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Research Centre in Physical Activity Health and Leisure

Associations of cardiovascular risk factors and physical activity with autonomic function.

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"Somewhere over the rainbow skies are blue, and the dreams that you dare to dream
really do come true..."

Over the Rainbow sing on the movie "The Wizard of Oz", 1939.

To my mom, dad, brother, sister and Mario
for being my life.

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Resumo

O sistema nervoso autónomo é extremamente importante para uma apropriada interacção entre os meios interno e externo. Este sistema permite que o organismo responda de forma correcta a estímulos. Adicionalmente, o sistema nervoso autónomo tem um papel fundamental na manutenção da homeostasia através do sistema nervoso simpático e parassimpático.

Estudos anteriores sugerem que desvios no funcionamento do sistema autónomo são determinantes num leque alargado de doenças cardíacas e não cardíacas e no despoletar de arritmias. Contudo, e apesar de a investigação nesta área ser considerável, os factores que afectam a função autonómica cardíaca são ainda pouco conhecidos e percebidos.

Esta tese foi realizada com o objectivo de avaliar as relações entre função autonómica cardíaca e factores de risco cardiovasculares tais como: inflamação, consumo de ácidos gordos *trans*, componentes do síndrome metabólico e actividade física. Para atingir estes objectivos, cinco estudos foram efectuados:

1. Actividade física vigorosa e modulação vagal em jovens adultos.
2. Benefícios por atingir as recomendações de actividade física vigorosa bem como a moderada: evidências da complexidade da frequência cardíaca e modulação cardíaca vagal.
3. Proteína C reactiva de alta sensibilidade está associada a uma redução da modulação vagal e baixos níveis de actividade física em jovens adultos.
4. Consumo de ácidos gordos *trans* e variabilidade da frequência cardíaca em duas populações de adultos, jovens e idosos.
5. Associações entre os componentes do síndrome metabólico, actividade física e função autonómica cardíaca.

Com base nos nossos dados é possível concluir que a actividade física está positivamente associada a uma mais favorável função autonómica cardíaca. Contrariamente, inflamação, consumo de ácidos gordos *trans* 18:2 e os componentes do síndrome metabólico estão associados a uma menos favorável função autonómica cardíaca.

Abstract

The autonomic nervous system is extremely important for appropriate interaction between internal and external environments. It allows the body to respond properly to stimuli. Moreover, it plays a crucial role in the maintenance of homeostasis, regulating the complex interplay between the parasympathetic and sympathetic branches.

Evidence suggests that deviations in autonomic system function are determinant in a wide range of cardiac and non-cardiac disorders and in the onset of adverse events such as arrhythmias. However, and despite a considerable amount of research in this field, the factors that affect cardiac autonomic function are still not well known or understood.

This thesis was set out to evaluate cardiac autonomic function in healthy subjects, with the aim of assessing its relationships with cardiovascular risk factors such as inflammation, *trans*-fatty acid consumption, components of metabolic syndrome, and physical activity. To accomplish these goals, five studies were carried out on:

1. Vigorous physical activity and vagal modulation in young adults.
2. Benefits of achieving vigorous, as well as moderate, physical activity recommendations: evidence from heart rate complexity and cardiac vagal modulation.
3. High levels of C-reactive protein are associated with reduced vagal modulation and low physical activity in young adults.
4. *Trans*-fatty acid consumption and heart rate variability in older and younger adults.
5. Associations between metabolic syndrome components, physical activity, and cardiac autonomic function.

Based on our experimental work, it is possible to conclude that factors like physical activity seem to be associated with more favourable cardiac autonomic function. Conversely, factors like inflammation, 18:2 *trans*-fatty acid consumption, and the components of metabolic syndrome seem to be associated with less favourable cardiac autonomic function.

List of Abbreviations

ANS- Autonomic Nervous System

cANS- Cardiac autonomic function

PA- Physical activity

HR- Heart rate

HRV- Heart rate variability

MSNA- Muscle sympathetic nerve activity

SDNN- Standard deviation of the R-R intervals

rMSSD- The square root of the mean squared differences of successive R-R intervals

NN50- The number of interval differences between successive R-R intervals greater than 50 ms

PNN50- The proportion derived by dividing NN50 by the total number of R-R intervals

SDANN- The standard deviation of the average R-R intervals calculated over short periods over the course of 24 hours

PSP- Power spectrum density

VLF- Very low frequency

LF- Low frequency

HF- High frequency

nu- Normalized units

ULF- Ultra low frequency

SampEn- Sample entropy

SD1- Poincaré plot the short diameter of an ellipse

SD2- Poincaré plot the long diameter of an ellipse

DFA- Detrended fluctuation analysis

n-3PUFA- Polyunsaturated n-3 fatty acids

TFA- *Trans*-fatty acid

CHO- Total cholesterol

LDL- Low-density lipoprotein

HDL- High-density lipoprotein

TRG- Triglycerides

Glu- Blood glucose

CRP- C-reactive protein

IL-6- Interleukin-6

MetS- Metabolic syndrome

Chapter 1. General Introduction and Theoretical Background

1. General Introduction

The physiological activities of the cardiovascular system are controlled by the autonomic nervous system (ANS), through a complex interplay between the parasympathetic and sympathetic divisions (Guyton and Hall 2006; Vinik, Maser et al. 2011). The ANS is extremely important for appropriate interaction between internal and external environments, allowing the human body to generate adequate responses. Impaired ANS function (e.g. increased sympathetic activity and/or suppressed parasympathetic activity) and improper autonomic responsiveness to stimulus are associated with a number of unhealthy behaviours and a wide range of diseases (Carnethon and Craft 2008). In fact, excessive sympathetic activation and diminished parasympathetic modulation are not only markers of an unhealthy cardiovascular system, but, in part, are also prevalent and potent risk factors for adverse cardiovascular events, including mortality (Curtis and O'Keefe 2002). Indeed, imbalanced ANS function is associated with the pathogenesis of several arrhythmias (Johnson, Gray et al. 2004). Therefore, it is important to know and understand how a balanced ANS function can be maintained or even improved and to be aware of which factors may impair its function and possibly lead to disease and/or adverse events. It is equally important to know which factors, after a disease or disorder is established, improve ANS function.

It is believed that the mechanisms by which traditional risk factors, such as smoking, an unhealthy diet, obesity, and a sedentary lifestyle, predispose people to adverse events are multifaceted (Curtis and O'Keefe 2002). However, sympathetic activation and a reduction in parasympathetic modulation seem to be one important final common pathway where a significant portion of cardiovascular risk is conferred (Curtis and O'Keefe 2002). Additionally, it is possible that ANS imbalance is a significant factor in both the aetiology and clinical course of cardiovascular disease (Vinik, Maser et al. 2011). Factors that lead to inappropriate ANS function as activation of the sympathetic nervous system are expected to be harmful, whereas factors that augment parasympathetic modulation are seen as beneficial. However, and despite a considerable amount of research in this field, the factors that affect ANS function are still not well known or understood.

In this context, there is a need to understand and know the risk factors capable of unbalancing or balancing the ANS function of healthy subjects. Therefore, this thesis was set out to evaluate cardiac autonomic (cANS) function in healthy adults,

with the aim of assessing cardiovascular risk factors that may be positively or negatively associated with cANS function, as well as the relationship between cANS function and physical activity (PA). We have chosen a population of healthy university students, since the university period is a unique time in young adults' lives. It is normally accompanied by considerable lifestyle changes, leading, probably, to subsequent increases in cardiovascular risk factors either in the short or long term (Brandao, Pimentel et al. 2008). We followed our sample for three years (2008-2010).

The aims of this thesis and the original articles on which this thesis is based are the following:

- 1) to analyse the association of PA elements (as intensity) with cANS function in young adults:

(I) Soares-Miranda L, Sandercock G, Valente H, Vale S, Santos R, and Mota J. Vigorous physical activity and vagal modulation in young adults. *Eur J Cardiovasc Prev Rehabil* 16: 705-711, 2009;

(II) Soares-Miranda L, Sandercock G, Vale S, Silva P, Moreira C, Santos R, Mota J. Benefits of achieving vigorous as well as moderate physical activity recommendations: evidence from heart rate complexity and cardiac vagal modulation. *Journal Sport Science* May 25:1-8 [Epub ahead of print]

- 2) to study the associations between inflammation, cANS function, and physical activity in young adults:

(III) Soares-Miranda L, Negrão CE, Antunes-Correa LM, Nobre TS, Silva P, Santos R, Vale S, Mota J. High levels of C-reactive protein are associated with reduced vagal modulation and low physical activity in young adults. *Scandinavian Journal of Medicine & Science in Sports*. 2010 Jul 6 [Epub ahead of print].

- 3) to examine the relationship between cANS function and *trans*-fatty acid consumption in a young and old adult population:

(IV) Soares-Miranda L, Imamura F, Sattelmair J, Lemaitre RN, Stein PK, Siscovick DS, Mota J, Mozaffarian D. *Trans*-fatty acid consumption and heart rate variability in older and younger adults. (submitted-under review).

- 4) to evaluate associations between the components of metabolic syndrome and cANS function in a population of young adults, over time.

(V) Soares-Miranda L, Sandercock G, Vale S, Santos R, Abreu S, Moreira C, Mota J. Associations between metabolic syndrome components, physical activity and cardiac autonomic function (submitted-under review).

2. Theoretical Background

2.1. Autonomic nervous system

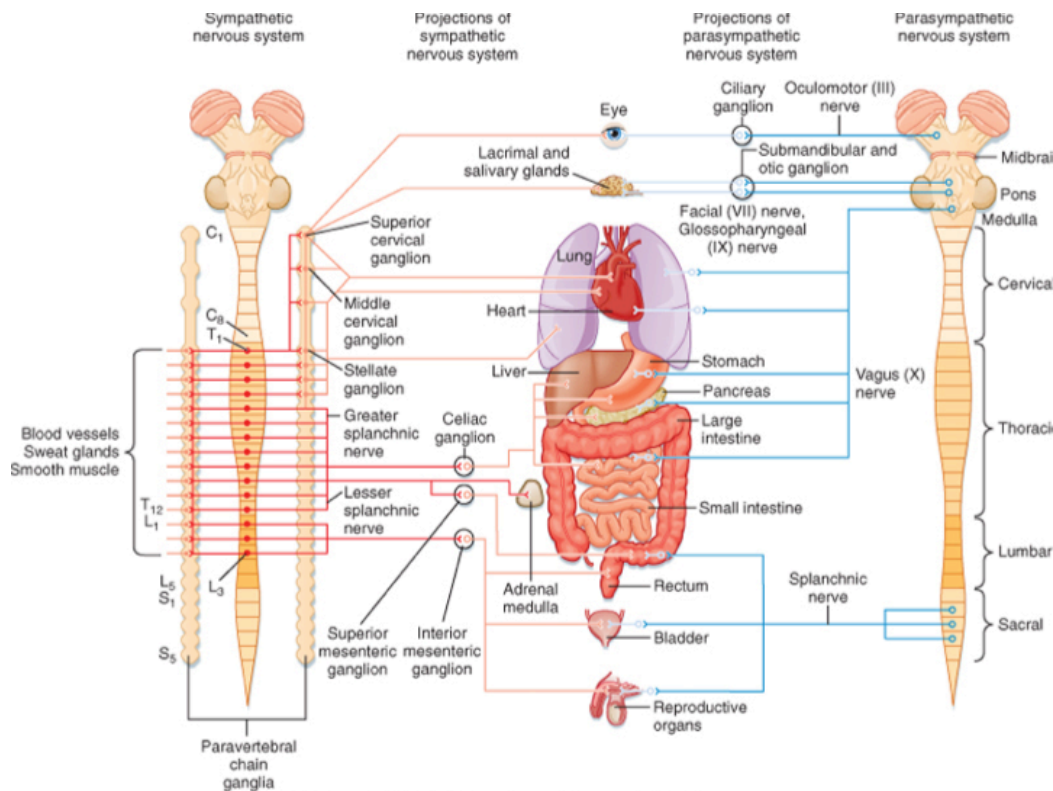
The autonomic nervous system (ANS) is the division of the nervous system that controls automated body functions like heart rate (HR), blood pressure, digestion, and metabolism, to name a few (Carnethon and Craft 2008). It participates in the generation of proper and coordinated responses to either external or internal stimuli and also leads to compensatory action when signals require it (Koeppen and Stanton 2008). Therefore, the ANS has a crucial role in assisting the human body to maintain the homeostasis needed for its correct function (Koeppen and Stanton 2008).

The ANS can be divided into two branches: the sympathetic and the parasympathetic, which work in a coordinated way, at times performing reciprocally and others synergistically, to provide a tight level of control over their target organs (Figure 1) (Koeppen and Stanton 2008). Both branches are tonically active, providing some degree of nervous input to a given tissue at all times (McCorry 2007). Without this tonic activity, nervous input would only be able to increase.

In general, stimulation of the sympathetic branch exerts facilitatory effects on the organism, preparing it for action (Vaseghi and Shivkumar 2008), by inducing an excitatory state. In response to a stimulus, the sympathetic nervous system leads to coordinated changes in organs and tissues throughout the body, including: an increase in the delivery of oxygenated blood to working muscles; an increase in both heart and myocardial contractility, so that the heart pumps more blood per minute; vasoconstriction that redirects or redistributes blood away from inactive tissue toward working muscles; bronchodilatation in the lungs, which facilitates the movement of air in and out of the lungs, so that oxygen uptake and the elimination of carbon dioxide are maximized; and an increase in the rate of glycogenolysis and lipolysis (McCorry 2007; Koeppen and Stanton 2008). Conversely, the parasympathetic nervous system prevails during resting conditions; it is related to vegetative and restorative functions that promote digestion, the storage of substrates, and anabolism (Koeppen and Stanton 2008). The parasympathetic system decreases HR; increases salivary secretion, to facilitate the swallowing of food; stimulates gastric and intestinal motility; increases secretion, to process ingested food and facilitate the absorption of nutrients and promotes pancreatic secretion (McCorry 2007; Koeppen and Stanton 2008). These branches are different in many ways (Vaseghi and

Shivkumar 2008), and the interplay between them is complex and susceptible to control at numerous levels. Central pathways that influence autonomic activity include the spinal cord, brainstem, and hypothalamus (Koeppen and Stanton 2008).

Figure 1: Schematic showing the sympathetic and parasympathetic pathways. Sympathetic pathways are shown in red and parasympathetic pathways in blue. Preganglionics are shown in darker shades and postganglionics in lighter shades. (Used with permission, this figure was published in: *Berne & Levy Physiology sixth edition, Mosby Elsevier 2008*)



The functional ANS units of both the sympathetic and parasympathetic branches are two-neuron motor pathways that consist, each, of a preganglionic neuron and a postganglionic neuron (Koeppen and Stanton 2008). The preganglionic is positioned in the central nervous system, and the axon of this neuron travels to an autonomic ganglion located outside the central nervous system, where it synapses with the postganglionic neuron (McCorry 2007; Koeppen and Stanton 2008). The

postganglionic neuron innervates the effector organ or tissue (McCorry 2007; Koeppen and Stanton 2008), and the axon terminals of these neurons contain multiple varicosities, so that when a neuron is stimulated (by the arrival of an action potential), these varicosities release neurotransmitters that diffuse throughout the interstitial fluid in the synaptic cleft and bind to specific receptors located in the cell membrane of the effector tissue/organ (McCorry 2007). The binding of the neurotransmitters leads to a series of tissue-specific biochemical events within cells, which alters the effector tissue/organ activity (McCorry 2007). All parasympathetic and preganglionic sympathetic neurons have as a neurotransmitter acetylcholine, and most postganglionic sympathetic neurons release norepinephrine (McCorry 2007; Koeppen and Stanton 2008). Besides acetylcholine, sympathetic preganglionic neurons can release enkephalin, substance P, luteinizing hormone-releasing hormone, neurotensin, and somatostatin (McCorry 2007; Koeppen and Stanton 2008). The norepinephrine receptors on target organs are named alpha (α) and beta (β) (McCorry 2007; Koeppen and Stanton 2008), while the acetylcholine receptors are named muscarinic and nicotinic (McCorry 2007; Koeppen and Stanton 2008). The actions that those neurotransmitters mediate are listed, for some target organs, in Table 1.

Both branches innervate many tissues and organs, and they typically have opposite effects. An example of a dually innervated organ is the heart (Thayer and Lane 2007). The heart has an innate ability to start contraction, and its automaticity is an essential physiological function in the human body (Mangoni and Nargeot 2008). After denervation, the intrinsic sinus node rate is about 95 to 110 beats per minute (Guyton and Hall 2006). However, under supine resting conditions, the sinus node rate is about 60 to 70 beats per minute (Goldberger, Stein et al. 2010). In fact, in this condition, little sympathetic influence exists, and the concentration of catecholamine is low, so that the major influence is from the vagus nerve (parasympathetic), which slows the sinus node rate (Goldberger, Stein et al. 2010). Besides its influence on HR, ANS also has effects on: atrioventricular conduction velocity; myocardial contractility; coronary vasculature; and various cardiac electrophysiological parameters, including refractory periods, fibrillation-defibrillation thresholds, automaticity, and triggered activity after potentials (Levick 2003; Koeppen and Stanton 2008). Indeed, the sympathetic and parasympathetic arms of the ANS play a key role in the regulation of cardiovascular homeostasis. Quick variations in cardiovascular function, which are essential to the preservation of body homeostasis,

are mediated by the ANS and depend on neural reflexes primarily integrated within the brainstem (Michelini and Stern 2009). Additionally, receptors in the cardiovascular system are able to detect variations in pressure, volume, flow, blood gases, pH, temperature, and movement and send information about such changes for neural processing and, subsequently, autonomic reaction (Michelini and Stern 2009).

Table 1: Responses of some effector organs to Autonomic Nerve Impulses (Used with permission, this table was adapted from: *Berne & Levy Physiology sixth edition, Mosby Elsevier 2008*)

<i>Effector Organs</i>	<i>Adrenergic Impulse</i>	<i>Cholinergic Impulse</i>
Heart		
Sinoatrial node	Increase in heart rate	Decrease in heart rate
Atria	Increase contractility and conduction velocity	Decrease in contractility
Atrioventricular node	Increase in automaticity and conduction velocity	Decrease in conduction velocity, AV block
His-Purkinje system	Increase in automaticity and conduction velocity	Little effect
Ventricles	Increase in contractility, conduction velocity, automaticity, and rate of idioventricular pacemakers	Slight decrease in contractility
Arterioles		
Coronary	Constriction (α_1); dilation (β_2)	Dilation
Skin and mucosa	Constriction	Dilation
Skeletal muscle	Constriction (α_1); dilation (β_2)	Dilation
Cerebral	Constriction (slight)	Dilation
Pulmonary	Constriction (α_1); dilation (β_2)	Dilation
Abdominal viscera, renal	Constriction (α_1); dilation (β_2)	----
Salivary glands	Constriction	Dilation
Veins (systemic)	Constriction (α_1); dilation (β_2)	
Lung		
Bronchial muscle	Relaxation+	Contraction
Bronchial glands	Inhibition (?)	Stimulation
Kidney	Rennin secretion	----
Adrenal medulla	----	Secretion of epinephrine and norepinephrine
Liver	Glycogenolysis, gluconeogenesis	Glycogen synthesis
Pancreas		
Acini	Decreased secretion	Secretion
Islets (beta cells)	Decreased secretion	----
Fat cells	Lipolysis	----

It has been suggested that deviations in ANS function, characterized by predominance of sympathetic activity and/or reduction of parasympathetic activity, are determinant in a wide range of cardiac and non-cardiac disorders (Goldberger, Stein et al. 2010; Thayer, Yamamoto et al. 2010). Excessive sympathetic activation and diminished vagal modulation not only are markers of an unhealthy cardiovascular system, but, in part, are also the origin of adverse cardiovascular events (Curtis and O'Keefe 2002). Impaired ANS function is associated with the pathogenesis of several arrhythmias, including supraventricular tachycardias, atrioventricular block, and ventricular arrhythmias associated with myocardial ischemia and sudden death (Johnson, Gray et al. 2004). Thus, it appears that sympathetic activation can trigger malignant arrhythmias, while vagal activity may exert a protective effect (Vaseghi and Shivkumar 2008). Hence, it is believed that vagal activity, by improving cardiac electrical stability, has a cardioprotective effect, while sympathetic activity may have the opposite effect (Billman 2002). Indeed, the direct electrical stimulation of sympathetic cardiac nerves decreases the ventricular fibrillation threshold, inducing ventricular arrhythmias (Billman 2002). Moreover, evidence indicates that low parasympathetic modulation is associated with cardiovascular morbidity and mortality (La Rovere, Bigger et al. 1998; Chumaeva, Hintsanen et al. 2010). In addition, imbalanced ANS function has been suggested to be associated with obesity, insulin sensitivity, inflammation, diabetes, hypertension, and metabolic syndrome (Carnethon and Craft 2008). For example, even though autonomic dysfunction is an established complication of diabetes, it may also play a role in the onset of this disease (Carnethon and Craft 2008). Furthermore, imbalanced ANS function seems to wield an adverse effect on the cardiovascular system, inducing cardiac hypertrophy, arterial remodelling, and endothelial dysfunction (Kanaley, Goulopoulou et al. 2009). Parasympathetic activity is reduced and sympathetic hyperactivity is present in pathologies as heart failure (Negrao and Middlekauff 2008) and coronary heart disease (Malpas 2010), with consequences for disease progression, as well as survival.

2.2. Measurement of autonomic nervous system function in humans

As mentioned, increased sympathetic and reduced parasympathetic activity have been linked with higher risk of sudden death, vulnerability to arrhythmias, and several health disorders and diseases (Lahiri, Kannankeril et al. 2008; Goldberger, Stein et al. 2010). Consequently, the evaluation and study of ANS function has gained importance in highlighting the factors that may influence it. The location of the ANS makes it difficult to conduct a direct, simple physiological measure to assess it (Freeman 2006). The most broadly-used methods imply assessment of an end-organ response, such as heart rate variability (HRV) and muscle sympathetic nerve activity (MSNA), which are both objective and reliable tools for assessing ANS function (Hautala, Kiviniemi et al. 2009). Thus, ANS function can be non-invasively measured by using HRV that captures both parasympathetic (vagal) modulation of the heart, as well as global cANS function and is a widely used method, especially in large population studies. MSNA is an invasive methodology that only gives information on sympathetic function and is used mostly in animals and small studies with adults, where more direct and invasive methodologies can be used. Some other markers have been proposed to reflect autonomic activity, including HR, HR recovery, blood pressure variability and others. However, in the following paragraphs, we will only review HRV and MSNA in more detail, since they are the most-used methods (Lahiri, Kannankeril et al. 2008).

2.2.1. Heart rate variability

HRV is a non-invasive method of assessing cANS function (Billman 2009). HR varies on a beat-to-beat basis (Moss and Stern 1996), and even at rest, it oscillates regularly (Kleiger, Stein et al. 2005). Quantification of HR period fluctuations over time is termed HRV or R-R intervals variability. The rhythm of the heart is controlled by the sinoatrial node, which is modulated by both the sympathetic and parasympathetic branches of the ANS. The continuous modulation of the sympathetic and parasympathetic branches results in variations in HR, even at rest. The HR accelerates during inspiration and decelerates during expiration; the expression used to illustrate spontaneous fluctuation of the R-R intervals during respiration is respiratory sinus arrhythmia.

Anomalous HRV is considered an independent risk factor for mortality (Tsuiji,

Venditti et al. 1994; Brouwer, van Veldhuisen et al. 1996; La Rovere, Pinna et al. 2003). Low HRV, indicative of sympathetic predominance and parasympathetic reduction, has prognostic value for all-cause mortality (Tsuji, Venditti et al. 1994) and sudden cardiac death (Brouwer, van Veldhuisen et al. 1996; La Rovere, Pinna et al. 2003). The mechanism by which reduced HRV is related to mortality is probably related to increased sympathetic activity and decreased parasympathetic activity, which reduces the threshold for ventricular fibrillation (Vanoli, De Ferrari et al. 1991; 1996; Saffitz 2008).

HRV can be evaluated by time-domain, frequency-domain, and non-linear indices (Task 1996). It can also be based on either short-term (e.g. 5 to 20 minutes) or long-term (e.g. 24-hour) recordings. Long-term measures mainly reflect longer-term circadian differences in HRV, as well as daytime and nighttime parasympathetic respiratory variation. Total HRV is modulated by sympathetic, parasympathetic, and endocrine influences but mostly expresses circadian rhythm, which is not considered a vagal marker *per se*. On the other hand, short-term measures obtained under laboratory conditions, where subjects are usually in supine positions, do not capture circadian or sleep-related variations, but reflect mainly resting parasympathetic (vagal) variation in HR. Standardized conditions are crucial to short-term HRV measures (Task 1996).

2.2.1.1. Time-domain methods

Time-domain methods are applied directly to series of successive R-R intervals (Figure 2). The indices of time-domain analysis are easily calculated with mathematical methods. A very commonly used and useful index is the standard deviation of the R-R intervals (SDNN) (Equation 1). This measure summarizes all variation in R-R intervals.

Equation 1:

$$SDNN = \sqrt{\frac{1}{N-1} \sum_{j=1}^N (RR_j - \overline{RR})^2}$$

Additionally, other very used measures derived from time-domain methods are: rMSSD (Equation 2), the square root of the mean squared differences of successive R-R intervals; NN50, the number of interval differences between successive R-R intervals greater than 50 ms; and PNN50 (Equation 3), the proportion derived by dividing NN50 by the total number of R-R intervals.

