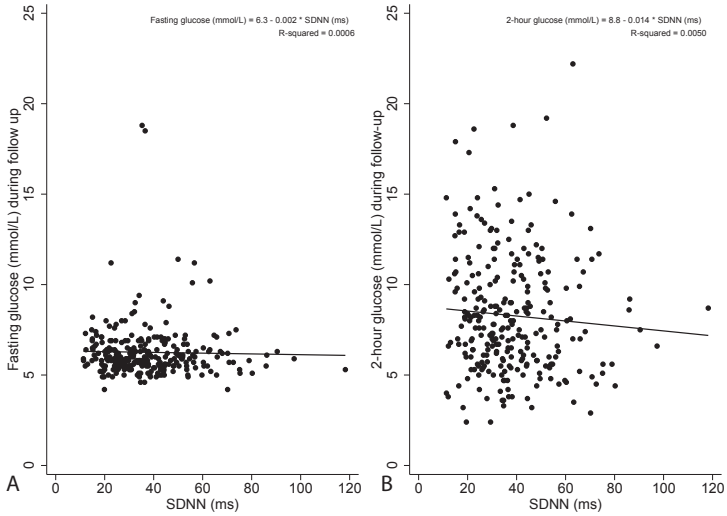

Figure. Scatter plots of the standard deviation of all normal-to-normal intervals (SDNN; ms) at baseline, and fasting (A) and 2-h (B) glucose concentrations (mmol/L) at the last available follow-up study visits.

plasma glucose concentrations (mmol/L) at the time of the last available follow-up. The associations between parameters of autonomic function and glucose concentrations were small, and only the relationship between the EI difference and fasting glucose was statistically significant after adjustment (mean increase was 0.20 mmol/L (95% CI: 0.03, 0.36) per SD increase in EI difference). All other associations were not significant after adjusting for age, gender, BMI, hypertension, prevalent CVD, cardiac medication, smoking, physical activity, follow-up duration, and baseline glucose and insulin concentrations. The summary score of the autonomic function tests also showed no relationship. For every SD of the summary score, the adjusted difference in fasting glucose concentration at follow-up was 0.09 (−0.09, 0.27) mmol/L, with a 2-h glucose concentration of 0.09 (−0.25, 0.43) mmol/L (Table 4).

Autonomic function and incidence of DM2 in participants with normal glucose tolerance at baseline

In the subgroup of 182 participants with NGT at baseline, 24 developed DM2. Sixteen cases were detected at the first follow-up visit and eight at the second follow-up (Table 5). ORs were close to 1 and comparable to those for the total study population. In addition, there was no association between the summary score of autonomic function and DM2 incidence in participants with NGT at baseline (adjusted OR: 1.13, 95% CI: 0.60, 2.15). For participants with IFG or IGT at baseline, the OR for DM2 per SD of the summary score was 1.43 (0.72, 2.85), indicating that reverse causation may have been involved. Of the 182 participants who had NGT at baseline, 72 reached the composite

Table 4: Associations between parameters of autonomic function with fasting and 2-hour glucose concentrations at follow-up

		Difference in fasting glucose (mmol/L) (95% CI)[1]		Difference in 2-hour glucose (mmol/L) (95% CI)[1]	
	SD	Crude	Adjusted[2]	Crude	Adjusted[2]
Mean NN (ms)	153	−0.08 (−0.26, 0.10)	0.03 (−0.14, 0.20)	−0.11 (−0.53, 0.28)	0.18 (−0.15, 0.50)
SDNN (ms)	17	−0.04 (−0.22, 0.14)	0.08 (−0.08, 0.25)	−0.23 (−0.63, 0.16)	0.12 (−0.20, 0.45)
Ln LF power (ms^2)	1	−0.01 (−0.19, 0.17)	0.08 (−0.10, 0.25)	−0.24 (−0.64, 0.15)	−0.06 (−0.39, 0.27)
Ln HF power (ms^2)	1	0.10 (−0.08, 0.28)	0.14 (−0.02, 0.31)	−0.14 (−0.54, 0.26)	0.18 (−0.14, 0.50)
EI-difference (ms)	108	0.19 (0.01, 0.36)	0.20 (0.03, 0.36)	−0.29 (−0.68, 0.11)	0.00 (−0.33, 0.33)
BRS (ms/mmHg)	6	0.09 (−0.09, 0.28)	0.14 (−0.04, 0.31)	−0.28 (−0.69, 0.14)	0.06 (−0.27, 0.40)
RR max (ms)	98	−0.19 (−0.37,−0.02)	−0.04 (−0.20, 0.12)	−0.32 (−0.71, 0.07)	0.00 (−0.32, 0.31)
RR max/min (ms)	0.2	−0.10 (−0.27, 0.08)	0.00 (−0.17, 0.17)	−0.19 (−0.57, 0.21)	0.13 (−0.19, 0.46)
SBP difference (mmHg)	15	0.02 (−0.16, 0.21)	0.01 (−0.16, 0.18)	−0.23 (−0.64, 0.20)	−0.04 (−0.37, 0.29)
Summary score	6	−0.08 (−0.27, 0.10)	0.09 (−0.09, 0.27)	−0.25 (−0.66, 0.16)	0.09 (−0.25, 0.43)

[1] Difference (95% CI) in glucose concentrations per SD increase of parameters of autonomic function
[2] Adjusted for age, sex, BMI, hypertension, prevalent CVD, cardiac medication, smoking, physical activity, follow-up duration, family history of DM, and baseline glucose and insulin concentrations

Table 5: Odds ratios and 95% CI for the incidence of type 2 diabetes per SD of parameters of autonomic function in the individuals with normal glucose tolerance at baseline

	N	Events	SD	Model 1	Model 2	Model 3	Model 4
Mean NN (ms)	168	23	154	0.95 (0.61, 1.48)	0.95 (0.60, 1.48)	0.78 (0.46, 1.33)	0.79 (0.39, 1.58)
SDNN (ms)	168	23	18	1.01 (0.64, 1.57)	1.02 (0.65, 1.61)	0.98 (0.59, 1.61)	0.99 (0.55, 1.78)
Ln LF power (ms^2)	168	23	1	1.16 (0.75, 1.80)	1.19 (0.75, 1.90)	1.26 (0.75, 2.11)	1.39 (0.75, 2.58)
Ln HF power (ms^2)	168	23	1	0.91 (0.58, 1.42)	0.91 (0.58, 1.44)	0.84 (0.52, 1.36)	0.87 (0.48, 1.59)
EI-difference (ms)	173	24	108	0.95 (0.61, 1.48)	0.95 (0.59, 1.50)	1.01 (0.60, 1.70)	1.16 (0.65, 2.08)
BRS (ms/mmHg)	159	22	5	0.91 (0.56, 1.46)	0.87 (0.53, 1.42)	0.81 (0.46, 1.43)	0.89 (0.43, 1.84)
RR max (ms)	172	24	99	0.77 (0.46, 1.27)	0.76 (0.45, 1.27)	0.69 (0.39, 1.22)	0.74 (0.38, 1.45)
RR max/min (ms)	172	24	0.2	1.04 (0.69, 1.59)	1.05 (0.67, 1.65)	1.05 (0.64, 1.73)	1.08 (0.63, 1.84)
SBP difference (mmHg)	156	21	15	1.29 (0.82, 2.05)	1.29 (0.81, 2.06)	1.11 (0.62, 1.98)	0.89 (0.44, 1.79)
Summary score	162	23	6	0.99 (0.64, 1.55)	1.00 (0.63, 1.59)	1.03 (0.61, 1.73)	1.13 (0.60, 2.15)

Model 1: crude
Model 2: adjusted for age and sex
Model 3: adjusted for age, sex, BMI, hypertension, prevalent CVD, cardiac medication, smoking, physical activity, follow-up duration and family history of DM
Model 4: adjusted for age, sex, BMI, hypertension, prevalent CVD, cardiac medication, smoking, physical activity, follow-up duration, family history of DM, and baseline glucose and insulin concentrations

endpoint of IFG/IGT/DM2 (24 cases at the first follow-up and 48 at the second). All ORs were non-significant (Table 6). Linearregression analyses with parameters of autonomic

function as determinants, and fasting and 2-h glucose at the last available follow-up visit as outcomes, also showed no significant associations (data not shown).

Table 6: Odds ratios and 95% CI for the incidence of IFG, IGT or DM2 per SD of parameters of autonomic function in individuals with normal glucose tolerance at baseline

	N	Events	SD	Model 1	Model 2	Model 3	Model 4
Mean NN (ms)	168	66	154	0.98 (0.72, 1.34)	0.98 (0.72, 1.34)	1.08 (0.77, 1.51)	1.16 (0.80, 1.69)
SDNN (ms)	168	66	18	0.92 (0.67, 1.27)	0.91 (0.66, 1.26)	0.90 (0.63, 1.28)	0.90 (0.62, 1.31)
Ln LF power (ms^2)	168	66	1	0.79 (0.58, 1.09)	0.76 (0.54, 1.07)	0.75 (0.52, 1.07)	0.71 (0.48, 1.05)
Ln HF power (ms^2)	168	66	1	1.01 (0.74, 1.38)	1.01 (0.73, 1.40)	0.98 (0.69, 1.38)	1.04 (0.72, 1.50)
EI-difference (ms)	173	71	108	0.91 (0.67, 1.24)	0.91 (0.66, 1.26)	0.80 (0.56, 1.15)	0.78 (0.53, 1.14)
BRS (ms/mmHg)	159	68	5	0.93 (0.68, 1.29)	0.96 (0.69, 1.33)	0.88 (0.61, 1.26)	0.94 (0.63, 1.38)
RR max (ms)	172	69	100	0.87 (0.63, 1.20)	0.86 (0.62, 1.20)	0.93 (0.67, 1.31)	1.01 (0.71, 1.44)
RR max/min (ms)	172	69	0.2	0.84 (0.61, 1.16)	0.83 (0.59, 1.17)	0.84 (0.57, 1.23)	0.86 (0.59, 1.27)
SBP difference (mmHg)	156	63	15	1.02 (0.74, 1.40)	1.01 (0.73, 1.39)	0.97 (0.66, 1.43)	0.92 (0.61, 1.39)
Summary score	162	63	6	0.75 (0.54, 1.03)	0.72 (0.51, 1.02)	0.72 (0.50, 1.04)	0.75 (0.50, 1.12)

Model 1: crude
Model 2: adjusted for age and sex
Model 3: adjusted for age, sex, BMI, hypertension, prevalent CVD, cardiac medication, smoking, physical activity, follow-up duration and family history of DM
Model 4: adjusted for age, sex, BMI, hypertension, prevalent CVD, cardiac medication, smoking, physical activity, follow-up duration, family history of DM, and baseline glucose and insulin concentrations

DISCUSSION

The present study aimed to examine the association between autonomic function and the incidence of DM2 in a homogeneous Caucasian population aged 50–75 years. Non-significant associations were observed between nine parameters of autonomic function (mean NN interval, SDNN, LF power, HF power, EI difference, BRS, RRmax, RRmax/min and SBP difference) and DM2 after a median follow-up duration of 9.2 (range: 4.5–11.1) years. In addition, a summary score of all nine functions was not associated with incident DM2. The associations between parameters of autonomic function and fasting and 2-h glucose concentrations at the last available follow-up visit were small and non-significant, with the exception of an unexpected positive association between EI difference and fasting glucose during follow-up. To exclude the influence of higher glucose concentrations on autonomic function, two analyses were performed in individuals with NGT at baseline, one with DM2 and another with a composite endpoint including IFG, IGT and DM2 as outcomes. There were no significant associations between any of these parameters of autonomic function and DM2 incidence.

An important strength of the present study was its prospective design. Cross-sectional studies fail to provide information on causal relationships between autonomic function and glucose tolerance, particularly because glucose and insulin concentrations can also influence autonomic function.[17-20] The present prospective study was able to investigate the temporal relationship between autonomic function and the development of DM2. By adjusting the analyses for baseline glucose and insulin concentrations and performing the analyses in individuals with NGT at baseline, it was possible to correct for the possible effect of baseline glucose and insulin levels on autonomic function (reverse causation).

Another strength was the use of OGTTs for the diagnosis of DM2 at follow-up, as this test is more reliable than self-reporting for diagnosis of DM2 and also identifies diabetics who have not yet been diagnosed by their physicians.[21] Furthermore, we used an extensive set of parameters of autonomic function that can be divided in three categories. Four parameters (EI-difference, RRmax, RRmax/min and SBP difference) are part of the Ewing test battery.[22] These tests evaluate cardiovascular autonomic reflexes and are indicative of sympathetic (EI-difference and RRmax) or parasympathetic (EI-difference, RRmax, RRmax/min and SBP difference) integrity. The value of Ewing tests has been extensively evaluated and the tests are used in clinical practice for the assessment of (diabetic) neuropathy.[23] The second category of autonomic function tests are the heart rate (variability) parameters: mean NN, SDNN, LF power and HF power. The mean NN interval is the reciprocal of mean heart rate and is indicative of the sympathovagal balance, with shorter NN intervals representing more sympathetic activation.[24] Heart rate variability is the result of autonomic modulations of the sympathetic and parasympathetic nervous system, with the purpose to buffer blood pressure.[25, 26] Finally, baroreflex sensitivity is defined as the reflex-induced change in interbeat interval in milliseconds per millimeter of Hg blood pressure change and describes the functioning of the baroreflexes in short term regulation of arterial blood pressure.[27] The use of three categories of autonomic function parameters enabled us to evaluate several aspects of the autonomic nervous system and is therefore an important strength of our study.

On the other hand, one limitation of the present study was the small sample size of 298 participants, which may have resulted in insufficient power. The proportion of IFG and IGT cases at baseline was high because of oversampling of such individuals in the study population, and this contributed to the 94 cases of DM2 found at follow-up. Yet, despite the small sample size, overall there were no indications of any association between autonomic function and incidence of DM2. The associations with fasting and 2-h glucose concentrations were small, and the limits of the 95% CI excluded large effects. For this reason, it is not believed that associations would be detected with larger

sample sizes and power. The present limited sample size was in large part the result of non-participation in the follow-up visits (140 of the 438 non-diabetic participants at baseline did not participate, 51 died, 29 moved and, for 59 the reason for not attending was unknown). In fact, the 298 participants included in our analyses were healthier and had better autonomic function than the 140 who were lost to follow-up. The association between autonomic function and DM2 among non-participants may have been stronger than among participants, and this may have led to underestimation of the true association between autonomic function and DM2 in our analyses. However, even with a hypothetical worst-case scenario in which all participants who did not return for follow-up developed DM2, analysis would still not have found a significant association between parameters of autonomic function and incidence of DM2 (data not shown).

Several cross-sectional studies have shown an association between autonomic function and glucose tolerance.[5-9] Four previous studies prospectively investigated the association between autonomic function and incident DM2. Shigetoh et al found a significant OR (5.39, 95% CI: 1.34, 21.8) for the development of DM2 in individuals with a heart rate ≥80 beats/min as compared with a heart rate <60 beats/min (n=614, number of incident cases of DM2 not reported).[28] However, it is unclear whether individuals with known DM2 at baseline were excluded from this study and therefore the results may be biased. An analysis on the Chicago Heart Association Detection Project in Industry showed an association for 1 SD increase in heart rate (12 beats/min) and diabetes mortality (n=14992, 400 cases of diabetes mortality): OR=1.21, 95% CI: 1.03-1.41) in individuals aged 35-49 years. Heart rate was also associated with non-fatal DM2, but this association attenuated after adjustment for BMI and post load glucose concentrations at baseline.[29] Since we only investigated non-fatal DM2 and not diabetes mortality, these results are comparable to our study. The ARIC study (n=8185, 1063 incident cases of DM2) showed an association of resting heart rate with DM2 after adjustment for confounding including baseline glucose (relative risk per SD (9.7 beats/min) = 1.06 (95% CI 1.00, 1.13). A comparable association was observed in individuals with normal fasting glucose at baseline (RR=1.13, 95% CI: 1.04, 1.22). In line with our study, there were no associations between LF and HF power and SDNN with DM2.[30] The ARIC study is comparable with the Hoorn study in design. An important difference is the inclusion of more ethnic groups (mainly black, 19%) in the ARIC study. The influence of ethnicity on autonomic function is unknown, but differences in glucose metabolism have been shown between ethnic groups.[31] The CARDIA study (n=3295, 98 incident cases of DM2) showed an association between low heart rate recovery and incident DM2 in individuals with poor fitness (OR=3.27, 95% CI 1.34, 7.94), but not in fit persons.[32] In our study, there was no association in either fit or unfit participants after stratification for regular physical activity (defined as at least five days a week moderately active) (data not shown). However, the population of the

CARDIA study is much younger than the population of the Hoorn study (18-30 years versus 50-75 years). It is possible that physical activity is an important effect modifier in young individuals, but is less important in older age due to an entirely different risk profile. Slow heart rate recovery may reflect decreased parasympathetic activity.[33] Our study did not investigate heart rate recovery, which is defined as the rapid decrease in heart rate following cessation of exercise, but a previous study showed a significant association between heart rate variability and heart rate recovery.[34] Therefore it would be expected that the association of heart rate recovery and heart rate variability with DM2 would be comparable. However, the results of the CARDIA study are not adjusted for glucose concentrations at baseline. Since glucose concentrations at baseline are a strong predictor for the development of DM2, results may suffer from reverse causation.[35] Overall, the results from previous prospective studies are inconsistent. Furthermore, our study used more parameters of autonomic function than the previous studies. It has been shown that relying on one parameter may result in over- or underestimation of the autonomic function.[22] This may explain the incidental significant results that were found earlier. In our study we examined several aspects of the autonomic nervous system and we consistently found no evidence for an association with DM2.

The results of cross-sectional studies have identified an association between autonomic function and glucose tolerance that may be bidirectional. Some studies suggested that autonomic dysfunction may not only be a complication of DM2, but may also be involved in its development. However, in our prospective analysis there was no association between autonomic function and DM2. This suggests that autonomic dysfunction is solely a consequence of glucose concentrations and is not involved in the development of DM2. Such a link was proposed in 1986 by Landsberg, who postulated that the sympathetic activation associated with obesity is mediated by insulin resistance and concomitant hyperinsulinaemia.[36] Experimental studies in humans showed that hyperinsulinaemia caused by the infusion of insulin enhanced sympathetic activation and depressed vagal activation.[17, 18] This decreased autonomic function may be the result of sympathetic activation caused by insulin acting in the hypothalamus.[37] Also hyperglycaemia has been associated with sympathetic activation.[19, 38] These studies indicate that hyperinsulinaemia and hyperglycaemia may be the underlying factor of the previously reported cross-sectional association between impaired glucose metabolism and sympathetic activation.

In conclusion, there is no evidence from the present study of any association between autonomic function and the incidence of DM2 and fasting glucose concentrations in the follow-up of a homogeneous Caucasian population aged 50–75 years. These results suggest that previously observed associations between autonomic function and glucose metabolism in cross-sectional settings may have simply reflected reverse causation.

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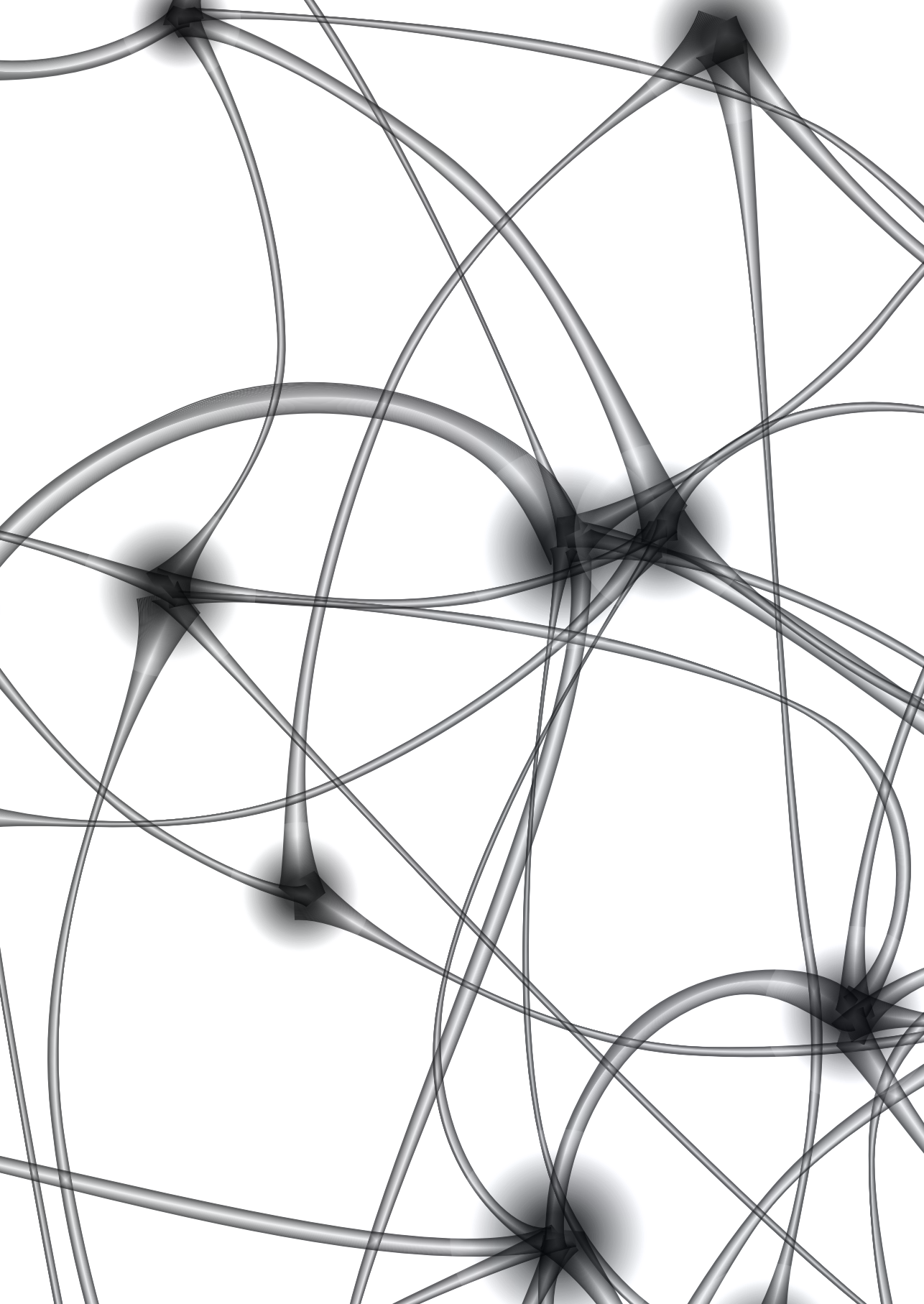

4

The role of insulin resistance in the association between body fat and autonomic function

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ABSTRACT

Background and Aim

Excess body fat is associated with altered autonomic function. We investigated whether this association is mediated by insulin resistance.

Methods and Results

Cross-sectional analysis of a subgroup of the Netherlands Epidemiology of Obesity study with measurements of autonomic function (heart rate variability calculated as mean interbeat interval, standard deviation of all normal intervals (SDNN), low frequency (LF) power and high frequency (HF) power). We measured BMI (kg/m^2), total body fat (%) and waist circumference (cm), and calculated the HOMA-index of insulin resistance (HOMA-IR). We examined the association between body fat and heart rate variability with multivariate linear regression analysis. To investigate whether the association was mediated by insulin resistance, we additionally adjusted for HOMA-IR.

After exclusion of participants with glucose lowering medication (n=19), 466 participants were included. Per SD of BMI, the difference in SDNN was −2.7% (95% CI: −5.5, 0.1) in the multivariate model. Additional adjustment for HOMA-IR attenuated this association to −1.2% (95% CI: −4.2, 1.7), suggesting that 55% of the association between BMI and SDNN was mediated by HOMA-IR. All measures of body fat were associated with mean interbeat interval, SDNN and LF power. Depending on the parameter of body fat or heart rate variability, 29-81% of the association was mediated by HOMA-IR.

Conclusion

In this cross-sectional study, body fat was associated with heart rate variability. This association may at least partially be mediated by insulin resistance. Future studies should investigate whether a reduction in obesity and insulin resistance may prevent the adverse cardiovascular consequences of altered autonomic function.

INTRODUCTION

Excess body fat is associated with altered function of the autonomic nervous system and sympathetic activation.[1,2] Altered autonomic function is an independent risk factor for cardiovascular events in populations with prevalent cardiovascular disease,[3,4] but also for a first event in the general population.[5] The mechanism underlying the association between body fat and altered autonomic function is not elucidated. We hypothesize that insulin resistance may be the underlying factor (Figure 1).

Obesity, especially abdominal obesity, is a risk factor for insulin resistance.[6,7] Insulin resistance has also been associated with altered autonomic function in several cross-sectional studies.[8-12] Based on the results of these cross-sectional results, it remained unclear whether insulin resistance alters autonomic function, or vice versa. We however recently showed that autonomic function was not prospectively associated with the incidence of type 2 diabetes mellitus during a median follow up of 9.2 years.[13] This suggests that the autonomic nervous system may not be involved in the development of insulin resistance, and supports a pathophysiological mechanism in which insulin resistance is the first abnormality in obesity, resulting in sympathetic activation. This mechanism is further supported by the physiological reactions of the autonomic nervous system to infusions of blood glucose and insulin in humans.[14,15]

Therefore, our aim was to study the role of insulin resistance as a mechanism underlying the association between body fat and autonomic function (Figure 1). To that extent, we investigated the association between measures of body fat and parameters of autonomic function and whether this association was mediated by insulin resistance.

METHODS

Study design and population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based prospective cohort study comprising 6673 persons with an oversampling of individuals with a BMI $\geq$ 27 kg/m^2. The study design and data collection of the NEO study have been described previously.[16] In short, persons aged between 45 and 65 years with a self-reported BMI of 27 kg/m^2 or higher were included between September 2008 and October 2012. In addition, all inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited irrespective of their BMI, allowing for a reference distribution of BMI.

The baseline visit was performed in the NEO study centre in the Leiden University Medical Centre, during which an extensive physical examination was performed. The present study is a cross-sectional analysis of baseline measurements of the participants with a measurement of heart rate variability. We additionally excluded participants who were using oral glucose lowering medication or insulin. The NEO study was approved by the Medical Ethical Committee of the Leiden University Medical Centre and all participants gave their informed consent.

Data collection

Participants reported their highest level of education in ten categories according to the Dutch educational system. We grouped these data into low education (defined as no education, primary education or lower vocational training) versus high education (used as the reference category). Tobacco smoking was categorized into current, former or never (reference) smokers. Self-reported pre-existing cardiovascular disease was defined as myocardial infarction, angina, congestive heart failure, stroke, or peripheral vascular disease. Participants reported the frequency and duration of their physical activity using the Short Questionnaire to Assess Health-enhancing physical activity (SQUASH) questionnaire. We calculated the total energy expended during physical activity in hours per week of metabolic equivalents (MET-h/week).

Body fat

We measured height and weight without shoes and one kilogram was subtracted to correct for the weight of clothing. Body Mass Index (BMI) was calculated by dividing the weight in kilograms by the height in meters squared. Total body fat was measured using a bio-impedance device (TBF-310, Tanita International Division, United Kingdom). We measured the circumference of the waist with a flexible tape in the middle of the distance between the lowest rib and the crista iliaca and used this as a measure of abdominal fat mass.

Insulin resistance

Venous blood was sampled after an overnight fast for at least 10 hours. Plasma glucose concentrations were measured with the enzymatic and colorimetric method (Roche Modular Analytics P800, Roche Diagnostics, Mannheim, Germany) and serum insulin concentrations were determined by an immunometric method (Siemens Immulite 2500, Siemens Healthcare Diagnostics, Breda, the Netherlands). We calculated the updated Homeostasis Model Assessment Insulin Resistance (HOMA-IR), a measure of insulin resistance which corresponds well to estimates of insulin resistance derived from the hyperinsulinemic euglycemic clamp, by entering fasting glucose and fasting insulin in a Microsoft Excel spreadsheet available on the internet.[17]

Heart rate variability

Ten Actiheart devices, accelerometers combined with heart rate monitors, were randomly distributed to NEO participants. We used the data from the heart rate monitor to calculate heart rate variability (HRV) parameters as indices of autonomic function. We selected only the Actiheart recordings with the least noise (<1%) for manual reparation and a valid estimation of heart rate variability. Two standard ECG electrodes (H98SG, Tyco Healthcare, Germany) were placed at the level of the second intercostal space, one electrode on the sternum and one ten centimetres to the left of the first electrode. The Actiheart was set up for a recording of four days. During this period, all interbeat intervals (IBIs) were stored in milliseconds. Participants were asked to perform their normal daily activities. For each participant with an Actiheart measurement, we excluded the first 12 hours of the recording because participants were not performing their normal activities during that period due to their visit to the NEO study centre. Then, we determined whether the quality of the IBI data was sufficient for heart rate variability analysis, by the detection of artefacts and non-sinus beats in the data. We calculated the percentage noise by dividing the number of artefacts and non-sinus beats by the total number of IBIs for each 24-h period using a moving average with a 1-h window. We selected the 24-h period with the least artefacts and non-sinus beats for reparation and subsequent HRV analysis, with a maximum of 1% artefacts/non-sinus beats. The raw IBI data were cleaned by removing artefacts and non-sinus beats using a semi-automatic Matlab program (designed by ACM). An overview of this procedure is shown in Figure 2. The Actiheart stores timings of all QRS complexes. Based on these timings, three types of non-sinus beats could be detected: missed QRS complexes, extra QRS complexes and QRS complexes that were detected too early (premature complexes) or too late (post-mature complexes). We repaired non-sinus beats if they either added up to or could be divided in interpolated intervals that differed <5% from the mean of the ten preceding intervals. Artefacts and non-sinus beats that could not be repaired by the algorithm were deselected from the recordings by hand.

Heart rate variability parameters were calculated from the repaired IBI data according to a procedure that was described and applied previously.[18,19] In short, HRV was calculated using a 5-min moving window and all valid 5-min values were averaged. We calculated the mean interbeat interval and standard deviation of all normal intervals (SDNN). Prior to spectral analysis of the recordings, the tachogram was processed with adjustment for linear trends, tachogram tapering and zero padding. A test of stationarity was performed, and we excluded non-stationary episodes from the analysis. For calculation of low frequency power (LF power) and high frequency power (HF power), a fast Fourier transformation was employed.

Statistical analysis

In the NEO study individuals with a BMI of 27 kg/m^2 or higher were oversampled. To correctly represent associations in the general population, adjustments for the oversampling of individuals with a BMI ≥ 27 kg/m^2 were made. This was done by weighting individuals towards the BMI distribution of participants from the Leiderdorp municipality, whose BMI distribution was similar to the BMI distribution of the general Dutch population.[20] All results were based on weighted analyses.

Baseline characteristics were calculated as mean (standard deviation, SD), median (25th-75th percentile) or as percentage and were stratified for sex-specific tertiles of BMI. Differences in baseline characteristics between NEO participants with and without measurement of heart rate variability were tested with Student t-test for continuous variables and Chi-square for categorical variables. The HOMA-IR and heart rate variability parameters were ln-transformed. Serum insulin concentrations below the detection limit of the assay (2.0 mU/L) were imputed using multiple imputation methods for left censored data, with ten imputation datasets. We standardized the measures of body fat using sex-specific standard deviations. We used linear regression analysis to calculate regression coefficients of the associations between measures of body fat and HRV parameters (the total association, A+B in Figure 1). We adjusted for age, sex, tobacco smoking, ethnicity, education, physical activity, prevalent cardiovascular disease and cardiac medication. Regression coefficients were expressed as percentage difference in heart rate variability with 95% confidence intervals per weighted standard deviation of measures of body fat.

As a last step in our analyses, we included HOMA-IR into our model to investigate whether the total association between body fat and heart rate variability was mediated by insulin resistance (the indirect association, A in Figure 1). To that end, we calculated what percentage of the total association between body fat and heart rate variability was

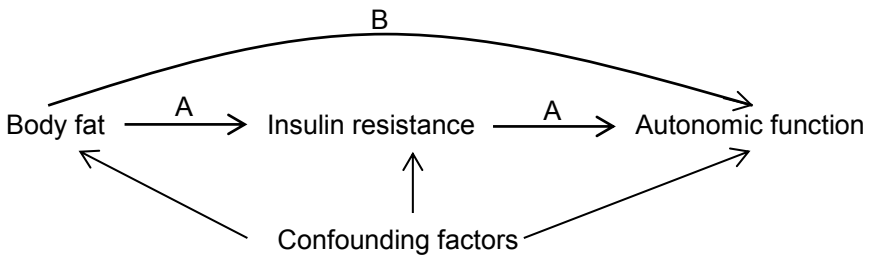

Figure 1. Hypothesized path diagram. Confounding factors: age, sex, tobacco smoking, ethnicity, education, physical activity, prevalent cardiovascular disease and cardiac medication. A, indirect association between body fat and autonomic function (mediated by insulin resistance) B, direct association between body fat and autonomic function A + B, total association between body fat and autonomic function.

mediated by HOMA-IR. The percentage mediated association was calculated as (1 - (direct association / total association) * 100). The remaining coefficient after adjustment for HOMA-IR represents the direct association between body fat and heart rate variability, B in Figure 1.

Before adding HOMA-IR as mediator to the model, we tested for interaction between determinant and mediator by adding an interaction term of body fat and HOMA-IR to our linear regression model.

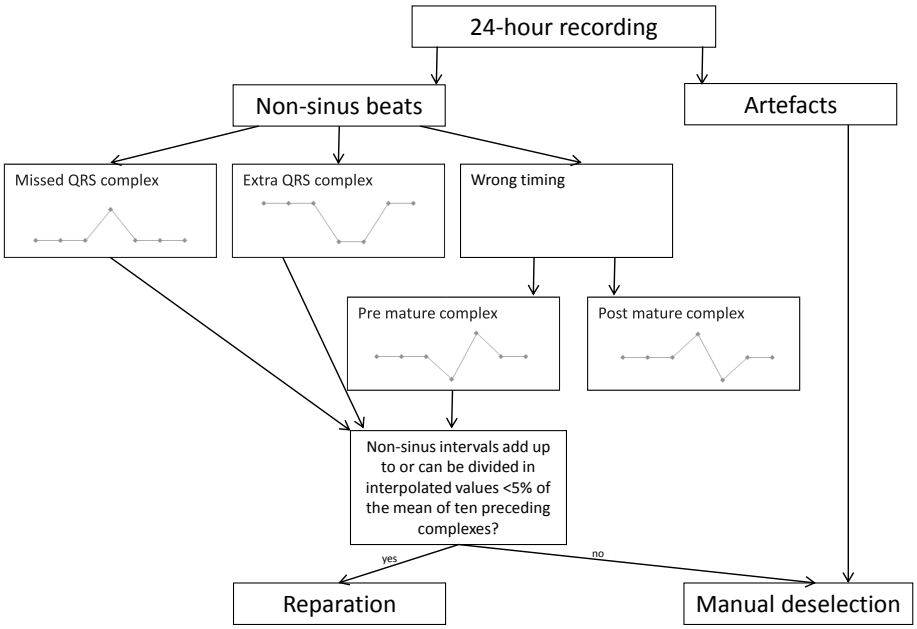

Figure 2. Data processing of interbeat intervals for HRV analysis

RESULTS

Baseline characteristics

From the 6673 participants included in the NEO study, an Actiheart device was carried by 955 participants to estimate physical activity. In 50 of this 955, the recording failed due to technical problems (e.g. broken battery, detached electrodes) or incorrect use by the participant (e.g. early removal). From the 905 remaining recordings, we selected 485 recordings with maximum quality of IBI data (<1% artefacts) to use for heart rate variability analysis. After exclusion of participants with oral glucose lowering medication or insulin (n=19), the present analysis included 466 participants. Participants included in our study were more often men (56%) than excluded NEO participants (43%). Other

baseline characteristics did not differ between included and excluded participants (mean age was 56 (SD: 6) years in both groups, mean BMI was 27 (4) kg/m^2 in included participants and 26 (4) kg/m^2 in excluded participants, median HOMA-IR was 0.8 (25th-75th percentile: 0.4-1.2) in included and 0.6 (0.4-1.1) in excluded participants). Table 1 shows the baseline characteristics of the study population stratified by sex-specific tertiles of BMI.

Table 1: Baseline characteristics of NEO study participants with data on autonomic function, stratified by sex-specific tertiles of BMI (n=466)

	BMI cut-off (kg/m^2)		
	Men: 20-27	**Men: 28-30**	**Men: 29-47**
Variable	**Women: 19-28**	**Women: 29-31**	**Women: 32-47**
Sex (% men)	57	59	43
Age (years)	56 (6)	56 (6)	56 (6)
Smoking			
- Former (%)	56	51	54
- Current (%)	15	20	18
Education (% low)	16	23	27
Physical activity (MET-hours/week)	121 (73-166)	123 (88-158)	108 (68-146)
Prevalent cardiovascular disease (%)	9	10	8
Use of antihypertensive medication (%)	19	32	40
Use of lipid lowering medication (%)	9	18	17
Body mass index (kg/m^2)			
- Men	25 (2)	30 (1)	34 (4)
- Women	25 (2)	30 (1)	35 (4)
Waist circumference (cm)			
- Men	93 (8)	106 (5)	117 (11)
- Women	85 (9)	98 (5)	109 (9)
Total body fat (%)			
- Men	23 (4)	29 (3)	34 (6)
- Women	37 (5)	42 (2)	47 (3)
Mean IBI (ms)	844 (788-901	805 (747-909)	816 (744-878)
SDNN (ms)	48 (41-56)	47 (39-57)	46 (36-55)
LF power (ms^2)	928 (655-1300)	947 (609-1402)	775 (531-1144)
HF power (ms^2)	218 (142-323)	242 (159-386)	232 (147-398)
HOMA-IR	0.5 (0.3-1.1)	1.0 (0.7-1.4)	1.3 (0.8-2.0)

Data are presented as mean (SD), median (25th-75th percentile) or percentage. Results were based on weighted analyses.

NEO, Netherlands Epidemiology of Obesity; BMI, Body mass index; MET, metabolic equivalent of task; IBI, interbeat interval; SDNN, standard deviation of all normal intervals; LF, low frequency; HF, high frequency; HOMA-IR, HOMA-index of insulin resistance

Body mass index, insulin resistance and heart rate variability

The associations of BMI (per 4 kg/m^2 for men and 5 kg/m^2 for women) and parameters of heart rate variability are shown in Table 2. BMI was associated with mean IBI and LF power, with and without adjustment for confounding variables. There was also an association with SDNN, although not significant. We found no association between BMI and HF power. After additional adjustment for HOMA-IR as mediating factor, the association between BMI and parameters of heart rate variability attenuated. For mean IBI, 40% of the association was mediated by HOMA-IR, for SDNN 55% and for LF power 37% of the total association was mediated by HOMA-IR.

An interaction term of BMI and HOMA-IR was added to all models to test for interaction. For mean IBI the regression coefficient was 0.00025 (95% CI: −0.00243, 0.00292, *P*-value 0.856), for SDNN the coefficient was −0.00014 (95% CI: −0.00775, 0.00749, *P*-value 0.972), for LF power 0.00166 (95% CI: −0.015555, 0.01887, *P*-value 0.849) and for HF power 0.003379 (−.01342, 0.02100, *P*-value 0.665).

A sensitivity analysis excluding all participants with self-reported cardiovascular disease showed similar results. In the multivariate analyses and per standard deviation of BMI, the difference in mean IBI was −1.8 (95% CI: −2.9, −0.8), the difference in SDNN was −3.4 (95% CI: −6.4, −0.6), in LF power was −8.0 (95% CI: −15.0, −1.4), and in HF power was 1.7 (95% CI: −5.0, 8.0).

Total body fat and heart rate variability

Table 2 shows the associations between total body fat (per 6%) and parameters of heart rate variability. Total body fat was associated with mean IBI and non-significantly associated with SDNN and LF power. Total body fat did not associate with HF power. Of the total association between body fat and heart rate variability, 34% (mean IBI), 67% (SDNN) and 81% (LF power) was mediated by HOMA-IR.

An interaction term of total body fat and HOMA-IR was added to all model to test for interaction, showing regression coefficients of 0.0003192 (95% CI: −0.0014396, 0.0020781, *P*-value 0.360) for mean IBI, −0.0013493 (95% CI: −0.005757, 0.0030584, *P*-value 0.547) for SDNN, −0.0052708 (95% CI: −0.0145081, 0.0039666, *P*-value 0.262) for LF power and −0.0033135 (95% CI: −0.0137051,; 0.0070782, *P*-value 0.651) for HF power.

Repeating the analyses without participants with self-reported cardiovascular disease yielded similar results: per standard deviation of total body fat, the difference in mean IBI was −1.9 (95% CI: −3.0, −0.8), the difference in SDNN was −3.0 (95% CI: −6.2, 0.1), in LF power was −5.8 (95% CI: −11.7, 1.7), and in HF power was 0.8 (95% CI: −7.5, 8.5).

Waist circumference and heart rate variability

The regression coefficients for the associations between waist circumference (per 11 cm for men and 12 cm for women) and parameters of heart rate variability are shown in Table 2. Waist circumference was associated with mean IBI, SDNN and LF power. No association was found for HF power. The total association between waist circumference and heart rate variability was for 29% (mean IBI), 29% (SDNN) and 34% (LF power) mediated by HOMA-IR.

The interaction term of waist circumference and HOMA-IR was non-significant in all models, mean IBI: −0.00019 (95% CI: −0.00119; 0.00081, *P*-value 0.713), SDNN: −0.00027 (95% CI: −0.00309; 0.00256, *P*-value 0.854), LF power: 0.00045 (95% CI: −0.00598; 0.00688, *P*-value 0.890), HF power: 0.00414 (95% CI: −0.00311; 0.01138, *P*-value 0.262).