Equation 2:

$$RMSSD = \sqrt{\frac{1}{N-1} \sum_{j=1}^{N-1} (RR_{j+1} - RR_j)^2}.$$

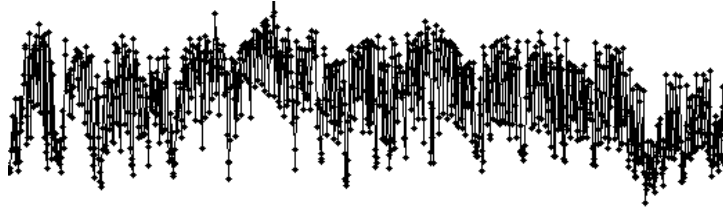
Equation 3:

$$pNN50 = \frac{NN50}{N-1} \times 100\%.$$

All these measures, when derived from short-term variation, estimate high frequency variations in the heart (Task 1996) and reflect vagal modulation of the heart, since these indices suppress with parasympathetic blockade (Lahiri, Kannankeril et al. 2008).

When assessing HRV with long-term recordings, there is greater opportunity to apply mathematical formulas (Moss and Stern 1996). Two measures arise: the SDNN index, which is an index of the SDNN, and the standard deviation of the average R-R intervals calculated over short periods over the course of 24 hours (SDANN). Additionally, in long-term recordings, time-domain measures like the SDNN, SDNN index, and SDANN mainly reflect circadian differences in HRV, while rMSSD reflects the average of daytime and nighttime parasympathetic respiration variation.

Figure 2: Time series of R-R intervals. HRV was assessed with participants in supine position and matching their breathing to a metronome-paced frequency of 12 breaths•min⁻¹ and the last 5 minutes of the 20 minutes were utilized for calculation of HRV variables (short-term).



2.2.1.2. Frequency-domain methods

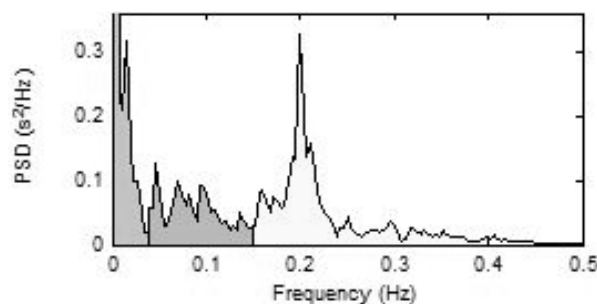
Frequency-domain methods are also called spectral methods, and they are based on power spectrum density (PSD) estimates that are calculated from R-R intervals series. PSD estimation can be done using either fast Fourier transformation, or parametric autoregressive modelling. Spectral analysis of a sequence of R-R intervals is used to provide information on how power is distributed as a function of frequency (Figure 3) (Lahiri, Kannankeril et al. 2008).

Short-term recordings (2 to 5 minutes) have three power spectral bands: very low frequency (VLF – <0.04 Hz), low frequency (LF – 0.04-0.15 Hz), and high frequency (HF – 0.15-0.40 Hz) (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996; Moss and Stern 1996). The physiological meaning of the VLF component is not well defined (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). Vagal activity is the main source of the HF component, resulting from respiratory sinus arrhythmia. However, in respect to the LF component, no agreement exists. It is mainly seen as resulting from both sympathetic and parasympathetic activity (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). The HF and LF components are usually presented as absolute values (ms²), but they may also be calculated as normalized units (nu) (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). When normalized, the effect of total power on their values is minimized. The LF/HF

ratio has been suggested as an index of sympathovagal balance; nevertheless, not all literature agrees with this (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996) .

In long-term recordings (24-hours), four power spectral bands are reported, along with total power: ultra-low frequency (ULF – $< 0.003\text{Hz}$), VLF ($0.003\text{-}0.04\text{ Hz}$), LF ($0.04\text{-}0.15\text{ Hz}$), and HF ($0.15\text{-}0.40\text{ Hz}$) (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996; Moss and Stern 1996). The physiological modulation of ULF is not totally clear. However, it appears that it is mainly determined by circadian rhythms and may also be modulated by the rennin-angiotensin system (Goldberger, Stein et al. 2010). Additionally, reduced ULF is a significant predictor of events such as myocardial infarction, cardiomyopathy, valvular heart disease, congestive heart failure, and mortality (Kleiger, Stein et al. 2005). The significance of VLF is not clear either. However, it is believed that it may reflect parasympathetic and rennin-aldosterone activity (Goldberger, Stein et al. 2010). As in short-term recordings, HF reflects parasympathetic influences (vagally mediated), and the LF component seems to reflect both sympathetic and parasympathetic modulation.

Figure 3: A power spectrum of short-term HRV calculated from the R-R intervals shown in figure 1.



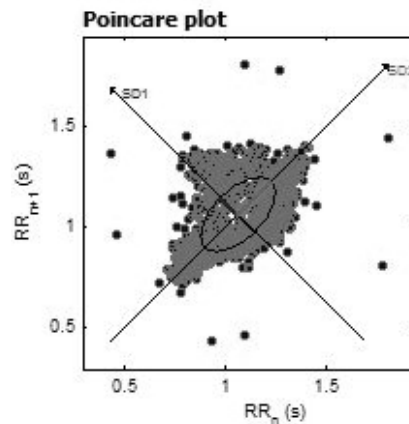
2.2.1.3. Non-linear methods

Non-linear methods are based on the assumption that the mechanisms involved in the genesis of HRV are non-linear (Task Force of the European Society of

Cardiology and the North American Society of Pacing and Electrophysiology 1996). They try to quantify the structure or complexity of R-R intervals (Kleiger, Stein et al. 2005). The non-linear proprieties of HRV can be explored using measures such as Poincaré plot (Brennan, Palaniswami et al. 2001; Carrasco, Gaitan et al. 2001), approximate and sample entropy (SampEn) (Richman and Moorman 2000), detrended fluctuation analysis (Peng, Havlin et al. 1995; Penzel, Kantelhardt et al. 2003), correlation dimension (Guzzetti, Signorini et al. 1996), and recurrence plotting (Webber and Zbilut 1994). Since numerous algorithms have been developed, we will revise only the ones used in this thesis in more detail.

Poincaré plot (Figure 4) is one frequently used non-linear method. It can be represented graphically, and it can reveal abnormal R-R intervals patterns (Kleiger, Stein et al. 2005). There are two Poincaré plot axes: the short diameter of an ellipse (SD1) and the long diameter of an ellipse (SD2). They reflect the deviation of instantaneous beat-to-beat R-R intervals variability. SD12 is the axes ratio and gives information on the organization of heart rate patterns, based on the ratio of the axes of an ellipse, fitted to the scatter plot of R-R intervals.

Figure 4: Poincaré Plot calculated from the R-R intervals shown in figure 1.



SampEn is another non-linear method and is used to quantify the complexity of variations in R-R intervals (Lipsitz and Goldberger 1992). SampEn is largely independent of record duration and exhibits consistency in situations where other measures do not (Richman and Moorman 2000). A lower value for sample entropy

indicates more self-similarity in the time series, as well as lower complexity (Lipsitz and Goldberger 1992). This measure may be of particular interest, since it is considered capable of measuring HR complexity and, thus, may be indicative of the flexibility or adaptability of the cardiovascular autonomic system to internal or external stressors (Lipsitz 1995), such as exercise. Its calculation is given by the following equation.

Equation 4:

$$\text{SampEn}(m, r, N) = \ln(C^m(r) / C^{m+1}(r)), \text{ being } m=2 \text{ and } r=0.2 \text{ SDNN}.$$

Detrended fluctuation analysis (DFA) is a non-linear measure that reflects the randomness or correlativeness of R-R intervals patterns. A totally random R-R intervals pattern has a α value of 0.5, whereas a totally correlated pattern has a value of 1.5 (Kleiger, Stein et al. 2005; Stein and Reddy 2005).

2.2.2. Muscle sympathetic nerve activity (Microneurography)

Direct measurement of MSNA in the peroneal nerve with the microneurography technique is suggested as the “golden standard” for measuring the degree of sympathetic activity (Hautala, Kiviniemi et al. 2009). Additionally, microneurography is the only available method to directly assess efferent post-ganglionic muscle sympathetic nerve activity in humans (Grassi and Esler 1999; Freeman 2006). It involves the insertion of a tungsten microelectrode in a nerve fascicle (Grassi and Esler 1999). The electrode is attached to a pre-amplifier, and the signal is recorded by a polygraph (Grassi and Esler 1999). Additionally, with this technique, both visual and acoustic identification of sympathetic bursts are allowed (Grassi and Esler 1999). Further, sympathetic nerve activity measured from muscles correlates well with sympathetic nerve activity of the heart (Barretto, Santos et al. 2009). Thus, increased MSNA is a marker of increased cardiac sympathetic activity (Barretto, Santos et al. 2009).

Studies using this technique have been mainly conducted on patients, particularly heart failure patients, in whom sympathetic hyperactivation is typical (Negrao and Middlekauff 2008). In fact, studies have shown that higher levels of

MSNA in heart failure patients have consequences for disease progression, as well as survival, and can even predict mortality (Negrao and Middlekauff 2008; Barretto, Santos et al. 2009). Examples of recording results are shown in the following pictures.

Figure 5: Direct recording of muscle sympathetic discharge in a heart failure patient (87 burts/min).

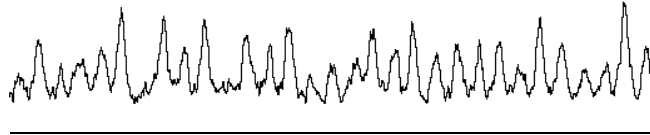
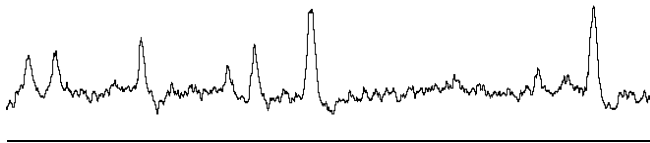


Figure 6: Direct recording of muscle sympathetic discharge in a healthy person (21 burts/min).



2.3. Cardiovascular risk factors and autonomic function

Numerous cardiovascular risk factors, including age, gender, obesity and fat distribution, lifestyle (including physical activity (PA) and nutrition), metabolic parameters, and components of metabolic syndrome (MetS), may be capable of influencing ANS function. This section will review some of those factors.

2.3.1. Age and gender

Ageing is associated with significant changes in several physiological functions, which occur during the normal process of ageing. ANS function is not an exception. Indeed, studies have suggested that ageing is associated with a progressive decrease in HRV and a decrease in the complexity of HR dynamics (Pikkujamsa, Makikallio et al. 1999; Stein, Barzilay et al. 2009). Additionally, it has been suggested that ageing is associated with an increase in MSNA (Kemi and Wisloff 2010).

Therefore, ageing appears to be associated with a decrease in vagal modulation and an increase in sympathetic activity (Malpas 2010).

Gender is considered, by some authors, to be capable of influencing ANS function but no agreement exists. Studies have suggested that women, compared to men, have lower levels of vagal modulation and lower sympathetic activity (Matsukawa, Sugiyama et al. 1998) and that the influence of gender tends to diminish after menopause and with age (Matsukawa, Sugiyama et al. 1998). However, several other studies have failed to find differences between genders and have suggested that several other factors can dissolve those differences (Filaire, Portier et al. 2010; Dangardt, Volkmann et al. 2011).

2.3.2. Obesity and fat distribution

Obesity is characterized by marked sympathetic activation (Malpas 2010) and, thus, ANS impairment. There has been some controversy concerning the effects of obesity and overweight on ANS function and vice versa. Consequently, it is still not clear whether impaired ANS function contributes to obesity or is, instead, a consequence of it. In healthy adults, increased body mass index (BMI) appears to be negatively associated with cANS function, as measured by HRV (Jensen-Urstad, Jensen-Urstad et al. 1998; Molino, Fiorentini et al. 2009). Additionally, healthy non-obese males to whom a diet to gain weight was given had increased sympathetic activity that correlated with the magnitude of their body weight and fat gain (Gentile, Orr et al. 2007). Furthermore, it has been reported that the central-thigh adiposity ratio is associated with lower cardiac parasympathetic modulation (Christou, Jones et al. 2004), and large amounts of visceral fat have been suggested to activate the sympathetic and/or deactivate the parasympathetic nervous system (Lindmark, Lonn et al. 2005). In fact, it has been suggested that upper-body fat, compared with lower-body fat, is more closely associated with cardiovascular disease and metabolic abnormalities of metabolic syndrome (Vague 1956; Kaplan 1989). Moreover, Jean Vague has claimed that the complications commonly found in obese patients are closely related to fat distribution, rather than to excessive weight *per se* (Vague 1956; Despres, Lemieux et al. 2001). Hence, it seems that not only the level of obesity, but mainly fat distribution, influences ANS function. The mechanisms that lead to the

aforementioned facts are unclear, and the role of ANS is uncertain. It is possible that deregulation of the ANS is a primary event that promotes adipose distribution, which would have a detrimental impact on insulin and adipocyte-derived proteins regulation as leptin and adiponectin (Lindmark, Lonn et al. 2005). However, it is also plausible that visceral fat accumulation due to genetic and environmental factors leads to insulin and leptin resistance, which may lead to ANS imbalance (Lustig 2006). The associations between obesity, fat distribution, and cANS function remain uncertain and require further investigation (Yakinci, Mungen et al. 2000).

2.3.3. Lifestyle factors

Several lifestyle factors are capable of influencing ANS function, including diet, PA, and smoking, among others. Physical inactivity and unhealthy diet are considered major lifestyle risk factors for the development of several diseases, including cardiovascular disease (Ignarro, Balestrieri et al. 2007).

2.3.3.1. Physical activity

PA is defined as any body movement that results in the contraction of skeletal muscles that, consequently, increase energy expenditure (ACSM 2010). Exercise is a type of PA that consists of planned, structured, and repetitive bodily movement done to improve or maintain one or more components of physical fitness (ACSM 2010). It is well established that regular PA improves a number of health outcomes and reduces all-cause mortality (Lee 2010). In fact, regular PA, mainly of the endurance type, seems to increase parasympathetic activity and decrease sympathetic activity (Krieger, Da Silva et al. 2001; Carter, Banister et al. 2003; De Angelis, Wichi et al. 2004; Swain and Franklin 2006; Billman 2009), leading to chronic adaptations of cardiac autonomic regulation (Billman 2002) and having antiarrhythmic effects (Hull, Vanoli et al. 1994; Billman 2002). Importantly, this seems to be true not only for healthy populations, but also for patients (Billman 2002). The mechanisms by which regular PA leads to changes in cardiac autonomic control are not totally understood. However, evidence suggests that regular PA is associated with increased vascular compliance and higher levels of arterial restructuring (Krieger, Da Silva et al. 2001;

Green, O'Driscoll et al. 2008). Indeed, areas containing baroreceptors are also remodelled, which may increase their afferent activity, resulting in higher parasympathetic outflow (Green, O'Driscoll et al. 2008). Moreover, animal data suggested that endurance PA is capable of modifying behaviour in the cardio-respiratory centres of the brain, decreasing sympathetic and increasing parasympathetic outflow (Nelson, Juraska et al. 2005; Billman and Kukielka 2007). Remodelling associated with regular PA appears to affect both branches of the ANS (Carter, Banister et al. 2003; Green, Spence et al. 2010). Increased production of nitric oxide is another attractive hypothesis to explain the improvements of cANS function balance with regular PA (Chowdhary, Vaile et al. 2000; Waki, Kasparov et al. 2003; Chowdhary, Marsh et al. 2004). In fact, a recent study has suggested that genetic variations in the endothelial nitric oxide synthase gene may explain, in part, the inter-individual variability in the parasympathetic adaptations induced by regular PA (Silva, Neves et al. 2011). Additionally, it has been proposed that regular PA is capable of inducing neuroplastic changes in areas of the central nervous system (Nelson, Juraska et al. 2005; Michelini and Stern 2009), as the brainstem and hypothalamic neural circuits that influence cardiovascular autonomic function (Michelini and Stern 2009). Other mechanisms suggested by animal studies involve increased acetylcholine content and choline acetyltransferase activity in the heart and the improvement of β -adrenergic receptor balance (Billman and Kukielka 2007). Cardiovascular adaptations to PA depend on its intensity, frequency, and duration. However, the exact dose of exercise needed for cardiac protection is not completely known (Lee 2010).

The positive association between PA and vagal outflow, measured by HRV, has emerged by comparing athletes or very active individuals with sedentary controls (Melanson 2000; Middleton and De Vito 2005). However, observational and interventional studies analyzing the relationship between PA and HRV in more generalizable populations have provided heterogeneous results. Some of these studies have found positive associations between PA and HRV (Melanson and Freedson 2001; Rennie, Hemingway et al. 2003; Buchheit, Simon et al. 2004; Buchheit, Simon et al. 2005; Felber Dietrich, Ackermann-Liebrich et al. 2008; Gilder and Ramsbottom 2008; Sandercock, Hardy-Shepherd et al. 2008), while others have failed to find any relation (Loimaala, Huikuri et al. 2000; Leicht, Allen et al. 2003; Buchheit and Gindre

2006; Greiser, Kluttig et al. 2009; Kluttig, Schumann et al. 2010). There are some possible explanations for the differing results. First, in most of such studies, PA has been assessed indirectly via questionnaires, which have inherent bias. Additionally, the mixed results may be due to differences in group allocation methods. None of these studies have used a priori objective assessment of PA to create groups. Furthermore, some interventional studies have been mainly low-intensity and of short duration. Longer interventions and higher intensities may be needed to achieve physiological adaptations. Nevertheless, it remains unclear from these studies how different elements of PA (such as intensity) are associated with HRV.

It is believed that the central nervous system regulates cardiovascular autonomic functioning via a reciprocal method. This means that improved vagal modulation is traditionally associated with reduced sympathetic activity (Hautala, Kiviniemi et al. 2009). Curiously, sympathetic measurements of MSNA in healthy subjects, under resting conditions, have provided heterogeneous results on the association between MSNA and aerobic fitness (Hautala, Kiviniemi et al. 2009). The controversy over these results remains inconclusive. However, it might be possible, according to some authors, that in healthy subjects, the effects of aerobic fitness on the behaviour of MSNA is non-linear (Hautala, Kiviniemi et al. 2009). Nevertheless, future studies should confirm this theory (Hautala, Kiviniemi et al. 2009).

2.3.3.2. Nutritional factors

Studies analyzing nutritional factors and ANS function are scarce. Existing studies focus mainly on fatty acid consumption and cANS function, assessed by HRV. Consumption of fish and polyunsaturated n-3 fatty acids (n-3PUFA) appears to be associated with more favourable HRV values in older adults (Mozaffarian, Stein et al. 2008). Additionally, intervention studies have found that n-3 PUFA has a positive effect on HRV in healthy men (Christensen, Christensen et al. 1999; Dyerberg, Eskesen et al. 2004), elderly (Holguin, Tellez-Rojo et al. 2005), and coronary patients (Villa, Calabresi et al. 2002). Thus, one mechanism that underlies the protective cardiac effect of n-3 PUFA may involve cANS function. Conversely, one study measuring MSNA in young adults observed that resting MSNA remained unchanged

after n-3 PUFA supplementation (Monahan, Wilson et al. 2004). More studies are needed to analyse those associations.

Trans-fatty acid (TFA) consumption is associated with increased risk for coronary heart disease (Mozaffarian, Katan et al. 2006). Moreover, the several isomers of TFA that exist may have different health effects. Several studies have suggested that dietary TFA, mainly 18:2 TFA (*trans*-18:2), is associated with increased risk of sudden cardiac death (Lemaitre, King et al. 2002; Lemaitre, King et al. 2006), an outcome not strongly related to blood lipid abnormalities (Mozaffarian, Aro et al. 2009). However, potential mechanisms for the relationship between TFA and sudden cardiac death remain uncertain. It has been suggested that dietary fatty acids interact with biological membranes, which are lipid bilayers made up of two hydrophilic surfaces and a hydrophobic core, changing their configuration (Katz 2002). Proteins imbedded in the bilayer as, for example, enzymes, receptors, carriers, pumps, and voltage-gated ion channels, mediate most biological activities (Katz 2002). Thus, some have suggested that TFA may modulate cardiac membrane ion channel function (Katz 2002), having proarrhythmic properties and affecting cardiovascular electrophysiology (Chiuve, Rimm et al. 2009; Siddiqui, Harvey et al. 2009). However, the relationship of TFA consumption with cANS function is not well established. It is tempting to suggest that if a relationship between TFA consumption and HRV or HR exists, this could elucidate novel potential mechanisms whereby TFA may influence coronary heart disease and sudden death. Relatively little is known about this topic. A small (N=79) 8-week intervention, where a diet rich in TFA was given to one group of male subjects (Dyerberg, Eskesen et al. 2004), showed that daily 20g TFA dietary supplementation tended to reduce HRV and increase HR by 3 beats per minute, while no changes were observed in the control group. The association between TFA consumption and cANS function remains under-studied.

Consumption of fruits and vegetables reduces the risk of coronary heart disease. Only one study has used HRV to assess the association between fruits, vegetables, and cANS function (Park, Tucker et al. 2009). This study suggests that increased consumption of green leafy vegetables leads to favourable changes in cANS modulation (Park, Tucker et al. 2009).

Evidence suggests that Mediterranean dietary patterns are associated with reduced risk of death from all causes, including death due to cardiovascular disease (Mitrou, Kipnis et al. 2007). Only one study has assessed the association between cANS function and Mediterranean diet. Positive associations were observed between Mediterranean dietary patterns and cANS function, as measured by HRV (Dai, Lampert et al. 2010).

2.3.4. Metabolic parameters

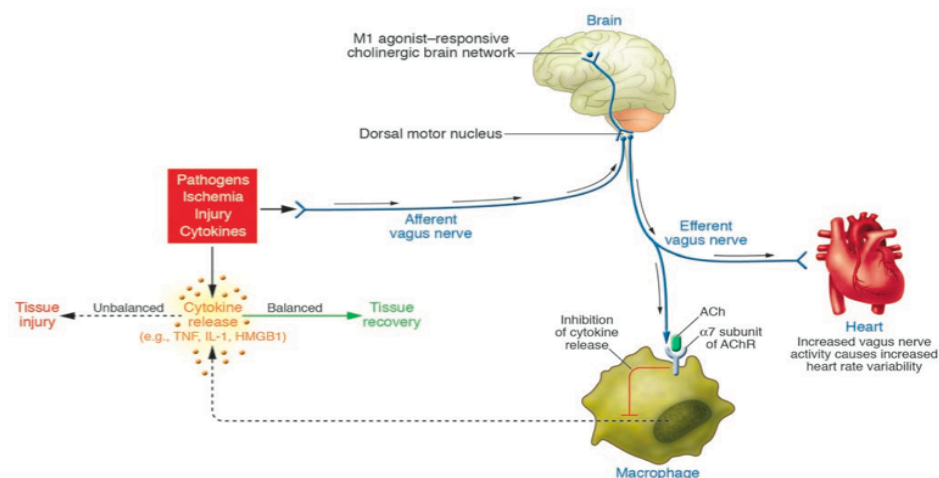
Metabolic parameters as total cholesterol (CHO), low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides (TRG), blood glucose (GLU), C-reactive protein (CRP), and interleukin-6 (IL-6) have been associated with ANS function. Accordingly, TRG (Colhoun, Francis et al. 2001), CHO (Thayer, Yamamoto et al. 2010), and LDL (Thayer, Yamamoto et al. 2010) appear to be inversely associated with ANS function, assessed by HRV. Conversely, it seems that increased HDL is positively associated with HRV (Jensen-Urstad, Jensen-Urstad et al. 1998). Nevertheless, some studies have failed to find associations between these metabolic parameters and HRV (Kluttig, Kuss et al. 2010). Therefore, more studies are needed to analyse those associations.

IL-6 is a pro-inflammatory cytokine that has a central role in inflammatory responses (Saadeddin, Habbab et al. 2002). CRP is a marker of systemic inflammation formed in the liver in response to IL-6 (Vinik, Maser et al. 2011). CRP has been identified as an independent predictor of cardiovascular disease and all-cause mortality (Blake, Rifai et al. 2003; Ridker 2003). These inflammatory markers have been associated with cANS function.

Studies have suggested that vagal activity may play a role in immune response, preventing inflammation through a mechanism called the “nicotinic or cholinergic anti-inflammatory pathway” (Figure 7) (Ulloa 2005). It has been suggested that acetylcholine release by the vagus nerve can inhibit the production of pro-inflammatory cytokines from macrophages by interacting with the alpha 7-nicotinic acetylcholine receptor subunit ($\alpha 7nAChR$) on monocytes and macrophages (Wang, Yu et al. 2003; Ulloa 2005).

This pathway can be activated experimentally by electrical or mechanical stimulation of the vagus nerve (Bernik, Friedman et al. 2002; Wang, Yu et al. 2003) or through administration of an $\alpha 7$ agonist, to inhibit inflammatory cytokine production, which prevents inflammation and increases survival (Huston and Tracey 2011). Animal studies have reported that electrical stimulation of the vagus nerve inhibits the release of pro-inflammatory cytokines (Borovikova, Ivanova et al. 2000). In humans, studies conducted in healthy middle-aged and elderly subjects have reported a negative association between inflammation and vagal modulation (Sajadieh, Nielsen et al. 2004; Araujo, Antelmi et al. 2006; Madsen, Christensen et al. 2007; Sloan, McCreath et al. 2007; Lampert, Bremner et al. 2008; Stein, Barzilay et al. 2008; von Kanel, Nelesen et al. 2008; Thayer and Fischer 2009). This association remains under-studied in healthy young subjects. Moreover, it seems that regular exercise is a strategy to reduce inflammation (Milani, Lavie et al. 2004; Panagiotakos, Pitsavos et al. 2005; Stewart, Flynn et al. 2007) and leads to higher levels of vagal modulation (Rennie, Hemingway et al. 2003; Soares-Miranda, Sandercock et al. 2009). These two factors, lower inflammation and higher vagal modulation, are believed to prevent against the development of cardiovascular disease and, thus, help maintain a healthy cardiovascular system.