Table 2: Percentage difference (95% CI) in autonomic function per standard deviation of body fat measure (n=466)

Body fat measure (SD)	Model	Mean IBI	SDNN	LF power	HF power
BMI (m: 4, w: 5 kg/m^2)	Age and sex adjusted	−1.9 (−3.1, −0.8)	−3.3 (−6.3, −0.4)	−7.9 (−14.3, −1.8)	−0.1 (−6.8, 6.2)
	Multivariate[1]	−1.6 (−2.7, −0.6)	−2.7 (−5.5, 0.1)	−7.0 (−13.5, −0.9)	0.3 (−5.9, 7.0)
	+ HOMA-IR as mediator[2]	−1.0 (−2.2, 0.3)	−1.2 (−4.2, 1.7)	−4.4 (−11.0, 1.8)	3.9 (−3.1, 11.5)
Total body fat (6%)	Age and sex adjusted	−2.1 (−3.4, −0.8)	−2.9 (−6.1, 0.1)	−4.9 (−11.4, 1.3)	0.1 (−7.3, 8.2)
	Multivariate[1]	−1.9 (−3.0, −0.8)	−2.4 (−5.4, 0.4)	−4.3 (−10.8, 1.9)	0.8 (−6.7, 9.0)
	+ HOMA-IR as mediator[2]	−1.3 (−2.5, 0.0)	−0.8 (−3.9, 2.2)	−0.8 (−7.4, 5.4)	4.9 (−4.3, 15.0)
Waist circumference (m: 11, w: 12 cm)	Age and sex adjusted	−2.3 (−3.6, −1.0)	−4.5 (−7.8, −1.4)	−8.5 (−15.9, −1.6)	−1.2 (−9.1, 6.2)
	Multivariate[1]	−2.1 (−3.3, −0.9)	−4.0 (−7.1, −0.9)	−7.5 (−14.7, −0.8)	−1.1 (−9.1, 6.3)
	+ HOMA-IR as mediator[2]	−1.5 (−2.9, −0.1)	−2.8 (−6.1, 0.4)	−5.0 (−12.1, 1.7)	2.3 (−5.9, 11.1)

Results were based on weighted analyses. CI, confidence interval; SD, standard deviation; BMI, body mass index; IBI, interbeat interval; m, men; w, women; +, additionally adjusted for; SDNN, standard deviation of all normal intervals; LF, low frequency; HF, high frequency, HOMA-IR, Homeostatic model assessment of insulin resistance.

[1]Multivariate model adjusted for: age, sex, smoking, ethnicity, education, physical activity, prevalent cardiovascular disease and cardiac medication

[2]Regression coefficients reflect the direct associations between body fat measures and measures of autonomic function, after adjustment for HOMA-IR as mediating variable

Repeating the analyses without participants with self-reported cardiovascular disease yielded similar results: per standard deviation of waist circumference, the difference in mean IBI was −2.4 (95% CI: −3.6, −1.2), the difference in SDNN was −4.8 (95% CI: −8.1, −1.6), in LF power was −8.3 (95% CI: −15.9, −1.1), and in HF power was −2.7 (95% CI: −11.3, 5.2).

DISCUSSION

In our middle-aged population, measures of body fat (BMI, waist circumference, total body fat) were associated with autonomic function measured as long term heart rate variability (mean NN interval, SDNN, LF power). These associations were most pronounced for waist circumference. Depending on the parameter of body fat or heart rate variability, 29 to 81% of the total association seemed to be mediated by insulin resistance as assessed by HOMA-IR. There was no association between body fat and autonomic function, as measured with HF power.

The results on mean NN interval, SDNN and LF power are in line with previous studies on the association between body fat and parameters of sympathetic activation.[1;21-23] Our findings may add to those studies that insulin resistance is a possible mechanism underlying the association between body fat and autonomic function. An explanation for the lack of association with HF power may be that this spectral component is more indicative of the parasympathetic and not directly of the sympathetic outflow.(24)

A strength of the present study is the extensive phenotyping of the NEO participants including the measurement of total body fat and waist circumference, which more accurately quantify body fat and abdominal fat accumulation than BMI does. However, our study also has limitations that need to be considered. The cross-sectional observational nature of our study is a limitation. We cannot exclude the possibility that our outcome (sympathetic activation) may have influenced our determinant (body fat), and so there may be reverse causation in our study. Another limitation is the use of the Homeostasis Model Assessment Insulin Resistance as a measure of insulin resistance instead of the hyperinsulinaemic euglycaemic clamp, but the correlation with the clamp is very high.[25] Also the use of the Actiheart for measurement of heart rate variability may be a limitation, but the agreement of Actiheart with the often used Holter electrocardiogram is high.[26] With regard to the statistical analysis, we used parametric mediation analysis assuming linear models for the outcome and the mediator. This is a valid method to evaluate mediation in absence of mediator-outcome confounding and interaction between the exposure and the mediator under study.[27] It needs to be taken into account that add-

ing a possible mediating variable into a regression model could introduce bias in the estimation of the direct and indirect association if there is residual mediator-outcome confounding or interaction between determinant and mediator.[27] In our study, we could only identify low-grade systemic inflammation as a potential residual confounder between insulin resistance (mediator) and autonomic function (outcome). Not adjusting for inflammation might have resulted in an overestimation of both the direct association of body fat with sympathetic activation and the association mediated by insulin resistance. However, it may well be that inflammation is not a common cause of insulin resistance and sympathetic activation, but rather an intermediate factor between insulin resistance and sympathetic activation. In that case, adjusting for inflammation would result in an underestimation of the true association in our mediation analyses. To examine the presence of interaction between determinant and mediator, we tested for interaction by adding an interaction term of body fat measures and HOMA-IR to our linear regression models. All were non-significant, meaning that it is unlikely that adding HOMA-IR as a mediator to our models has resulted in bias in the estimation of the direct and indirect association. In addition, estimating direct and indirect associations is affected if the variability in the measurement of determinant and mediator is of different magnitude. In the NEO study, 100 participants were examined for the second time approximately three months after their baseline visit. From this repeated measurements, we calculated intraclass correlation coefficients (ICCs). The ICCs of body fat measures and HOMA-IR were both high (e.g. 0.940 (0.921-0.959) for total body fat and 0.749 (0.675-0.822) for HOMA-IR). This suggests that it is unlikely that a difference the variability in the measurement of determinant and mediator has influenced our results.

Although cross-sectional, our results indicate that insulin resistance may be a mechanism underlying in the association between body fat and autonomic function (Figure 1). This finding is supported by evidence on three associations. First, excess body fat is a well-established risk factor for insulin resistance.[6,7] Second, the association between body fat and autonomic function has been shown in several previous studies.[8-12] Adipose tissue secretes a number of hormones (e.g. leptin, adiponectin, resistin, visfatin) and inflammatory markers (e.g. TNF-α, IL-6). Thereby, the adipose tissue is able to directly stimulate the central sympathetic nervous system in the hypothalamus, or to induce a state of low-grade inflammation that can also stimulate the sympathetic nervous system.[28] Third, insulin resistance has been associated with autonomic function in several cross-sectional studies.[8-12] We know from experimental studies that higher levels of circulating insulin elevate sympathetic outflow both in the euglycaemic or hyperglycaemic state.[14,15] The stimulation of the sympathetic nervous system seems at least in part attributable to the central action and peripheral action of insulin.[29] However, also after the diagnosis of diabetes (and the onset of beta-cell failure), autonomic function

deteriorates.[30] Therefore, it seems unlikely that the impairment of autonomic function is due to hyperinsulinaemia alone. Likely, also the hyperglycaemia and an abundance of advanced glycation end-products in insulin resistant individuals play a role in inducing autonomic dysfunction by damaging the nerves or interfering with chemical signalling.

In conclusion, in this cross-sectional study body fat was associated with autonomic function measured as heart rate variability. This association may at least partially be mediated by insulin resistance. Future studies should investigate whether a reduction in obesity and insulin resistance may prevent the adverse cardiovascular consequences of altered autonomic function.

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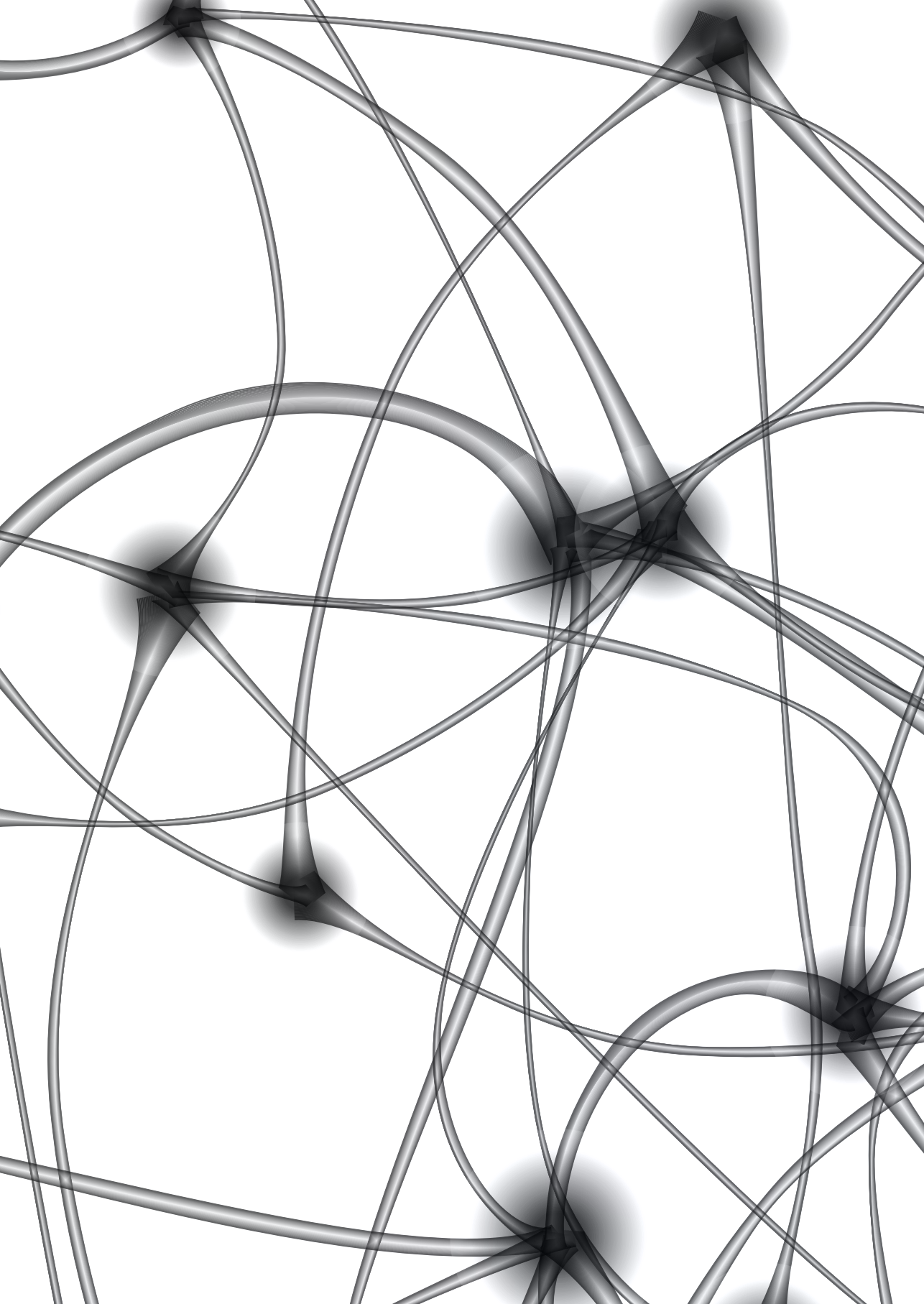

5

Heart rate variability and first cardiovascular event in populations without known cardiovascular disease: meta-analysis and dose-response meta-regression

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ABSTRACT

Aims

Heart rate variability (HRV) is associated with cardiovascular disease (CVD) in individuals with known CVD. It is less clear whether HRV is associated with a first cardiovascular event. Therefore, we performed a meta-analysis to study the association between HRV and incident cardiovascular events in populations without known CVD.

Methods and results

We performed a meta-analysis and dose-response meta-regression of studies assessing the association between HRV and CVD. We searched Pubmed, Embase, Web of Science, Cochrane library, ScienceDirect and CINAHL up to December 2011 for eligible studies. We selected studies that used the standard deviation of the normalized N-N interval (SDNN), low frequency (LF) or high frequency (HF) spectral component as a measure of HRV. Primary outcomes were (non)fatal cardiovascular events.

Eight studies with a total number of 21988 participants were included. The pooled relative risk comparing the lowest level to the highest level of SDNN was 1.35 (95% CI 1.10, 1.67). The pooled relative risks for LF and HF were 1.45 (95% CI 1.12, 1.87) and 1.32 (95% CI 0.96, 1.81), respectively. In a meta-regression, the predicted RR of incident CVD of the 10th and 90th HRV (SDNN) percentile compared to the 50th percentile were 1.50 (95% CI 1.22, 1.83) and 0.67 (95% CI 0.41, 1.09).

Conclusion

In conclusion, low HRV is associated with a 32-45% increased risk of a first cardiovascular event in populations without known CVD. An increase in SDNN of 1% results in an approximately 1% lower risk of fatal or non-fatal CVD.

INTRODUCTION

Cardiovascular disease (CVD) is one of the main causes of morbidity and mortality in industrialized countries. Cardiac autonomic dysfunction is a risk factor for the development of CVD that can be measured non-invasively using heart rate variability (HRV).[1] Physiologically, HRV is thought to be the result of adaptive changes in heart rate caused by the sympathetic and parasympathetic nervous system, with the purpose to buffer blood pressure.[2] Less compensatory change (low HRV) suggests a decreased adaptive autonomic nervous system and is thought to be associated with increased morbidity. HRV has long been known to be associated with morbidity and mortality after myocardial infarction.[3] This finding has been confirmed in many studies.[4, 5] Besides acute myocardial infarction and cardiac death, also occurrences of congestive heart failure and arrhythmias have been associated with reduced HRV.[6, 7]

Although HRV is associated with cardiovascular events in individuals with known CVD, fewer studies have been published on the association between HRV and the risk of CVD in a population without known CVD. Therefore, the aim of this meta-analysis was to assess the association between HRV and CVD in individuals without known CVD at baseline.

METHODS

Search Strategy

A systematic literature search was performed, aimed at identifying epidemiological studies with data on the association between HRV and CVD. We searched the electronic databases Pubmed, Embase, Web of Science, Cochrane library, ScienceDirect and CINAHL from their inception up to December 2011. No language restriction was set in advance. The reference lists of all relevant identified articles were screened for additional potentially relevant articles.

Eligibility criteria

Initial selection of studies by title and abstract was performed by two reviewers (S.H. and K.B.G.) independently. Disagreements were resolved by consensus. Population-based studies in adults assessing the association between HRV and CVD were eligible for inclusion. Abstracts of meetings and unpublished results were not eligible. Since the aim of the study was to assess the association between HRV and incident CVD, cohorts restricted to patients with CVD at baseline were excluded. Furthermore, we did not include studies in our meta-analysis if they presented a measure of HRV that was only used

in one or two studies. To be included, studies should have expressed the association between HRV and CVD as relative risks (RRs) (risk ratios, odds ratios or hazard ratios). Maximally adjusted RRs and confidence intervals (CIs) for the association between HRV and CVD were extracted. Data were extracted in duplicate (S.H. and K.B.G.), using a standard data extraction sheet.

Measures of HRV

HRV can be expressed using several measures. In order to calculate different measures of HRV, intervals between adjacent normal R waves (NN intervals) are measured. The standard 12-lead ECG is a common method to measure these intervals. In the time domain analyses, a variety of statistical variables are calculated from NN intervals. The most commonly used time domain variables are the standard deviation of all N-N intervals (SDNN) and the square root of the mean of the sum of the squares of differences between adjacent R-R intervals (RMSSD).[8]

The frequency domain is another method used to calculate HRV, of which the low frequency (LF) and the high frequency (HF) spectral components are common measures. In the frequency domain method, cyclic fluctuations of RR intervals are quantified by the frequency of the fluctuation using Fourier transformation or auto regression spectral analysis.[1] Typically, HF fluctuations are thought to be caused by respiration and are effectuated by parasympathetic outflow. LF fluctuations are probably the result of a blood pressure resonance phenomenon mediated by the baroreflexes and are effectuated by both sympathetic and parasympathetic fluctuations in outflow.[2, 9]

Study endpoints

The outcome of interest was the occurrence of a first fatal or nonfatal cardiovascular event. In this meta-analysis we defined fatal or non-fatal CVD as myocardial infarction, coronary revascularization procedures, hospitalization for unstable angina pectoris, congestive heart failure, arrhythmia (atrial/ventricular), arterial peripheral vascular disease, stroke (ischemic/haemorrhagic) or death from these events.[10]

Risk of bias assessment

For the aim of the present meta-analysis, risk of bias assessment focused on design elements that could potentially bias the association between HRV and CVD: (i) ascertainment that the outcome of interest was not present at baseline, (ii) adequacy of follow up (loss-to-follow up of less than five percent was considered adequate), (iii) effect estimates having been adjusted for potential confounding factor, (iv) adequacy of endpoint verification. Endpoint verification was considered adequate when diagnoses

were confirmed using medical records or death certificates. Self-report was considered to present a high risk of bias.

Statistical analyses

According to the data available in included articles, HRV was categorized based on percentiles (median, tertiles or quartiles). When papers presented their results using an author defined cut-off value, we calculated the accompanying percentiles under the assumption of a normal distribution of HRV. If authors calculated a RR for one SD change in HRV, we recalculated this RR for categories based on quartiles assuming a normal distribution of HRV and a linear association on a logarithmic scale between HRV and CVD. For each study the highest HRV category was used as the reference and for all studies the lowest HRV category was compared with the highest category. We performed a random effects meta-analysis based on maximally adjusted relative risks of the highest versus the lowest categories and their accompanying standard error. Heterogeneity was quantified using the I^2 statistics. We performed two sensitivity analyses; one excluding populations with only diabetes patients and one separating the different measurement durations of HRV (short measurements of 10 seconds to three minutes versus longer measurements of 24 hours).

Secondly, we performed a meta-regression to assess a dose-response slope for the association between HRV and CVD. We used a random effects model to take the between-study variation into account. For each individual study, a weight was calculated based on the standard error. The 50th percentile was used as the reference category.[11] Based on the slope from the random effects regression, we predicted risk ratios comparing the 10th and the 90th percentile to the 50th percentile. Statistical analyses were performed with Stata Statistical Software (Statacorp, USA), version 12.

RESULTS

Literature search

We identified 3613 publications by database search (PubMed, n=2062; EMBASE, n=516; Web of Science, n=83; ScienceDirect, n=9; CINAHL, n=152; Cochrane, n=791). After checking for duplicates, we retrieved 3395 unique publications. After screening of title and abstract, 3309 studies were excluded and 86 papers were retrieved for detailed assessment. Sixty-three papers did not meet the eligibility criteria, 23 studies were assessed in duplicate for inclusion. Of these papers, several were based on the same cohort. Four papers were based on the ARIC study[12-15], two on the Framingham Heart study[16, 17], three on the Cardiovascular Health study[18-20], three on the Ohasama study[21-23]

and two on a cohort from Finland.[24, 25] To avoid multiple inclusions of the same study subjects, for each study we only included the paper with the largest sample size in our meta-analysis. As a result, nine papers were excluded. Furthermore, six studies were excluded because they presented a measure of HRV that was only used in one or two studies.[23, 26-30] Finally, a total of eight studies were included in this meta-analysis that used SDNN, LF or HF as a measure of HRV (Figure 1).

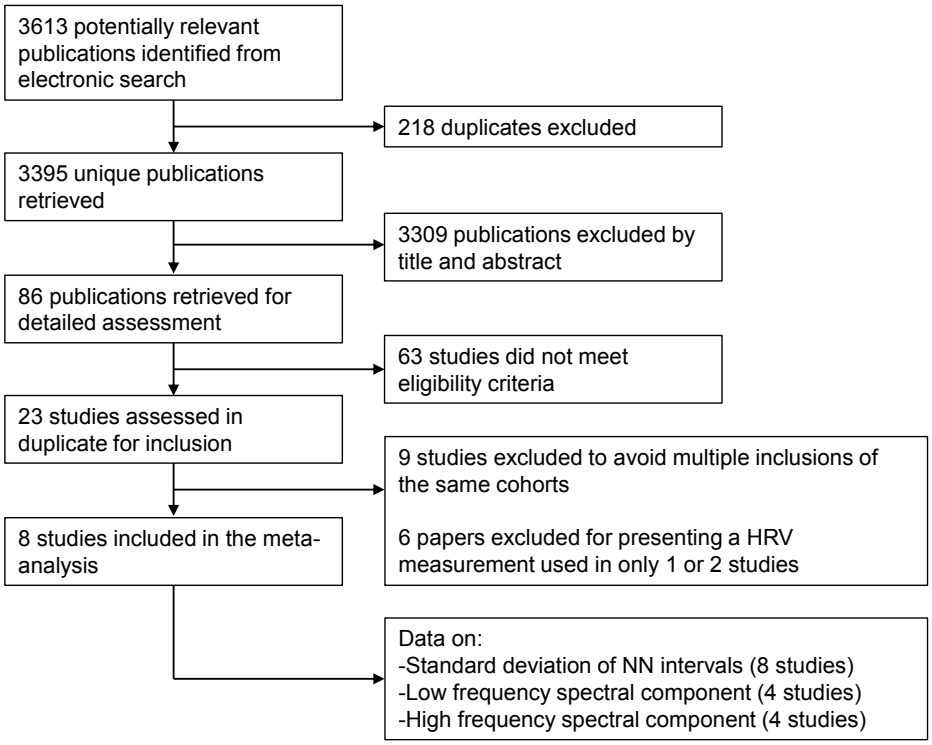

Figure 1. Flow chart of study selection

Study characteristics

Characteristics of the included studies are presented in Table 1. All included studies were prospective cohort studies. Two cohorts presented data separately for diabetics and non-diabetics.[15, 31] A total of 21988 participants were included in the analyses. The size of the study populations ranged from 159 to 11647.[15, 31] The percentage of women varied from 0% to 64%.[32, 33] Mean follow-up duration ranged from 3.5 to 15 years.[17, 32] Four studies defined their endpoint as cardiovascular death.[25, 32-34] Other studies used a combined endpoint, namely: congestive heart failure, hospitalization for angina pectoris and cardiovascular death[17]; MI, coronary revascularization and cardiovascular death[15]; MI, arterial peripheral vascular disease, stroke, congestive heart failure, hospitalization

for angina pectoris and cardiovascular death[20] and MI, coronary revascularization, arterial peripheral vascular disease, stroke and cardiovascular death.[31]

Risk of bias

CVD at baseline was assessed in all studies except in one that did not give a clear description of this assessment.[32] Six studies mentioned loss-to-follow-up; in five of them this was <5%.[15, 20, 31-33] The other study mentioned a loss-to-follow up of 5.6%.[34] Two studies did not give any information about loss-to-follow up.[17, 25] HRV was assessed prior to the outcome in all eight papers. The instrument used to measure NN intervals was clearly described in all studies, but one study lacked data on the assessment of HRV from the recordings.[34] Diagnosis of CVD was verified by medical records, death certificates or general practitioner's registries in all studies. Seven studies adjusted for potential confounders, one study only presented unadjusted risk estimates.[20] For details of adjustments see Table 1.

Standard deviation of NN intervals and CVD

All eight studies reported on the association between SDNN and fatal and nonfatal CVD. Two studies presented data for two separate groups of patients (diabetic and non-diabetic). The reported relative risks of fatal or non-fatal CVD comparing low to high HRV ranged from 0.78 to 1.99. One study reported a relative risk below 1.0[34] and one study an estimate of 1.02.[33] In the other six studies the relative risks were all higher than 1.26. In a random effects meta-analysis, a low SDNN was associated with an increased risk of fatal or non-fatal CVD: RR 1.35 (95% CI 1.10, 1.67). The I_2 of the analysis was 60.2% (Figure 2).

We performed a sensitivity analysis excluding the risk estimates of the two populations with diabetes.[15, 31] This analysis showed a similar pooled relative risk of fatal or non-fatal CVD of 1.32 (95% CI 1.04, 1.68). Furthermore, we distinguished between different measurement durations. This analysis indicated effect modification of the association between HRV and fatal or non-fatal CVD by duration of the measurement, although the numbers of studies per subgroup were small. The association was stronger for HRV measured over 2-24 hours (RR for CVD 1.70, 95% CI 1.33, 2.17) than HRV measurements over 2-3 minutes (RR 1.35, 95% CI 1.08, 1.69) or measurements shorter than approximately 30 seconds (RR 0.97, 95% CI 0.74, 1.28).

Finally, for studies that defined their outcome solely as cardiovascular death (n=4) pooled RR of 1.05 (95% CI 0.83, 1.33) was shown.

Table 1. Study characteristics

	Study population	Measure of HRV	Duration of measurement	Endpoints	N	Mean follow-up (year)	Women (%)	Age range (year)	Ajusted for
Kop 2010	Cardiovascular Health Study, USA	SDNN, LF, HF	24 h	MI, per.vasc, stroke, CHF, hosp. AP., and cardiovasc. death	908	13.3	59	62-80	Not adjusted
Liao 2002	Atherosclerosis Risk in Community Study, USA	SDNN, LF(nu), HF(nu)	2 min	MI, cor. revasc., per. vasc., stroke and cardiovascular death	11647	8	42	48-60	Age, sex, ethnicity, centre, smoking, mean heart rate
Gerritsen 2001	Hoorn study, the Netherlands	SDNN, LF, HF	3 min	Cardiovasc. death	159	7.9*	52	57-71	Age, sex, glucose tolerance status
Makikallio 2001	General population Turku, Finland	SDNN	24 h	Cardiovasc. death	232	10	47	ND	Age, sex. CHF, VES, previous MI, AP, cardiac medication
De Bruyne 1999	Rotterdam Study, the Netherlands	SDNN	10 s	Cardiovasc. death	5272	4	60	60-78	Age, sex, DBP, BMI, DM, negative T-waves, mean R-R interval, Q-Tc interval
Dekker 1997	Zutphen Study, the Netherlands	SDNN	30 s	Cardiovasc. death	878	15	0	40-60	Age, BMI, SBP, total cholesterol, smoking
Bernstein 1997	The Bronx Ageing Study, USA	SDNN	10-40 RR intervals	Cardiovasc. death	391	8.5	64	75-85	Age, sex, DM, hypertension, smoking, history CVD, BMI, cancer, cholesterol, self-health index, medication
Tsuji 1996	Framingham Heart Study, USA	SDNN, LF, HF	2 h	MI, CHF, hosp. AP., and cardiovascular death	2501	3.5	56	26-53	Age, sex, SVP, smoking, DM, SBP, LVH, medication

AP, angina pectoris; BMI, body mass index; cardiovasc. death, cardiovascular death as defined by authors; CHF, congestive heart failure; cor. revasc, coronary revascularization procedures; CVD, cardiovascular disease; DBP, diastolic blood pressure; DM, diabetes mellitus; HF, high-frequency spectral component; hosp. ap, hospitalization for angina pectoris; LF, low-frequency spectral component; LVH, left ventricular hypertrophy; MI, myocardial infarction; ND, not defined; nu, normalized units; per. vasc, arterial peripheral vascular disease; SBP, systolic blood pressure; SDNN, standard deviation of N–N intervals; SVP, supraventricular premature beats; VES, ventricular extra systoles; *, median.

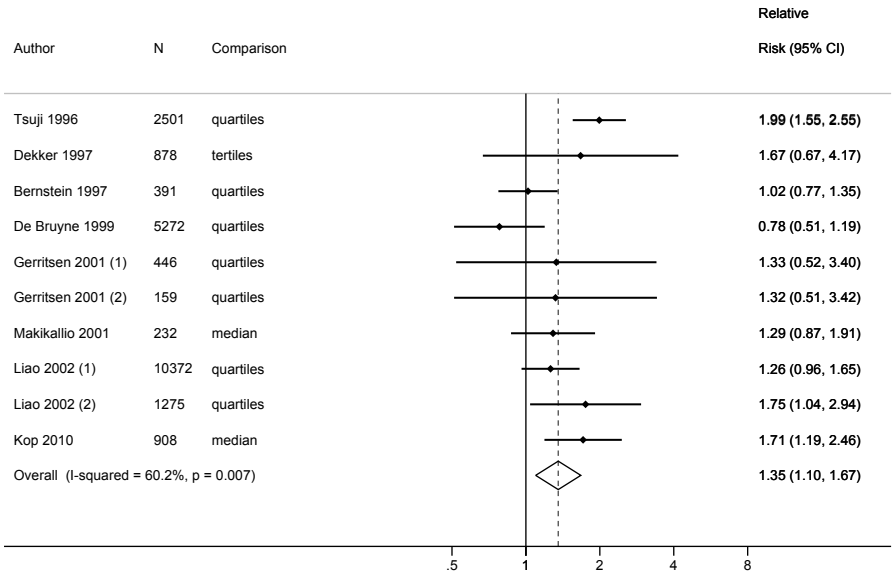

Figure 2. Meta-analysis comparing risk of fatal and non-fatal cardiovascular disease in low versus high heart rate variability measured as standard deviation of NN intervals

Low frequency spectral component and CVD

Four studies used the LF spectral component as a measure of HRV, with a total number of 15215 participants. All these studies used combined endpoints to define CVD. Two studies presented results for the diabetic as well as the non-diabetic population.[15, 31] The RR for the lowest LF compared to the highest LF category ranged from 1.01 to 1.77. The

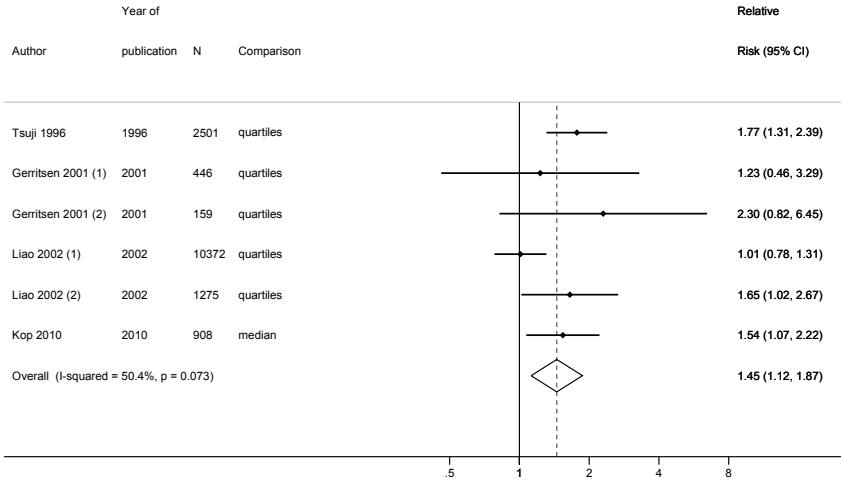

Figure 3. Meta-analysis comparing risk of fatal and non-fatal cardiovascular disease in low versus high heart rate variability measured as low frequency spectral component

pooled RR was 1.45 (95% CI 1.12, 1.87), indicating that a low LF was associated with a higher risk of fatal and non-fatal CVD. I_2 of this analysis was 50.4% (Figure 3).

High frequency spectral component and CVD

The HF spectral component was used as a measure of HRV in the same studies that reported on the LF spectral component. The RRs for the association between HF and HRV ranged from 0.83 to 1.87. Lower HF was associated with a higher risk of fatal and non-fatal CVD (RR: 1.32, 95% CI: 0.96, 1.81), I_2=67% (Figure 4).

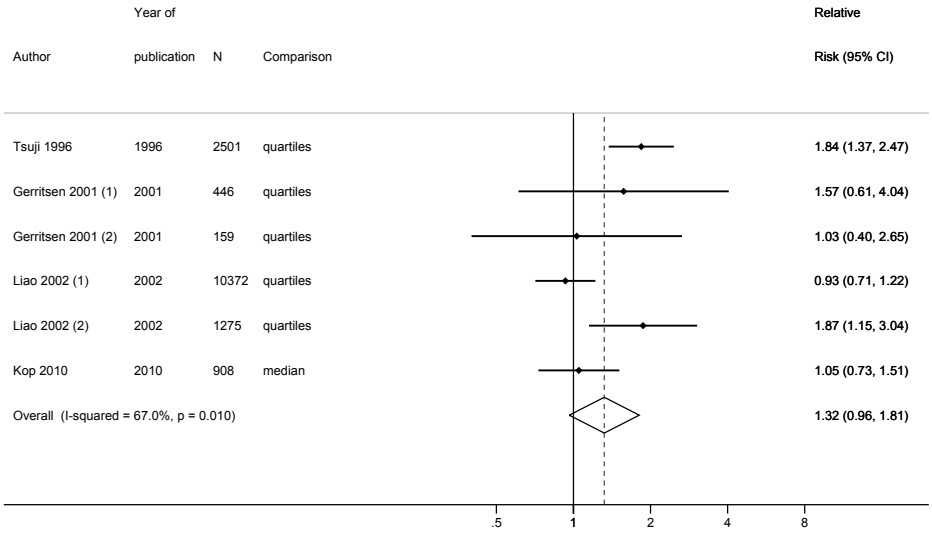

Figure 4. Meta-analysis comparing risk of fatal and non-fatal cardiovascular disease in low versus high heart rate variability measured as high frequency spectral component

Meta-regression of SDNN and CVD

We performed a meta-regression to assess a dose-response relationship between HRV and CVD for studies with SDNN as a measure for HRV. We accepted a linear relationship between HRV and CVD, since the model including a quadratic term did not perform better than the linear model (p from likelihood ratio = 0.75). The random effects meta-regression showed an increasing risk of cardiovascular events for lower HRV (slope = −0.01, p=0.011). The predicted RR of fatal or non-fatal CVD of the 10th HRV percentile compared with the 50th percentile was 1.50 (95% CI 1.22, 1.83); the predicted RR of the 90th percentile compared with the 50th percentile was 0.67 (95% CI 0.41, 1.09). These results indicate that an increase in SDNN of one percent results in an approximately one percent lower risk of the development of fatal or non-fatal CVD (Figure 5).

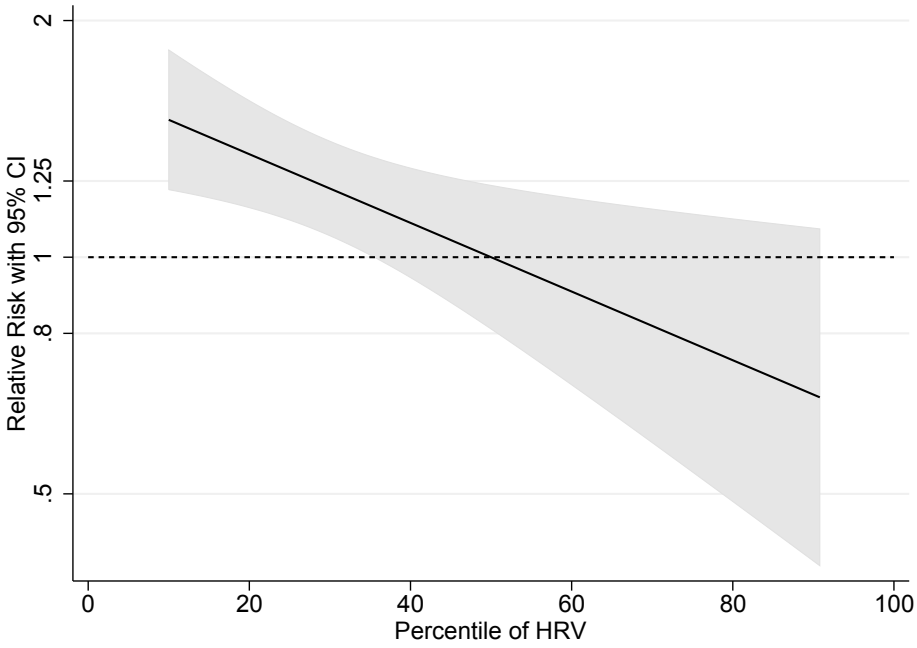

Figure 5. Dose-response meta-regression for the association between heart rate variability measured as standard deviation of NN intervals and fatal and non-fatal cardiovascular disease

DISCUSSION

Main results

The present meta-analysis was performed to assess the association between HRV and a first cardiovascular event in a population without known CVD. The study showed that individuals with low HRV have approximately a 40% increased risk of fatal or non-fatal CVD compared with individuals with high HRV. Our study is the first meta-analysis and dose-response meta-regression to show that low HRV is also associated with CVD in a population without known CVD.

Strengths and limitations

The present study has several strengths. First, studies included in our analyses were cohorts with a large total number of participants, which adds to the robustness of the results. Outcome ascertainment at baseline and verification of endpoints were considered adequate in the majority of the studies. Furthermore, our findings were consistent across three different measures of HRV (SDNN 1.35, LF 1.45 and HF 1.32).

A potential weakness of our study is the adjustment for possible confounders in some studies. One study did not present adjusted RRs.[20] Furthermore, none of the studies adjusted for physical activity and respiration, whereas only one study adjusted for mean heart rate. Studies have been published that suggest that these factors should be taken into account.[35-37]

Another potential limitation is the limited number of included studies, especially in the analysis of the LF and HF spectral component (n=4). Despite the low number of studies however, the direction and magnitude of the pooled RRs of fatal and non-fatal CVD for these two measures of HRV were in line with the pooled risk of the studies using SDNN.

Clinical implications and future research

HRV is the result of changes in heart rate caused by fluctuations in sympathetic and parasympathetic outflow. These fluctuations vary in length. An example of slow fluctuations is circadian variation, whereas faster fluctuations in heart rate are due to sympathetic and parasympathetic responses to minor hemodynamic changes. Respiration causes a cyclic variation in intrathoracic pressure and thereby venous return, which leads to fluctuations in cardiac output and arterial blood pressure. The arterial baroreflex reacts to these fluctuations in arterial blood pressure by producing fluctuations in parasympathetic and sympathetic autonomic outflow that induce compensatory modulations in peripheral resistance, cardiac contractility and heart rate.[2, 9] Reduced compensatory change (low HRV) suggests a less adaptive autonomic nervous system. However, the physiological interpretation of HRV, especially in the LF spectral band, remains controversial. Whereas the dependence of HF power on respiration-related parasympathetic outflow has repeatedly been confirmed, there are indications that LF power is not a measure of sympathetic outflow. Some investigators have found that LF is a reflection of baroreflex function instead of cardiac sympathetic innervation.[38]

It is known that after a cardiovascular event, individuals with lowered HRV have an increased risk of cardiovascular morbidity and mortality compared to subjects with high HRV.[3-7, 39, 40] These results indicate that a less adaptive autonomic nervous system is a marker of cardiovascular risk. Furthermore, it shows that the autonomic nervous system becomes less sensitive for minor hemodynamic changes after a first cardiac event, which might be a direct cause of a secondary cardiovascular event. Our meta-analysis shows that HRV is also associated with cardiovascular morbidity and mortality in the general population. This suggests that decreased fluctuations in heart rate, caused by inadequate autonomic nervous system circulatory control, is also a sign of less favourable health in otherwise healthy individuals. Furthermore, our results seem to show effect modification of the association between HRV and CVD by duration of the

measurement. This finding might indicate that a decrease in long term fluctuations in heart rate is more important for the risk of CVD than short term fluctuations. However, since the numbers our stratified analyses are very small, this result must be interpreted with caution.

A possible mechanism underlying our findings is low grade inflammation. It has been suggested that autonomic imbalance could activate inflammation by influencing the bone marrow and lymphoreticular system.[41] Increased inflammation is associated with higher risk of CVD. Another possible explanation for the association between HRV and a first cardiovascular event is that individuals with low HRV already suffer from subclinical or silent cardiovascular disease. These individuals are expected to have both a lower HRV and a higher risk of a following CVD event.

HRV measurement is inexpensive, easy to assess and non-invasive. It is potentially a useful predictor of CVD in both healthy and diseased individuals. Improved identification of individuals at risk for the development of CVD may result in more targeted and adequate prevention strategies. Future research should focus on the clinically applicability of the existing knowledge about HRV, for example by aiming to find cut-off points and determine the value of HRV in addition to established risk factors. When investigating HRV for risk stratification in clinical practice, the possible influence of respiration and mean heart rate should be taken in account.

Conclusion

In conclusion, our meta-analysis shows that individuals with a low HRV have 32-45% increased risk of CVD compared with individuals with a high HRV. Our study is the first meta-analysis to show that low HRV is also associated with a first cardiovascular event in a population without known CVD. It therefore supports the hypothesis that cardiac autonomic dysfunction is associated with a higher risk of CVD. Whether HRV might be clinically useful in individual risk identification should be assessed in future research.