Figure 7: Cholinergic anti-inflammatory pathway. (Used with permission, this figure was published in: Tracey KJ. Physiology and immunology of the cholinergic anti-inflammatory pathway. *J Clin Invest.* Feb 2007;117(2):289-296.) (Tracey 2007).



MetS is defined as a cluster of several cardiovascular risk factors and is a pro-inflammatory and pro-thrombotic state (Third Report of The National Cholesterol Education Program 2001). MetS is associated with the development of type 2 diabetes and increased odds of developing cardiovascular disease and negative changes in ANS function (Lorenzo, Okoloise et al. 2003). Although genetic determinants cannot be ignored, the primary contributors to MetS are adverse lifestyle factors (Carnethon and Craft 2008). Insulin resistance has been suggested as the possible core of MetS pathophysiology (Mancia, Bousquet et al. 2007). However, insulin resistance has a multifactorial origin, and the question of which mechanisms underlie it remains unanswered (Mancia, Bousquet et al. 2007). Nevertheless, ANS imbalance may be a determinant of its development and progression (Mancia, Bousquet et al. 2007). It is plausible that changes in autonomic function precede insulin resistance in the initiation of MetS (Mancia, Bousquet et al. 2007). Autonomic dysfunction is present in lean subjects, with small increases in plasma insulin (Kanaley, Gouloupoulou et al. 2009) and after brief exposure to hyperglycemia, or even in patients with normal plasma glucose values (Carnethon, Jacobs et al. 2003; Carnethon, Prineas et al. 2006). Nevertheless, which problem appears first and triggers the cycle of metabolic aberrations is still unclear (Vinik, Maser et al. 2011).

Studies on ANS (assessed by HRV) and MetS have suggested that they are negatively associated (Liao, Sloan et al. 1998; Hemingway, Shipley et al. 2005; Stein, Barzilay et al. 2007; Min, Min et al. 2008; Gehi, Lampert et al. 2009; Koskinen, Kahonen et al. 2009; Chang, Yang et al. 2010). However, these studies have been mainly conducted on middle-aged or elderly subjects (Liao, Sloan et al. 1998; Hemingway, Shipley et al. 2005; Stein, Barzilay et al. 2007; Min, Min et al. 2008; Gehi, Lampert et al. 2009; Chang, Yang et al. 2010), and most have not adjusted their analyses for PA (Liao, Sloan et al. 1998; Stein, Barzilay et al. 2007; Min, Min et al. 2008; Koskinen, Kahonen et al. 2009), a potentially important confounder. Furthermore, there are no longitudinal studies addressing this topic in healthy young adults. Indeed, prospective studies are needed to answer some of the aforementioned questions. Therefore, the associations between MetS and HRV in young, healthy populations are under-studied.

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Chapter 2. Experimental Work

3. Experimental Work

This thesis was conducted in Porto Cohort, a longitudinal study with university students that started in 2008 and finished in 2010. Additionally, one study was also done in Cardiovascular Health Study (CHS), a longitudinal study carried out in older adults.

The basic characteristics of the samples are shown in table 1. A detailed description concerning variables assessment, samples studied and statistical procedures, are given in each paper in the following pages.

Table 2. Summary of the characteristics of the sub-studies.

<i>Study</i>	<i>Sample Size</i>	<i>Age</i>	<i>Variables studied</i>
<ul style="list-style-type: none"> Vigorous physical activity and vagal modulation in young adults. 	84	18-20 years	PA (accelerometer); HRV
<ul style="list-style-type: none"> Benefits of achieving vigorous as well as moderate physical activity recommendations: evidence from heart rate complexity and cardiac vagal modulation. 	168	19-21 years	PA (accelerometer); HRV
<ul style="list-style-type: none"> High levels of C-reactive protein are associated with reduced vagal modulation and low physical activity in young adults. 	80	19-21 years	HRV, hs-CRP, PA (accelerometer);
<ul style="list-style-type: none"> <i>Trans</i>-fatty acid consumption and heart rate variability in older and younger adults. 	160 (young adults) + 1076 (older adults from CHS study)	18-21 years ≥65 years	HRV, <i>Trans</i> -fatty acids intake (24-hour recall and Food Frequency Questionnaire), Plasma phospholipids <i>trans</i> -fatty acids

<ul style="list-style-type: none"> Associations between metabolic syndrome components, physical activity and cardiac autonomic function. 	163	18-21 years	HRV, hs-CRP, PA (accelerometer), SBP, TRG, HDL, Glu WC
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HRV= heart rate variability, WC= waist circumference, PA = physical activity, SBP= systolic blood pressure, DBP= diastolic blood pressure, TRG= Triglycerides, HDL= high-density lipoprotein, hs-CRP= high sensitivity C-reactive protein, LDL= low-density lipoprotein.

Paper I

Vigorous physical activity and vagal modulation in young adults.

Soares-Miranda L, Sandercock G, Valente H, Vale S, Santos R, Mota J.

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Original Scientific Paper

Vigorous physical activity and vagal modulation in young adults

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Background Aerobic exercise leads to reduced sympathetic and increased cardiac vagal modulation, providing an antiarrhythmic effect. The optimal exercise intensity to promote this adaptation remains undefined. The aims of the present investigation were twofold. First, to examine differences in heart rate variability (HRV) measures in participants with different levels of objectively measured physical activity (PA). Second, to identify the characteristic of PA which most influences the cardiac autonomic nervous system (cANS) function in young adults.

Methods Cross-sectional evaluation of 84 adults examining relationships between PA amount and intensities, measured by accelerometry, cANS function derived from HRV. Groups were created based on tertiles of PA and analysis of covariance was used to assess between-group differences in HRV. Stepwise regression analysis was used to determine the characteristic of PA, which best predicted vagal HRV indices.

Results There were significantly higher levels of vagal HRV indices in the most active group compared with the least active group. Regression analysis revealed that the number of bouts of vigorous PA undertaken was the best predictor of the vagal HRV indices assessed.

Conclusion This study suggests that vagal modulation is enhanced with high levels of PA and that it is the number of bouts of vigorous PA that is most closely associated with cANS function. *Eur J Cardiovasc Prev Rehabil* 00:000–000 © 2009 The European Society of Cardiology

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Keywords: cardiac autonomic function, heart rate variability, physical activity

Introduction

Until 1995, the Center for Disease Control (CDC) and American College of Sports Medicine (ACSM) recommendations on physical activity (PA) and public health both recommended that adults should partake in moderate PA to maintain cardiovascular health and reduce the risk of coronary heart disease. When updated in 2007, the guidelines were changed to explicitly recommend the inclusion of vigorous PA [1]. Epidemiological evidence suggests that vigorous PA plays an important role in providing cardioprotective benefits [2,3].

Heart rate variability (HRV) is a noninvasive method to assess cardiac autonomic nervous system (cANS) function. Low HRV is indicative of a shift towards sympathetic predominance, and has prognostic value for all-cause mortality [4] and sudden cardiac death [5,6]. The mechanism by which HRV is related to mortality is probably because of an increased sympathetic activity reducing the threshold for ventricular fibrillation [7–9].

Aerobic exercise leads to a chronic reduction in sympathetic activity and increased vagal tone [2], providing an antiarrhythmic effect [10]. The optimal exercise dose to promote autonomic benefits remains undefined.

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The positive association between PA and vagal outflow measured by HRV is adequately demonstrated by comparing athletes or other very active individuals with sedentary controls [11,12]. Longitudinal studies also show improved HRV after exercise training in sedentary participants [13,14].

Five cross-sectional studies have used a three-group comparison to analyse the relationship between HRV and PA in more generalizable populations, but have provided heterogeneous results [12,15–18]. The mixed results may be because of the different group allocation methods and none of these studies have used a priori objective assessment of PA to create groups.

The aims of this study were twofold. First, to examine differences in HRV between participants grouped by objectively measured moderate and vigorous PA. Second, to examine which elements of objectively measured PA show the strongest association with HRV in a sample of young adults.

Methods

Participants

A total of 84 healthy university students volunteered to participate. Exclusion criteria were: known cardiovascular illness, the taking of any medication or supplements that could influence the control of cANS.

Written informed consent was obtained. The study was approved by the local ethics committee and conducted in accordance to the declaration of Helsinki.

Procedures

All procedures were performed between 08.00 and 11.00 h. Participants were wearing light clothing without shoes. Mass and body fat percentage were assessed Tanita Inner scan, BC-532 (Tanita, Hoofddorp, The Netherlands). Stature was measured using a stadiometer Seca model 708 (Seca, Hamburg, Germany). Body mass index (kg/m^2) was calculated. Waist circumference was measured mid-way from the lower rib margin to the anterior superior iliac crest, using a nonmetallic tape without significant compression [19].

A capillary sample of blood was taken from the ear lobe, after an overnight fast. The sample was analysed using the Cholestech LDX cassette (Cholestech, Hayward, California, USA). The Cholestech LDX shows good agreement with laboratory measures for population-based risk factor screening [20] and meets the criteria set by lipid standardization panel for accuracy and precision of cholesterol measurements [21].

A standard 24-h dietary recall was applied to evaluate food intake. Twenty-four hour recall is the most common

dietary assessment method in large-scale studies because of its easy way to administer [22]. Food intake was converted to nutrient values by the Food Processor Plus (ESHA, Salem, Oregon, USA), participants were asked how typical the 24-h recall was of their regular eating pattern.

Free-living PA was assessed with a uniaxial ActiGraph accelerometer (model GT1M; ActiGraph, Fort Walton Beach, Florida, USA). The device provides valid and reliable measures of vertical body acceleration [23] and provides estimates of energy expenditure comparable with other accelerometers and room calorimetry [24]. Participants were instructed to wear the monitor on the iliac crest of the right hip with an elastic belt; to remove it for sleep and in any activity that may cause damage to the monitor. PA was monitored in 15 s epochs for up to seven consecutive days. A minimum recording period of 5 days was necessary for PA measurement to be considered adequate [25]. Freedson *et al.*'s [26] cut points were used to analyse data through a Mahuffe activity analyser (MRC Epidemiology Unit, Cambridge, UK). Counts were transformed to average minutes per day in light (< 1951 counts/min), moderate (1952–5724 counts/min), vigorous (5725–9498 counts/min) and very vigorous (> 9499 counts/min) PA. Daily time spent in moderate-to-vigorous PA (MVPA) was calculated. An exercise bout was defined as 10 min of continuous PA. Ten minutes was chosen because recommendations of the ACSM/AHA emphasizes the need of bouts of PA lasting 10 or more minutes [1].

Recordings of R–R interval data were made at rest in a quiet room, in the supine position, using the Polar Advantage NV (Polar Electro OY, Kempele, Finland). Verbal instructions and a practice period of paced breathing were provided. Participants matched their breathing to a metronome-paced frequency of 12 breaths/min. Participants were asked to avoid strenuous exercise and caffeine or alcohol consumptions for 24 h before examination.

The Polar Advantage heart monitor is comparable with the more conventional ECG devices for R–R interval measurement [27,28]. R–R interval data were downloaded into Polar Precision Performance Software SW (Polar Electro OY). Ectopic beats or arrhythmias were excluded from the analysis. The last 5 min of the 20 min was utilized for calculation of HRV variables. The R–R interval were analysed using time domain, frequency domain and Poincaré plot techniques using HRV Analysis Software 1.1 for Windows developed by The Biomedical Signal Analysis Group, University of Kuopio, Finland.

Time domain measures included mean R–R interval, square root of the mean of the squares of successive R–R interval differences (RMSSD) and standard deviation of

the R–R interval (SDNN). R–R intervals were also used to produce Poincaré Plot, SD1 (Poincaré plots descriptor: deviation of instantaneous beat-to-beat R–R interval variability is short diameter of ellipse) and SD2 (Poincaré plots descriptor: deviation of instantaneous beat-to-beat R–R interval variability is long diameter of ellipse) values [29–31].

Nonparametric (fast Fourier transform) method was used to obtain frequency domain measures of HRV. Short-term HRV recording enabled the computation of the three-frequency bands: very-low frequency (0.00–0.04 Hz), low frequency (LF: 0.004–0.15 Hz), and high frequency (HF: 0.15–0.4 Hz). Only LF and HF were considered in our analysis, as the very-low frequency component is considered a dubious measure because of its uncertain physiological meaning and interpretation [7]. A normalized HF value computed as HF/LF + HF was also reported.

Statistical analysis

Data were tested for normality of distribution using the Kolmogorov–Smirnov test. Owing to skewed distributions, HF and LF were transformed using natural logarithms (ln) before parametric analyses.

Participants were grouped according to tertile of minutes spent in MVPA with 1 as the lowest. MVPA was used because ACSM/American Heart Association suggest combinations of moderate and vigorous PA to reach recommendations [1].

Descriptive statistics (means and standard deviations) were used to characterize the sample. One-way analysis of

variance, with Bonferroni correction, was used to identify differences between tertiles. Between-tertile comparisons for HRV measures were made using analysis of covariance adjusting for sex. Post-hoc comparisons and Bonferroni correction were used to identify between-group differences.

Stepwise linear regression was used to determine which elements of PA (bouts of moderate PA, bouts of vigorous PA, minutes of vigorous PA per week and minutes of moderate PA per week) accounted for most variance in RMSSD, SD1 and HF. The level of significance was set at *P* value less than 0.05. Data were analysed using the SPSS version 15.0 (SPSS Inc., Chicago, Illinois, USA).

Results

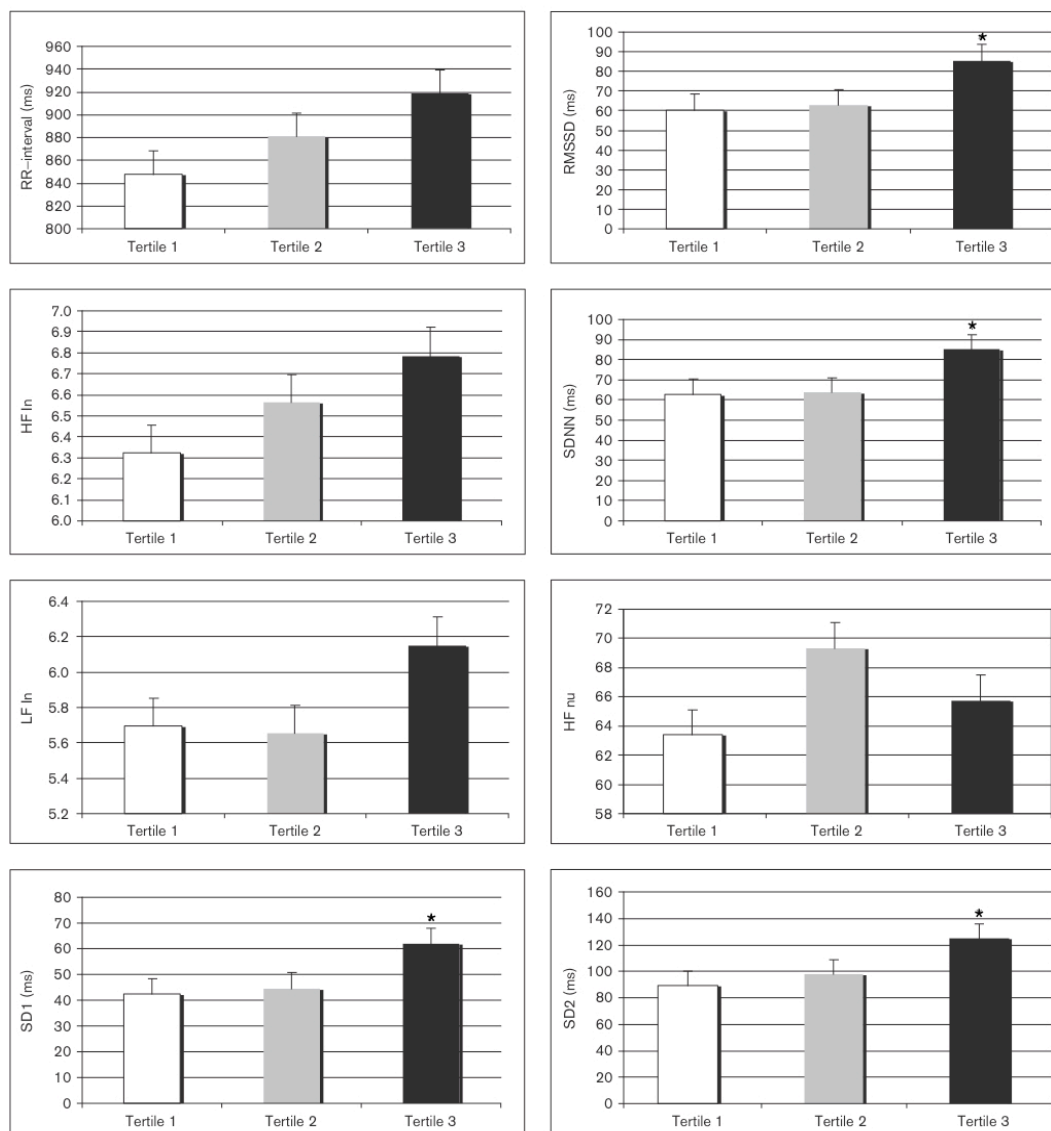
Table 1 shows the descriptive characteristics of the sample and between-tertile differences. Body fat percentage was significantly lower in the highest tertile of PA when compared with the lowest tertile. Steps, MVPA, moderate PA in a week and 10-min bouts of moderate PA were significantly higher in tertile 3 when compared with tertiles 2 and 1. In addition, those variables were significantly higher in tertile 2 compared with tertile 1. Ten-minute bout of vigorous activity and average of light and vigorous PA in a week were significantly higher in tertile 3 when compared with tertiles 1 and 2. Sedentary time was significantly lower in the most active tertile and was significantly lower in tertile 2 than tertile 1. No significant between-group differences were found in sex, age, weight, height, body mass index, waist circumference, low-density lipoprotein, high-density lipoprotein, Glu, omega 3 or bouts of very vigorous PA (Fig. 1).

Table 1 Descriptive characteristics by tertile of habitual physical activity (mean \pm SD)

	Tertile 1	Tertile 2	Tertile 3
Sex (male/female)	8/19	11/17	17/12
Smoke status (yes/no)	4/23	4/24	9/20
Age	19.81 \pm 1.00	19.32 \pm 0.55	19.55 \pm 1.12
Height (m)	167.43 \pm 8.94	167.54 \pm 9.14	171.19 \pm 9.39
Weight (kg)	64.89 \pm 13.88	65.49 \pm 9.31	67.98 \pm 10.38
BF %	23.47 \pm 7.24	22.64 \pm 7.73	18.17 \pm 7.67*
BMI (kg/m ²)	22.99 \pm 3.7	23.28 \pm 2.40	23.12 \pm 2.41
WC (cm)	81.33 \pm 11.65	81.00 \pm 6.27	80.14 \pm 7.28
LDL (mg/dl)	119.59 \pm 42.34	101.51 \pm 29.19	104.52 \pm 34.42
HDL (mg/dl)	43.81 \pm 11.88	48.61 \pm 15.91	45.38 \pm 13.53
Glu (mg/dl)	79.19 \pm 5.88	80.75 \pm 8.86	78.62 \pm 6.38
TRG/HDL (mg/dl)	2.04 \pm 1.03	1.38 \pm 0.52*	1.58 \pm 0.72
MVPA (min/week)	162.18 \pm 35.25	245.25 \pm 21.06*	400.79 \pm 105.87***
Light PA (min/week)	376.41 \pm 104.18	430.86 \pm 132.63	490.21 \pm 117.78*
Moderate PA (min/week)	159.81 \pm 35.00	239.25 \pm 19.99*	372.93 \pm 81.98***
Vigorous PA (min/week)	2.33 \pm 4.23	5.61 \pm 6.64	25.07 \pm 28.32*
10-min bout, moderate (bout/week)	2.74 \pm 2.47	5.43 \pm 2.89*	10.00 \pm 5.59***
10-min bout, vigorous (bout/week)	0.00 \pm 0.00	0.07 \pm 0.26	0.31 \pm 0.60*
10-min bout, very vigorous (bout/week)	0.00 \pm 0.00	0.00 \pm 0.00	1.10 \pm 4.41
Sedentary (min/week)	2704.93 \pm 419.86	3039.54 \pm 445.48***	2827.14 \pm 371.49
Omega 3 (g/day)	0.68 \pm 0.75	0.56 \pm 0.32	0.64 \pm 0.46

Analysis of variance with Bonferroni post-hoc for continuous variables and χ^2 test for categorical variables. BF, body fat; BMI, body mass index; Glu, glucose; HDL, high-density lipoprotein; LDL, low-density lipoprotein; light PA, average of light physical activity during the week; moderate PA, average of moderate physical activity during the week; MVPA, moderate-to-vigorous PA; PA, physical activity; TRG/HDL, triglycerides/high-density lipoprotein ratio; vigorous PA, average of vigorous physical activity during the week; WC, waist circumference. **P* < 0.05 compared with tertile 1. ***P* < 0.05 compared with tertile 2. ****P* < 0.05 compared with tertile 3.

Fig. 1



Time and frequency domain measures and Poincaré plots of heart rate variability in participants grouped according to tertile of free-living physical activity. HF ln, high frequency powering ln; HF nu, high frequency normalized unit; LF ln, low frequency powering ln; RMSSD, square root of the mean of the squares of successive R-R interval differences; R-R interval, the mean length of the filtered R-R intervals; SD1, deviation of instantaneous beat-to-beat R-R interval variability is short diameter of ellipse; SD2, deviation of instantaneous beat-to-beat R-R interval variability is long diameter of ellipse; SDNN, standard deviation of all normal-to-normal R-R intervals. * $P < 0.05$ compared with tertile 1; ANCOVA controlling for sex, comparison with Bonferroni post-hoc.

Analysis of covariance showed significant differences between PA tertiles in RMSSD, SDNN, SD1 and SD2. Post-hoc tests showed higher values for all vagal indices

(R-R interval, RMSSD, HF ln, SDNN, SD1) in tertile 3 compared with tertile 1, but there were no other significant between-group differences.

Stepwise regression using bouts of moderate and vigorous PA showed that only the number of bouts of vigorous PA predicted RMSSD ($\beta = 30.3 \pm 10.2$, $P = 0.004$) and SD1 ($\beta = 21.7 \pm 7.2$, $P = 0.004$). There were no significant predictors of HF. Using minutes of vigorous and moderate PA per week, only weekly vigorous PA predicted RMSSD ($\beta = 0.962 \pm 0.32$, $P = 0.003$) and SD1 ($\beta = 0.685 \pm 0.23$, $P = 0.003$).

Stepwise regression with collinearity diagnostics including all PA variables was performed to determine the best overall predictors of SD1 and RMSSD. Number of weekly vigorous PA bouts best predicted RMSSD ($\beta = 40.86 \pm 12.9$, $P = 0.002$) and SD1 ($\beta = 29.06 \pm 9.2$, $P = 0.002$).

Discussion

The aims of this study were twofold. First, to examine differences in HRV measures in participants grouped by objectively measured PA. Second, to determine the characteristic of PA most strongly associated with vagal measures of cANS function in young adults. The following discussion will address each aim in turn.

Differences in heart rate variability between participants grouped by physical activity

Studies using three groups of participants drawn from the general population are relatively scarce [12,15–18] and can be grouped according to their findings. Most studies [12,15,16] show enhanced vagal modulation in moderately and highly active groups compared with low-active groups. Buchheit *et al.* [17,18] demonstrated an alternative relationship between self-reported PA and HRV in which moderate PA is associated with higher HRV in young [17] and middle-aged [18] participants. Generalization from these studies is difficult as the young active group [17] reported extremely high PA levels and the active middle-aged participants [18] were highly trained. These and all previous studies have used self-report to create groups, although two studies have used accelerometry to verify activity levels [16,18].

This study is the first to group participants according to objectively measured PA. Using this methodology, we provide results that differ from those previously published. At present, we found that only the most active group had significantly higher levels of vagal modulation compared with the least active group. This suggests that some vigorous PA is necessary to enhance vagal outflow.

These findings differ from those suggesting that moderate PA is associated with enhanced HRV [17,18] and from studies which show differences between low and moderate activity groups without further differences between low and highly active individuals [12,15,16]. Recently, Sandercock *et al.* [15] found that the most

active participants showed bradycardia but without correspondingly higher vagal HRV indices. The study, however, used the Baecke questionnaire total activity score [32] which has two shortcomings. First, it relies on self-report, which may lead to recall bias and unreliable estimation of PA in some participants. Second, and more importantly, the Baecke score measures only the total amount of reported PA and gives no information about the type or intensity. Therefore, the present data differ in some way from findings of all previous studies.

Differences in findings are most probably because of the use of objectively measured MVPA. By creating groups in this way, we have shown a positive dose-response relationship, which suggests that vigorous PA is necessary to significantly enhance cardiac vagal control as measured by HRV.

As it seems that vigorous PA is needed to significantly modify vagal cANS activity, it may also be the case that elements of objectively measured PA have a different relationship with HRV than those assessed by self-report. Given this possibility, the second aim of this study was to ascertain which elements or characteristics of PA are best associated with vagal outflow.

Elements of physical activity associated with vagal indices of heart rate variability

This is the first study to demonstrate that, whether expressed as the total number of bouts or as total weekly minutes, vigorous PA showed the highest association with vagal HRV indices RMSSD and SD1. Vigorous PA is more strongly associated with vagal HRV measures than moderate PA. These results support previous findings in adults showing vigorous, not moderate, PA was associated with higher HF power in adult males [33] and adolescents [34,35].

The finding that bouts (10 min) of vigorous PA are most effective in improving cardiac vagal modulation confirm previous findings showing that endurance-type exercise interventions, including bouts of vigorous PA improve cardiac vagal modulation [36–38]. Levy *et al.* [36] found that 45 progressive bouts of PA over 6 months significantly increased HRV. Melanson and Freedson [37] demonstrated that an 8-week intervention with 30 min cycling bouts at 70–80% heart rate reserve lead to significant increases in pNN50 (percentage of successive intervals differing more than 50 ms), RMSSD and HF. Lee *et al.* [38] and Carter *et al.* [39] both found that 12 weeks of vigorous (40–65 min) exercise bouts increased vagal modulation. Our study supports such results, as we found that the number of 10-min bouts of vigorous PA best predicted vagal HRV indices. As expected, vigorous PA was a better predictor of heart variability measures.

These findings indicate that a certain duration of vigorous PA is needed to produce physiological adaptations. Vigorous PA plays an important cardioprotective role [2], possibly through autonomic adaptation as shown here [2]. It has been hypothesized that vigorous PA would result in higher levels of autonomic adaptation compared with moderate intensity PA of equal energy expenditure [2]. This hypothesis is supported by the findings from regression analysis, but not from our between-group comparisons.

The major strength of this study is the use of objective measures of PA in-group allocation. One major limitation of this study is the cross-sectional design, which prevents us from making causal inferences. Accelerometers cannot provide valid information regarding specific activities, as cycling and swimming and no qualitative data on PA were recorded. Therefore, we are unable to identify specific activities, which produced bouts of intense PA. A combined objective and subjective approach would overcome this limitation in future research. Nevertheless, accelerometry is rapidly becoming the method of choice for PA monitoring because of its objectivity, validity and reliability in providing information on amount and intensity of PA. A full discussion of accelerometry is beyond the scope of this study (for recent reviews on this topic, see Refs [24,40]).

The results presented here are unique in showing that vigorous PA is most strongly associated with cardiac vagal modulation in young adults. It seems that the PA stimulus required to elicit changes in cANS function should last at least 10 min and needs to be performed at a vigorous intensity.

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Conflicts of interest: none declared.

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Paper II

**Benefits of achieving vigorous as well as moderate physical activity
recommendations: evidence from heart rate complexity and cardiac vagal
modulation.**

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Benefits of achieving vigorous as well as moderate physical activity recommendations: Evidence from heart rate complexity and cardiac vagal modulation

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Abstract

The aim of this study was to examine differences in traditional heart rate variability measurements and heart rate complexity (sample entropy) in young adults grouped by objectively measured achievement of either moderate or both moderate and vigorous physical activity recommendations. Of 168 young adults tested (86 females, 82 males; age 20.5 ± 1.2 years), 119 achieved only recommendations for moderate physical activity (moderate group) and 49 achieved recommendations for both moderate and vigorous physical activity (vigorous group). Analysis of covariance controlling for sex, weekly minutes of moderate physical activity, and percentage of body fat was used to assess between-group differences in heart rate variability and heart rate complexity. Logistic regression analysis was used to determine the group characteristics that best predicted high heart rate complexity and vagal indices of heart rate variability. The majority of the autonomic measures were higher ($P < 0.05$) in the vigorous group, and regression analysis showed that vigorous physical activity was the only multivariate predictor of higher heart rate complexity and higher heart rate variability. Young adults engaged in regular vigorous physical activity were more than twice as likely to have high heart rate complexity than those involved in predominantly moderate exercise. These findings suggest that vigorous physical activity is more closely associated with high heart rate complexity than moderate physical activity in young adults.

Keywords: Heart rate variability, exercise, intensity, entropy, vagal modulation

Introduction

Low heart rate variability measured in the time or frequency domain indicates a shift towards sympathetic cardiac predominance and is an independent indicator for all-cause mortality (Tsuiji et al., 1994) and sudden cardiac death, especially in patients (Brouwer et al., 1996; La Rovere et al., 2003).

Cross-sectional studies show that greater overall levels of physical activity and aerobic fitness are associated with higher values of traditional indices of heart rate variability (Buchheit et al., 2005; Melanson, 2000; Sandercock, Hardy-Shepherd, Nunan, & Brodie, 2008). What is not clear from these studies is how different elements of physical activity (such as intensity) are associated with heart rate variability.

There is also reasonable consensus that traditional time and frequency domain heart rate variability indices respond positively to aerobic training inter-

ventions (Sandercock, Bromley, & Brodie, 2005) due probably to their close association with vagal autonomic activity (Platasa & Gal, 2006). Non-linear indices of heart rate variability may complement traditional measures by providing additional information on the complexity, or randomness, of a time series (Pikkujämsä et al., 1999; Tulppo et al., 2005). One such measure, sample entropy, may be of particular interest, since it is considered to be able to measure heart rate complexity, and thus be indicative of the flexibility or adaptability of the cardiovascular autonomic system to adapt to internal or external stressors (Lipsitz, 1995) such as exercise. Low heart rate complexity is indicative of autonomic pathology (Pincus & Goldberger, 1994) and is associated with the onset of atrial fibrillation (Vikman et al., 1999), ventricular fibrillation (Makikallio et al., 1999b), and tachycardia (Makikallio et al.,

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1997). Resistance exercise (Heffernan, Fahs, Shin-sako, Jae, & Fernhall, 2007) and mixed (resistance and aerobic) training (Karavirta et al., 2009) can increase sample entropy, but no such effect has been shown with aerobic training (Kanaley et al., 2009).

The American College of Sports Medicine and the American Heart Association physical activity guidelines recommend accumulation of 30 min or more of moderate-intensity aerobic physical activity on 5 days per week or 20 min of vigorous-intensity aerobic physical activity on 3 days per week to promote health (Haskell et al., 2007). Vigorous physical activity may convey greater health benefits than light or moderate activities (Kemi & Wisloff, 2010; Swain & Franklin, 2006) but the optimal exercise dose to promote maximal cardiovascular health benefits remains unclear.

The aim of this study was to determine if there are differences in traditional heart rate variability indices and heart rate complexity (measured by sample entropy) in young adults who achieve only moderate or both moderate and vigorous physical activity recommendations.

Materials and methods

Study population

A total of 168 healthy university students volunteered to participate in the study. Exclusion criteria were cardiovascular diseases assessed by clinical examination and any medication or supplements that could influence the control of cardiac autonomic function. The study was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each individual who participated in the study.

Procedures

All procedures were performed between 08.00 and 11.00 am. Participants wore light clothing without shoes. Body mass and percent body fat were assessed by a Tanita Inner scan (BC-532, Tanita, Hoofddorp, Netherlands). Stature was measured using a stadiometer (model 708, Seca, Hamburg, Germany). Waist circumference was measured midway from the lower rib margin to the anterior superior iliac crest, using a non-metallic tape without significant compression (Graham et al., 2007). A capillary sample of blood was taken from the ear lobe, after an overnight fast. The sample was analysed using a Cholestech LDX[®] cassette (Cholestech, Hayward, CA, USA). The Cholestech LDX[®] shows good agreement with laboratory measures for population-based risk factor screening (Shemesh, Rowley, Shephard, Piers, & O'Dea, 2006) and meets the criteria set by the Lipid

Standardization Panel for accuracy and precision of cholesterol measurements (Allison, Pavlinac, & Wright, 2007).

A standard 24-h dietary recall was applied to evaluate food intake. Such 24-h recall is the most commonly used dietary assessment method in large-scale studies due to ease of administration (Biro, Hulshof, Ovesen, & Amorim Cruz, 2002). Food intake was converted to nutrient values by Food Processor Plus[®] (ESHA, Salem, OR, USA), and participants were asked how typical the 24-h recall was of their regular eating pattern. Free-living physical activity was assessed with a uniaxial Acti-Graph accelerometer (model GT1M, Fort Walton Beach, FL, USA). This device provides valid and reliable measures of vertical body acceleration (Brage, Wedderkopp, Franks, Andersen, & Froberg, 2003) and estimation of energy expenditure (Rothney, Schaefer, Neumann, Choi, & Chen, 2008). Participants were instructed to wear the monitor on the iliac crest of the right hip with an elastic belt and to remove it for sleep and during any activity that might damage the monitor. Physical activity was monitored in 15-s epochs for up to seven consecutive days. A minimum recording period of 5 days (4 weekdays and 1 weekend day) was necessary for the physical activity measurement to be considered adequate (Esliger, Copeland, Barnes, & Tremblay, 2005). Freedson and colleagues' cut points were used to analyse the data via a Mahuffe activity analyser (Freedson, Melanson, & Sirard, 1998). Counts were transformed to average minutes per day in light ($<1951 \text{ counts} \cdot \text{min}^{-1}$), moderate ($1952\text{--}5724 \text{ counts} \cdot \text{min}^{-1}$), vigorous ($5725\text{--}9498 \text{ counts} \cdot \text{min}^{-1}$), and very vigorous ($>9499 \text{ counts} \cdot \text{min}^{-1}$) physical activity.

Recordings of R-R intervals were made at rest in a quiet room, in the supine position, using the Polar Advantage NV (Polar Electro OY, Finland). Verbal instructions and a practice period of paced breathing were provided. Participants matched their breathing to a metronome-paced frequency of 12 breaths $\cdot \text{min}^{-1}$. Participants were asked to avoid strenuous exercise, caffeine and alcohol consumption, and smoking in the 24-h prior to the examination. The Polar Advantage heart monitor is comparable to the more conventional ECG devices for R-R intervals measurement (Nunan et al., 2009; Radespiel-Troger, Rauh, Mahlke, Gottschalk, & Muck-Weymann, 2003). The R-R intervals data were downloaded into the Polar Precision Performance Software SW. The last 5 min of the 20 min was utilized for the calculation of variables of heart rate variability. The R-R intervals were analysed using the Kubios HRV Software v.2.0 for Windows (Biomedical Signal Analysis Group, University of Kuopio, Finland). Standard deviation of the R-R intervals (SDNN), and

square root of the mean of the squared differences of successive R-R intervals (RMSSD) were calculated. Non-parametric analysis (Fast Fourier transform) was used to obtain the high frequency band of heart rate variability. Only the high frequency band and high frequency normalized variables (HF nu) were considered in our analysis, since high frequency activity allows quantification of vagal modulation (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Furthermore, the low frequency/high frequency ratio (LF/HF ratio) was calculated as a measure that might express sympathovagal balance. The R-R intervals were also used to produce Poincaré plot axes: the short diameter of ellipse (SD1) and the long diameter of ellipse (SD2) (Braun et al., 1998; Gilder & Ramsbottom, 2008; Mourot et al., 2004). Poincaré plot variables were normalized by dividing the absolute value by the mean R-R intervals and then multiplying by 1000 (SD1 nu, SD2 nu) (Tulppo, Makikallio, Seppanen, Laukkanen, & Huikuri, 1998). Sample entropy was used to quantify the complexity of variations in R-R intervals (Lipsitz & Goldberger, 1992). Sample entropy (SampEn) is largely independent of record duration and exhibits consistency when other measures do not (Richman & Moorman, 2000). A lower value for sample entropy indicates more self-similarity in the time series as well as less complexity (Lipsitz & Goldberger, 1992). Its calculation by the Kubios HRV software is given in equation (1):

$$\text{SampEn}(m, r, N) = \ln(C^m(r)/C^{m+1}(r)) \quad (1)$$

where $m = 2$ and $r = 0.2$ SDNN.

Statistical analysis

Data were tested for normality of distribution using the Kolmogorov-Smirnov test. Due to skewed distributions, high frequency and low frequency/high frequency ratio were logarithmically transformed before parametric analyses.

Participants were grouped according to whether they achieved specific elements of the current American College of Sports Medicine and the American Heart Association recommendations for physical activity. Specifically, the moderate group achieved the recommendations for moderate physical activity (150 min per week), while the vigorous group achieved 150 min of moderate physical activity but also achieved at least 60 min of vigorous physical activity per week.

Descriptive statistics (means and standard deviations) were used to characterize the sample. An independent samples *t*-test was used to identify between-group differences in continuous variables; chi-square was used for categorical variables. Between-group comparisons

were also made using analysis of covariance (ANCOVA) adjusted for sex, minutes of moderate physical activity per week, and percent body fat.

Sample entropy, standard deviation of the R-R intervals, square root of the mean of the squared differences of successive R-R intervals, Poincaré plot short diameter of ellipse normalized, Poincaré plot long diameter of ellipse normalized, high frequency and high frequency normalized were dichotomized by median split to enable analysis by logistic regression. Separate, univariate logistic regression models were created to determine which variables should be included in the final model. Logistic regression adjusted for sex was used to determine the best predictor of heart rate variability indices.

Statistical significance was set at $P < 0.05$. Data were analysed using SPSS version 15.0.

Results

Table I provides the descriptive characteristics of the sample, and between-group differences. Percent body fat and heart rate were significantly lower in the vigorous than in the moderate group. Moderate physical activity and vigorous physical activity were both significantly higher in the vigorous than in the moderate group. No significant between-group differences were observed for age, waist circumference, total cholesterol, high-density lipoprotein, low-density lipoprotein, glucose or omega-3.

Table I. Descriptive characteristics of the participants (mean \pm s).

	Moderate group (<i>n</i> = 119)	Vigorous group (<i>n</i> = 49)
Sex (M/F)	46/73	36/13*
Smoking status (yes/no)	10/109	5/42
Age (years)	20.5 \pm 1.1	20.8 \pm 1.5
Percent body fat	21.62 \pm 7.08	19.00 \pm 6.09*
Waist circumference (cm)	76.12 \pm 9.35	78.50 \pm 7.74
Total cholesterol (mg \cdot dl ⁻¹)	154.76 \pm 35.04	153.14 \pm 31.53
HDL cholesterol (mg \cdot dl ⁻¹)	50.58 \pm 14.81	48.20 \pm 13.19
LDL cholesterol (mg \cdot dl ⁻¹)	89.15 \pm 28.67	89.43 \pm 30.21
Glucose (mg \cdot dl ⁻¹)	84.10 \pm 7.22	86.50 \pm 6.88
Moderate PA (min \cdot week ⁻¹)	256.18 \pm 86.82	357.85 \pm 105.45*
Vigorous PA (min \cdot week ⁻¹)	10.76 \pm 10.35	70.04 \pm 9.35*
Omega 3 (g/day)	0.65 \pm 0.57	0.74 \pm 0.49
Resting heart rate (beats \cdot min ⁻¹)	71 \pm 12	65 \pm 17*

Note: HDL = high-density lipoprotein, LDL = low-density lipoprotein, Glu = glucose, Moderate PA = average of moderate physical activity during the week, Vigorous PA = average of vigorous physical activity during the week. * $P < 0.05$ compared with moderate group, *t*-test for independent samples and chi-square test for categorical variables.

ANCOVA showed significant between-group differences in standard deviation of the R-R intervals, square root of the mean of the squared differences of successive R-R intervals, Poincaré plot short diameter of ellipse normalized, high frequency (vigorous group = 4612 ms², moderate group = 3207 ms²; $P=0.035$), and sample entropy. Heart rate variability indices were significantly higher in the vigorous group compared with the moderate group, with the exception of the low frequency/high frequency ratio and high frequency normalized (Figure 1).

Table II shows the results of the logistic regression analysis. Only sex, vigorous physical activity/week, and physical activity recommendation groups (moderate and vigorous groups) were significantly related to the dichotomized measure of sample entropy.

Males were nearly twice as likely than females to have sample entropy values above the median split value. Members of the vigorous group were also more than twice as likely to display such values. Moderate physical activity did not predict sample entropy. These findings were used to select variables for inclusion in the final multivariate model to predict sample entropy; in this case, sex, and recommendation group.

Logistic regression analysis (Table III) adjusted for sex showed that members of the vigorous group were more likely to have higher (above median split) vagal and sample entropy values than their counterparts in the moderate group. This was not the case, however, for high frequency, high frequency normalized, and Poincaré plot short diameter of ellipse normalized.

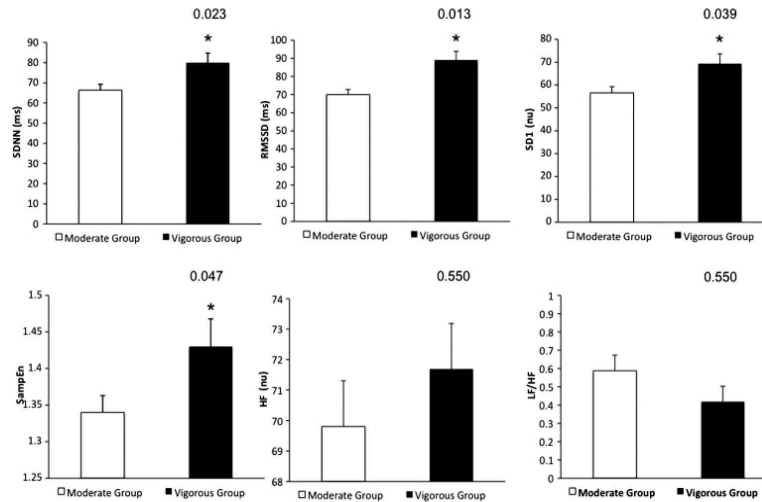


Figure 1. Between-group differences in heart rate variability assessed in the time domain, the frequency domain, and using non-linear techniques. *Time domain*: SDNN = standard deviation of the R-R intervals; RMSSD = square root of the mean of the squared differences of successive R-R intervals. *Frequency domain*: HF nu = normalized high frequency. *Non-linear techniques*: SD 1 nu = Poincaré plot short diameter of ellipse normalized; SampEn = sample entropy, LF/HF = low frequency/high frequency ratio. * $P < 0.05$ compared with moderate group; ANCOVA controlling for sex and minutes of moderate physical activity per week and percent body fat.

Table II. Univariate logistic regressions models to determine the association of sex, minutes of vigorous physical activity per week, minutes of moderate physical activity per week, and moderate versus vigorous physical activity groups with sample entropy.

	N	Variable	OR	P	95% CI
Model 1	168	Sex (female)	1.961	0.032	(1.062–3.622)*
Model 2	168	Vigorous physical activity	1.010	0.048	(1.001–1.021)*
Model 3	168	Moderate physical activity	1.001	0.375	(0.998–1.004)
Model 4	168	PA group (moderate group)	2.145	0.029	(1.082–4.251)*

Note: Sex – referent group female; Physical activity group – referent group moderate; Vigorous physical activity – continuous variable (min); Moderate physical activity – continuous variable (min); OR = odds ratio; CI = confidence interval. * $P < 0.05$.

Table III. Logistic regression analysis predicting heart rate variability indices.

	N	Recommendation PA group		
		OR	P	95% CI
SampEN	168	2.145	0.029	(1.082–4.251)
SDNN (ms)	168	2.428	0.018	(1.163–5.071)
RMSSD (ms)	168	2.131	0.040	(1.035–4.339)
HF (ms ²)	168	2.022	0.056	(0.982–4.167)
HF (nu)	168	1.222	0.583	(0.598–2.498)
SD1 (nu)	168	1.672	0.167	(0.806–3.468)
SD2 (nu)	168	2.222	0.031	(1.075–4.592)

Note: OR=odds ratio; CI=confidence interval; reference category moderate group (accomplishes only moderate recommendations for physical activity). Adjusted for sex.

SampEn=sample entropy; SDNN=standard deviation of the R-R intervals; RMSSD=square root of the mean of the squared differences of successive R-R intervals; HF=high frequency; HF nu=normalized high frequency; SD 1 nu=Poincaré plot short diameter of ellipse normalized; SD2 nu=Poincaré plot long diameter of ellipse normalized.

Discussion

The aim of this study was to examine differences in traditional heart rate variability indices and heart rate complexity in young adults grouped by objectively measured achievement of either moderate or both moderate and vigorous physical activity recommendations. Habitual physical activity offers protection against cardiovascular morbidity and mortality but the exact exercise dose needed is not clear (Lee, 2010). We observed that accomplishing moderate and vigorous physical activity recommendations was associated with a better heart rate variability profile and greater heart rate complexity than accomplishing moderate recommendations only. The present findings support the belief that vigorous physical activity may provide additional benefits to those offered by moderate physical activity in young adults.

Vigorous exercisers had higher values for standard deviation of the R-R intervals, square root of the mean of the squared differences of successive R-R intervals, Poincaré plot short diameter of ellipse normalized, high frequency, and sample entropy than moderate exercisers. There was, however, no significant difference in high frequency normalized or low frequency/high frequency ratio, despite a trend for the vigorous group to have higher and lower values of high frequency normalized and low frequency/high frequency ratio respectively. In the logistic regression analyses, we found that vigorous exercisers were more likely to have higher sample entropy, standard deviation of the R-R intervals, square root of the mean of the squared differences of

successive R-R intervals, and Poincaré plot long diameter of ellipse normalized than participants achieving moderate physical activity recommendations only. When measured at rest, standard deviation of the R-R intervals, square root of the mean of the squared differences of successive R-R intervals, and Poincaré plot are predominantly vagally mediated indices, suggesting higher total vagal outflow in the vigorous group compared with the moderate group.

The loss of protective vagal reflexes is related to ventricular tachycarrhythmias (Billman, 2009; Vaseghi & Shivkumar, 2008) and improvements in vagal indices are independent protectors against sudden death (Billman, 2009; Cole, Blackstone, Pashkow, Snader, & Lauer, 1999). Our data could suggest a mechanism by which vigorous physical activity can confer additional cardioprotection to that afforded by achieving moderate physical activity recommendations only. The vigorous group also had higher sample entropy, indicating greater heart rate complexity compared with the moderate group.

Interestingly, the vigorous group had lower resting heart rate (longer R-R intervals) than the moderate group. This difference in heart rate may explain why we observed significant differences in absolute values of high frequency but not in normalized values, as the first index is mediated by R-R intervals. Such differences led us to express high frequency and Poincaré plot as indices corrected for R-R intervals. The lower heart rate in the vigorous group support these results, since it has been suggested that lower heart rate can be due to a reduction in sympathetic modulation and an increase in parasympathetic modulation (Carter, Banister & Blaber, 2003; De Angelis et al., 2004), reinforcing the higher cardiac vagal dominance in the vigorous group already suggested by the heart rate variability variables. A lower heart rate may also be the product of a lower intrinsic heart rate (Katona, McLean, Dighton, & Guz, 1982), which can also be modulated by exercise training (Carter et al., 2003).

Our results are in agreement with a previous study in which we found that the most active of three groups (based on objective physical activity monitoring) had significantly higher levels of these vagal heart rate variability indices compared with the least active group (Soares-Miranda et al., 2009). Several other studies (Buchheit et al., 2005; Melanson, 2000; Sandercock et al., 2008) have also shown higher values of vagal heart rate variability indices in moderately and highly active groups compared with less active individuals. The present data are in broad agreement with these studies but also highlight the potential importance of meeting recommendations for vigorous physical activity in addition to moderate recommendations.

Some previous studies have found no such positive association between physical activity and heart rate variability (Greiser et al., 2009; Loimaala, Huikuri, Oja, Pasanen, & Vuori, 2000). There are two possibilities for this discord between studies. All these studies physical activity was assessed indirectly via questionnaires, which have inherent bias. Second, one study involved a relatively short intervention, so a longer intervention may be needed to achieve physiological adaptations.

The mechanisms by which exercise produces changes in autonomic control are not clear. However, the above-mentioned cardioprotective effects of vigorous physical activity can be observed via associations with more favourable heart rate variability profiles (Buchheit et al., 2005; Sandercock et al., 2008; Soares-Miranda et al., 2009), which imply greater myocardial electrical stability and a higher myocardial fibrillation threshold (Billman, 2009). Regular physical activity is associated with increased vascular compliance and greater arterial restructuring (Green, O'Driscoll, Joyner, & Cable, 2008). Areas containing baroreceptors are also remodelled, which may increase their afferent activity resulting in a higher parasympathetic outflow (Green et al., 2008). Animal data suggest that endurance exercise is capable of modifying behaviour in the cardiorespiratory centres, decreasing sympathetic and increasing parasympathetic outflow (Billman & Kukielka, 2007; Nelson, Juraska, Musch, & Iwamoto, 2005). Remodelling associated with regular physical activity appears to affect both branches of the autonomic nervous system (Carter et al., 2003; Green, Spence, Halliwill, Cable, & Thijssen, 2011). Changes in vagal outflow have already been well illustrated, showing changes in absolute time and frequency domain values of heart rate variability due to training (Carter et al., 2003; Sandercock et al., 2008). Both branches of the autonomic nervous system constantly interact in a complex way to control heart rate, and our work provides additional information on heart rate complexity measured through sample entropy.

Higher sample entropy suggests superior heart rate complexity; the randomness or entropy of the time series. A lack of complexity may be indicative of a diminished capacity to adapt to physiological stress (Pikkujämsä et al., 1999). Complex dynamics are needed to preserve the cardiovascular system's capacity to adapt to internal and external perturbations (Lipsitz, 1995). Biological signals such as heart rate are not regular and even during steady-state conditions such signals have significant complexity (Lipsitz, 1995). Goldberger suggested that many disease conditions could be seen as leading to a breakdown in complexity resulting in more periodic ordered behaviours (Sharma, 2009). Complexity analysis may provide valuable additional information

about the genesis of heart rate behaviour when combined with traditional heart rate variability indices, and information not forthcoming from traditional heart rate variability indices (Pikkujämsä et al., 1999). Our data suggest that vigorous physical activity is more strongly associated with heart rate complexity than moderate physical activity in young adults. Young adults who engaged in regular vigorous physical activity were more than twice as likely to have a higher sample entropy, standard deviation of the R-R interval, and square root of the mean of the squared differences of successive R-R intervals than those involved in predominantly moderate activities. Engaging in vigorous physical activity may be important because chronic diseases, ageing (Beckers, Verheyden, & Aubert, 2006; Lipsitz, 1995; Pikkujämsä et al., 1999), and cardiac autonomic neuropathy are associated with reduced heart rate complexity (Khandoker, Jelinek, & Palaniswami, 2009). Low complexity can also predict atrial fibrillation (Tuzcu, Nas, Börklü, & Ugur, 2006), cardiac arrhythmia, and even sudden death (Lipsitz & Goldberger, 1992). Reduced complexity may also indicate a lower capacity for adaptation to the demands and unpredictable changes of daily life (Lipsitz & Goldberger, 1992).