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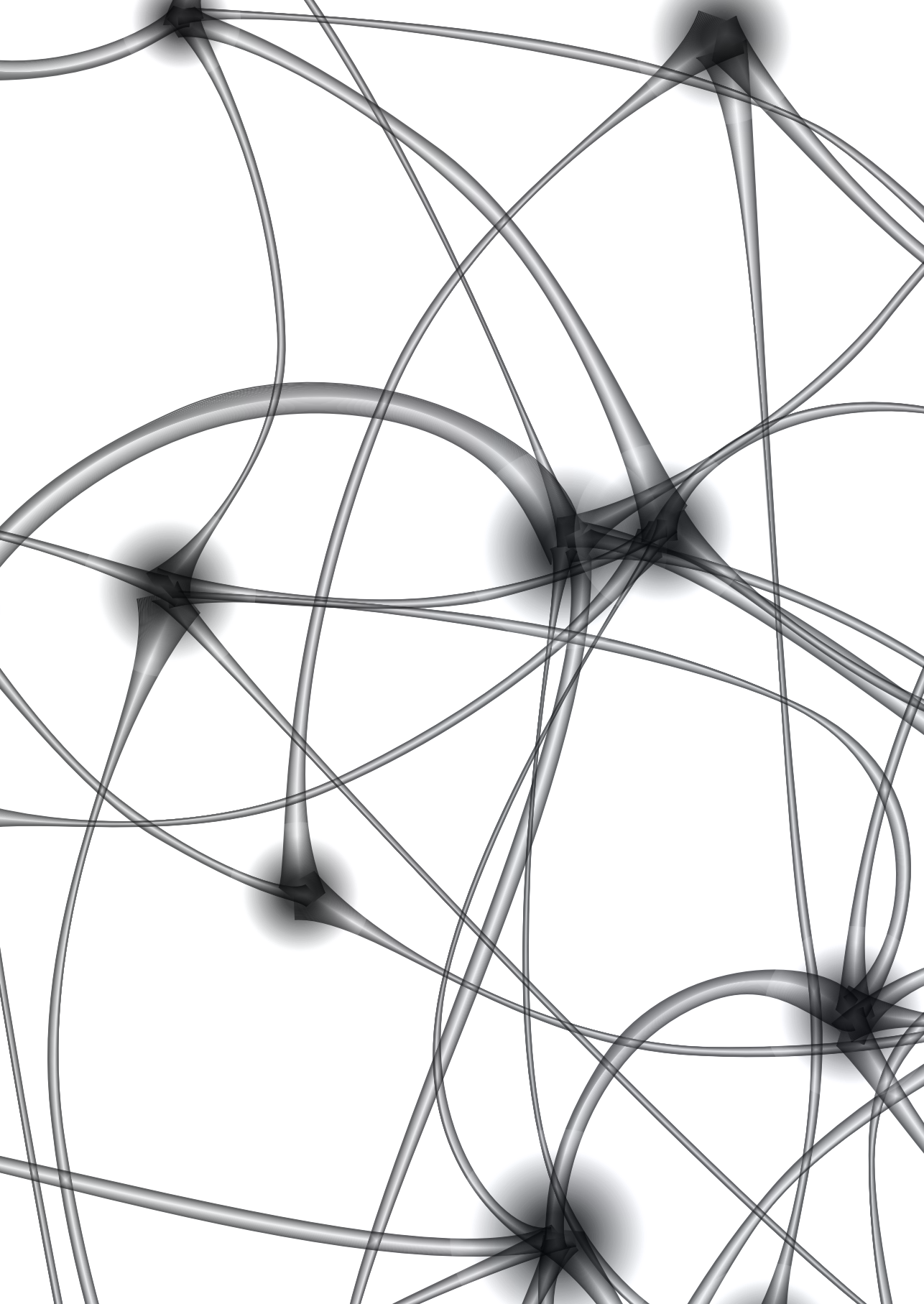

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Sympathetic nervous system activation and lipid metabolism: the NEO study

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ABSTRACT

Introduction

Mouse studies have revealed an important role for the sympathetic nervous system (SNS) in lipid metabolism, which may offer novel therapeutic modalities to treat dyslipidaemia. However, the role of the SNS in lipid metabolism in humans is insufficiently studied. Therefore, we aimed to study the association between SNS activation, measured as heart rate or heart rate variability, and serum triglyceride, non-high-density lipoprotein concentrations, high-density lipoprotein concentrations, and intrahepatic triglyceride content.

Methods

This is a cross-sectional analysis of baseline data of the Netherlands Epidemiology of Obesity study. Heart rate was estimated from a 10 second 12-lead electrocardiogram and parameters of heart rate variability were calculated over 24 hours. Fasting serum cholesterol concentrations were measured. Serum triglycerides were measured after fasting and 30 and 150 min after a liquid mixed meal. Intrahepatic triglyceride content was measured by ^{1}H-magnetic resonance spectroscopy. We performed linear regression analyses and adjusted for age, sex, smoking, education, physical activity, meal time, heart rate lowering medication, lipid lowering medication, body mass index, total body fat and HOMA-IR.

Results

After exclusion of individuals with missing data (n=496), 6174 participants were included: 44% men, mean (SD) age: 56 (6) years and mean BMI 26 (4) kg/m^2. Per 10 beats/min, total cholesterol concentration was 0.06 mmol/L (95% CI: 0.02, 1.10) higher and fasting serum triglyceride concentration was 5.8% (3.9, 7.8) higher. Resting heart rate was not associated with serum HDL-cholesterol or LDL-cholesterol concentrations. Postprandial triglyceride concentrations were 5.2% (3.5, 6.9) higher 30 minutes, and 4.4% (2.6, 6.3) higher 150 minutes after the liquid mixed meal. Intrahepatic triglyceride content was 13.1% (7.7, 18.7) higher after adjustment for confounding factors. Associations with heart rate variability were similar.

Conclusion

SNS activation, measured as higher resting heart rate or lower heart rate variability, was associated with higher fasting total cholesterol and higher serum triglyceride concentrations, and with higher intrahepatic triglyceride content. These results imply that the SNS is involved in lipid metabolism in humans, and that dyslipidaemia may be targeted by lowering SNS activation

INTRODUCTION

The autonomic nervous system is an involuntary nervous system with a sympathetic and a parasympathetic branch. Heart rate is a reflection of autonomic nervous system function (sympathovagal balance) that can be estimated as the intrinsic heart rate multiplied by a sympathetic factor and a vagal factor.[1, 2] Heart rate variability is a manifestation of activity of the baroreflex, which purpose is to buffer blood pressure by adaptions in sympathetic and parasympathetic outflow.[3, 4] A shift in sympathovagal balance towards increased activity of the sympathetic nervous system (SNS) results in a higher heart rate and lower heart rate variability, which are associated with both cardiovascular risk factors and cardiovascular events.[5-8] The mechanism underlying the increased cardiovascular risk due to SNS activation has not been elucidated. Since dyslipidaemia is a prominent risk factor for cardiovascular disease, alterations in lipid metabolism possibly play a role.

Lipid metabolism is influenced by the SNS in several ways. First, the SNS innervates the adipose tissue.[9] Sympathetic stimulation of adipose tissue induces intracellular lipolysis, thereby increasing the amount of glycerol and free fatty acids in the circulation.[10] Second, sympathetic stimulation of the liver enhances hepatic de novo lipogenesis.[11] Experimental studies showed that selective sympathetic denervation of the liver in rats decreased both hepatic lipogenesis and decreased hepatic very low-density lipoprotein (VLDL)-triglyceride secretion.[12, 13] This indicates that activation of the SNS stimulates both lipogenesis and the secretion of VLDL-triglycerides in the liver.

In healthy individuals, activation of the SNS is an adaptive response, aimed at temporarily providing lipids as a substrate for energy metabolism in muscles during fasting or 'flight-fight-or-fright actions'. However, chronic SNS activation may result in an prolonged increased lipid production, by stimulation of lipolysis in adipose tissue and of de novo lipogenesis in the liver, even if lipids are not necessary as a substrate for energy metabolism. This may result in higher fasting serum triglycerides and non-high-density lipoprotein (non-HDL) cholesterol concentrations and possibly a lower HDL-cholesterol concentration in the circulation (Figure). Some previous studies have reported these associations.[14-16] However, no previous study has investigated the association between heart rate and postprandial serum triglyceride concentrations after a mixed meal. Furthermore, in addition to an increased concentration of lipids in the circulation, the chronic activation of the SNS may also result in accumulation of lipids in the liver.[17] Evidence on this association is also lacking. Therefore, we hypothesized that SNS activation is associated with a disadvantageous lipid metabolism, specifically with triglyceride levels in the circulation and the liver. To this extent, we studied the associa-

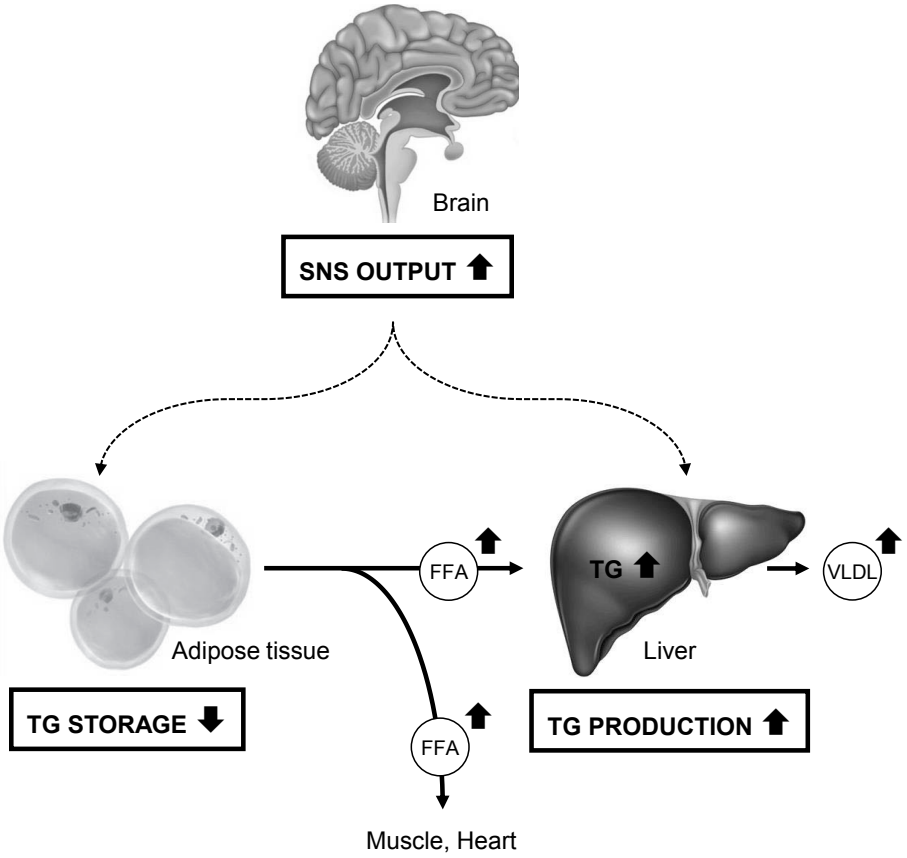

Figure. Sympathetic control of lipid metabolism
SNS=sympathetic nervous system; TG=triglyceride; FFA=free fatty acid; VLDL=very low-density lipoprotein; LDL=low-density lipoprotein

tions of resting heart rate and heart rate variability with fasting and non-fasting serum lipid concentrations and intrahepatic triglyceride content in a population-based study.

METHODS

Study design and population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based prospective cohort study of 6673 individuals aged 45 to 65 years with an oversampling of individuals with a BMI ≥ 27 kg/m^2. The study design and data collection of the NEO study have been described previously.[18] In short, persons with a self-reported BMI of 27 kg/m^2 or higher were included between September 2008 and October 2012 from the greater area of

Leiden, the Netherlands. In addition, all inhabitants from one municipality (Leiderdorp) were invited irrespective of their BMI, allowing for a reference distribution of BMI. The present study is a cross-sectional analysis using the baseline measurements of the NEO study.

Participants were invited for a baseline visit at the NEO study centre of the Leiden University Medical Centre. Prior to this study visit, participants fasted overnight for at least 10 hours and completed a questionnaire on demographic, lifestyle, and clinical data in addition to questions on diet and physical activity. The participants were asked to bring all their current medication to the study centre. At the baseline visit, the participants underwent an extensive physical examination, including fasting and postprandial blood sampling, and a resting standard 10-second electrocardiogram (ECG). Heart rate variability and intrahepatic triglyceride content were measured in random subsamples of the baseline population. The NEO study was approved by the Medical Ethical Committee of the Leiden University Medical Centre. Before inclusion, all participants gave their informed consent.

Data collection

Participants reported their highest level of education in 10 categories according to the Dutch educational system. We defined low education as no education, primary education or lower vocational training) and used high education as the reference category in the analyses. Self-identified ethnicity of participants was reported in eight categories that we grouped into white (reference) and other. Tobacco smoking was categorized into current, former or never (reference) smokers. We estimated alcohol intake in grams per week by questionnaire. We measured physical activity using the Short Questionnaire to Assess Health-enhancing physical activity questionnaire and calculated the metabolic equivalent of task (MET) hours per week.[19]

We measured height and weight without shoes and one kilogram was subtracted to correct for the weight of clothing. Body Mass Index (BMI) was calculated by dividing the weight in kilograms by the height in meters squared. Total body fat was measured with a bio-impedance device (TBF-310, Tanita International Division, United Kingdom). Blood was sampled from the antecubal vein after an overnight fast of at least 10 hours, and 30 minutes and 150 minutes after consumption of a liquid mixed meal (400 mL containing 600 kcal, 16 % of energy (En%) derived from protein, 50 En% carbohydrates, and 34 En% fat). In the fasting blood samples, serum concentrations of triglycerides and total cholesterol were measured with the colorimetric method and HDL-cholesterol concentrations were measured with the homogenous HDLc method (Roche Modular Analytics P800, Roche Diagnostics, Mannheim, Germany). LDL-cholesterol concentra-

tions were calculated with the Friedewald formula.[20] Serum triglyceride concentrations were also measured 30 minutes and 150 minutes after the liquid mixed meal. Plasma glucose concentrations were measured with the enzymatic and colorimetric method (Roche Modular Analytics P800, Roche Diagnostics, Mannheim, Germany) and serum insulin concentrations were determined by an immunometric method (Siemens Immulite 2500, Siemens Healthcare Diagnostics, Breda, the Netherlands). Serum insulin concentrations below the detection limit of the assay (2.0 mU/L) were imputed using multiple imputation methods for left censored data, with 10 imputation datasets.[21] We calculated the updated Homeostasis Model Assessment Insulin Resistance (HOMA-IR), by entering fasting glucose and fasting insulin in a Microsoft Excel spreadsheet available on the internet.[22]

Heart rate and heart rate variability

To estimate the heart rate, a resting 12-lead ECG was obtained using a Mortara Eli-350 electrocardiograph (Mortara Instrument Inc., Best, Netherlands). Mean resting heart rate was calculated using the Matlab-based program BEATS.[23]

The Actiheart device, an accelerometer combined with a heart rate monitor (CamNtech Ltd, Cambridge, United Kingdom), was worn by a random subsample (14%) of the baseline population. We used the data from the heart rate monitor to calculate heart rate variability parameters. We selected only the Actiheart recordings with the least noise (<1%) for manual reparation and a valid estimation of heart rate variability. The heart rate data were processed to remove artefacts and non-sinus beats, and consequently heart rate variability was calculated over a 24 hour period according to a procedure that was described and applied previously.[2, 24, 25] In short, heart rate variability was calculated using a 5-minute moving window and all valid 5-minute values were averaged. We calculated the mean interbeat interval, the standard deviation of all normal intervals (SDNN) and the root mean square of the successive differences (RMSSD). Prior to spectral analysis of the recordings, the tachogram was processed with adjustment for linear trends, tachogram tapering and zero padding. A test of stationarity was performed, and we excluded non-stationary episodes from the analysis. For calculation of low frequency power (LF power) and high frequency power (HF power), a fast Fourier transformation was employed.

Intrahepatic triglyceride content

A random subsample (35%) of participants without contra-indications underwent proton (1H)-MR spectroscopy of the liver to assess hepatic triglyceride content. All imaging was performed on an MR system operating at a field strength of 1.5 Tesla (Philips Medical Systems, Best, Netherlands). Hepatic ^{1}H-MR spectra were obtained as

described previously.[26] In short, ^{1}H-MRS (magnetic resonance spectroscopy) of the liver was performed with an 8 mL voxel positioned in the right lobe of the liver, avoiding gross vascular structures and adipose tissue depots. Sixty-four averages were collected with water suppression. Spectra were obtained with an echo time of 26 milliseconds and a repetition time of 3000 milliseconds. Data points (1024) were collected using a 1000 Hz spectral line. Without changing any parameters, spectra without water suppression, with a repetition time of 10 seconds, and with four averages were obtained as an internal reference. ^{1}H-MRS data were fitted using Java-based magnetic resonance user interface software (jMRUI version 2.2, Leuven, Belgium), as described previously.[27] Intrahepatic triglyceride content relative to water was calculated as (signal amplitude of triglyceride) / (signal amplitude of water) * 100.

Statistical analyses

In the NEO study, individuals with a BMI of 27 kg/m^2 or higher were oversampled. To correctly represent associations in the general population, adjustments for the oversampling of individuals with a BMI ≥ 27 kg/m^2 were made.[28] This was done by weighting individuals towards the BMI distribution of participants from the Leiderdorp municipality, whose BMI distribution was similar to the BMI distribution of the general Dutch population.[29, 30] All results were based on weighted analyses. Consequently, the results apply to a population-based study without oversampling of participants with a BMI ≥ 27 kg/m^2.

Baseline characteristics were expressed as mean (standard deviation), median (25th- 75th percentiles) or as percentage by tertiles of heart rate. We performed weighted linear regression analysis with heart rate per 10 beats/min or heart rate variability per standard deviation as a determinant. As outcome variables we used total cholesterol, LDL-cholesterol, HDL-cholesterol, fasting and postprandial (30 min and 150 min) serum triglyceride concentrations (mmol/L) and intrahepatic triglyceride content (%). The serum triglyceride concentrations and intrahepatic triglyceride content were ln-transformed. Regression coefficients were expressed as mmol/L or as percentage difference with 95% confidence intervals for ln-transformed variables. We adjusted our results for age, sex, tobacco smoking, education, physical activity, heart rate lowering medication and lipid lowering medication. The analyses including intrahepatic triglyceride content were also adjusted for time since ingestion of the mixed meal and alcohol intake. Additionally we adjusted for BMI and total body fat, and in a last step for HOMA-IR.

RESULTS

Baseline characteristics

The NEO study included 6673 participants. We consecutively excluded participants with missing data on heart rate (n=89), lipid concentrations (n=44), education (n=64), physical activity (n=116), body composition (n=31), HOMA-IR (n=148) and other confounders (n=7). The main analyses therefore included 6174 participants. Compared with excluded participants, included participants were more often men (44% versus 40%). Other variables were comparable: age was 56 (6) years and BMI was 26 (4) kg/m^2 in both excluded and included participants. Total cholesterol was 5.6 (1.1) mmol/L in excluded and 5.7 (1.1) mmol/L in included participants and fasting serum triglyceride concentration was 1.1 (0.8) mmol/L versus 1.2 (0.8) mmol/L.

Table 1 shows the weighted baseline characteristics of the 6174 participants included in our analyses, stratified by tertiles of heart rate. Participants with a higher heart rate had more body fat and were more often women.

Table 1 Characteristics of the study population by tertiles of heart rate

Beats/min	**30-59**	**60-67**	**68-157**
Age (year)	55 (6)	55 (6)	56 (6)
Sex (% men)	51	41	39
BMI (kg/m^2)	26 (4)	26 (4)	27 (5)
Total body fat (%) - Men - Women	 24 (5) 36 (6)	 26 (6) 37 (7)	 28 (7) 40 (7)
Tobacco smoking - Current (%) - Former (%)	 17 45	 16 48	 15 43
Education level (% low)	17	19	23
MET hour/week	124 (84-161)	123 (79-157)	109 (68-143)
Heart rate lowering medication (%)	14	11	11
Lipid lowering medication (%)	10	10	14
Fasting triglycerides (mmol/L)	1.0 (0.7-1.3)	1.0 (0.7-1.5)	1.2 (0.8-1.7)
30 min triglycerides (mmol/L)	1.1 (0.9-1.5)	1.2 (0.9-1.7)	1.4 (1.0-1.9)
150 min triglycerides (mmol/L)	1.6 (1.1-2.1)	1.6 (1.1-2.3)	1.8 (1.3-2.6)
Total cholesterol (mmol/L) LDL cholesterol (mmol/L)	5.6 (1.0) 3.5 (0.9)	5.7 (1.0) 3.6 (1.0)	5.8 (1.1) 3.6 (1.0)
HDL cholesterol (mmol/L)	1.6 (0.5)	1.6 (0.5)	1.5 (0.4)
HOMA-IR	0.5 (0.3-0.9)	0.6 (0.3-1.1)	0.8 (0.5-1.4)

Data are expressed as mean (standard deviation), mean (25th-75th percentile) or percentage
Results are based on weighted analyses (n=6174)

Heart rate and cholesterol

Per 10 beats/min, the total cholesterol concentration was 0.09 mmol/L higher (95% CI 0.05, 0.13) (Table 2). After adjustment for confounding factors, including BMI, total body fat and HOMA-IR, the difference was 0.06 mmol/L (0.02, 1.10). There was a positive association between heart rate and LDL-cholesterol concentration, and an inverse association between heart rate and HDL-cholesterol concentration. These associations attenuated after adjustment for confounding variables.

Table 2 Difference in cholesterol concentrations per 10 bpm in heart rate

		Total cholesterol (mmol/L)	HDL cholesterol (mmol/L)	LDL cholesterol (mmol/L)
Model 1	Crude	0.09 (0.05, 0.13)	−0.02 (−0.03, 0.00)	0.04 (0.01, 0.08)
Model 2	+ age, sex	0.06 (0.03, 0.10)	−0.04 (−0.06, −0.03)	0.03 (0.00, 0.07)
Model 3	+ confounders*	0.08 (0.04, 0.11)	−0.04 (−0.05, −0.02)	0.04 (0.01, 0.08)
Model 4	+ BMI, TBF	0.07 (0.03, 0.11)	−0.01 (−0.02, 0.00)	0.02 (−0.01, 0.06)
Model 5	+ HOMA-IR	0.06 (0.02, 0.10)	0.00 (−0.01, 0.02)	0.02 (−0.02, 0.05)

*Confounders: smoking, education, physical activity, heart rate lowering medication and lipid lowering medication

BMI, body mass index; TBF, total body fat; HOMA-IR, updated Homeostasis Model Assessment Insulin Resistance

Results are based on weighted analyses (n=6174)

Heart rate and triglycerides

Heart rate was associated with fasting serum triglyceride concentration (Table 3). Per 10 beats/min, fasting serum triglyceride concentration changed with 7.8% (5.4, 10.3). After adjustments for confounders this was 5.2% (3.5, 6.9). Heart rate was also associated with postprandial serum triglyceride concentrations (30 minutes and 150 minutes after a liquid mixed meal) in multivariate analyses. However, when we additionally adjusted for fasting serum triglyceride concentration, these results attenuated (Table 3).

Heart rate and intrahepatic triglyceride content

In the NEO study, a random 2354 participants underwent magnetic resonance spectroscopy of the liver. After exclusion of missing data on heart rate (40), intrahepatic triglyceride content (n=266), education (n=17), physical activity (n=44), HOMA-IR (n=53) and other confounders (n=5), the analyses on intrahepatic triglyceride content included 1929 participants. Included participants were more often male (47%) than excluded participants (42%) and had a slightly higher intrahepatic triglyceride content (2.8% (1.4-6.4) versus 1.9% (1.1-5.0)). Age and body mass index were not different.

Heart rate was associated with intrahepatic triglyceride content. Per 10 beats/min, intrahepatic triglyceride content changed with 20.3% (13.0, 28.1). Part of this association was explained by confounders including BMI, total body fat and HOMA-IR. After all adjustments, this difference was 13.1% (7.7, 18.7) per 10 beats/min (Table 4).

Table 3 Percentage difference in triglyceride concentrations per 10 bpm in heart rate

		Fasting	30 minutes	150 minutes
Model 1	Crude	7.8 (5.4, 10.3)	6.6 (4.6, 8.7)	5.8 (3.6, 8.0)
Model 2	+ age, sex	9.4 (7.0, 11.8)	8.0 (5.9, 10.0)	7.3 (5.2, 9.5)
Model 3	+ confounders*	9.5 (7.2, 11.9)	8.1 (6.1, 10.1)	7.4 (5.3, 9.5)
Model 4	+ BMI, TBF	7.0 (4.7, 9.3)	6.1 (4.1, 8.0)	5.4 (3.4, 7.5)
Model 5	+ HOMA-IR	5.9 (3.9, 8.0)	5.2 (3.5, 6.9)	4.4 (2.6, 6.3)
Model 6	+ fasting TG		0.6 (−0.1, 1.2)	−0.1 (−1.0, 0.7)

*Confounders: smoking, education, physical activity, heart rate lowering medication and lipid lowering medication

BMI, body mass index; TBF, total body fat; HOMA-IR, updated Homeostasis Model Assessment Insulin Resistance; TG, triglycerides

Results are based on weighted analyses (n=6174)

Table 4 Percentage difference in intrahepatic triglyceride content per 10 bpm in heart rate

		Intrahepatic triglyceride content
Model 1	Crude	20.3 (13.0, 28.1)
Model 2	+ age, sex	22.7 (15.5, 30.4)
Model 3	+ confounders*	21.5 (14.5, 29.0)
Model 4	+ BMI, TBF	13.5 (7.7, 19.5)
Model 5	+ HOMA-IR	13.1 (7.7, 18.7)

*Confounders: smoking, education, physical activity, heart rate lowering medication and lipid lowering medication, meal time, alcohol consumption

BMI, body mass index; TBF, total body fat; HOMA-IR, updated Homeostasis Model Assessment Insulin Resistance

Results are based on weighted analyses (n=1929)

Heart rate variability and lipids

Of the 6673 participants included in the NEO study, an Actiheart device was carried by 955 participants. In 50 of this 955, the recording failed due to technical problems (e.g. broken battery, detached electrodes) or incorrect use by the participant (e.g. early removal). From the 905 remaining recordings, we selected 485 recordings with maximum quality of IBI data (<1% artefacts) to use for heart rate variability analysis. After exclusion of participants with missing data on education (n=5), physical activity (n=4), HOMA-IR (n=9) and other confounders (n=2), the present analysis included 465

participants. Of this subgroup, 147 participants were also included in the subgroup with data on intrahepatic triglyceride content. Included participants were comparable with excluded participants, only the percentage men was higher (56% in included versus 43% in excluded participants).

The associations between heart rate variability parameters and lipid concentrations in the circulation were comparable to the associations using heart rate as a determinant. Increased heart rate variability (reflecting reduced SNS activity) was associated with lower total cholesterol and serum triglyceride concentrations. Unfortunately, due to the smaller sample size, these results were all non-significant (Appendix).

DISCUSSION

This is the first study that shows a positive association between activation of the SNS, measured by heart rate and heart rate variability, and intrahepatic triglyceride content. Furthermore, we identified an association of SNS activity not only with fasting serum triglyceride concentrations, but also with serum triglycerides concentrations 30 and 150 minutes after a standardized mixed meal. Finally, we observed a small positive association between heart rate and serum fasting total cholesterol concentrations. The positive association of heart rate with LDL-cholesterol, and the inverse association with HDL-cholesterol attenuated after adjustment for confounders. The results from this study suggest that sympathetic nervous system activity is associated with alterations in lipid metabolism.

Studies in rodents clearly indicate as role for the autonomic nervous system in lipid metabolism, as vagatomy increases cholesterol concentrations in the circulation, whereas sympathectomy decreases cholesterol concentrations.[31] However, previous studies on activation of the SNS and cholesterol concentrations in humans have produced conflicting results. We found a positive association of heart rate, reflecting SNS activity, with total cholesterol. This has also been shown in some earlier studies.[14-16] We did not find an association of heart rate with LDL-cholesterol and HDL-cholesterol after adjustment for confounding variables. This is contradicting some studies,[14-16] but is in line with another study.[32] In line with our results, some earlier studies observed an association between activity of the SNS and serum triglyceride concentrations.[14, 16, 33] No study has yet evaluated the association between heart rate and postprandial serum triglycerides concentrations 30 and 150 minutes after a liquid mixed meal. We showed that heart rate was also associated with postprandial triglyceride concentrations, but that this association could be explained by higher fasting triglyceride concentrations

with higher heart rate. In addition to cross-sectional studies, one study prospectively investigated the relation of heart rate with changes in serum triglyceride and HDL-cholesterol concentrations over two years.[34] An association between heart rate and a decrease in HDL-cholesterol concentrations was observed in this study, possibly indicating that increased SNS activation precedes alterations in lipid metabolism. However, no prospective association between heart rate and serum triglycerides concentrations was found. Only one previous study investigated the association of SNS activation, measured as low heart rate variability, with intrahepatic triglyceride content and reported lower heart rate variability in individuals with non-alcoholic fatty liver disease compared with controls.[35] We extend this finding by showing a positive association between heart rate and intrahepatic triglyceride content in the general population. Per 10 beats/min in heart rate, the difference in intrahepatic triglyceride content was 13.1% (7.7, 18.7), independent of confounders including body mass index, total body fat and HOMA-IR.

The most important strength of the present study is the study size of 6174 participants with data on lipid concentrations in the circulation. Especially the association of heart rate with postprandial serum triglyceride concentrations after a standardized liquid mixed meal has not been published yet. Since most individuals are in a postprandial state for the larger part of the day, and elevated postprandial serum triglyceride concentrations are associated with increased cardiovascular risk, this seems to be an important finding.[36, 37] Furthermore, intrahepatic triglyceride content measured by MRS, was available in 1929 participants. This technique is expensive and time-consuming, and therefore usually not available in large cohorts such as the NEO study. A limitation of our study is the cross-sectional design which may have resulted in residual confounding and reverse causation. Because parameters of SNS activation and lipid concentrations were assessed at the same time, we cannot make any statements on the longitudinal association between both variables. Furthermore, the absence of the measurement of free fatty acids is a limitation. Another limitation of the present study may be that we did not measure brown adipose tissue volume present in the body or any markers for its activity. Besides an effect on white adipose tissue and the liver, stimulating lipolysis and de novo lipogenesis, the SNS also innervates brown adipose tissue. SNS activation stimulates the uptake of VLDL-triglycerides in brown adipose tissue for production of heat through the mechanism of uncoupling, i.e. non-shivering thermogenesis.[38] As a result, SNS activation of brown adipose tissue may (partly) counteract the triglyeride-raising and cholesterol-raising effect of SNS activation of white adipose tissue and liver. On the other hand, a divergence of autonomic nervous system activity towards different organs has also been proposed, implying that increased SNS activity to white adipose tissue or the liver does not necessarily have to be accompanied by increased activity towards brown adipose tissue, especially not at room temperature.[39]

Stimulation of the SNS seems to be regulated by the hypothalamus, for example by release of neuropeptide Y.[12] In addition, peripherally produced hormones such as leptin and glucagon-like peptide-1 may stimulate the hypothalamus and consequently the SNS.[40, 41] Activation of the SNS influences lipid metabolism in several ways. Experimental studies showed that selective sympathetic denervation of the liver in rats decreased both hepatic lipogenesis and decreased hepatic VLDL-triglyceride secretion.[12, 13] This indicates that SNS activation to the liver stimulates both lipogenesis and the secretion of VLDL-triglycerides. Furthermore, in white adipose tissue, stimulation of the SNS induces lipolysis, thereby increasing the amount of glycerol and free fatty acids in the circulation.[10] Since in a fasting state most triglycerides in the circulation are produced by the liver, increased lipogenesis could explain our results on heart rate and the serum fasting triglyceride concentration.

On the mechanism underlying the positive association we found between heart rate and intrahepatic triglyceride content we can only speculate. In rats, sympathetic hepatic denervation or adrenalectomy decreased VLDL-triglyceride production, but increased intrahepatic triglyceride content.[13] This finding is not in line with our results, that show a higher intrahepatic triglyceride content with a higher heart rate. Increased SNS activation could possibly induce the accumulation of intrahepatic triglyceride by the secretion of leptin and the inhibition of adiponectin. High leptin and low adiponectin are associated with both a higher SNS activation and a higher intrahepatic triglyceride content.[43]

Postprandial serum concentrations of triglycerides in the circulation are strongly influenced by insulin resistance. Insulin stimulates the expression of lipoprotein lipase in adipose tissue, which results in increased uptake and storage of free fatty acids in adipocytes.[42] In an insulin resistant state, clearance of lipids from the circulation is therefore inhibited. However, in our study we still observed approximately 5% higher postprandial serum triglyceride concentrations 30 and 150 minutes after a meal, independent of insulin resistance measured as HOMA-IR. This may reflect an association between SNS activation and serum postprandial triglyceride concentrations independent of insulin resistance. Triglycerides, especially postprandial serum triglycerides concentrations, are strongly associated with cardiovascular risk.[36, 37] Therefore, elevated postprandial serum triglycerides concentrations may be a mechanism underlying the association between increased SNS activation and cardiovascular disease.

The strength of the associations of heart rate with cholesterol concentrations is modest. The difference in total cholesterol per 10 beats/min was 0.06 mmol/L after adjustments for confounders. This difference is clinically irrelevant. The observed associations of

heart rate with serum triglyceride concentrations and intrahepatic triglyceride content, however, may be of clinical relevance. Per 10 beats/min, fasting triglyceride concentrations were 5.8% higher. For a participant with a fasting triglyceride concentration of 1.0 mmol/L, this would imply an additional 0.06 mmol/L, independent of all confounding variables. The difference in intrahepatic triglyceride content was 13.1% per 10 beats/min in heart rate. In a participant with 5% intrahepatic triglycerides, this would correspond to 0.7% more liver fat.

In conclusion, SNS activation, measured as higher resting heart rate or lower heart rate variability, was associated with higher fasting total cholesterol, higher fasting and postprandial serum triglyceride concentrations, and with higher intrahepatic triglyceride content. These results imply that the SNS is involved in lipid metabolism in humans, and that dyslipidaemia may be targeted by lowering SNS activation. Future research should elucidate whether abnormalities in lipid profile are a mechanism underlying the association between SNS activation and cardiovascular disease.

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APPENDIX

E-Table 1 Difference in cholesterol concentrations per standard deviation in SDNN

		Total cholesterol (mmol/L)	HDL cholesterol (mmol/L)	LDL cholesterol (mmol/L)
Model 1	Crude	−0.02 (−0.14, 0.09)	0.00 (−0.06, 0.06))	0.01 (−0.11, 0.13)
Model 2	+ age, sex	0.05 (−0.09, 0.18)	0.04 (−0.02, 0.10)	0.06 (−0.06, 0.19)
Model 3	+ confounders*	0.07 (−0.06, 0.20)	0.03 (−0.02, 0.09)	0.08 (−0.05, 0.20)
Model 4	+ BMI, TBF	0.08 (−0.05, 0.20)	0.02 (−0.03, 0.07)	0.09 (−0.03, 0.21)
Model 5	+ HOMA-IR	0.09 (−0.04, 0.21)	0.00 (−0.05, 0.06)	0.10 (−0.03, 0.22)

*Confounders: smoking, education, physical activity, heart rate lowering medication and lipid lowering medication
SDNN, standard deviation of all normal intervals; BMI, body mass index; TBF, total body fat; HOMA-IR, updated Homeostasis Model Assessment Insulin Resistance
Results are based on weighted analyses (n=465)

E-Table 2 Difference in triglyceride concentrations per standard deviation in SDNN

		Fasting triglycerides (%)	Triglycerides 30 minutes (%)	Triglycerides 150 minutes (%)
Model 1	Crude	−2.9 (−11.3, 4.9)	−2.7 (−9.9, 4.0)	−2.4 (−9.6, 4.3)
Model 2	+ age, sex	−5.6 (−14.9, 2.9)	−4.8 (−12.6, 2.5)	−4.3 (−11.7, 2.7)
Model 3	+ confounders*	−4.4 (−12.9, 3.5)	−4.2 (−11.5, 2.6)	−4.5 (−11.4, 2.0)
Model 4	+ BMI, TBF	−3.7 (−12.1, 4.0)	−3.6 (−10.7, 3.1)	−3.9 (−10.6, −2.5)
Model 5	+ HOMA-IR	5.9 (3.9, 8.0)	−0.8 (−6.5, 4.5)	−1.7 (−7.3, 3.5)
Model 6	+ fasting TG		−0.8 (−2.6, 1.0)	−1.1 (−4.2, −1.9)

*Confounders: smoking, education, physical activity, heart rate lowering medication and lipid lowering medication
SDNN, standard deviation of all normal intervals; BMI, body mass index; TBF, total body fat; HOMA-IR, updated Homeostasis Model Assessment Insulin Resistance; TG, triglycerides
Results are based on weighted analyses (n=465)

E-Table 3 Difference in cholesterol concentrations per standard deviation in RMSSD

		Total cholesterol (mmol/L)	HDL cholesterol (mmol/L)	LDL cholesterol (mmol/L)
Model 1	Crude	−0.11 (−0.22, 0.00)	0.01 (−0.04, 0.06)	−0.07 (−0.18, 0.04)
Model 2	+ age, sex	−0.10 (−0.22, 0.02)	0.01 (−0.05, 0.06)	−0.06 (−0.17, 0.06)
Model 3	+ confounders*	−0.07 (−0.17, 0.04)	0.01 (−0.03, 0.06)	−0.02 (−0.13, 0.09)
Model 4	+ BMI, TBF	−0.06 (−0.17, 0.05)	0.01 (−0.04, 0.05)	−0.02 (−0.12, 0.09)
Model 5	+ HOMA-IR	−0.05 (−0.16, 0.06)	−0.01 (−0.06, 0.03)	−0.01 (−0.11, 0.10)

*Confounders: smoking, education, physical activity, heart rate lowering medication and lipid lowering medication
RMSSD, root mean square of the successive differences; BMI, body mass index; TBF, total body fat; HOMA-IR, updated Homeostasis Model Assessment Insulin Resistance
Results are based on weighted analyses (n=465)

E-Table 4 Difference in triglyceride concentrations per standard deviation in RMSSD

		Fasting triglycerides (%)	Triglycerides 30 minutes (%)	Triglycerides 150 minutes (%)
Model 1	Crude	−5.8 (−13.6, 1.4)	−5.4 (−12.2, 0.9)	−4.8 (−11.0, 1.0)
Model 2	+ age, sex	−5.6 (−13.9, 2.1)	−5.2 (−12.4, 1.6)	−4.4 (−11.0, 1.9)
Model 3	+ confounders*	−5.6 (−13.8, 2.0)	−5.5 (−12.6, 1.3)	−4.8 (−11.7, 1.6)
Model 4	+ BMI, TBF	−5.8 (−13.8, 1.6)	−5.6 (−12.5, 0.8)	−4.9 (−11.5, 1.4)
Model 5	+ HOMA-IR	−2.2 (−7.3, 2.6)	−2.7 (−7.4, 1.9)	−1.7 (−6.4, 2.8)
Model 6	+ fasting TG		−0.7 (−2.3, 0.8)	0.3 (−2.3, 3.0)

*Confounders: smoking, education, physical activity, heart rate lowering medication and lipid lowering medication

RMSSD, root mean square of the successive differences; BMI, body mass index; TBF, total body fat; HOMA-IR, updated Homeostasis Model Assessment Insulin Resistance; TG, triglycerides

Results are based on weighted analyses (n=465)

E-Table 5 Difference in cholesterol concentrations per standard deviation in LF power

		Total cholesterol (mmol/L)	HDL cholesterol (mmol/L)	LDL cholesterol (mmol/L)
Model 1	Crude	−0.02 (−0.14, 0.09)	0.01 (−0.04, 0.06)	−0.01 (−0.12, 0.10)
Model 2	+ age, sex	0.06 (−0.08, 0.20)	0.06 (0.00, 0.12)	0.05 (−0.07, 0.18)
Model 3	+ confounders*	0.07 (−0.07, 0.21)	0.05 (−0.01, 0.10)	0.06 (−0.07, 0.18)
Model 4	+ BMI, TBF	0.08 (−0.06, 0.21)	0.03 (−0.03, 0.08)	0.07 (−0.06, 0.19)
Model 5	+ HOMA-IR	0.09 (−0.05, 0.22)	0.01 (−0.04, 0.06)	0.08 (−0.05, 0.20)

*Confounders: smoking, education, physical activity, heart rate lowering medication and lipid lowering medication

LF, low frequency; BMI, body mass index; TBF, total body fat; HOMA-IR, updated Homeostasis Model Assessment Insulin Resistance

Results are based on weighted analyses (n=465)

E-Table 6 Difference in triglyceride concentrations per standard deviation in LF power

		Fasting triglycerides (%)	Triglycerides 30 minutes (%)	Triglycerides 150 minutes (%)
Model 1	Crude	−4.2 (−11.1, 2.2)	−4.1 (−10.8, 2.3)	−4.3 (−11.1, 2.0)
Model 2	+ age, sex	−7.6 (−15.7, −0.1)	−6.6 (−14.1, 0.5)	−6.5 (−14.0, 0.5)
Model 3	+ confounders*	−5.5 (−13.3, 1.7)	−5.0 (−12.1, 1.7)	−6.1 (−13.4, 0.7)
Model 4	+ BMI, TBF	−5.1 (−13.0, 2.2)	−4.6 (−11.7, 2.0)	−5.8 (−13.1, 1.1)
Model 5	+ HOMA-IR	0.6 (−5.2, 6.7)	−0.3 (−5.9, 5.1)	−2.5 (−3.4, 7.9)
Model 6	+ fasting TG		−0.7 (−2.6, 1.1)	−2.4 (−5.9, 1.0)

*Confounders: smoking, education, physical activity, heart rate lowering medication and lipid lowering medication

LF, low frequency; BMI, body mass index; TBF, total body fat; HOMA-IR, updated Homeostasis Model Assessment Insulin Resistance; TG, triglycerides

Results are based on weighted analyses (n=465)

E-Table 7 Difference in cholesterol concentrations per standard deviation in HF power

		Total cholesterol (mmol/L)	HDL cholesterol (mmol/L)	LDL cholesterol (mmol/L)
Model 1	Crude	−0.06 (−0.17, 0.05)	0.03 (−0.02, 0.09)	−0.06 (−0.17, 0.05)
Model 2	+ age, sex	−0.08 (−0.21, 0.04)	0.01 (−0.05, 0.06)	−0.06 (−0.17, 0.06)
Model 3	+ confounders*	−0.04 (−0.16, 0.08)	0.01 (−0.04, 0.07)	−0.02 (−0.13, 0.09)
Model 4	+ BMI, TBF	−0.04 (−0.16, 0.08)	0.01 (−0.04, 0.06)	−0.02 (−0.13, 0.09)
Model 5	+ HOMA-IR	−0.03 (−0.15, 0.09)	−0.01 (−0.05, 0.04)	−0.01 (−0.12, 0.10)

*Confounders: smoking, education, physical activity, heart rate lowering medication and lipid lowering medication

HF, high frequency; BMI, body mass index; TBF, total body fat; HOMA-IR, updated Homeostasis Model Assessment Insulin Resistance

Results are based on weighted analyses (n=465)