There is good evidence that aerobic training increases time domain and spectral heart rate variability measures (Sandercock et al., 2005) but less is known about the trainability of heart rate complexity. In healthy individuals, heart rate complexity increases with resistance training (Heffernan et al., 2007) and combined aerobic/resistance training (Karavirta et al., 2009). Kanaley and colleagues (2009), however, found no change in sample entropy in obese middle-aged men and women following aerobic training, probably due to the low intensity of the intervention. The present data show a favourable cross-sectional association between physical activity and sample entropy. We do not know the exact nature of the vigorous physical activity undertaken by study participants, but according to the cut-points used and the placement of the accelerometers we must assume that this was predominantly aerobic, endurance-type exercise. These findings extend the notion that vigorous physical activity is important to promote cardiovascular health and is associated with general health benefits (Kemi & Wisloff, 2010) and cardioprotection (Wisloff, Ellingsen, & Kemi, 2009). Given the association between heart rate complexity and events such as atrial (Vikman et al., 1999) and ventricular fibrillation (Makikallio et al., 1999b) as well as mortality (Makikallio et al., 1999a), vigorous physical activity in addition to moderate physical activity may be an important cardioprotective health behaviour.

A strength of the present study resides in the objective measurement of physical activity using

accelerometers. To our knowledge, this is the first study in a healthy young population to address the achievement of physical activity recommendations and its effects on heart rate variability and heart rate complexity. A major limitation of the study, however, is the cross-sectional design, which prevents us from making causal inferences. Another weakness is that accelerometers cannot provide valid information regarding specific activities, as cycling and swimming and no qualitative data on physical activity were recorded. Furthermore, comparisons between studies may be difficult due to different cut-offs used to define physical activity intensities. Nevertheless, accelerometry is rapidly becoming the method of choice for physical activity monitoring due to its objectivity, validity, and reliability in providing information on both the amount and intensity of physical activity.

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Paper III

**High levels of C-reactive protein are associated with reduced vagal modulation
and low physical activity in young adults.**

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High levels of C-reactive protein are associated with reduced vagal modulation and low physical activity in young adults

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The purpose of this study was to examine the relationship between cardiac autonomic control derived from heart rate variability (HRV), high-sensitivity C-reactive protein (hs-CRP) and physical activity (PA) levels measured using accelerometers. A total of 80 healthy university students volunteered to participate in this study (20.56 ± 0.82 years, 1.36 ± 1.5 mg/L of hs-CRP). The participants were divided into groups based on tertiles of hs-CRP. Analysis of covariance adjusted to PA was used to assess group differences in HRV. Associations between hs-CRP, HRV indices and PA were analyzed using Pearson's correlation. The

participants at the highest tertile of hs-CRP (tertile 3) had lower cardiac vagal modulation (SDNN, tertile 1 = 78.05 ± 5.9 , tertile 2 = 82.43 ± 5.9 , tertile 3 = 56.03 ± 6.1 ; SD1, tertile 1 = 61.27 ± 5.3 , tertile 2 = 62.93 ± 5.4 , tertile 3 = 40.03 ± 5.5). In addition, vagal indices were inversely correlated with hs-CRP but positively correlated with PA (SDNN $r = -0.320$, SD1 $r = -0.377$; SDNN $r = 0.304$, SD1 $r = 0.299$; $P < 0.05$). Furthermore, the most physically active subjects had lower levels of hs-CRP and the highest levels of vagal modulation.

Atherothrombosis is known as a dynamic chronic inflammatory process of the vessel wall that starts in childhood and progresses asymptotically (Viles-Gonzalez et al., 2004). However, later in life it can lead to coronary artery disease, stroke, transient ischemic attack and peripheral arterial disease (Viles-Gonzalez et al., 2004). C-reactive protein (CRP) is an acute phase protein that increases during inflammation. CRP, measured by a high-sensitivity assay, is considered a marker of inflammation and a predictor of cardiovascular events in patients as well as in healthy individuals (Ridker et al., 1997; Koenig et al., 1999; Ridker et al., 2001; Danesh et al., 2004). Thus, CRP determination has been suggested as a strategy in the assessment of the risk of cardiovascular diseases (Pearson et al., 2003). It has been proposed that CRP can promote atherothrombotic development (Pasceri et al., 2000; Zwaka et al., 2001).

Heart rate variability (HRV) is a non-invasive method used to assess cardiac autonomic control. Low HRV is indicative of a shift toward sympathetic predominance and has a poor prognosis associated with all-cause of mortality (Tsuiji et al., 1994), and sudden cardiac death (La Rovere et al., 2003; Brouwer et al., 1996). Moreover, it has been reported

that acetylcholine release by the vagal nerve can inhibit the production of pro-inflammatory cytokines from macrophages (Ulloa, 2005). In fact, in clinical trials, a negative association between inflammation and vagal modulation has been demonstrated (Sajadieh et al., 2004; Araujo et al., 2006; Madsen et al., 2007; Sloan et al., 2007; Lampert et al., 2008; Stein et al., 2008; Thayer & Fischer 2009). However, these studies were conducted in middle-aged or elderly subjects. Thus, the association between inflammation and decreased vagal modulation in healthy young subjects, when the development of atherosclerosis may be in the subclinical stage, remains unknown. Moreover, it appears that regular exercise is a strategy to reduce CRP and other inflammatory markers (Milani et al., 2004; Panagiotakos et al., 2005; Stewart et al., 2007). Furthermore, it is known that regular exercise is conducive to higher levels of vagal modulation (Rennie et al., 2003; Soares-Miranda et al., 2009). These two factors, lower inflammation and higher vagal modulation, are believed to be preventive regarding the development of cardiovascular disease.

In the present study, we tested the hypothesis that higher levels of inflammation are related to lower

levels of physical activity (PA) and lower levels of vagal modulation.

Materials and methods

Study population

A total of 80 healthy university students volunteered to participate in this study (20.56 ± 0.82 years, 1.36 ± 1.5 mg/L of hs-CRP). Exclusion criteria were cardiovascular diseases assessed by clinical examination and any medication or supplements that could influence the control of cardiac autonomic function. Smokers were considered eligible for the participation in this study, but they were instructed not to smoke on the morning of the evaluations. The study was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each individual who participated in the study.

Procedures

All procedures were performed between 08:00 and 11:00 hours. Participants were wearing light clothing without shoes. Body mass was assessed by a Tanita Inner scan, BC-532 (Tanita, Hoofddorp, the Netherlands). Stature was measured using a stadiometer Seca model 708 (Seca, Hamburg, Germany). Body mass index (kg/m^2) was calculated. Waist circumference was measured midway from the lower rib margin to the anterior superior iliac crest, using a non-metallic tape without significant compression (Graham et al., 2007). Blood pressure, via a sphygmomanometer, was recorded using a Colin 8800P Monitor (Colin, Texas, USA) from the left arm in the seated position after a standardized 10-min rest period. Three measures were performed; the final value was the average of the last two. A capillary sample of blood was taken from ear lobe, after an overnight fast. The sample was analyzed using a Cholestech LDX[®] cassette (Cholestech, Hayward, California, USA). The Cholestech LDX[®] shows good agreement with laboratory measures for population-based risk factor screening (Shemesh et al., 2006) and meets the criteria set by the lipid standardization panel for accuracy and precision of cholesterol measurements (Allison et al., 2007). CRP was measured with a high-sensitivity assay using Cholestech, which has a high correlation with a nephelometric reference method according to the manufacturer ($R = 0.98$).

A standard 24-h dietary recall was applied to evaluate food intake. The 24-h recall is the most commonly dietary assessment method in large-scale studies due to its easy administration (Biro et al., 2002). Food intake was converted to nutrient values by *Food Processor Plus*[®] (ESHA, Salem, Oregon, USA), and participants were asked how typical the 24-h recall was of their regular eating pattern.

Free-living PA was assessed using a uniaxial ActiGraph accelerometer (model GT1M, Fort Walton Beach, Florida, USA). This device provides valid and reliable measures of vertical body acceleration (Brage et al., 2003) and an estimation of energy expenditure (Rothney et al., 2008). Participants were instructed to wear the monitor on the iliac crest of the right hip with an elastic belt and to remove it for sleep and in any activity that may cause damage to the monitor. PA was monitored in 15 s epochs for up to seven consecutive days. A minimum recording period of 5 days was necessary for the PA measurement to be considered adequate (Esliger et al., 2005). Freedson's (1998) cut points were used to analyze the data using a Mahuffe activity analyzer (Freedson et al., 1998). Counts were transformed to average minutes per day in light

(< 1951 counts/min), moderate (1952–5724 counts/min), vigorous (5725–9498 counts/min) and very vigorous (> 9499 counts/min) PA. Daily time spent in moderate-to-vigorous physical activity (MVPA) was calculated.

Recordings of R-R interval data were made at rest in a quiet room, in the supine position, using the Polar Advantage NV (Polar Electro OY, Kempele, Finland). Verbal instructions and a practice period of paced breathing were provided. Participants matched their breathing to a metronome-paced frequency of 12 breaths/min. Participants were asked to avoid strenuous exercise and caffeine or alcohol consumption for a 24 h before the examination. The Polar Advantage heart monitor is comparable to the more conventional ECG devices for R-R interval measurement (Radespiel-Troger et al., 2003; Nunan et al., 2009). The R-R interval data was downloaded into the Polar Precision Performance Software SW. Ectopic beats or arrhythmias were excluded from the analysis. The last 5 min of the 20 min was utilized for the calculation of HRV variables. The R-R intervals were analyzed using time domain, frequency domain and Poincaré plot techniques, using Kubios HRV Software 2.0 for Windows developed by The Biomedical Signal Analysis Group, University of Kuopio, Finland. Time domain measures included mean R-R interval, square root of the mean of the squares of successive R-R interval differences (RMSSD), standard deviation of the R-R interval (SDNN), percentage of heart period differences > 50 ms (PNN50) and number of pairs of adjacent R-R intervals differing by more than 50 ms in the entire recording (NN50). R-R intervals were also used to produce Poincaré Plot, SD1 (Poincaré plots' descriptor: deviation of instantaneous beat-to-beat R-R interval variability is the short diameter of ellipse) and SD2 (Poincaré plots' descriptor: deviation of instantaneous beat-to-beat R-R interval variability is the long diameter of ellipse) values (Braun et al., 1998; Mourot et al., 2004; Gilder & Ramsbottom 2008). Non-parametric (Fast Fourier transform) was used to obtain the frequency domain measures of HRV. Short-term HRV recording enabled the computation of the three-frequency bands: very-low frequency (VLF 0.00–0.04 Hz), low frequency (LF 0.04–0.15 Hz) and high frequency (HF 0.15–0.4 Hz). Only LF and HF were considered in our analysis because the VLF component is considered a dubious measure due to its uncertain physiological meaning and interpretation (Task, 1996). LF is considered to provide information about both sympathetic and parasympathetic modulation; thus, we used HF in the analyses because it provided quantification of vagal modulation. Furthermore, the LF:HF ratio was calculated as a measure that expresses sympathovagal balance.

Statistical analysis

Data were tested for normality of distribution using the Kolmogorov-Smirnov test. Because of skewed distributions, HF was logarithmically transformed before parametric analyses. In an exploratory analysis, no differences in the studied variables were found between genders. Participants were grouped according to tertiles of hs-CRP. Tertile 3 corresponds to the highest tertile of hs-CRP and tertile 1 to the lowest (tertile 1 ≤ 0.530 ; tertile 2 > 0.530 and ≤ 1.12 ; tertile 3 > 1.12). Descriptive statistics (means and standard deviations) were used to characterize the sample. One-way analysis of the variance, with Bonferroni's *post hoc* test, was used to identify differences between tertiles. The chi-squared test was used for categorical variables. Between-tertile comparisons for HRV measures were made using analysis of the covariance (ANCOVA) adjusting for MVPA. The analysis was adjusted to MVPA, given the significant differences of hs-CRP found

between tertiles. *Post hoc* comparisons and Bonferroni's correction were used to identify between-group differences. Associations between hs-CRP, HRV indices and MVPA were analyzed through Pearson's correlation. The level of significance was set at $P < 0.05$. Data were analyzed using SPSS version 15.0.

Results

The descriptive characteristics of the sample and between-tertile differences are shown in Table 1. MVPA was significantly lower in the highest tertile of hs-CRP compared with the lowest tertile of hs-CRP. There were no significant differences between tertiles for any of the other studied parameters (Table 1).

ANCOVA showed significant differences between hs-CRP tertiles in RMSSD, SDNN, NN50, PNN50, HF and SD1. *Post hoc* tests showed lower values for all vagal indices (R-R interval, RMSSD, NN50, PNN50, HF, SDNN and SD1) in tertile 3 compared with tertile 1. In addition, RMSSD, SDNN, NN50, PNN50, SD1 and SD2 were significantly lower in tertile 3 compared with tertile 2 (Fig. 1). The R-R interval and high-frequency normalized unit tended to be lower in tertile 3. Moreover, heart rate, LF/HF and LFnu were higher in tertile 3.

Pearson's correlation analyses indicated significant associations between hs-CRP, HRV indices and MVPA (Table 2). Vagal indices were inversely correlated with hs-CRP and positively associated with MVPA.

Discussion

This study investigates the relationship between cardiac autonomic function, high-sensitivity CRP and objectively measured PA levels, in healthy young individuals. The results of the present study provide evidence for the so-called "nicotinic anti-inflammatory pathway," as individuals in the highest tertile of hs-CRP have lower cardiac vagal modulation. In addition, vagal indices were inversely correlated with hs-CRP in this healthy young sample and the most physically active subjects where those who had lower levels of hs-CRP and higher levels of vagal modulation.

Although our study does not allow discrimination of cause and effect between vagal activity, hs-CRP and PA, the anti-inflammatory properties of acetylcholine suggests that vagal modulation can inhibit the production of pro-inflammatory markers.

Previous studies have shown inverse relationships between vagal indices of HRV and inflammatory markers in middle-aged and elderly subjects (Sajadieh et al., 2004; Araujo et al., 2006; Madsen et al., 2007; Sloan et al., 2007; Lampert et al., 2008; Stein et al., 2008; Thayer & Fischer, 2009). Our study is in line with these studies, and it extends this notion to young individuals. Despite the fact, that, in one previous study the individuals were divided according to age and included a group of young healthy subjects, the investigators failed to find an association between vagal modulation and inflammatory markers in the youngest group (Araujo et al., 2006). However, they demonstrated that SDNN was associated with hs-CRP in particular age groups. None of

Table 1. Descriptive characteristics of C-reactive protein by tertile (mean \pm SD)

	C-Reactive Protein		
	First tertile ($n = 28$)	Second tertile ($n = 26$)	Third tertile ($n = 26$)
hs-CRP (mg/L)	0.382 \pm 0.08	0.833 \pm 0.204*	2.943 \pm 1.77*†
Age (years)	20.57 \pm 0.81	20.41 \pm 0.59	20.79 \pm 1.03
Gender (M/F)	15/13	12/14	8/18
Smoke status (yes/no)	9/19	7/19	4/22
Systolic blood pressure (mmHg)	112.64 \pm 11.65	116.58 \pm 10.18	113.60 \pm 9.27
Diastolic blood pressure (mmHg)	60.29 \pm 8.01	64.29 \pm 6.56	62.88 \pm 7.00
Body mass index (kg/m ²)	23.50 \pm 3.05	23.38 \pm 2.62	24.06 \pm 3.15
Waist circumference (cm)	77.74 \pm 9.38	77.35 \pm 7.96	78.40 \pm 9.04
Total cholesterol (mg/dL)	154.11 \pm 33.04	155.38 \pm 36.11	167.65 \pm 37.73
LDL cholesterol (mg/dL)	91.72 \pm 32.43	89.25 \pm 33.27	97.94 \pm 26.42
HDL cholesterol (mg/dL)	48.89 \pm 13.36	49.81 \pm 15.91	51.73 \pm 15.21
Triglycerides (mg/dL)	67.46 \pm 35.36	81.62 \pm 43.82	89.81 \pm 35.15
Blood glucose (mg/dL)	86.32 \pm 7.44	84.00 \pm 6.56	85.69 \pm 6.07
MVPA (min/week)	258.73 \pm 119.41	224.46 \pm 82.05	117.80 \pm 87.76*
Omega 3 (g/day)	0.72 \pm 0.39	0.58 \pm 0.44	0.71 \pm 0.52

* $P < 0.05$ significantly different from tertile 1.

† $P < 0.05$ significantly different from tertile 2; ANOVA with Bonferroni's *post hoc* for continuous variables and chi-squared test for categorical variables. hs-CRP, high-sensitivity C-reactive protein; MVPA, moderate to vigorous physical activity; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ANOVA, analysis of variance.

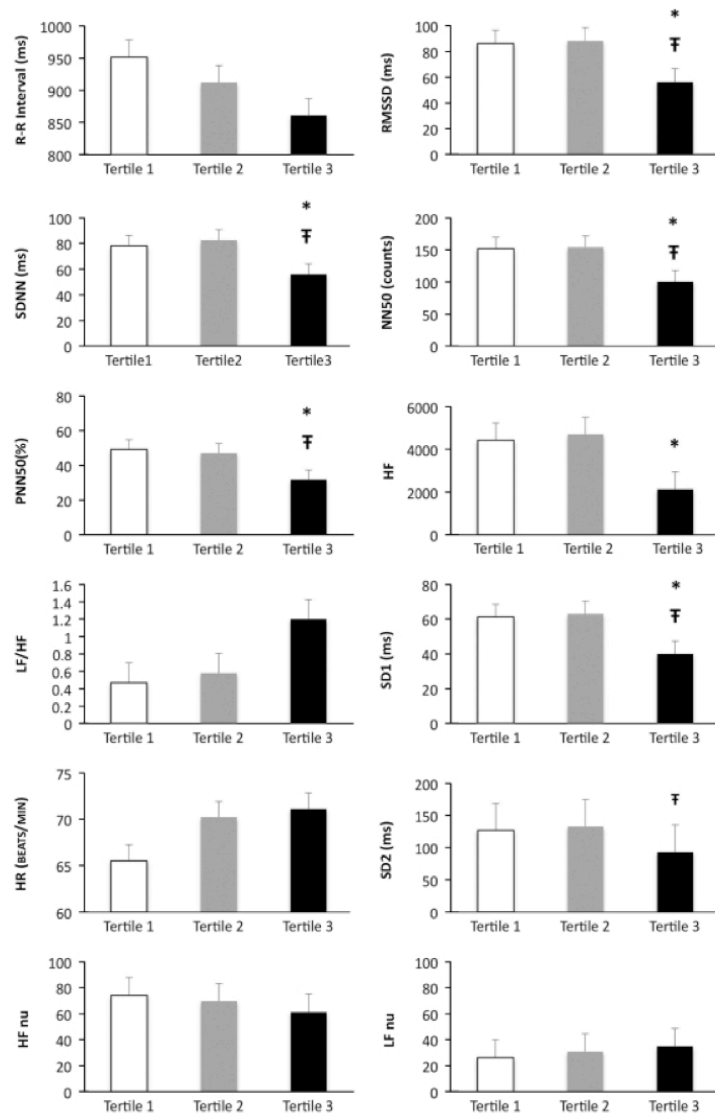


Fig. 1. Time and frequency domain measures and Poincaré plots of heart rate variability in participants according to C-reactive protein by tertile. Data from Porto University Students collected in 2009. R-R interval, the mean length of the filtered R-R intervals; SDNN, standard deviation of all normal-to-normal R-R intervals; RMSSD, square root of the mean of the squares of successive R-R interval differences; SD1, deviation of instantaneous beat-to-beat R-R interval variability is the short diameter of ellipse; SD2, deviation of instantaneous beat-to-beat R-R interval variability is the long diameter of ellipse; HF, high frequency power; LF/HF, ratio low frequency-high frequency; PNN50, percentage of heart period differences > 50 ms; NN50, number of pairs of adjacent R-R intervals differing by more than 50; HR, heart rate; LFnu, low-frequency normalized unit; HFnu, high-frequency normalized unit. * $P < 0.05$ compared with tertile 1, † $P < 0.05$ compared with tertile 2, ‡ analysis of the covariance controlling for moderate-to-vigorous physical activity, comparison with Bonferroni's *post hoc*.

the other HRV indices were associated with hs-CRP. There are at least four possibilities to explain the controversy between studies. First, there is the low cut-off point for hs-CRP (1.5 mg/dL). Second, PA

levels were not taken into consideration in their study, and it has been well documented that PA influences HRV and CRP levels (Rennie et al., 2003; Panagiotakos et al., 2005; Stewart et al., 2007;

hs-CRP, vagal modulation and physical activity

Table 2. Pearson's correlations between hs-CRP, heart rate variability indices and MVPA

	R-R interval	SDNN	RMSSD	NN50	PNN50	Log HF	LF/HF	SD1	SD2	HR	MVPA
hs-CRP (mg/L)	-0.347**	-0.320**	-0.378**	-0.393**	-0.447**	-0.424**	0.395**	-0.377**	-0.256*	0.273*	-0.286*
MVPA (min/week)	0.309*	0.304*	0.298*	0.202	0.245*	0.268*	0.002	0.299*	0.349	-0.283*	

Data from Porto University Students collected in 2009.

* $P < 0.05$.

** $P < 0.01$.

hs-CRP, high-sensitivity C-reactive protein; R-R interval, the mean length of the filtered R-R intervals; SDNN, standard deviation of all normal-to-normal R-R intervals; RMSSD, square root of the mean of the squares of successive R-R interval differences; SD1, deviation of instantaneous beat-to-beat R-R interval variability is short diameter of ellipse; SD2, deviation of instantaneous beat-to-beat R-R interval variability is the long diameter of ellipse; HF log, high-frequency powering log; HR, heart rate; PNN50, percentage of heart period differences > 50 ms; NN50, number of pairs of adjacent R-R intervals differing by more than 50 ms; MVPA, moderate to vigorous physical activity; LF/HF, ratio low frequency-high frequency.

Soares-Miranda et al., 2009). Third, frequency or time domain analyses of HRV are not always assessed.

To the best of our knowledge, the present study is the first to analyze the association of hs-CRP, cardiac autonomic function and PA in young healthy adults. We found that higher levels of vagal modulation were associated with lower levels of inflammation and that individuals in the higher tertile of hs-CRP had lower values of vagal indices. Besides subjects with lower inflammation and higher vagal modulation were those that had more minutes per week of MVPA. This suggests that PA can be a therapeutic tool for inflammation and to maintain adequate vagal function. Our results are in line with those of Jae et al. (2009), because they found that subjects with higher cardiorespiratory fitness had the lowest levels of CRP and higher vagal modulation. Moreover, we have added new information as our study was conducted in young adults, and habitual PA was assessed objectively. Using this method, we were able to show that the most active individuals demonstrated both higher levels of vagal modulation and lower levels of inflammation. Furthermore, previous studies had already shown that exercise could reduce hs-CRP in patients (Milani et al., 2004) and in healthy individuals (Stewart et al., 2007).

Additionally, significant negative correlations were observed between hs-CRP and vagal indices. Our results suggest an association between vagal indices and hs-CRP in young healthy subjects.

The results of the present study provide support for the role of the vagus nerve as an anti-inflammatory modulator. In fact, recent studies suggest that the stimulation of the vagus nerve can control inflammation in rodents (Bernik et al., 2002; Wang et al., 2003). Moreover, vagotomy can induce septic shock in these animals (Bernik et al., 2002). Vagal electric stimulation can reduce the levels of TNF in the serum, liver, lungs and heart (Wang et al., 2003). This is indicative that vagus plays an important role in the immune response of an organism. It has been

suggested that acetylcholine and nicotine can inhibit the production of pro-inflammatory cytokines from macrophages (Ulloa, 2005). This mechanism has been defined as the "nicotinic anti-inflammatory pathway" as acetylcholine can inhibit the production of pro-inflammatory cytokines from macrophages by a nicotinic acetylcholine receptor. Additionally, our results support the idea of Tracey et al. (2007) that the role attributed to exercise as capable of reducing and preventing cardiovascular disease, diabetes type 2 and other diseases, may be due to the fact that exercise increases cholinergic anti-inflammatory pathway activity.

Study limitations and strengths

One major limitation of this study is the cross-sectional design, which prevents us from making causal inferences. Furthermore, we only measured hs-CRP as a marker of inflammation and smokers were included in this study. Therefore, further studies using other pro-inflammatory markers, such as interleukin-6, tumor necrosis factor- α and adhesion molecules might provide further evidence on this matter. The major strength of this study is the use of objective measures of PA. To our knowledge, this is the first study, with a healthy population, and controlling HRV and hs-CRP for PA levels, which were measured directly using accelerometers, and for omega 3 intake.

Perspectives

Both inflammation and low levels of vagal modulation predict future illness (Tsuji et al., 1994; Libby 2006; Jae et al., 2009). In this respect, PA emerges as an important paradigm as it promotes a shift in the vagal/sympathetic balance in favor of vagal predominance in humans and can also reduce inflammation. A potential mechanism by which high levels of PA may promote these beneficiary changes is via the

"nicotinic anti-inflammatory pathway." The present data do not, however, provide sufficient information to fully support such a presumption. Further, physiological studies on this topic are warranted.

Key words: inflammation, heart rate variability, physical activity, accelerometer, young adults.

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Paper IV

***Trans*-fatty acid consumption and heart rate variability in older and younger adults.**

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***Trans*-fatty acid consumption and heart rate variability in older and younger adults.**

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Abstract

Background-*Trans*-fatty acid (TFA) consumption is associated with higher cardiac disease risk and *trans*-18:2 but not *trans*-18:1 in red blood cells membranes have been associated with sudden cardiac arrest risk. Abnormal heart rate variability (HRV) reflects autonomic dysfunction and predicts cardiac death. Relationships between TFA consumption and HRV remain understudied. We determined whether total TFA consumption as well as *trans*-18:1 and *trans*-18:2 isomers were independently associated with either increased or decreased HRV.

Methods and Results-In two separate cohorts comprising older US adults (Cardiovascular Health Study [CHS], age=72±5yrs) and young Portuguese adults (Porto, age=19±2yrs), we assessed habitual TFA intake by food frequency questionnaires (separately estimating *trans*-18:1 and *trans*-18:2) and multiple 24-hour recalls (estimating total TFA only), respectively. HRV was assessed by 24-hour Holter in CHS (N=1,076) and repeated short-term (5-min) in Porto (N=160). We used multivariate-adjusted linear regression to relate TFA consumption to HRV indices both cross-sectionally (CHS, Porto) and longitudinally (CHS).

Results-In CHS, each SD higher *trans*-18:2 consumption was associated with lower 24-hour standard-deviation-of-all-normal-to-normal-intervals (SDNN) both cross-sectionally (-12%, 95%CI=6-19%, p=0.001) and longitudinally (-15%, 95%CI= 4-25 %, p= 0.009), as well as with lower 24-hour SDANN, SDNN-index, and square-root-of-the-mean-of-the-squares-of-successive-NN-interval-differences (rMSSD) (p<0.05 each). Higher *trans*-18:1 consumption in CHS was associated with more favorable indices of 24-hour HRV, in particular time-domain indices (SDNN, SDANN, SDNN-index; p<0.05 each). In a Porto subset (n=40), estimated TFA consumption correlated with plasma phospholipid *trans*-18:2, but not *trans*-18:1, suggesting we captured the former. Each higher SD TFA consumption was associated with 4% lower 5-min SDNN (95%CI=1-8%, p=0.04), and 7% lower 5-min rMSSD (95%CI=1-13%, p=0.04). In both cohorts, no associations were seen between TFA consumption and other HRV indices such as high-frequency, low-frequency and non-linear measures.

Conclusions-*Trans*-18:2 consumption is associated with specific, less favorable indices of HRV in both older and young adults. *Trans*-18:1 consumption is associated with more favorable HRV indices in older adults. Our results support the need to investigate potential HRV-related mechanisms whereby *trans*-18:2 may increase arrhythmic risk.

Key Words-Electrophysiology, *trans*-fatty acids, heart rate variability, nutrition

Introduction

Increased dietary *trans*-fatty acids (TFA) adversely affect cardiovascular risk factors including markers of lipoprotein metabolism, inflammation, and endothelial function¹⁻³. The magnitude of the observed associations between TFA consumption and cardiovascular disease events cannot be explained exclusively by changes in circulating lipids^{2,4}. Moreover, several TFA isomers exist, each with potentially different dietary sources and health effects. In particular, higher plasma phospholipid and erythrocyte membrane 18:2 TFA (*trans*-18:2), are associated with higher risks of fatal ischemic heart disease and sudden cardiac death^{5,6}; however the latter outcome is not strongly related to blood lipid abnormalities¹. Potential mechanisms for the relationship between TFA and sudden cardiac death remain uncertain. Some have suggested that TFA may modulate cardiac membrane ion channel function⁷ or have proarrhythmic properties, affecting cardiovascular electrophysiology^{8,9}. However, relationships of TFA consumption with cardiac electrophysiologic measures are not well established.

Heart rate variability (HRV) indices are established measures of cardiac electrophysiology and autonomic function. The autonomic nervous system has a central role in maintaining normal cardiac rhythm¹⁰. For example, lower indices of the standard-deviation-of-all-normal-to-normal-R-R-intervals (SDNN) and ultra-low-frequency power (ULF) are associated with increased risk of cardiovascular events such as myocardial infarction, cardiomyopathy, valvular heart disease, congestive heart failure and mortality¹¹. Moreover, studies have been suggestive of a relationship between lower HRV and coronary heart disease, atrial fibrillation and heart failure¹². Furthermore, growing evidence has established heart rate (HR) as a marker of autonomic activity¹³ and higher resting HR has been associated with increased all-cause mortality, death from cardiovascular disease and sudden death¹³.

Relationships between TFA consumption and HRV or HR could elucidate novel potential mechanisms whereby TFA may influence coronary heart disease and sudden death. However, relatively little is known about this topic. A small (N=79) 8-week intervention study where a diet rich in TFA was given to one group of male subjects¹⁴, showed that daily 20g TFA dietary supplementation tended to reduce HRV and increase HR by 3 bpm, while HR and HRV were unaffected in the control group. We tested the hypothesis that habitual TFA consumption would be associated with less favorable indices of HRV in two separate cohorts, a population-based cohort of older US adults in the Cardiovascular Health Study (CHS) and a cohort of young adults in Portugal (Porto). Given prior work that *trans*-18:2 isomers are more strongly linked to cardiac death and inflammation than *trans*-18:1 isomers^{5,6,15-17}, we hypothesized that *trans*-18:2 may be

more strongly related to less favorable HRV. We therefore investigated estimated dietary consumption of *trans*-18:1 and *trans*-18:2 separately in CHS, and, in Porto in which only estimated total TFA consumption was available, whether total consumption was more closely linked to biomarkers of *trans*-18:1 or *trans*-18:2.

Methods

Design and Populations. The study design and recruitment of CHS have been described^{19, 20}. Briefly, 5,201 ambulatory, non-institutionalized men and women ≥ 65 years of age were randomly selected and enrolled from Medicare lists in 4 US communities (Forsyth County, North Carolina; Sacramento County, California; Washington County, Maryland; and Pittsburgh, Pennsylvania) in 1989-90. An additional 687 black participants similarly recruited and enrolled in 1992-93 could not be included in this analysis due to lack of baseline HRV measures. The institutional review committee at each centre approved the study, and all subjects provided written informed consent. A 24-hour Holter measure of HRV was obtained at baseline (N=1,294), and again five years later in the same subjects (N=781). We excluded subjects with atrial fibrillation or flutter, pacemakers, or markedly irregular rhythms (e.g., wandering atrial pacemaker or other non-sinus rhythm)²¹. Among these, we included participants with dietary data on TFA consumption (baseline N=1076 for time-domain and 1034 for frequency-domain and non-linear measures; and follow-up N=578 for time-domain and 544 for frequency-domain and non-linear measures). Data on plasma phospholipid TFA were available three years after baseline (1992-93). Because HRV data were not collected in that year, prospective relationships between plasma phospholipid TFA and HRV were determined in those who had valid HRV measures obtained 2 years later (N=461 for time-domain and 424 for frequency-domain and non-linear measures).

The Porto was established in 2008 to assess associations between lifestyle and HRV among young adults²². We recruited university students aged 18 to 21 years to participate in the longitudinal study from 2008-10. After excluding individuals with known cardiovascular illness or using any medication or supplements that could influence cardiac autonomic function and without information on TFA consumption were included in the analyses 160 subjects. We obtained written informed consent from all participants. The study was approved by the local ethics committee and conducted in accordance to the declaration of Helsinki.

Dietary Assessment of TFA Consumption and Plasma Phospholipids. In CHS, usual dietary intake was assessed at baseline (1989-90) by a validated picture-sort food frequency questionnaire (FFQ)²³, and dietary TFA consumption, including *trans*-18:2 and *trans*-18:1 isomers, were estimated using the Harvard food composition database¹⁸. In Porto, we assessed total TFA consumption by three annual 24-hour dietary recalls over 3 years. Portion sizes of food and drink consumed were estimated using food models and photos. Food consumption was converted to nutrient values, including total TFA consumption and energy intake, by *Food Processor Plus*®, (ESHA Research, USA) which uses the United States Department of Agriculture database²⁴. Several traditional Portuguese dishes were added using Portuguese food composition databases²⁵. Due to limited food composition data, estimates of TFA consumption in Porto were only available for total TFA and not for TFA isomers. We therefore evaluated correlations between total TFA consumption and plasma phospholipid TFA isomers in a subset of Porto (N=40) to identify which type of TFA isomer explained the greatest variability of total TFA consumption. In a subset of CHS (N=146), we assessed correlations between each dietary TFA isomer and plasma phospholipid levels of TFA isomers measured at baseline (1989-90), as previously described¹⁸.

Assessment of HRV. HRV can be evaluated by time-domain, frequency-domain and non-linear methods²⁶ as well as based on either short-term (e.g., 5 to 20-minutes) or long-term (e.g., 24-hour) recordings (**Table 1 - appendix**). Short-term measures, typically obtained at rest, do not capture circadian nor sleep-related variations, but reflect mainly resting parasympathetic (respiratory) variation in HR. Long-term measures can evaluate longer-term circadian differences in HRV as well as daytime and night-time baroreceptor and respiratory autonomic variation.

HRV assessment in CHS^{27,28} and Porto²² have been described previously. In CHS, a 2-channel 24-hour Holter recordings was obtained (Del-Mar-Medical Systems, Irvine, Calif), and HRV was determined at the Washington University School of Medicine HRV laboratory (GE-Marquette-Mars 800-Holter analyzer, Milwaukee, Wis). In Porto, R-R intervals were recorded during 20-minutes using Polar-Advantage NV (Polar-Electro-OY, Finland) in a quiet room. Participants were in the supine position and matched their breathing to a metronome-paced frequency of 12 breaths/min in the last 5-minutes of recording. The last 5-minutes were used to analyze HRV. The Polar-Advantage heart monitor is comparable to the more conventional ECG devices for R-R intervals measurement^{29,30}. R-R intervals were analysed using Kubios-HRV Software-1.1³¹.

Covariates. In CHS, participants completed a standardized questionnaire on medical history, health status, and lifestyle habits and underwent a clinic examination including standardized blood pressure and anthropometric measures¹⁹. Possible dietary confounders were estimated from responses to the FFQ. Usual leisure-time physical activity (PA) was assessed at baseline (1989-90) and at the third (1992-93) annual visit using a modified Minnesota Leisure-Time Activities questionnaire that evaluated frequency and duration of fifteen different activities during the prior two weeks³².

In Porto, anthropometric measures were obtained by standardized method at each of three visits. Habitual alcohol intake (yes or no) and smoking habits (yes or no) were assessed annually by questionnaire. Potential dietary confounders, such as omega-3 polyunsaturated fatty acids, fiber, and total energy intake, were estimated from the multiple 24-hour recalls. Free-living PA was objectively monitored for seven days prior to each visit by uniaxial accelerometer (model-GT1M, Fort Walton Beach, Florida). Freedson's (1998) cut-offs were used to analyse accelerometer data via Mahuffe-activity analyser³³, and daily-time spent in moderate-to-vigorous PA was calculated.

Statistical Analysis. In CHS, cross-sectional associations at baseline between estimated *trans*-18:1 and *trans*-18:2 consumption and HRV were evaluated by multivariable-adjusted linear regression. We also evaluated prospective associations between TFA consumption at baseline and HRV indices at the fifth visit. Multivariate models were adjusted for age, gender, race, education, income, clinical sites, smoking, body mass index (BMI), each of prevalent diabetes mellitus and coronary heart disease, prevalent hypertension, β -blocker use, anti-hypertensive medication, leisure-time PA, alcohol, total energy intake, energy-adjusted eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), 16:1 TFA (*trans*-16:1), energy-adjusted quintiles of fruit and energy-adjusted quintiles of vegetable consumption. We additionally assessed fiber consumption; levels of fibrinogen, C-reactive protein, triglycerides, LDL cholesterol, and HDL cholesterol; and use of digitalis and anti-arrhythmic medication as potential confounders or mediators. Inclusion of these variables did not appreciably alter results, and thus these were not included in the final analyses. TFA consumption in both cohorts was adjusted for total energy intake using the residual method³⁴. Missing covariate values (all<8%) were imputed by linear regression using age, race, gender, income, and prevalent cardiovascular disease. We also analysed, in CHS, relationships between plasma phospholipid TFA measured at the third annual visit and HRV measured at the fifth annual visit by linear regression analyses. Plasma

phospholipid TFA isomers were standardized to one standard deviation (SD) for mutual comparison.

We took advantage of repeated measures of diet and HRV in Porto (2008-09-10) by evaluating random-effects linear regression models, simultaneously assessing the multiple measures and taking into within-individual variability, and also by evaluating simple linear regression using the averages of the three measures of TFA consumption (g/day) and HRV to minimize within-person measurement error. Both methods provided similar results, and we present the results based on averages. We used multivariable-adjusted linear regression to examine cross-sectional associations over three years between TFA consumption and HRV indices as continuous variables, scaling the TFA measure to one SD to allow comparability. Multivariable models were adjusted for age, total energy intake, gender, smoking status, PA, alcohol intake, and BMI. We examined other potential confounders or mediators, including fiber intake, blood glucose and triglycerides, but these variables were not included in the final model, as they did not alter results. Missing accelerometer values for PA (8%) were imputed with a linear regression using nonmissing data from the other visits as well as age and gender.

Potential nonlinear associations between TFA consumption and SDNN were assessed using restricted cubic splines³⁵. All *p* values were two-tailed ($\alpha=0.05$). Analyses were performed using Stata-10.1(College-Station, TX)

Results

Table 1 shows the descriptive characteristics of both cohorts. At baseline, CHS, participants were 72 ± 5 years old, with average total TFA consumption of 1.6 ± 0.5 percent energy. In Porto, at baseline participants were 19 ± 2 years old, with average total TFA consumption of 0.7 ± 0.6 percent energy.

Table 1. Baseline characteristics of younger adults in the Porto and older adults in the Cardiovascular Health Study.

Characteristic	Porto	Cardiovascular Health Study
N	160	1,076
Age (Y)	19 ± 2	72 ± 5
Gender (% male)	48	46
Race (% white)	100	95
Education (% greater or equal to high school)	100	75
Body mass index (kg/m^2)	23 ± 3	27 ± 4
Prevalent diabetes mellitus (%)	0	15
Prevalent coronary heart disease (%)	0	24
Current smoking (%)	6	10
Leisure-time activity (kcal/wk)	*	$1392 \pm 1747^*$
Physical activity (min/day)	$61 \pm 29^*$	*
β blocker use (%)	0	14
Anti-hypertensive medication use (%)	0	43
Estimated dietary intake		
Total energy intake (g/d)	$2169 \pm 622^\dagger$	$2029 \pm 684^\ddagger$
Total <i>trans</i> -fatty acids intake (g/d)	$1.6 \pm 1.5^\dagger$	$3.7 \pm 1.2^\ddagger$
<i>Trans</i> -18:1 (g/d)	†	$2.0 \pm 0.7^\ddagger$
<i>Trans</i> -18:2 (g/d)	†	$0.16 \pm 0.06^\ddagger$
<i>Trans</i> -16:1 (g/d)	†	$0.08 \pm 0.03^\ddagger$
Total n-3 PUFAs (g/d)	$0.66 \pm 0.4^\dagger$	$0.28 \pm 0.2^\ddagger$
HRV indices		
SDNN (ms)	$67.1 \pm 29.9^\S$	$121.6 \pm 34.8 $
SDANN (ms)	§	$110.8 \pm 33.4 $
SDNN-index (ms)	§	$44.1 \pm 15.2 $

rMSSD (ms)	72.1±40.7§	26.5±16.9
TP (ms ²)	§	17537±67636
ULF (ms ²)	§	14509±27234
HF (ms ²)	3749.9±3785.1§	206.7±366.8
DFA1	§	1.05±0.18
SD12	§	0.27±0.1
HR (bpm)	69.0±9.1§	73.3 ±9.0

Values are mean±standard deviation (continuous variables) or percentage (categorical variables). For definition of HRV indices, see legend of table 1.

*Data estimated in Porto by accelerometer and in CHS cohort by questionnaire.

† Average of estimates from three 24-hour recalls. *Trans*-18:2 intake was not available for estimation in Porto, however total *trans*-fatty acids was only correlated with plasma phospholipids *trans*-18:2.

‡ Estimated with one FFQ

§ Estimated using short-term measures (5-min) some HRV indices are missing in Porto because there cannot be assessed in 5-min HRV.

|| Estimated using long-term measures (24-hour)

Cross-sectional Associations between TFA consumption and HRV at baseline in CHS

After multivariate adjustment, increased *trans*-18:2 consumption was cross-sectionally associated with decreases in several indices of 24-hour HRV in CHS (**Table 2**). Among time-domain measures, each one SD (0.06 g/day) of higher *trans*-18:2 consumption was associated with 12% lower SDNN and 12% lower standard-deviation-of-5-minute-average-N-N-intervals (SDANN) (both reflecting long-term circadian HRV), 11% lower SDNN-index (average-of-the-5-min-standard-deviations-of-N-N-intervals, reflecting combined sympathetic and parasympathetic modulation of HR) and 15% lower rMSSD(square-root-of –the-mean-of-the-squares-of-successive-R-R-intervals differences; reflecting mainly vagal modulation of HR) ($p<0.05$ each). Consistent with this, among frequency-domain measures, each one SD higher *trans*-18:2 consumption was associated with lower circadian HRV as reflected by 24% lower total power (TP), and 24% lower ultra-low-frequency power (ULF) ($p=0.001$ each). *Trans*-18:2 consumption was also associated with higher resting HR (+3.2 bpm per each one SD of consumption; $p=0.006$). In contrast at baseline, *trans*-18:1 consumption was cross-sectionally associated with higher HRV (**Table 2**).One SD (0.68 g/day) of higher *trans*-18:1 consumption was associated with 16% higher SDNN, 16% higher SDANN, 14% higher SDNN-index and 17% higher rMSSD ($p<0.05$ each); and 37% higher TP and 37% higher ULF ($p<0.001$ each); and lower resting HR (-2.85 bpm, $p=0.02$).

Neither *trans*-18:2 nor *trans*-18:1 consumption were associated with other HRV indices, including low-frequency high-frequency power ratio (LF/HF), normalized low-frequency power (nLF), normalized high-frequency power (nHF), Poincaré plot ratio (SD12), or the short-term fractal scaling exponent (DFA1).

Table 2. Multivariate-adjusted cross-sectional differences in heart rate variability (HRV) and heart rate per each one standard deviation higher intake *trans*-18:2 and *trans*-18:1 among older adults in the Cardiovascular Health Study.

HRV indices and heart rate	<i>Trans</i>-18:2 intake		<i>Trans</i>-18:1 intake	
	Multivariable-adjusted Difference (95%CI)*	<i>p</i>	Multivariable-adjusted Difference (95%CI)*	<i>p</i>
Time-domain (N=1,076)				
SDNN (ms)	-12% (-19, -6)	0.001	+16% (7, 25)	<0.001
SDANN (ms)	-12% (-19, -5)	0.001	+16% (7, 26)	<0.001
SDNN-index (ms)	-11% (-19, -3)	0.007	+14% (4, 24)	0.004
rMSSD (ms)	-15% (-25, -2)	0.023	+17% (2, 34)	0.028
Frequency-domain (N=1034)				
TP	-24% (-36, -11)	0.001	+37% (16, 60)	<0.001
ULF	-24% (-36, -11)	0.001	+37% (16, 61)	<0.001
LF/HF	-1% (-14, 15)	0.96	+1% (-13, 17)	0.90
nLF (nu)	-0.52 (-3.39, 2.35)†	0.72	+0.67 (-2.25, 3.58)†	0.65
nHF (nu)	-0.04 (-2.41, 2.33)†	0.98	+0.09 (-2.32, 2.50)†	0.94
Non-linear (N=1034)				
DFA1 (units)	-0.01 (-0.05, 0.04)†	0.77	0.01 (-0.04, 0.06)†	0.73
SD12	0% (-8, 8)	0.91	1% (-8, 8)	0.95
Heart Rate (bpm) (N=1,076)	+3.20 (0.92, 5.48)†	0.006	-2.85 (-5.17, 0.52)†	0.02

* Values represent percent difference, according to each one higher standard deviation of TFA intake, except for heart rate, nLF, nHF and DFA1 where actual differences are presented (†). Analysis adjusted for age (years), gender (male/female), race (white/nonwhite), education (< high school, high school, > high school), income (≤/ > \$ 25,000), clinical sites (four categories), smoking (never, former, current), body mass index (kg/m²), diabetes mellitus (yes/no), coronary heart disease (yes/no), hypertension (normal, borderline, hypertensive), β-blocker use (yes/no), other anti-hypertensive medication (yes, no), leisure-time physical activity (kcal/week), alcohol (drinks per week), and consumption of total energy (kcal/d), *trans*-16:1 fatty acids (mg/day), EPA and DHA (quintiles), fruits (quintiles), and vegetables (quintiles). All models are also mutually adjusted for consumption of *trans*-18:2 and *trans*-18:1 to investigate their independent effects. nu = normalized.

Longitudinal Associations between TFA consumption at baseline and HRV 5 years later in CHS

Longitudinal analyses in CHS (**Table 3**) were generally consistent with the cross-sectional analyses. Greater *trans*-18:2 consumption was associated with lower time- and frequency-domain circadian and vagal indices of HRV 5 years later, whereas higher *trans*-18:1 consumption was associated with several more favorable indices of HRV. Each one SD higher consumption of *trans*-18:2 at baseline was associated with lower time-domain measures 5 years later, including 15% lower SDNN, 15% lower SDANN, 14% lower SDNN-index and 19% lower rMSSD ($p < 0.05$ each). Conversely, each one SD higher *trans*-18:1 consumption at baseline was associated with higher time-domain measures 5 years later, including 19% higher SDNN and 19% higher SDANN and 15% higher SDNN-index, ($p < 0.05$ each).

Table 3. Multivariate-adjusted longitudinal differences in heart rate variability (HRV), 5 years after dietary assessment, per each one standard deviation higher intakes of *trans*-18:2 and *trans*-18:1 fatty acids among older adults in the Cardiovascular Health Study.

HRV indices	<i>Trans</i> -18:2 intake		<i>Trans</i> -18:1 intake	
	Multivariable-adjusted Difference (95%CI)*	<i>P</i>	Multivariable-adjusted Difference (95%CI)*	<i>P</i>
Time-domain (N=578)				
SDNN (ms)	-15% (-25, -4)	0.009	+19% (5, 34)	0.007
SDANN (ms)	-15% (-25, -3)	0.02	+19% (5, 36)	0.009
SDNN-index (ms)	-14% (-25, -1)	0.03	+15% (1, 32)	0.04
rMSSD (ms)	-19% (-33, -1)	0.04	+21% (-1, 48)	0.06
Frequency-domain (544)				
TP	-30% (-46, -10)	0.005	+44% (11, 86)	0.006
ULF	-30% (-47, -9)	0.008	+45% (11, 90)	0.007
LF/HF	10% (-12, 38)	0.38	-7% (-26, 17)	0.56
nLF (nu)	1.82 (-2.40, 6.04) †	0.40	-0.93 (-5.25, 3.39) †	0.67
nHF (nu)	-1.64 (-5.28, 2.00) †	0.38	+1.29 (-2.44, 5.02) †	0.50
Non-linear (N=544)				
DFA1 (units)	0.01 (-0.07, 0.09) †	0.79	-0.005 (-0.09, 0.08) †	0.90
SD12	-3% (-17, 7)	0.57	3% (-8, 18)	0.60

See Legend Table 3.

One SD higher *trans*-18:2 consumption was associated with 30 % lower TP and 30% lower ULF ($p<0.05$, each). On the contrary each one SD higher *trans*-18:1 consumption was associated with 44% higher TP and 45% higher ULF ($p<0.05$, each). Neither *trans*-18:2 or *trans*-18:1 were prospectively associated with LF/HF, nHF, nLF, nor non-linear indices of HRV. Additionally, *trans*-18:1 was not prospectively associated with rMSSD.

In a subset of CHS (N=146), estimated dietary consumption of each different TFA isomer was moderately correlated with the respective plasma phospholipid levels ($r=0.21$ for *trans*-18:1; $r=0.30$ for *trans*-18:2).

Longitudinal Associations between plasma phospholipid TFA levels in year 3 and HRV 2 years later in CHS

We investigated relationships between levels of plasma phospholipid TFA at year three and HRV indices two years later in CHS (N=461) (**Table 4**). Higher levels of plasma phospholipid *trans*-18:1 were associated with higher time-domain HRV measures including 5% higher SDNN ($p=0.006$) and 6% higher SDANN ($p=0.006$) and higher frequency-domain measures [10% higher TP and 11% ULF ($p=0.01$ each)]. Additionally plasma phospholipid *trans*-18:1 were associated with more favourable non-linear indices including higher DFA1 (+0.03 higher, $p=0.01$) and lower SD12 (4% lower, $p=0.03$). Levels of plasma phospholipid *trans*-18:2 were not significantly associated with any HRV indices.

Because greater erratic HRV can influence rMSSD, LF, HF, and LF/HF, we also evaluated these indices after excluding participants with higher (>median) DFA1. Results were generally similar for rMSSD although, statistical significance was reduced due to fewer numbers of participants. Directions of associations of TFA consumption with nLF, nHF, and their ratio were inverted, although these relations were still not statistically significant.

Table 4. Multivariate-adjusted longitudinal differences in heart rate variability (HRV) per one standard deviation higher levels of plasma phospholipid *trans*-18:2 and *trans*-18:1 fatty acids assessed two years earlier in the Cardiovascular Health Study.