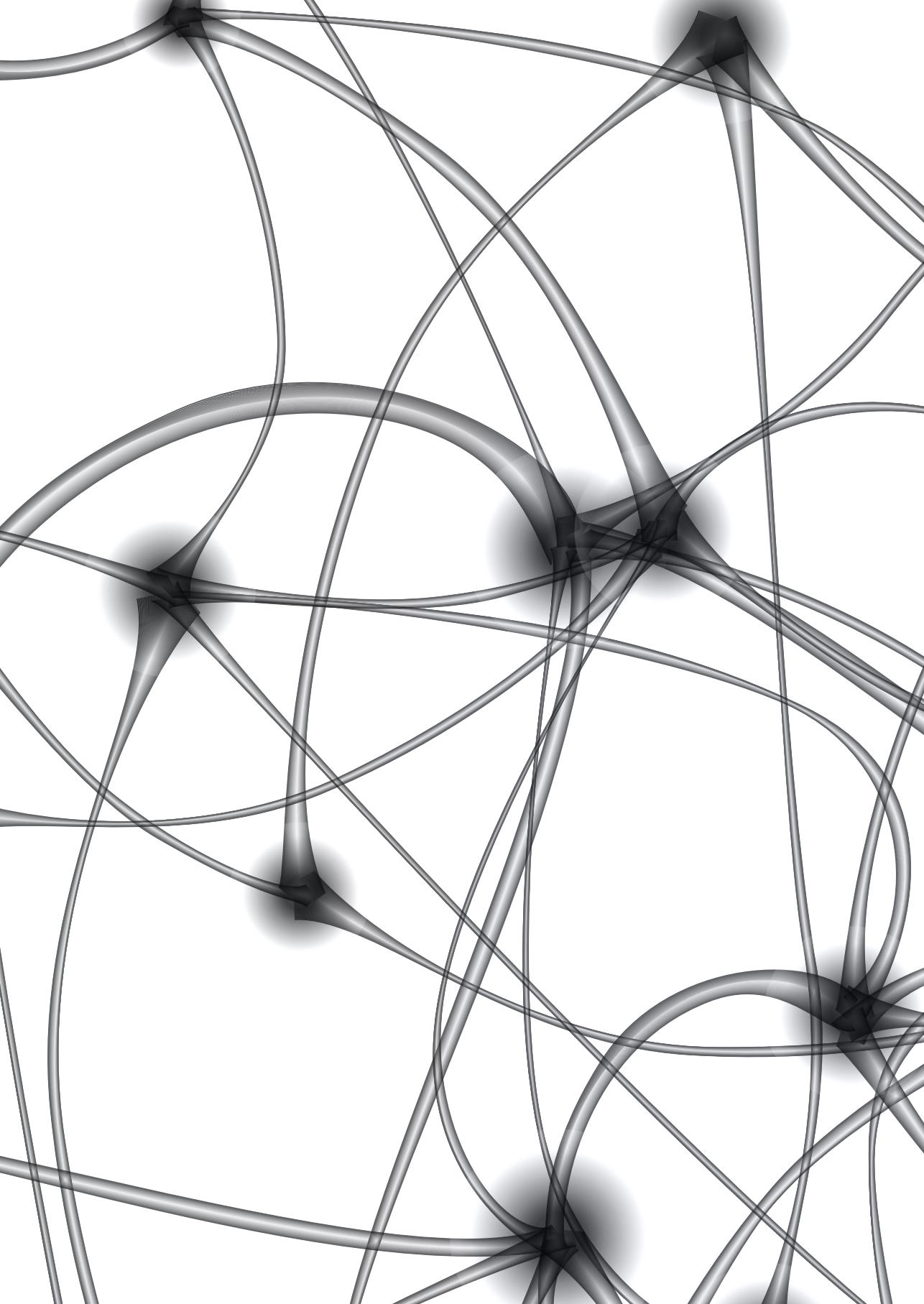
E-Table 8 Difference in triglyceride concentrations per standard deviation in HF power

		Fasting triglycerides (%)	Triglycerides 30 minutes (%)	Triglycerides 150 minutes (%)
Model 1	Crude	−6.7 (−14.2, 0.3)	−6.3 (−13.1, 0.0)	−6.3 (−13.1, 0.0)
Model 2	+ age, sex	−4.9 (−12.8, 2.5)	−4.7 (−11.9, 2.0)	−4.4 (−11.5, 2.3)
Model 3	+ confounders*	−4.6 (−12.7, 2.8)	−4.8 (−12.1, 1.9)	−4.6 (−12.1, 2.4)
Model 4	+ BMI, TBF	−5.4 (−13.4, −2.0)	−5.6 (−12.7, 1.0)	−5.2 (−12.6, 1.7)
Model 5	+ HOMA-IR	−0.7 (−6.4, 4.6)	−1.5 (−6.7, 3.4)	−1.6 (−7.4, 3.9)
Model 6	+ fasting TG		−0.9 (−2.3, 0.6)	−0.9 (−4.3, 2.4)

*Confounders: smoking, education, physical activity, heart rate lowering medication and lipid lowering medication

HF, high frequency; BMI, body mass index; TBF, total body fat; HOMA-IR, updated Homeostasis Model Assessment Insulin Resistance; TG, triglycerides

Results are based on weighted analyses (n=465)



7

General Discussion and Summary

The main objective of this thesis was to study the role of ANS function in the development of diabetes and cardiovascular disease. Based on earlier studies it has remained unclear whether impaired ANS function is a risk factor for the development of diabetes and cardiovascular disease, or merely a consequence of pre-existing disease. By using an epidemiological approach, this thesis showed that an impaired ANS function increased the risk of cardiovascular disease, but was not a risk factor for the occurrence of diabetes. In this discussion section we summarize our main results and discuss methodological considerations and possible implications of our findings.

MAIN FINDINGS

Obesity

In **chapter 2** we showed that body fat, in particular visceral fat, was associated with established and novel measures of activation of the sympathetic nervous system (heart rate, QTc and the ventricular gradient magnitude) in non-diabetic participants with structurally normal hearts. Our study expands results from previous studies on the association between body fat and ANS function by showing that visceral fat may be the most important fat depot for activation of the sympathetic nervous system.[1-3] These findings may be explained by the higher secretion rate of pro-inflammatory cytokines of visceral fat than of subcutaneous fat.[4-7] These cytokines can either directly stimulate the central nervous system, by crossing the blood-brain-barrier, or induce low-grade inflammations, resulting in sympathetic activation.[8, 9]

It must be noted, however, that our cross-sectional study does not permit conclusions on the direction of associations. Because body fat and sympathetic activation were measured at the same time, we cannot exclude the possibility that sympathetic nervous system activation may lead to visceral fat accumulation. However, our hypothesis that body fat influences sympathetic activation is supported by previous research that showed that a decrease in the amount of body fat by a hypocaloric diet or exercise attenuated sympathetic activation.[10-16] Because visceral fat is strongly associated with cardiovascular risk[17-20], our results suggest that activation of the sympathetic nervous system may be a mechanism underlying the association between visceral fat and cardiovascular disease.

Insulin resistance and type 2 diabetes mellitus

In **chapter 3** we showed that ANS function was not associated with incident type 2 diabetes mellitus in a prospective cohort study with 9.2 years of follow-up. Our study must be interpreted together with six previous studies that prospectively investigated

the association between ANS function and incident diabetes.[21-26] Two studies reported an association between resting heart rate and incident diabetes.[21, 22] However, these results were not adjusted for glucose or insulin concentrations at baseline and therefore impairments in glucose metabolism may have influenced ANS function. Another study reported an increased risk of mortality due to diabetes, but no increased risk of non-fatal diabetes.[23] This may suggest that impaired ANS function is associated with mortality in general instead of the development of diabetes. Two studies observed an association between impaired ANS function and incident diabetes only in subgroups of their population.[24, 25] In de CARDIA study, slow heart rate recovery was associated with diabetes in participants with poor fitness.[24] After stratification of our data on level of fitness, we could not replicate this finding. Possibly, fitness is an effect modifier only in a young population. The population of the CARDIA study was much younger than the population of the Hoorn study (18-30 years versus 50-75 years). The Australian Diabetes Obesity and Lifestyle study showed that higher heart rate was a risk factor for diabetes mainly in non-obese men.[25] However, the reference category in this analysis was chosen as <60 beats/min and the risk of diabetes was similar in the heart rate categories 60-70, 80-90 and >80 beats/min. Normal values for heart rate are defined as 60-80 beats/min. Possibly, individuals with a heart rate below 60 beats/min represented a specific group in this study with a low risk of disease, for example young athletes. Because the risk of diabetes was similar in all other categories of heart rate, the reference category may have strongly influenced the results and overestimated the association between heart rate and incident diabetes. Finally, one study reported an association between heart rate and incident diabetes (relative risk per SD (9.7 beats/min) = 1.06 (95%CI 1.00, 1.13). However, no associations were observed for parameters of heart rate variability.[26] Hence, taking our study and all the above described results together, there does not seem to be a large body of evidence for a causal role of the ANS in the development diabetes.

Several previous cross-sectional studies observed associations between impaired ANS function and impaired glucose metabolism.[27-31] The results from **chapter 3** suggest that these associations may reflect reverse causation, and that impaired ANS function is more likely a consequence of impaired glucose metabolism than a risk factor for type 2 diabetes. In **chapter 4** we hypothesized insulin resistance to be a mechanism underlying the association between body fat and ANS function. We showed (in line with **chapter 2**) that body fat was associated with ANS function measured as heart rate variability, and furthermore we showed that insulin resistance for a large part mediated the association between body fat and ANS function. Even though the latter results are based on cross-sectional data, our findings from **chapter 3 and 4** together support the existence of a pathophysiological mechanism in which insulin resistance or type 2 diabetes precedes impairment of the ANS.

Cardiovascular disease

In **chapter 5** we aimed to summarize the evidence on the association between ANS function and incident cardiovascular disease using meta-analysis and meta-regression. We investigated heart rate variability, divided in percentiles, as a risk factor for a first fatal or non-fatal cardiovascular event in populations without prevalent cardiovascular disease. Based on the nine cohort studies with a total of 21988 participants included in our analysis, persons in the lowest percentile of heart rate variability had a 32 to 45% increased risk of a first cardiovascular event, depending on the measure of heart rate variability. Hence, by studying incident cardiovascular disease in a population without previous events, we showed in this chapter that impaired ANS function may be involved in the development cardiovascular disease.

Subsequently, we hypothesized that impaired lipid metabolism may be a mechanism underlying the association between ANS function and the development of cardiovascular disease. Therefore, we studied the associations between activation of the sympathetic nervous system and lipid concentrations in both the circulation and the liver. **Chapter 6** showed that resting heart rate was positively associated with both fasting and postprandial serum triglyceride concentrations. Per 10 beats/min, fasting serum triglycerides were 5.9% (95% CI: 3.9, 7.8) higher. We were the first to investigate associations of ANS function with postprandial serum triglycerides and intrahepatic triglyceride content. We showed that postprandial triglycerides were 5.2% (3.5, 6.9) higher 30 minutes after the mixed meal, and 4.4% (2.6, 6.3) higher 150 minutes after the mixed meal. Furthermore, per 10 beats/min of resting heart rate, intrahepatic triglyceride content was 13.1% (7.7, 18.7) higher. Although this study was cross-sectional, the results are supported by experimental studies that show that invasive sympathetic denervation in rats is followed by abnormal lipid concentrations.[32, 33] It is furthermore well-established that dyslipidaemia, especially a high serum triglyceride concentration[34, 35], is associated with an increased risk of cardiovascular disease. Therefore, our results may imply that impaired ANS function increases cardiovascular risk by alterations in the lipid metabolism.

METHODOLOGICAL CONSIDERATIONS

Making causal inferences on the basis of observational studies is often challenging, because several sources of bias must be taken into account. In this thesis we used an epidemiological approach to diminish the effects of bias on our results. In the next paragraphs, limitations to the measurement of ANS function, residual confounding, loss to follow up and reverse causation are considered.

Interpretation of and limitations to the measurement of ANS function in this thesis

In this thesis, we used several measures of ANS function: resting heart rate, heart rate variability, baroreflex sensitivity, Ewing tests and electrocardiographic measures of sympathetic activation.

Heart rate, heart rate variability and baroreflex sensitivity are commonly used, non-invasive measures that reflect mixed sympathetic and parasympathetic outflow. In the interpretation of heart rate variability it is important to take the difference between autonomic tone and modulation in parasympathetic or sympathetic outflow into account. Heart rate variability does not correspond to parasympathetic or sympathetic tone (as does resting heart rate), but to modulations in efferent activity.[36, 37] Therefore, an increased high frequency spectral component of heart rate variability indicates increased modulation of parasympathetic activity, but not necessarily an increased parasympathetic tone. Parasympathetic modulation can be reduced when parasympathetic tone is abolished or when the parasympathetic nervous system is constantly stimulated, two very different scenarios. Constant stimulation of parasympathetic tone will decrease heart rate, but will also result in decreased high frequency power heart rate variability because there is no modulation in parasympathetic outflow.[38] Similarly, the low frequency spectral component also does not provide information about the sympathetic tone, but of modulation in sympathetic outflow.[39] Heart rate and heart rate variability therefore reflect different aspects of the ANS. Heart rate is indicative of autonomic tone, whereas heart rate variability is a manifestation of activity of the baroreflex, which purpose is to buffer blood pressure by adaptions in sympathetic and parasympathetic outflow.[39-41] Both an increased resting heart rate and decreased heart rate variability reflect impairment of the ANS.

In this thesis, heart rate variability was measured during spontaneous breathing. In spectral heart rate variability analysis, the high frequency peak of the spectrum can be attributed to respiration. However, when breathing is low frequent (6 breaths/minute), the respiration peak shifts to lower frequencies and thereby (partly) overlaps with the smaller low frequency peak that is caused by a blood pressure resonance phenomenon mediated by the baroreflexes. This will result in increased low frequency heart rate variability and an overestimation of the baroreflex sensitivity.[42] Because we mainly used free breathing recordings to estimate ANS function, we cannot exclude the possibility that our measurements are affected by slow breathing of our participants. Such random error in the measurement of heart rate variability may have diluted the observed associations in our studies. However, most individuals will have a respiration that is at high frequency (normal range 12-18/min) and therefore the influence of slow breathing is

probably limited in this thesis. Only in **chapter 3**, baroreflex sensitivity was systematically measured using deep breathing recordings. In this chapter, the sensitivity of the baroreflex is overestimated in all individuals and must be interpreted with care. Because other measures of ANS function showed similar associations with incident diabetes in this chapter, the deep breathing does not affect our conclusions.

A limitation of the measurement of ANS function in **chapter 2** may be the use of novel electrocardiographic measures of sympathetic activation, identified by three experimental studies in which the sympathetic nervous system was stimulated mechanically or pharmacologically.[43-45] The validity and reproducibility of some of these measures (e.g., the ventricular gradient or spatial QRS-T angle) is yet unknown and should be established in future studies. However, the results of these novel measures were similar to the results of well-known measures of ANS function (heart rate, heart rate variability) studied in this chapter. This indicates that the results of **chapter 2** are robust and may provide a first step towards the validation of these novel measures.

Residual confounding by physical activity and mental stress

All studies in this thesis are based on observational data and we aimed to adjust for all known and measured confounding variables. However, residual confounding may still be present when not all confounding variables are known or measured, or when there is measurement error in the confounding variables. In **chapter 5** we performed a meta-analysis and meta-regression. The nine included studies were adjusted for a varying range of confounding variables. However, none of the studies were adjusted for physical activity and mental stress. Because low physical activity and mental stress may impair ANS function and increase the risk of cardiovascular disease, there may have been residual confounding. This may have resulted in an overestimation of the true association between ANS function and the development of cardiovascular disease. The associations studied in **chapters 4 and 6** may also suffer from residual confounding by mental stress. In **chapter 4** we performed a mediation analysis. Mental stress may have been a residual confounding variable because it influences insulin resistance (the mediating variable) and autonomic nervous system function (the outcome). Not adjusting for a variable that may confound the association between the mediator and the outcome may result in an overestimation of the mediated effect. In case of residual confounding by mental stress, the association between body fat and autonomic nervous system function that is mediated by insulin resistance, may therefore have been overestimated. In **chapter 6** we investigated the association between ANS function and lipid metabolism. Because mental stress may also influence lipid concentrations, any residual confounding may have resulted in an overestimation of the true association. Hence, it is a limitation of these studies that we could not adjust for physical activity and mental stress. In addition,

because of the observational nature of our studies we cannot exclude residual confounding by unknown factors or measurement error in our confounding variables. However, it is unlikely that these factors were completely responsible for the observed associations. Furthermore, because our studies were adjusted for many confounders that are strongly correlated with the potential confounding variables physical activity and mental stress, such as age, sex, and body mass index, it is unlikely that any overestimation of the true associations would have been large.

Loss to follow up in cohort studies

In cohort studies, selection bias may occur when reasons for loss to follow-up are related to prognosis (informative censoring). The consequence of such bias is that the association between exposure and outcome among those selected for analysis differs from the association among those eligible.[46, 47] In the Hoorn study (**chapter 3**), 140 of the 438 participants at baseline did not participate in the follow-up investigation. Reasons for non-participation were death (n=51), moving to another region (n=29) or unknown (n=59). Participants included in our analyses appeared overall healthier at baseline than those who were lost to follow up. If the association between ANS function and type 2 diabetes mellitus among non-participants had been stronger than among participants, this would have led to an underestimation of the true association in our analyses. We performed a sensitivity analysis to investigate the potential effect of such selection bias. When we analysed a hypothetical "worst case scenario" of the complete population in which all the participants who did not participate in the follow-up investigation would have developed type 2 diabetes mellitus, there was still no association between ANS function and the incidence of diabetes. Selection bias due to differential loss to follow-up may also have played a role in **chapter 5**, because in two studies that were included in our meta-analysis, the percentage of participants loss to follow-up was unknown. However, in the other seven included studies, the percentage of loss to follow-up was small (≤ 5.6%) and therefore selection bias due to informative censoring could not have strongly influenced our results.

Reverse causation

Reverse causation may occur especially in cross-sectional studies. When the exposure and outcome variables are measured at the same time, the observed exposure may be a consequence of the outcome variable rather than a cause. In longitudinal studies reverse causation may also play a role when the outcome or disease of interest is already (subclinically) prevalent at baseline.

The results from **chapters 2 and 6** were based on cross-sectional analyses. Therefore, we cannot exclude the possibility that impaired ANS function has led to accumulation

of visceral fat, or that alterations in lipid metabolism have led to impaired ANS function, instead of vice versa. In these chapters we have to rely on current literature and experimental studies that support our hypothesized directions and conclusions that visceral fat most likely leads to impaired ANS function, whereas impaired ANS function may result in alterations in lipid metabolism.

In **chapters 3 and 5**, we studied associations of ANS function with incident diabetes and cardiovascular disease using prospective follow-up studies. By studying first events of diabetes and cardiovascular disease during follow-up of individuals without pre-existing disease at baseline, we minimized a potential influence of underlying disease and showed that impaired ASN function is a risk factor for cardiovascular disease, but not for type 2 diabetes.

CONCLUSIONS AND IMPLICATIONS

Despite the methodological challenges mentioned above, our results contributed to the unravelling of causal pathways between obesity, diabetes and cardiovascular disease. On the basis of the results from this thesis and the literature, the Figure shows the putative pathways between body fat, insulin resistance and diabetes, ANS function, lipid metabolism and cardiovascular disease.

The main conclusions of this thesis are that excess body fat, in particular visceral fat, is associated with activation of the sympathetic nervous system in individuals without diabetes and cardiovascular disease. Furthermore, this thesis showed that impaired ANS function is not a risk factor for the development of diabetes mellitus. The presented studies suggest that insulin resistance precedes the impairment of the ANS. This implies that impaired ANS function is merely a consequence of diabetes, and that insulin resistance may be a mechanism underlying the association between body fat and ANS function. Therefore, ANS function does not seem useful in the prevention of diabetes and this thesis emphasizes the importance of controlling of body weight and glycaemic status in the prevention of the development of diabetes. However, our research does imply that the ANS is affected by insulin resistance before the onset of diabetes. This may in part explain the increased risk of cardiovascular disease in insulin resistant individuals.[48-50]

The results from this thesis also show that impaired ANS function is a risk factor for the development of cardiovascular disease in populations without pre-existing cardiovascular disease. Furthermore, individuals with a higher heart rate have higher concentrations of cholesterol and triglycerides in the circulation and a higher intrahepatic triglyceride

content, suggesting that an altered lipid metabolism may be a mechanism underlying the association between ANS function and cardiovascular disease. Because ANS function is a risk factor for the development of cardiovascular disease, the ANS may be a target in the prevention of cardiovascular disease, or may be used to identify individuals at high risk of cardiovascular disease. Enhancing the ANS function may be useful in the treatment of dyslipidaemia and the prevention of a first cardiovascular event. Beta blockers are well-known drugs that decrease the effect of sympathetic neurotransmitters on β-sympathetic receptors. However, these drugs have many side effects and are therefore unlikely to play a role in the primary prevention of cardiovascular disease in otherwise healthy individuals. Both observational studies and randomized controlled trials have shown that a hypocaloric diet and exercise training improve the function of the ANS and inhibit sympathetic activation.[10-16] Lifestyle modifications may provide the best intervention to preserve the function of the ANS. Therefore, the results of this thesis emphasize the maintenance of a healthy body weight as the cornerstone in the prevention of diabetes and cardiovascular disease. Future studies should further elucidate the role of the ANS in the causal pathways between body fat, cardiovascular risk factors and cardiovascular endpoints. The ongoing collection of long-term follow-up data in participants of the NEO study will contribute to this in the near future.

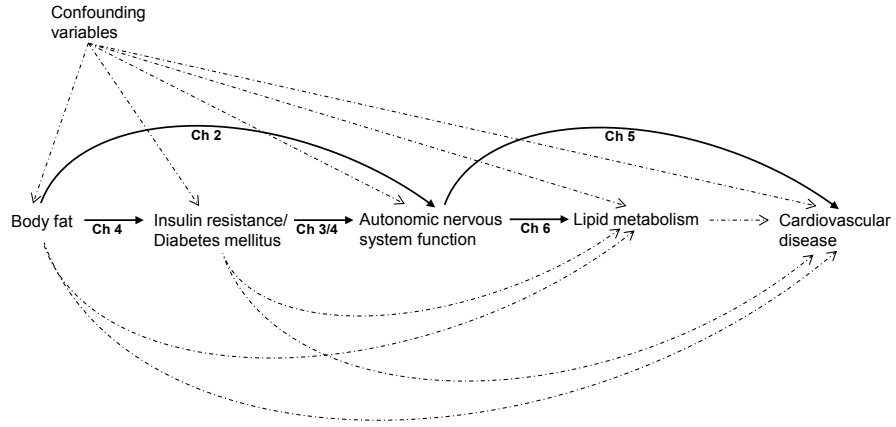


Figure. Hypothetical pathways between body fat, insulin resistance and diabetes, autonomic nervous system function, lipid metabolism and cardiovascular disease

Directional arrows indicate the flow of association. Confounding variables include age, sex, smoking status, social economic status, physical activity, mental stress

→ : results from this thesis; Ch=chapter

⇢ : evidence from literature

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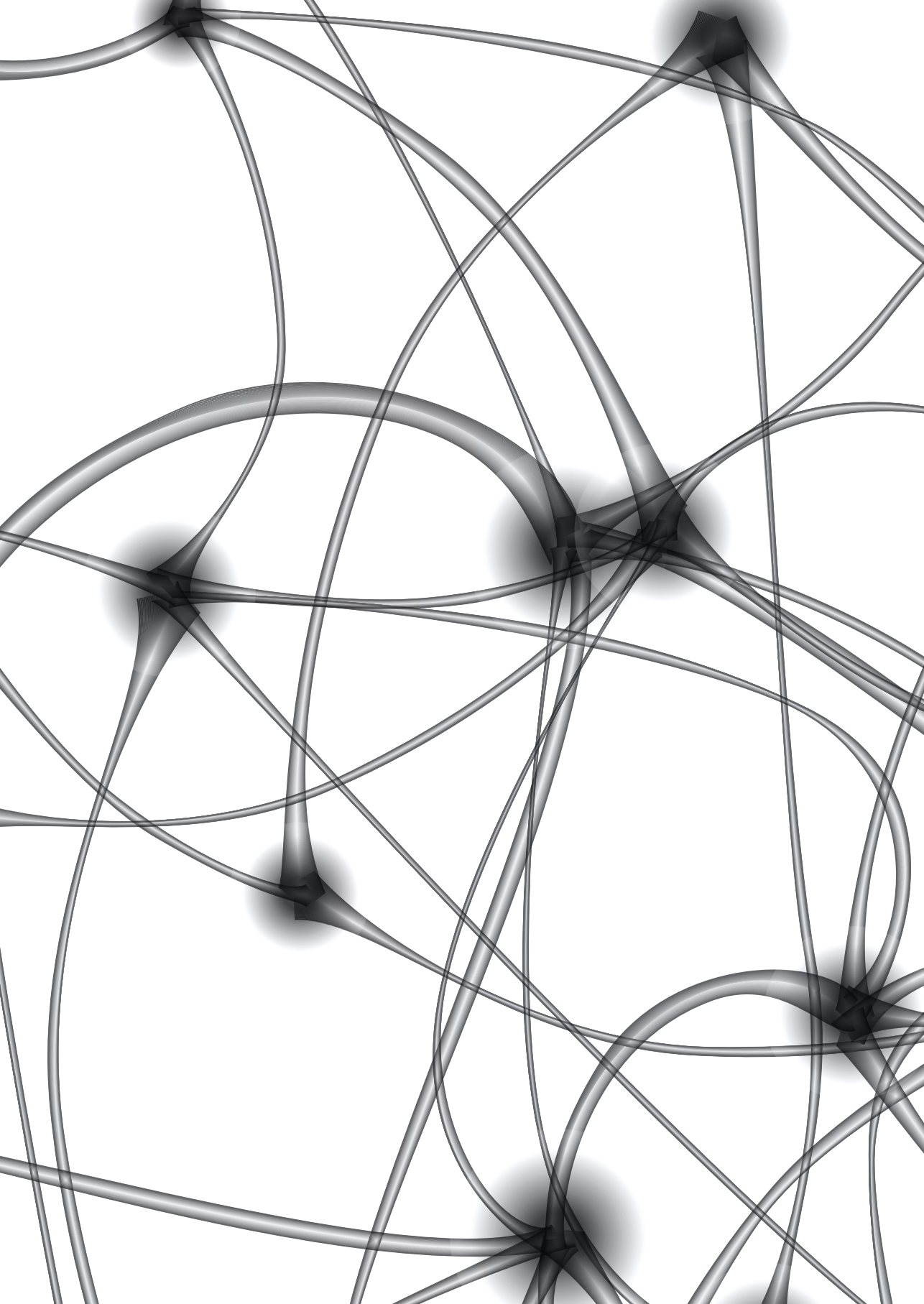


8

Nederlandse Samenvatting

Het autonome zenuwstelsel (AZS) reguleert de functie van organen en is belangrijk voor homeostase, het in evenwicht houden van het interne milieu van het lichaam. Het AZS wordt niet bewust aangestuurd, maar ontvangt informatie van onder andere de hersenen, de circulatie, de zintuigen en het limbisch systeem en vertaalt dit in uitgaande signalen. Deze signalen worden doorgegeven aan de rest van het lichaam door de twee takken van het autonome zenuwstelsel, het parasympathische en sympathische zenuwstelsel. Het parasympathische zenuwstelsel is vooral actief in rust, en zorgt onder andere voor een rustige hartslag en een actieve spijsvertering. Het sympathische zenuwstelsel is vooral actief tijdens perioden van activiteit, zoals lichamelijk inspanning of emotionele momenten. In gezonde mensen is er veel modulatie in parasympathische en sympathische signalen, afhankelijk van wat het lichaam nodig heeft, en deze modulatie is een teken dat het lichaam zich goed kan aanpassen. Als de werking van het autonoom zenuwstelsel verminderd is, is er minder modulatie en is het sympathisch zenuwstelsel dominant. Het is bekend dat mensen met obesitas, diabetes of hart- en vaatziekten vaak een slechtere AZS-functie hebben. Echter, het is onduidelijk of een slechtere AZS-functie alleen een gevolg is van diabetes en hart- en vaatziekten, of dat het AZS ook betrokken is bij het ontstaan van deze ziekten. Het doel van dit proefschrift was dan ook om te onderzoeken of AZS-functie een rol speelt in het ontstaan van diabetes en hart- en vaatziekten.

LICHAAMSVET

Oorspronkelijk werd gedacht dat weinig activiteit van het sympathische zenuwstelsel zou leiden tot de opslag van lichaamsvet. Tegenwoordig lijkt het echter waarschijnlijker dat een hoge activiteit van het sympathische zenuwstelsel en een lage activiteit van het parasympathische zenuwstelsel een rol spelen in het ontstaan van een overmaat aan lichaamsvet en ziekte. De resultaten van **hoofdstuk 2** sluiten aan bij deze hypothese. Dit hoofdstuk beschrijft het verband tussen verschillende maten van lichaamsvet en activiteit van het sympathische zenuwstelsel. De resultaten laten zien dat de hoeveelheid lichaamsvet, in het bijzonder visceraal vet (vet dat is opgeslagen rond de organen in de buik), samenhangt met activatie van het sympathische zenuwstelsel. Omdat een ziektegeschiedenis van diabetes en hart- en vaatziekten veel invloed heeft op het autonome zenuwstelsel, zijn in dit onderzoek alle mensen met diabetes of afwijkingen aan het hart uitgesloten van de analyse. De resultaten suggereren daarom dat lichaamsvet al invloed heeft op de activiteit van het sympathische zenuwstelsel vóórdat ziekten optreden. Een verklaring hiervoor zou kunnen zijn dat hormonen en inflammatoire stoffen die door vetcellen worden uitgescheiden mogelijk het sympathische zenuwstel kunnen stimuleren. Toekomstige studies moeten laten zien of het lichaamsvet daadwerkelijk

effect heeft op het ontstaan van hart- en vaatziekten door het beïnvloeden van de activiteit van het sympathische zenuwstelsel.

INSULINERESISTENTIE EN DIABETES

Een slechtere functie van het AZS is een bekende complicatie van type 2 diabetes mellitus. Verschillende onderzoeken hebben laten zien dat insulineresistentie (preklinische diabetes) samenhangt met de functie van het AZS. Het is echter onbekend of de AZS-functie een oorzaak is van het ontstaan van diabetes. In **hoofdstuk 3** zijn gegevens van de Hoorn studie gebruikt om deze vraag te beantwoorden. De resultaten laten zien dat de functie van het AZS geen invloed heeft op het ontstaan van de diabetes. Op basis van de resultaten van hoofdstuk 3 lijkt het waarschijnlijker dat de aanwezigheid van insulineresistentie het AZS beïnvloedt, dan andersom. In **hoofdstuk 4** borduren we verder op de resultaten van hoofdstuk 2 en 3 met als hypothese dat lichaamsvet de functie van het AZS beïnvloedt door bij te dragen aan een insulineresistente staat van het lichaam. De resultaten wijzen er inderdaad op dat insulineresistentie gedeeltelijk het verband tussen lichaamsvet en AZS functie verklaart.

HART- EN VAATZIEKTEN

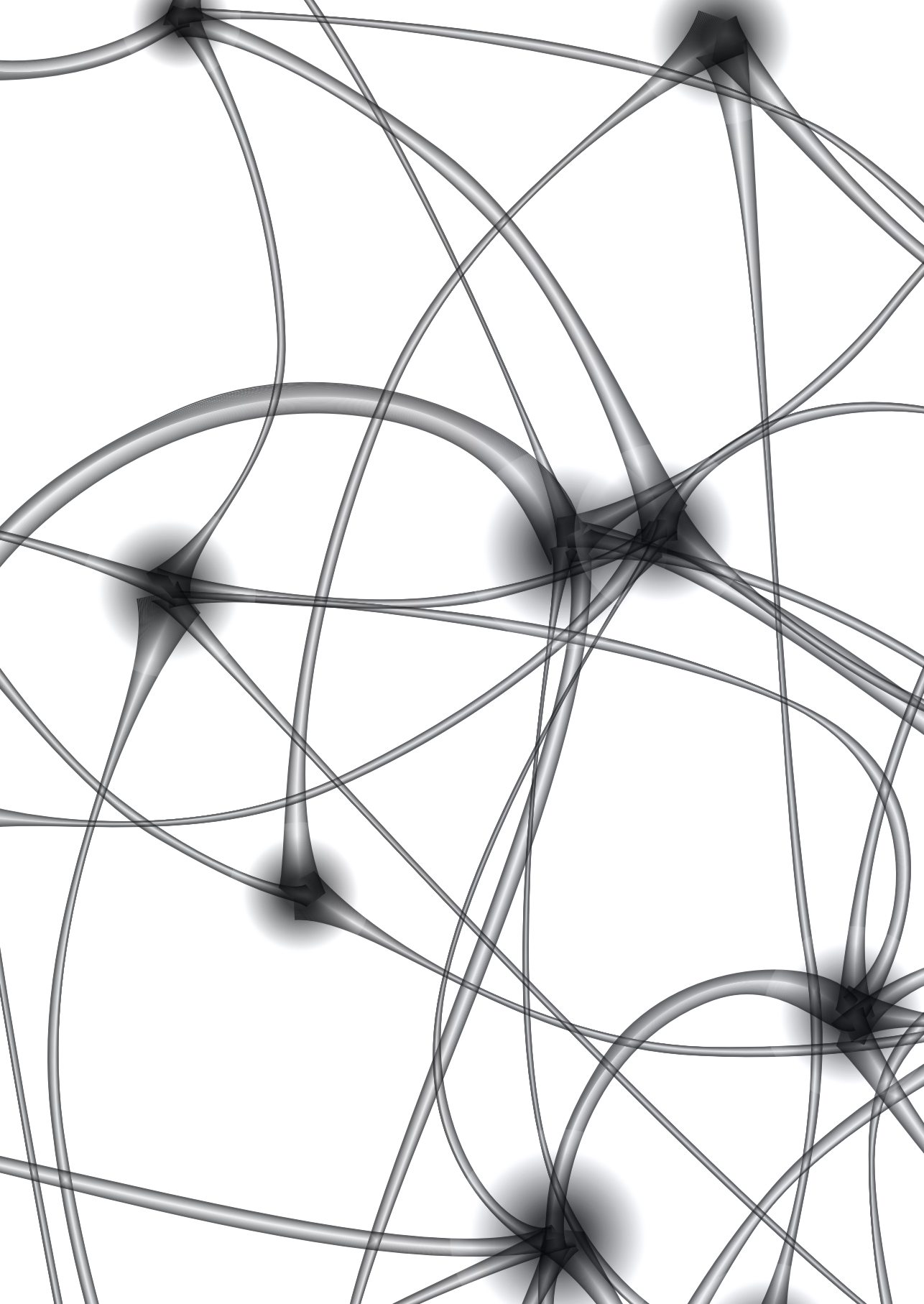
Het is bekend dat bij mensen met hart- en vaatziekten een slechtere functie van het AZS samenhangt met een slechtere prognose en meer sterfte. Het doel van **hoofdstuk 5** was om te onderzoeken of een slechtere AZS-functie ook een risicofactor is voor het ontstaan van hart- en vaatziekten in de algemene bevolking. Dit hoofdstuk is een meta-analyse waarin alle literatuur naar het verband tussen variabiliteit in hartslag en het ontstaan van hart- en vaatziekten is samengevat. Negen onderzoeken, waarin de functie van het AZS was gemeten als hartslagvariabiliteit, bleken geschikt voor deze meta-analyse. Afhankelijk van de gebruikte maat voor hartslagvariabiliteit hadden mensen met een slechtere functie van het AZS een 32 tot 45% hoger risico op het ontwikkelen van hart- en vaatziekten dan mensen met een betere functie van het AZS.

Een belangrijke vraag die tot op heden nog niet beantwoord kan worden, is hoe een slechtere functie van het AZS het risico op hart- en vaatziekten verhoogt. Uit de resultaten van **hoofdstuk 6** blijkt dat de functie van het AZS samenhangt met de concentratie van vetten in het bloed en in de lever. Deze resultaten suggereren dat een slechtere functie van het AZS mogelijk een verhogend effect heeft op de concentraties vetten in bloed en lever, en daardoor het risico op hart- en vaatziekten zou kunnen vergroten.

CONCLUSIES EN IMPLICATIES

De belangrijkste conclusies van dit proefschrift zijn dat overmatig lichaamsvet, vooral vet in de buik, samenhangt met activatie van het sympathische zenuwstelsel in mensen zonder diabetes of hart- en vaatziekten. Daarnaast laat dit proefschrift zien dat een slechtere functie van het AZS geen risicofactor is voor het ontstaan van diabetes. Uit de resultaten blijkt wel dat veel lichaamsvet mogelijk bijdraagt aan het ontstaan van insulineresistentie, waardoor de functie van het AZS zou kunnen verminderen. Dit proefschrift suggereert ook dat het AZS beïnvloed wordt door insulineresistentie vóór het ontstaan van diabetes. Mogelijk is dit een verklaring voor het verhoogde cardiovasculaire risico bij mensen met insulineresistentie. De resultaten van dit proefschrift tonen aan dat een slechtere functie van het AZS ook een risicofactor is voor het ontstaan van hart- en vaatziekten bij mensen zonder eerdere hart- en vaatziekten. De functie van het AZS hangt samen met de concentratie vetten in het bloed en in de lever, en mogelijk is een verandering in vetstofwisseling een verklaring voor het verband tussen AZS functie en hart- en vaatziekten.

Aangezien AZS-functie een risicofactor is voor hart- en vaatziekten, zou het gebruikt kunnen worden als aangrijpingspunt om mensen met een hoog risico te identificeren, en bij het voorkómen van hart- en vaatziekten. Wellicht zou het verbeteren van de AZS-functie hart- en vaatziekten kunnen voorkómen. Een groep van medicijnen die de activiteit van het sympathisch zenuwstelsel remt zijn bètablokkers. Helaas hebben deze medicijnen veel bijwerkingen en zijn daarom minder geschikt voor primaire preventie in mensen zonder gezondheidsklachten. Uit eerdere interventiestudies blijkt dat een gezond leefpatroon met voldoende beweging en een gezond voedingspatroon de functie van het AZS kan verbeteren. Dit proefschrift benadrukt daarom dat, op dit moment, een gezond gewicht het belangrijkste uitgangspunt is voor het voorkómen van hart- en vaatziekten. Toekomstige studies zijn nodig om de rol van AZS in de oorzakelijke verbanden tussen lichaamsvet, cardiovasculaire risicofactoren en cardiovasculaire eindpunten te ontrafelen. De continue verzameling van gegevens en analyse van het optreden van ziekten bij deelnemers van de NEO studie zal hier in de nabije toekomst aan bijdragen.



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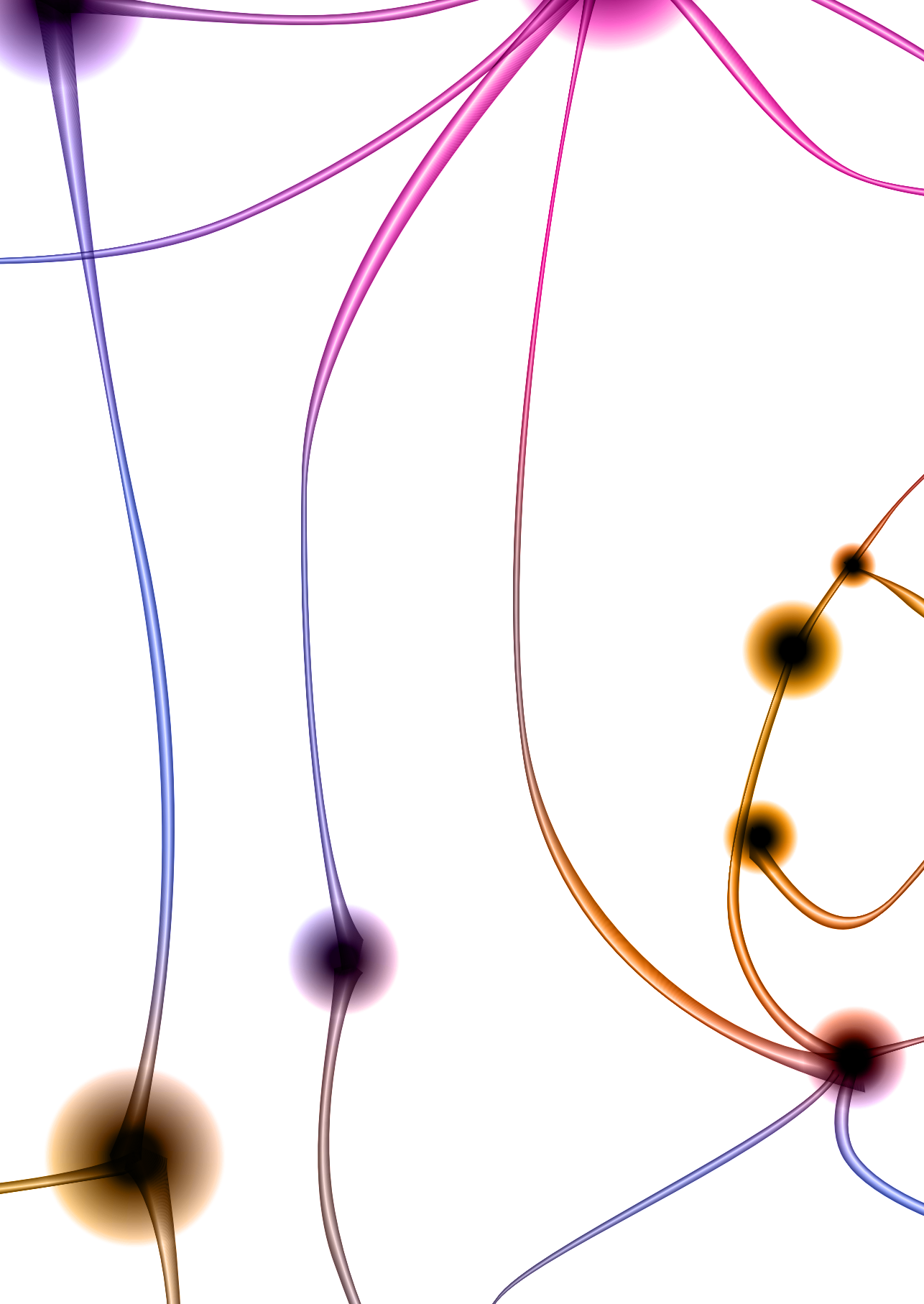
Tenslotte, lieve Naomi, bedankt voor je liefde en onvoorwaardelijk vertrouwen in mijn kunnen. Ik zou me geen beter thuis kunnen wensen. De laatste jaren hebben we veel toekomstplannen gemaakt, en ik kan niet wachten om die in de praktijk te gaan brengen!