HRV indices	Plasma phospholipid <i>trans</i> -18:2		Plasma phospholipid <i>trans</i> -18:1	
	Multivariable-adjusted difference (95%CI)*	<i>P</i>	Multivariable-adjusted difference (95%CI)*	<i>P</i>
Time-domain (N=461)				
SDNN (ms)	+1% (-3, 4)	0.73	+5% (2, 9)	0.006
SDNN-index (ms)	-1% (-5, 3)	0.69	+4% (-1, 8)	0.10
SDANN (ms)	+1% (-3, 5)	0.62	+6% (2, 10)	0.006
rMSSD (ms)	-1% (-6, 6)	0.88	-1% (-6, 6)	0.95
Frequency-domain (N=424)				
TP	+1% (-8, 8)	0.99	+10% (2, 19)	0.01
ULF	+1% (-7, 8)	0.98	+11% (2, 21)	0.01
LF/HF	+1% (-5, 8)	0.72	+7% (-1, 15)	0.06
nLF (nu)	-0.24 (-1.56, 1.07)†	0.71	+1.34 (-0.52, 2.74)†	0.06
nHF (nu)	-0.23 (-1.37, 0.90)†	0.69	-0.98 (-2.19, 0.22)†	0.11
Non-linear (N=424)				
DFA1 (units)	-0.008 (-0.033, 0.017)†	0.51	0.03 (0.008, 0.06)†	0.01
SD12	0% (-4, 4)	0.87	-4% (-8, 0)	0.03

* Values represent percent difference, according to each one higher standard deviation of each plasma phospholipids TFA, except for, nLF, nHF and DFA1 where absolute differences are presented (†). Analyses adjusted for age (years), gender (male/female), race (white/nonwhite), education (< high school, high school, > high school), income (≤/ > \$ 25,000), smoking (never, former, current), body mass index (kg/m²), diabetes mellitus (yes/no), coronary heart disease (yes/no), hypertension (normal, borderline, hypertensive), β-blocker use (yes/no), anti-hypertensive medication use (yes, no), leisure-time physical activity (kcal/d), alcohol use (drinks per week), and plasma phospholipid levels of *trans*-16:1 fatty acids, *trans*-18:1, *trans*-18:2, EPA and DHA. *Trans*-18:1 and *trans*-18:2 fatty acids were mutually adjusted.

Cross-sectional Associations between TFA consumption and HRV in Porto

Only total TFA consumption was measured in Porto. After multivariate adjustment, total TFA consumption was cross-sectionally associated with lower 5-min HRV indices in Porto (**Table 5**). Each 1 SD (1.5 g/d) of higher TFA consumption was related to lower values of time-domain indices, including 4% lower SDNN and 7% decreased rMSSD ($p=0.04$ each). RMSSD, reflects vagally-mediated respiratory responses. Consistent with these results, TFA consumption was also associated with a trend toward higher HR (+1.10 bpm, $p=0.07$), and lower HF (-11%, $p=0.08$) (also reflecting vagally-mediated HRV).

In a subset of Porto ($N=40$), total TFA consumption, estimated from multiple 24-hour recalls, was correlated with plasma phospholipids *trans*-18:2 ($r=0.32$, $p=0.04$), but not with *trans*-18:1 ($r=0.02$, $p=0.80$). This suggested that total TFA consumption as estimated by the dietary recall and food composition database largely reflected intake of *trans*-18:2.

Table 5. Multivariate-adjusted cross-sectional differences in heart rate variability (HRV) and heart rate per each one standard deviation higher intake of total *trans*-fatty acids among younger adults in Porto.

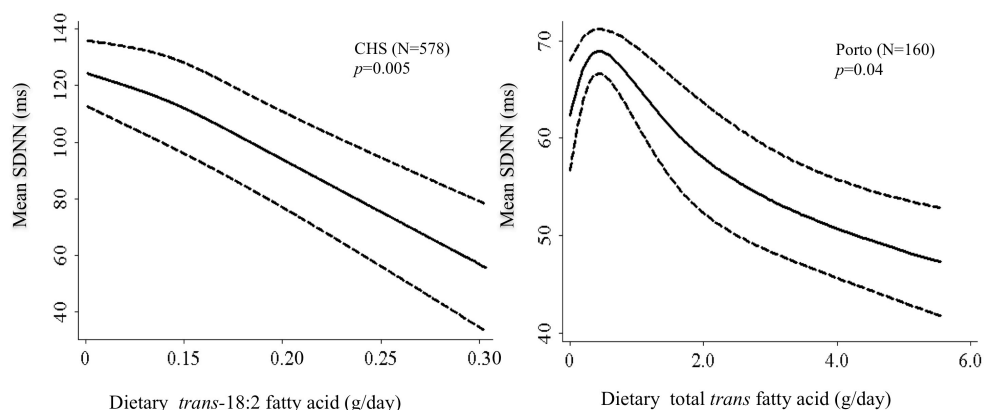
HRV indices and heart rate	Total <i>trans</i>-fatty acids intake	
	Multivariable-adjusted Difference (95%CI)*	<i>p</i>
Time-domain (N=160)		
SDNN (ms)	- 4% (-8, -1)	0.04
rMSSD (ms)	-7%(-13, -1)	0.04
Frequency-domain (N=160)		
HF (ms ²)	-11% (-22, 2)	0.08
Heart rate (bpm) (N=160)	+1.1 (-0.7, 2.3) †	0.07

* Values represent percent difference, according to each one higher standard deviation of total TFA intake, except for heart rate where the absolute differences presented (†). Analysis adjusted for age (years), gender, current smoking (yes/no) moderate to vigorous physical activity (min/day), alcohol use (yes/no), body mass index (kg/m²), and consumption of total n-3 PUFA (mg/day), dietary fiber (g/day) and total energy (kcal).

Linearity vs. non-linearity of relationships between TFA consumption and HRV

We found little evidence for nonlinearity of observed relationships. For example, in Porto higher total TFA consumption was monotonically associated with lower SDNN, and in CHS, higher *trans*-18:2 consumption was monotonically associated with lower SDNN (**Figure 1**).

Figure 1: Multivariable-adjusted associations of *trans*-fatty acid intake and mean standard deviation of the N-N intervals (SDNN), as assessed nonparametrically by means of restricted cubic splines. As would be expected due to differences between 24-hour vs. short-term (5-min) HRV, SDNN mean values were higher in CHS than in Porto.



Values are adjusted for age (years), gender (male/female), race (white/nonwhite), education (<high school, high school, >high school), income (\leq / $>$ \$25,000), clinical sites (four categories), smoking (never, former, current), body mass index (kg/m^2), diabetes mellitus (yes/no), coronary heart disease (yes/no), hypertension (normal, borderline, hypertensive), β -blocker use (yes/no), other anti-hypertensive medication (yes, no), leisure-time physical activity (kcal/week), alcohol use (drinks per week), and consumption of total energy (kcal/d), *trans*-16:1 fatty acids (mg/day), EPA and DHA (quintiles), fruits (quintiles), and vegetables (quintiles).

Discussion

Among older adults in CHS, higher *trans*-18:2 consumption was both cross-sectionally and prospectively associated with less favorable HRV. Similarly, in Porto, we observed cross-sectional associations of total TFA consumption that correlated most strongly with circulating phospholipid *trans*-18:2 and with less favorable HRV indices assessed annually over 3 years. To our knowledge, this study is the first to identify, in two distinct cohorts, a relationship between habitual dietary consumption of TFA, particularly *trans*-18:2, and unfavorable HRV measures. These results support emerging evidence that *trans*-18:2 in particular may increase cardiac risk. Compared with *trans*-18:1 fatty acids that appear largely derived from foods containing partially hydrogenated vegetable oils, *trans*-18:2 fatty acids are linked only to consumption of bakery foods and may be derived from other types of foods in the diet, for example related to oil deodorization or high-temperature cooking processes¹⁸. Thus, adverse health effects of *trans*-18:2 could have relevance for current policy efforts to reduce TFA exposure in populations.

TFA consumption was associated with specific, rather than all HRV indices. In CHS, *trans*-18:2 consumption was associated with less favorable HRV indices that reflect 24-hour circadian and vagal modulation (SDNN, ULF, SDANN, rMSSD). *Trans*-18:2 consumption was also associated with higher resting HR. In contrast, *trans*-18:2 consumption was not significantly associated with other HRV indices, including measures of erratic HRV (DFA1, Poincaré ratio) or indices such as nHF, nLF, and LF/HF that can be interpreted as reflecting beat-to-beat modulation of the heart rate in response to respiration or erratic rhythm, relative sympathetic modulation and sympathovagal balance respectively. Similarly, in Porto, TFA consumption was inversely associated with supine short-term measures including SDNN, rMSSD, and HF, and a trend toward higher resting HR.

Lower SDNN and ULF are significant predictors of clinical events including myocardial infarction, cardiomyopathy, mortality and arrhythmic mortality¹¹. Moreover, lower indices of rMSSD may suggest a relative reduction in parasympathetic activity¹¹. The loss of protective vagal reflexes is related to ventricular tachyarrhythmias^{36,37}. Furthermore, some evidence suggests that vagal activity may have a role in the immune response and lowering of inflammation, i.e., the “nicotinic anti-inflammatory pathway”³⁸. Higher resting HR is also an independent risk factor for sudden cardiac death, fatal cardiovascular disease, and all-cause mortality¹³.

In vitro and animal studies suggest that dietary fatty acids can alter the function of *trans*-membrane cell proteins, including cardiac ion channels⁷. Specific individual fatty acids appear to be preferentially incorporated into lipid rafts or caveolae that modulate membrane receptor

function⁷. Our findings support the need for further experimental investigation of how fatty acids in general, and TFA in particular, might affect cell membrane and ion channel functions. In one small (n=79), short-term (week) intervention, TFA consumption had adverse effects on both resting HR and time-domain measures of 24-hour HRV¹⁴. TFA consumption (6.8% of total energy) was from bakery products and comprised both *trans*-18:1 and *trans*-18:2 (55% and 5% of total fatty acids, respectively), limiting conclusions for specific effects of these different TFA isomers. Our findings are consistent with these results and support the possibility of adverse effects of *trans*-18:2 consumption at usual dietary levels of intake on HRV in free-living populations.

The associations of dietary *trans*-18:2 with HRV observed in CHS cohort could not be confirmed using plasma phospholipid TFA. Reasons for this inconsistency are unclear. The correlation between estimated dietary *trans*-18:2 and plasma phospholipid levels in CHS is not high ($r=0.30$), and known food sources of partially hydrogenated vegetable oils do not predict plasma phospholipid levels *trans*-18:2 levels well¹⁸ and the dietary estimate might conceivably include other dietary components associated with HRV. However, we adjusted the analyses for several possible confounders. Other possible explanations include limited power in the phospholipid analyses due to a smaller number of subjects, and potential non-dietary influences on phospholipid *trans*-18:2 levels, potentially related to metabolism or incorporation. Additionally, plasma phospholipid levels reflect diet in the prior few weeks, and variability in dietary *trans*-18:2 consumption over time might also have contributed to the absence of observed associations between plasma phospholipids *trans*-18:2 and HRV indices. Our findings support the need for further investigation of potential electrophysiologic effects of dietary *trans*-18:2 as well as determinants of their circulating levels and more precise estimates of intake.

In contrast to findings for *trans*-18:2, in CHS we found that dietary and plasma phospholipid *trans*-18:1 were associated with more favorable HRV measures including, abnormal heart rate patterns (DFA1 and SD12), circadian modulation (SDNN, SDANN, ULF), vagal activity (rMSSD), and resting HR. The different associations for *trans*-18:2 versus *trans*-18:1 are consistent with emerging evidence that these fatty acids may have different effects on some health outcomes. For example, our own and others' previous work has demonstrated generally positive associations of *trans*-18:2, and null or inverse associations of *trans*-18:1, with levels of systemic inflammation^{15,16} and risk of coronary heart disease including sudden cardiac arrest^{5,6,8,17,39}. A recent animal study found that *trans*-18:2 consumption increased biomarkers of endothelial dysfunction (ICAM-1) and oxidative stress⁹. Differences in health effects of *trans*-

18:1 and *trans*-18:2 deserve further attention, especially as most policy focus to date has been on partially hydrogenated vegetable oils that contain mostly *trans*-18:1.

Our analysis had several strengths. We evaluated the relationships between TFA consumption and HRV, including both short-term and 24-hour indices, in two separate cohorts. Information on dietary habits, HRV measures, and others risks were collected prospectively by standardized methods. We evaluated several HRV measures including time-domain, frequency-domain and non-linear HRV indices. We adjusted for several relevant confounders characterized prospectively using standardized methods, minimizing residual confounding. We reached similar conclusion about *trans*-18:2 consumption in older adults and younger adults in two distinct cohorts employing different methods for dietary and HRV assessments, suggesting that our findings may be generalizable to other populations.

We recognize some limitations. In both cohorts, residual confounding due to unknown or incompletely measured factors cannot be excluded, even though a range of covariates were available and evaluated as potential confounders. Inconsistency between CHS results for dietary and plasma phospholipid *trans*-18:2 was present. We evaluated multiple HRV indices, and some results could have been due to chance. However, the outcomes of the analyses were generally consistent in the two cohorts and with our prespecified hypotheses⁴⁰. Additionally, Porto had limited sample size and potential measurement errors from 24-hour dietary recall to assess TFA consumption. Despite the small sample size the serial assessments over three years would tend to reduce the error inherent to 24-hour recall and improve statistical power. Most participants were Caucasian.

In conclusion, estimated *trans*-18:2 consumption was associated with specific and less favorable indices of HRV in both young and older adults, whereas *trans*-18:1 consumption and blood levels were positively associated with some HRV indices in older adults. Because HRV is a potential mediator for heart disease and especially sudden death, our results indicate the need for further studies to characterize the dietary sources and potential electrophysiological roles of different TFA.

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Disclosures

None

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Appendix

Table 1: Measures of heart rate variability (HRV) in the Porto and Cardiovascular Health Study cohorts.

<i>Variable</i>	<i>Brief Physiologic Correlation</i>
<i>Analysis of Short-term recordings (5-min)</i>	
<i>Time-domain</i>	
SDNN (ms)	Combined sympathetic and parasympathetic modulation of the heart rate due to respiratory sinus arrhythmia, representing cardiac vagal control. When measured in a supine position with paced breathing, 5-min SDNN mostly γ parasympathetic activity. Higher values reflect higher parasympathetic (vagal) influence.
rMSSD (ms)	Mainly parasympathetic modulation of the heart rate due to respiratory sinus arrhythmia, representing cardiac vagal control. Higher values reflect higher parasympathetic (vagal) influence.
<i>Frequency-domain</i>	
HF (ms^2)	Vagal modulation of heart rate in response to respiration. Higher values reflect higher parasympathetic (vagal) influence or greater beat-to-beat variability due to erratic rhythm.
<i>Analysis of long-term recordings (24-hour)</i>	
<i>Time-domain</i>	
SDNN (ms)	Standard deviation of all N-N intervals. Reflects longer-term circadian differences and total HRV.
SDANN (ms)	Standard deviation of 5-min average of N-N intervals. Total circadian activity.
SDNN-index (ms)	Averaged 5-min SDNN. Reflects combined sympathetic and vagal activity but independent of circadian rhythm..
rMSSD (ms)	Root mean square of successive differences between N-N intervals. Reflects the average of daytime and night-time parasympathetic respiratory variation. Higher values reflect higher parasympathetic (vagal) influence or greater degree of erratic rhythm.

Frequency-domain

LF/HF	Has been proposed has an index of sympathovagal balance. However interpretation of this index is controversial.
nLF (nu)	Precise interpretation of this index is controversial. However there is evidence that normalized LF can be a measure of sympathetic modulation of heart rate. LF band is between 0.04 and 0.15 Hz.
nHF (nu)	Relative vagal modulation of heart rate in response to respiration. Higher values reflect higher parasympathetic (vagal) influence or greater degree of erratic rhythm. HF band is between 0.15 and 0.4 Hz.
ULF (ms ²)	Fluctuations in R-R intervals with underlying cycle length of >5-min and ≤24-hour. Predominantly circadian rhythm but other influences including activity and neuroendocrine rhythms. ULF band is below 0.003Hz
TP (ms ²)	Variance of all N-N intervals.
<i>Non-linear</i>	
DFA1	Randomness or correlatedness of the R-R intervals pattern. Totally random R-R intervals pattern has a value of 0.5, whereas a totally correlated pattern has a value of 1.5.
SD12 (Poincare Ratio)	Organization of heart rate patterns based on the ratio of the axes of an ellipse fitted to the scatter plot of N-N vs. N-N=1 intervals. Higher values can reflect a greater degree of erratic rhythm.

DFA1= short-term fractal scaling exponent, HF= high-frequency power, LF/HF ratio, nLF= normalized low-frequency power, nHF =normalized high-frequency power; SD12= Poincaré plot ratio, rMSSD=square-root-of -the-mean-of-the-squares-of-successive-R-R-intervals differences, SDNN=standard-deviation-of-the-R-R-intervals, SDANN= standard-deviation-of-5-minutes-average-of-the-R-R-intervals, SDNN-index, TP= total power, ULF= ultra-low-frequency power.

Paper V

**Associations between metabolic syndrome components, physical activity and
cardiac autonomic function.**

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(Submitted – Under review)

Associations between metabolic syndrome components, physical activity and cardiac autonomic function.

Metabolic syndrome and heart rate variability

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Abstract

Aim: We investigated the associations between metabolic syndrome (MetS) components and physical activity (PA) with cardiac autonomic nervous system estimated by heart rate variability (HRV) in young healthy adults followed over three years.

Methods: A total of 228 healthy participants volunteered to participate in the first year of a 3-year longitudinal study (2008 to 2010). PA was assessed annually by accelerometer. The MetS components considered in our analyses were waist circumference, systolic blood pressure, high-density lipoprotein, triglycerides, glucose, and C-reactive protein. These components are common components in the definition of MetS by the National Cholesterol Education Program - Adults Treatment Panel III and by the International Diabetes Federation. All components were standardized into standard deviation scores (SDS) to facilitate direct comparison of the different components and a linear mixed model was applied to analyse the repeated measures.

Results: The majority of the MetS components were associated with lower HRV indices and higher HR. Conversely, PA was associated with higher HRV and lower HR.

Conclusions: The results of this longitudinal study suggested that MetS components are associated with less favourable indices of HRV, even in a relatively healthy population of young adults. Given the observed increased prevalence of the MetS in younger populations, it remains imperative to promote the metabolic and autonomic benefits of exercise.

Key Words: cardiac autonomic function, heart rate variability, metabolic syndrome, young adults, inflammation, physical activity

Introduction

The Metabolic Syndrome (MetS) is as a group of risk factors that tend to cluster, increasing the risk of increasingly-prevalent cardiovascular disease [1]. Abdominal obesity, dyslipidemia, components of hypertension, impaired glucose metabolism, and pro-inflammatory state are generally accepted as MetS characteristics [2]. Although the genetic determinants cannot be ignored, the primary contributors to MetS are adverse lifestyle factors [3].

Autonomic changes are both linked to the development of type 2 diabetes and MetS, and even suggested as an underlying cause [4]. Autonomic dysfunction may be present after only a brief exposure to hyperglycemia or even in patients with normal plasma glucose values [4, 5]. Nevertheless, which problem appears first and triggers the cycle of metabolic changes is still unclear.

Heart rate variability (HRV) is commonly used as a measure of cardiac autonomic function. Low HRV indicates a shift toward sympathetic predominance and parasympathetic reduction, and has good prognostic value for cardiovascular disease outcomes including all-cause mortality [6], death from sudden cardiac arrest and other coronary heart diseases [7, 8]. Reduced HRV can also predict development and progression of coronary heart disease [9]. As a marker of cardiac autonomic activity heart rate (HR), has been increasingly considered in predicting cardiovascular disease risk [10].

Existing studies on HRV and MetS suggested that HRV is negatively associated with MetS [11-17]. These studies have mainly been conducted in middle age or elderly participants [11, 13-17], and most did not adjust the analyses for physical activity (PA) [11, 12, 14, 15], a potentially important confounder since PA can influence HRV and MetS. Additionally, none of these studies have objectively assessed PA, which may have created an inherent bias. To the best of our knowledge, only one study conducted in middle-aged participants has analysed the associations between HRV and components of MetS across their continuous range of values [17]. The remainder have analysed the relations between HRV and the number of components of MetS as categorical variable [11-16]. Furthermore, there are no longitudinal studies addressing this topic in healthy young adults. The purpose of this study was, therefore, to analyse the associations between MetS components (expressed as continuous variables), PA and cardiac autonomic function estimated by HRV in young healthy adults followed over three years.

Methods

A total of 228 volunteers participated in the first year of a 3-year longitudinal study (2008 to 2010). In the second year, 191 participants remained in the study; in the third year this number was 182. We excluded individuals with known cardiovascular illness, any medication or supplements that could influence the control of cardiac autonomic function. Participants without sufficient accelerometer and/or HRV data were excluded from this study. The present study represents analyses of data from 163 participants. We obtained written informed consent from all participants. The study was approved by the local ethics committee and conducted in accordance to the declaration of Helsinki.

Procedures

All procedures were performed annually over the 3 years; between 8 a.m. and 11 a.m. Participants were wearing light clothing without shoes. Body Mass was assessed using a Tanita Inner scan, BC-532 (Tanita, Hoofddorp, The Netherlands). Stature was measured using a stadiometer Seca model 708 (Seca, Hamburg, Germany). Body mass index (BMI, kg/m^2) was calculated. Waist circumference (WC) was measured midway from the lower rib margin to the anterior superior iliac crest, using a non-metallic tape without significant compression. Blood pressure, via sphygmomanometer, was recorded from the left arm in the seated position after a standardised 10-minute rest period with a Colin 8800P Monitor (Colin, TX, USA). Three measures were made; the final value being the average of the last two. A capillary sample of blood was taken from ear lobe after an overnight fast, annually. The sample was analysed using a Cholestech LDX® cassette (Cholestech, Hayward, California, USA) to measure total cholesterol (TC), triglycerides (TRG), glucose (Glu) and high-density lipoprotein (HDL). The Cholestech LDX® shows good agreement with laboratory measures for population-based risk factor screening [18] and meets the criteria set by the lipid standardization panel for accuracy and precision of cholesterol measurements [19]. C-reactive protein (CRP) was measured only in the two last years of the study (2009, 2010) in a subset of the sample (N= 89, 2009; N= 138, 2010), with a high-sensitivity (hs) assay using Cholestech LDX®, which has a high correlation with a nephelometric reference method according to the manufacturer (R= 0.98). Habitual alcohol intake (yes or no) and smoking habits (yes or no) were assessed annually by questionnaire.

Free-living PA was assessed annually using a uniaxial ActiGraph accelerometer (model GT1M, Fort Walton Beach, Florida, USA). This device provides valid and reliable

measures of vertical body acceleration and estimation of energy expenditure. Participants were instructed to wear the monitor on the iliac crest of the right hip with an elastic belt and to remove it for sleep and in any activity that may cause damage to the monitor. PA was monitored in 15 s epochs for up to seven consecutive days. A minimum recording period of five days was necessary for the PA measurement to be considered adequate [20]. Freedson's (1998) cut points were used to analyse the data via a Mahuffe activity analyser [21]. Counts were transformed to average minutes per day in light ($<1951 \text{ counts} \cdot \text{min}^{-1}$), moderate ($1952\text{-}5724 \text{ counts} \cdot \text{min}^{-1}$), vigorous ($5725\text{-}9498 \text{ counts} \cdot \text{min}^{-1}$) and very vigorous ($>9499 \text{ counts} \cdot \text{min}^{-1}$) PA. Daily time spent in moderate-to-vigorous physical activity (MVPA) was calculated.

Recordings of R-R intervals data were made at rest in a quiet room, with participants in the supine position, using a Polar Advantage NV (Polar Electro OY, Finland). Verbal instructions and a practice period of paced breathing were provided. Participants matched their breathing to a metronome-paced frequency of $12 \text{ breaths} \cdot \text{min}^{-1}$. Participants were asked to avoid strenuous exercise and caffeine or alcohol consumption for 24 hours prior to the examination. The Polar Advantage heart monitor is comparable to the more conventional ECG devices for R-R intervals measurement [22]. The R-R interval data were downloaded into the Polar Precision Performance Software SW. The last 5 minutes of the 20 minute recording were utilized for the calculation of HRV variables and HR. The R-R intervals were analysed using time domain, frequency domain and Poincaré plot techniques, using Kubios HRV Software 2.0 for Windows (The Biomedical Signal Analysis Group, University of Kuopio, Finland). Time domain measures included mean R-R intervals, square root of the mean of the squares of successive R-R intervals differences (RMSSD), standard deviation of the R-R intervals (SDNN), percentage of heart period differences $>50 \text{ ms}$ (PNN50), number of pairs of adjacent R-R intervals differing by more than 50 ms in the entire recording (NN50). R-R intervals were also used to produce Poincaré Plot, SD1 (Poincaré plots' descriptor: deviation of instantaneous beat-to-beat R-R intervals variability is short diameter of ellipse) and SD2 (Poincaré plots' descriptor: deviation of instantaneous beat-to-beat R-R intervals variability is long diameter of ellipse) values. Non-parametric (fast Fourier transform) was used to obtain the frequency domain measures of HRV. Short-term HRV recording enabled the computation of the three-frequency bands: very-low frequency (VLF $0.00\text{-}0.04 \text{ Hz}$), low frequency (LF $0.04\text{-}0.15 \text{ Hz}$), and high frequency (HF $0.15\text{-}0.4 \text{ Hz}$). Only HF were considered in our analysis because the VLF and LF component are considered dubious measures due to its uncertain physiological meaning and interpretation [23]. Furthermore, the LF:HF ratio was calculated as a measure to estimate sympathovagal balance.

Most HRV measures are predominantly under vagal control especially short-term measures obtained under laboratory conditions, where participants are usually supine [24]. Conversely, no coherent evidence exists on the information that HRV provides on sympathetic modulation.

Statistical Analyses

Descriptive statistics were used to characterize the sample. Means and standard error were used for continuous variables and frequencies for categorical variables. Data were tested for normality. Due to skewed distributions, HF and LF/HF were logarithmically transformed prior to analyses.

We considered the following MetS components in our analyses: WC, systolic blood pressure (SBP), TRG, HDL, Glu, and hs-CRP. These components are common components in the definition of MetS by the National Cholesterol Education Program - Adults Treatment Panel III and by the International Diabetes Federation. All components were standardized into standard deviation scores (SDS) to facilitate direct comparison of the different components (WC=8.64 cm, MVPA=35.94 min/week, SBP=12.70 mmHg, TRG= 36.27 mg/dl, Glu=7.78 mg/dl, HDL=15.40 mg/dl, hs-CRP=2.10 mg/l). Additionally MVPA was also included in the analyses since it is a potential confounder, and standardized into SDS. Thus, results are reported in SDS.

To analyse the associations between MetS components and MVPA with HRV indices we used mixed-effects linear regression analyses, in order to analyse repeated measures collected over 3 years, controlling for: sex, age, smoking status and alcohol intake. The analyses of the associations of hs-CRP with HRV were conducted separately, as data were available only in 2009 and 2010. MetS components were also mutually adjusted for each other and for MVPA. Longitudinal data typically need some structured covariance models since, such data possesses a hierarchical structure in the sense that repeated measurements are viewed as a separate level nested within an individual. This statistical analysis recognizes that multiple observations from the same subject are dependent making it highly suitable for longitudinal data.

The level of significance was set at $p < 0.05$. Data were analysed using Stata version 11.1

Results

Table 1 shows the descriptive characteristics of the sample over the three years of the study.

Table 1: Participants' characteristics at each year (mean \pm standard error).

	Total Sample		
	Year 1	Year 2	Year 3
Sex (M/F)	78/85	78/85	78/85
Age (years)	19.58 \pm 0.10	20.58 \pm 0.10	21.59 \pm 0.10
Smoke Status (yes/no)	14/149	15/148	16/147
Alcohol intake (no/yes)	5/158	5/158	3/160
BMI (kg/m²)	23.00 \pm 0.21	23.14 \pm 0.22	23.07 \pm 0.23
WC (cm)	79.98 \pm 0.64	76.74 \pm 0.70	78.60 \pm 0.7
MVPA (min/week)	61.06 \pm 2.41	61.63 \pm 2.50	60.27 \pm 2.31
SBP (mmHg)	113.82 \pm 1.07	113.67 \pm 1.03	116.42 \pm 0.86
DBP (mmHg)	67.95 \pm 0.65	63.20 \pm 0.76	64.78 \pm 0.54
Triglycerides (mg/dl)	67.30 \pm 2.57	74.22 \pm 2.70	81.09 \pm 3.09
Glucose (mg/dl)	79.59 \pm 0.61	84.80 \pm 0.56	81.92 \pm 0.59
HDL (mg/dl)	46.82 \pm 1.21	50.16 \pm 1.12	51.51 \pm 1.19
hs-CRP (mg/l)	-----	1.76 \pm 0.26	1.35 \pm 0.16
SDNN (ms)	67.59 \pm 2.13	69.60 \pm 2.27	67.94 \pm 2.30
RMSSD (ms)	72.14 \pm 2.79	74.23 \pm 2.91	73.34 \pm 3.12
PNN50 (%)	39.65 \pm 1.62	40.68 \pm 1.59	39.14 \pm 1.75
SD1 (ms)	51.29 \pm 1.98	53.37 \pm 2.08	52.79 \pm 2.34

HF (ms²)	3221.97±221.82	3430.79±238.43	3819±292.47
HF nu	69.95±1.40	69.91±1.33	70.65±1.17
LF/HF	0.72±0.13	0.63±0.96	0.53±0.50
HR (beats/min)	69.85±0.87	69.19±0.82	67.15±0.85

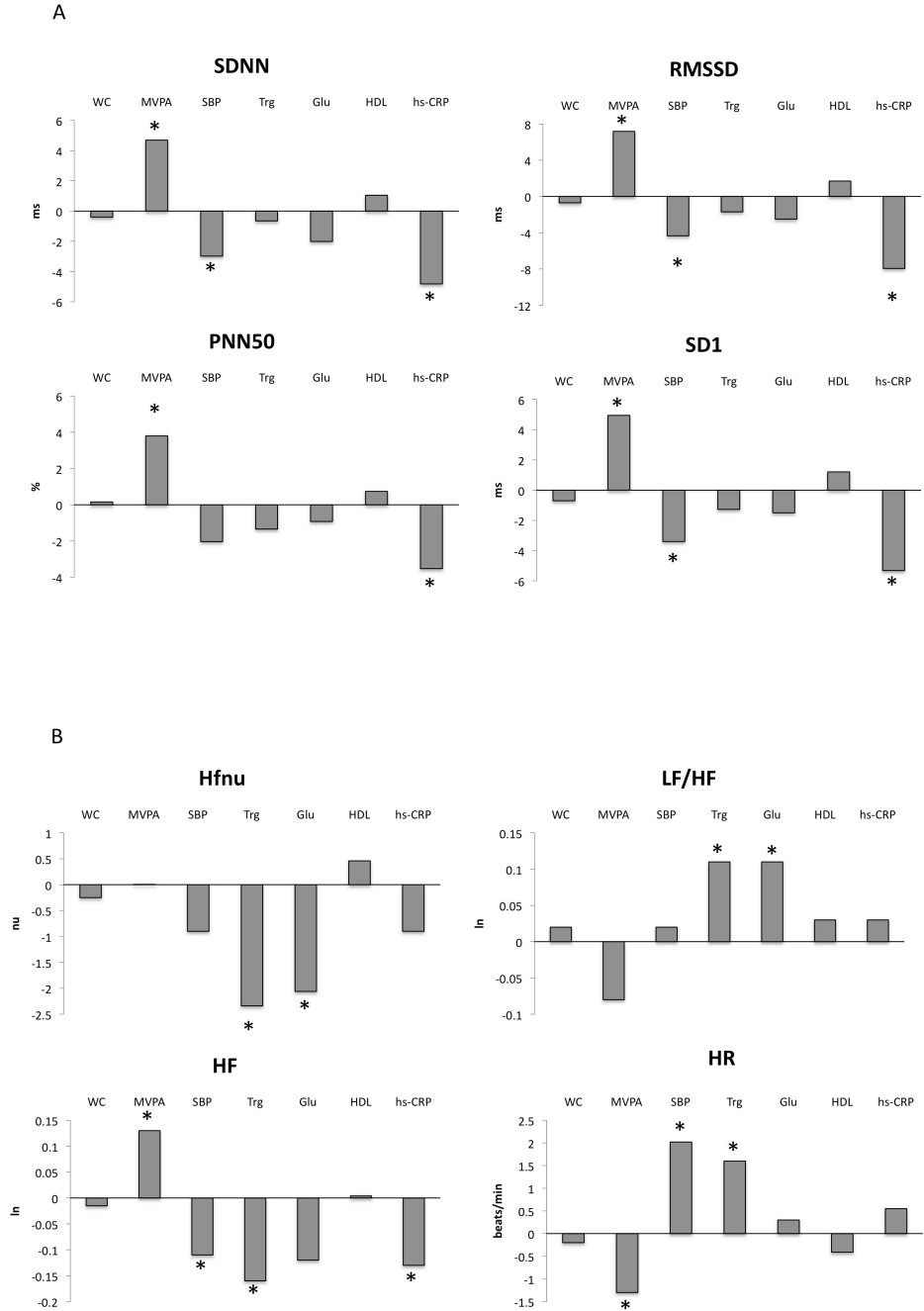
BMI= body mass index, WC= waist circumference, PA = physical activity, SBP= systolic blood pressure, DBP= diastolic blood pressure, TC= total cholesterol, HDL= high-density lipoprotein, hs-CRP= high sensitivity C-reactive protein, SDNN= standard deviation of the R-R interval, RMSSD= RR-interval, square root of the mean of the squares of successive R-R interval differences, PNN50= percentage of heart period differences>50 ms, SD1= normalized deviation of instantaneous beat-to-beat R-R interval variability is the short diameter of ellipse, HF = high frequency, HF nu = normalized high frequency, LF/HF= ratio low frequency-high frequency, HR= heart rate.

Figure 1 shows the associations between MetS components and MVPA with HRV indices, assessed annually over 3 year, following multivariate adjustment. Additionally, it shows the association between hs-CRP and HRV indices, assessed twice over 2 years.

The different MetS components were not associated with all HRV indices equally. After multivariate adjustment, one SDS higher in most MetS components was significantly associated with lower indices of HRV. In contrast, a one SDS higher MVPA was associated with more favourable HRV indices. All further associations are expressed as the change in the dependent variable according to a one SDS higher in the predictor as above.

Higher MVPA was associated with higher respiratory-vagal mediated HRV indices: SDNN, RMSSD, PNN50, SD1, HF and lower HR. Higher SBP was associated with lower vagal indices SDNN, RMSSD, SD1, HF and higher HR. Additionally, higher TRG was associated with lower vagal indices (HFnu and HF), conversely it was associated with higher LF/HF index, a sympathovagal balance index and with higher HR. Higher Glu was associated with lower values of HFnu and higher LF/HF. One SDS higher hs-CRP was associated with significantly lower value for indices of parasympathetic modulation (SDNN, RMSSD, PNN50, SD1 and HF).

Figure 1 A and B: Fixed effects of components of metabolic syndrome, high sensitivity C-reactive protein and physical activity on heart rate variability indices. Linear mixed model effects were performed with adjustments for sex, age, smoking status and alcohol intake.



Graphic represents differences in HRV and HR according to 1 higher standard deviation of each MetS components, PA, and hs-CRP. (hs-CRP data available only over 2 years); * $p < 0.05$.

Discussion

We examined the associations of repeated measurements of MetS components and PA with cardiac autonomic function estimated by HRV. In this cohort of healthy young adults, higher levels of MetS components were generally associated with a less favourable HRV profile. Conversely, and as expected, MVPA was associated with more favourable HRV values. Additionally, hs-CRP was associated with lower HRV indices assessed annually over 2 years. To our knowledge, this is the first longitudinal study in young healthy adults to analyse the associations between MetS components, and HRV indices while adjusting for MVPA and including hs-CRP in the analyses since MetS is considered a pro-inflammatory state [2].

As expected a positive association was observed between MVPA and HRV, this relationship is largely confirmatory since, it is in agreement with several previous studies [25, 26].

The inverse association between SBP, TRG and Glu and vagally-mediated indices is in agreement with previous studies showing that hypertension, dyslipidaemia, obesity, type 2 diabetes and impaired glucose are associated with lower HRV [17, 27]. The present results are largely confirmatory of those from studies where MetS and HRV values were inversely associated [11-17] however, we present new important information on that theme by controlling for MVPA and by including hs-CRP in the analyses. The majority of MetS components were inversely associated with vagal indices in the present population of young healthy adults, suggesting that those MetS components may be associated with lower vagal modulation even in a healthy population free of disease. Importantly, loss of protective vagal reflexes is related to ventricular tachyarrhythmia [28] and higher resting HR is an independent risk factor for sudden cardiac death, fatal cardiovascular disease, and all-cause mortality [29]. Moreover, lower vagal modulation is associated with increased risk of cardiovascular disease and death [30].

The negative association between hs-CRP and HRV, found in this study supports previous findings by our group [31]. Our previous study suggested that the inverse association observed between vagally mediated HRV indices and hs-CRP could be explained by the role that the vagus may play in the immune responses. It has been suggested that acetylcholine can inhibit the production of pro-inflammatory cytokines from macrophages [32]. This

mechanism has been defined as the '*nicotinic (or cholinergic) anti-inflammatory pathway*' as acetylcholine (the principle vagal neurotransmitter) can inhibit the production of pro-inflammatory cytokines from macrophages by a nicotinic acetylcholine receptor [32]. The present data reinforce the idea that PA may have an important role in the association between hs-CRP and HRV suggested in our previous work. In the statistical model with hs-CRP, MVPA remained significant and associated with higher values of vagal mediated indices (data not shown).

Several theories have been suggested regarding the relationship between autonomic function and MetS and PA. First, that the autonomic nervous system may facilitate mechanisms associated with the development of type 2 diabetes and MetS [3]. The idea that autonomic nervous system dysfunction might facilitate the onset of type 2 diabetes is reasonable if it is considered that the pancreas is innervated by parasympathetic fibers and these fibers stimulate the β -cells to release insulin in response to circulating glucose. On the contrary, sympathetic activation will reduce this secretion [4]. If, therefore, elevated levels of plasma glucose persist, even below the diabetic range, they may possibly damage the peripheral fibers. Consequently sympathetic activity will increase along with a decrease in parasympathetic control [4]. Secondly, it is believed that exercise has anti-inflammatory properties and, therefore, provide protection against cardiovascular diseases and type 2 diabetes due to its capability of increasing vagus activity [33]. Moreover, it is plausible that the mechanism by which exercise confers these protective effects may be, at least in part, due to exercise-induced increase in cholinergic anti-inflammatory pathway activity [33]. Since exercise is associated with higher vagal activity, it may also be that regular physical activity leads to an improved activity in the cholinergic anti-inflammatory pathway, decreasing cytokine production and diminishing the damage and metabolic disorganization mediated by chronic low grade systemic inflammation that is characteristic of the MetS [33].

We found no relationship between WC and HDL with either HRV indices or with HR. The reason for these null observations are not clear, but could be due to the very low incidence of obesity and overweight in this population. The fact that this population's mean value for HDL was >40 mg/dl is a potential reason for the lack of association with HRV and HR.

To the best of our knowledge this is the first longitudinal study in a healthy young population analysing the associations of MetS with HRV adjusting for PA and including a marker of inflammation as hs-CRP. The strength of this study was the longitudinal design with repeated measures analysed by a linear mixed model. Additionally, the objective

measurement of PA using accelerometers is other strength of the study. One limitation of the study comes from the fact that the population volunteered rather than randomly selected to participate in the study. This may have created an inherent bias toward healthy volunteers and may explain the relatively low levels of obesity and healthy HDL profiles observed. Future work should use less healthy people or group at least a bigger range of individuals.

In conclusion, the results of this longitudinal study suggest that components of the MetS including hs-CRP are associated with less favourable indices of HRV, even in a population free of clinical type 2 diabetes or full-blown MetS. This study confirms that objectively assessed PA is associated with more favourable indices of HRV. Given the observed increased prevalence of the MetS in ever younger populations, it remains imperative to promote the metabolic and autonomic benefits of exercise.

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Declaration of Competing Interests: Nothing to declare

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Chapter 3. General Discussion and Conclusions

4. General Discussion

The major findings of our experimental work suggested a positive association between PA and cANS function on one hand, and a negative association of inflammation, *trans*-fatty acid consumption, and MetS components with cANS function, on the other.

Physical activity and cardiac autonomic function:

We observed that PA was positively associated with cANS function in healthy young individuals. Our results suggested that in young adults, objectively measured PA was associated with more favourable HRV indices. In these studies, we observed that the most active groups had significantly higher levels of vagal modulation, compared with the least active groups. Furthermore, vigorous PA was more strongly associated with vagal indices of HRV than moderate PA. In addition, 10-minute bouts of vigorous PA were the best predictors of vagal HRV indices. We also observed that complying with moderate and vigorous PA recommendations, rather than only moderate recommendations, was associated with higher vagal indices of HRV and higher HR complexity. Several other studies (Melanson 2000; Rennie, Hemingway et al. 2003; Buchheit, Simon et al. 2005; Sandercock, Hardy-Shepherd et al. 2008) have also shown better HRV profiles in moderately and highly active groups, compared with less-active individuals. The present data broadly agreed with and confirmed such studies by adding information on objectively measured PA. What was not totally clear from those studies was how PA intensity was associated with HRV in young adults. Our results suggested that vigorous PA is more closely associated with more favourable cANS function than moderate PA. These results highlight the potential importance of vigorous PA as an anti-arrhythmic tool in young adults, since higher vagal modulation is believed to have an important role in reducing susceptibility to malignant arrhythmias (Billman 2009). It is believed that vigorous PA is important to promote cardiovascular health and is associated with more general health benefits (Kemi and Wisloff 2010) and cardioprotection (Wisloff, Ellingsen et al. 2009). Engaging in high-intensity PA seems to lead to greater health benefits than engaging in low-intensity PA (Kemi and Wisloff 2010). Thus, exercise intensity may dictate

magnitude of adaptation (Kemi and Wisloff 2010). Our results are in line with these facts and are supportive of and supported by them.

Inflammation and cardiac autonomic function:

Our results suggested an association between PA, inflammation, and vagal indices of HRV. Individuals in the highest tertile of high-sensitivity C-reactive protein (hs-CRP) had lower vagal modulation. Additionally, the most physically active subjects had lower levels of hs-CRP and the highest levels of vagal modulation. The so-called “nicotinic/cholinergic anti-inflammatory pathway” can support these results. It is believed that the vagus nerve can modulate innate immune response and prevent inflammation (Ulloa 2005). This mechanism is based on the assumption that acetylcholine can inhibit the production of pro-inflammatory cytokines from macrophages by binding to a nicotinic acetylcholine receptor (Ulloa 2005). Previous studies have shown inverse relationships between vagal indices of HRV and inflammatory markers in middle-aged and elderly subjects (Sajadieh, Nielsen et al. 2004; Araujo, Antelmi et al. 2006; Madsen, Christensen et al. 2007; Sloan, McCreath et al. 2007; Lampert, Bremner et al. 2008; Stein, Barzilay et al. 2008; Thayer and Fischer 2009). Our results are in line with those studies and extend the existent knowledge to young healthy individuals. Additionally, our findings are in line with a recent study that observed that subjects with higher cardio-respiratory fitness had the lowest levels of CRP and higher vagal modulation (Jae, Heffernan et al. 2009). We were able to replicate these results later, when using HRV to study the association of MetS with cANS over the course of three years. Similarly, we found a negative association between hs-CRP and HRV, and we observed that higher levels of PA were associated with higher levels of vagal modulation and lower levels of inflammation. These results support Tracey et al.’s (Tracey 2007) assertion that the role of exercise in reducing and preventing cardiovascular disease, type 2 diabetes, and other types of diseases may be related to the fact that exercise increases cholinergic anti-inflammatory pathway activity. It is plausible that one mechanism by which exercise confers those protective effects may be, at least in part, related to exercise-induced increase in cholinergic anti-inflammatory pathway activity (Tracey 2007). However,

our data do not provide sufficient information to fully support those ideas, and, hence, further physiological studies are needed on this topic.

Trans-fatty acid consumption and cardiac autonomic function:

We found that TFA consumption was associated with HRV indices. In a population of young adults, we observed cross-sectional associations of total TFA consumption, which correlated most strongly with circulating phospholipid *trans*-18:2, with less favourable HRV indices assessed annually over 3 years. Similarly, among older adults in the CHS cohort, higher *trans*-18:2 consumption was both cross-sectionally and prospectively associated with less favourable HRV, whereas higher *trans*-18:1 consumption was both cross-sectionally and prospectively associated with higher HRV. Previous work has generally demonstrated positive associations of *trans*-18:2, and null or inverse associations of *trans*-18:1, with levels of systemic inflammation (Mozaffarian, Pischon et al. 2004; Mozaffarian, Rimm et al. 2004) and risk of coronary heart disease, including sudden cardiac arrest (Roberts, Wood et al. 1995; Lemaitre, King et al. 2002; Baylin, Kabagambe et al. 2003; Lemaitre, King et al. 2006; Chiuve, Rimm et al. 2009). Thus, our results are in line with these studies, since we observed opposite directions of association between HRV indices and *trans*-18:2 and *trans*-18:1 consumption. *Trans*-18:2 consumption was associated with specific, less favourable indices of HRV in both older and young adults, and *trans*-18:1 consumption was associated with more favourable HRV indices in older adults. Our results support the growing evidence that *trans*-18:2, in particular, may increase cardiac risk and suggest the need to investigate potential HRV-related mechanisms whereby *trans*-18:2 may increase arrhythmic risk.

Metabolic syndrome and cardiac autonomic function:

We observed, when examining associations of repeated measures collected over three years, that higher levels of MetS components were generally associated with a less favourable HRV profile. Conversely, and as expected, PA was associated

with more favourable HRV values, reinforcing and supporting the findings of our previous studies. Additionally, hs-CRP was associated with lower HRV indices.

Studies have observed that Mets and HRV are inversely associated (Liao, Sloan et al. 1998; Hemingway, Shipley et al. 2005; Stein, Barzilay et al. 2007; Min, Min et al. 2008; Gehi, Lampert et al. 2009; Koskinen, Kahonen et al. 2009; Chang, Yang et al. 2010), and our results largely confirm those from these studies however, we present new important information on that theme by controlling for PA and by including hs-CRP in the analyses. Our results suggested that components of the MetS including hs-CRP are associated with less favourable indices of HRV, even in a population free of clinical type 2 diabetes or full-blown MetS. Given the observed increased prevalence of the MetS in younger populations, it remains imperative to promote the metabolic and autonomic benefits of exercise.

In summary, the aforementioned results gathered by this thesis increase the amount of information available on factors that may favourably and unfavourably affect cANS balance. They have important implications, because an imbalanced cANS function may lead to electrical instability of the heart, which can, in turn, lead to arrhythmia. It is now widely accepted that reductions in vagal control are associated with augmented risk of sudden death and that cardiac sympathetic hyperactivity increases the development of lethal arrhythmias. Furthermore, an imbalanced cANS function is associated with a broad range of diseases, including type 2 diabetes, hypertension, obesity, and MetS.

5. Conclusions

Based on our experimental work, it is possible to conclude that PA, inflammation, *trans*-fatty acid consumption, and the components of MetS are associated with cANS function. Factors such as PA seem to be associated with more favourable cANS function. Conversely, factors such as inflammation, *trans*-18:2 consumption, and MetS components seem to be associated with less favourable HRV and can be considered harmful to cANS function.

Considering our purpose and based on our findings, it seems reasonable to emphasize the following conclusions:

1. PA is positively associated with cANS function. Vigorous PA is associated with more favourable HRV profiles in young adults than moderate PA.
2. Inflammation is inversely associated with vagal modulation in young, healthy adults, and PA is associated with lower inflammation and higher vagal modulation. The nicotinic anti-inflammatory pathway may modulate this association.
3. *Trans*-18:2 consumption is associated with specific, less favourable indices of HRV in both older and younger adults, whereas, *trans*-18:1 consumption is associated with more favourable HRV indices in older adults.
4. MetS components are associated with less favourable indices of HRV in a population of young adults.

6. Perspectives for future research

The findings of this thesis enhance the knowledge on the factors that are positively and negatively associated with cANS function in a young healthy population. There are some points in our results that should be addressed, to highlight interesting directions for future research. Future research should focus on the study of the nicotinic anti-inflammatory pathway as a possible mechanism for the anti-inflammatory properties of regular PA. Moreover, mechanistic studies should attempt to clarify whether impaired ANS function is a consequence or a cause of disorders and diseases, including type 2 diabetes, MetS, obesity, and hypertension. Future studies should also focus on the relationship between *trans*-fat isomers and cardiovascular health.

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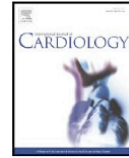
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Chapter 4. Appendix



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Letter to the Editor

Effects of exercise training on neurovascular responses during handgrip exercise in heart failure patients

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Exaggerated central sympathetic outflow typifies chronic heart failure (HF) [1]. Augmented plasma catecholamine levels, norepinephrine spillover and muscle sympathetic nerve activity (MSNA) have been demonstrated in HF patients [2,3]. In addition, HF patients have reduced skeletal muscle blood flow (FBF) [4,5], which may explain, at least in part, the skeletal myopathy and exercise intolerance in HF patients [1].

Exercise training markedly reduces MSNA and muscle vasoconstriction in HF patients [6,7]. However, previous studies were limited to the effects of exercise training on resting neurovascular control. It remains unknown whether exercise training improves sympathetic outflow and muscle vascular resistance during physiological manoeuvres, such as exercise. This is an important question, since previous observations strongly suggest that altered muscle afferent reflex control contributes to the increased sympathetic nerve activity and exercise intolerance in HF [8,9].

We tested the hypothesis that exercise training would reduce MSNA and increase FBF during handgrip exercise in HF patients.

The study included thirty-five clinically stable HF patients aged 40 to 75 years old, ejection fraction $\leq 40\%$ from our data base (1998–2004) of previous randomized studies [7,8] in a 1:1 ratio of the Unit of Cardiovascular Rehabilitation and Exercise Physiology, Heart Institute,

Medical School, University of São Paulo. Seventeen age-matched healthy individuals in our data base were used as controls. MSNA had been recorded from the peroneal nerve and FBF had been measured by venous occlusion plethysmography [6,7]. Exercise was elicited by isometric handgrip exercise (30% of MVC) for three minutes [3].

Exclusion criteria were: unstable angina, a recent myocardial infarction, severe chronic obstructive pulmonary disease, uncontrolled systemic arterial hypertension, and/or neurological or orthopaedic disabilities. The exercise group underwent 4 months of supervised exercise training. The study was approved by The Human Subject Protection Committee of the Heart Institute (InCor) and Clinical Hospital, University of São Paulo, Medical School.

Initial differences were tested by one-way ANOVA with repeated measures. Two-way ANOVA with repeated measures was used for between-group comparison. When significance was found, Scheffé's post hoc comparison test was performed. The level of significance was set at $p < 0.05$.

Baseline characteristics of the exercise-trained and untrained HF patients and normal controls are shown in Table 1. There were no significant differences between HF patients and normal controls in gender, heart rate, systolic and mean arterial pressure. Exercise-trained HF patients were older than normal controls. Body weight and BMI were greater in normal controls than in both HF groups. HF patients had lower peak VO_2 ($p < 0.001$), left ventricular ejection fraction ($p < 0.001$) and higher MSNA ($p = 0.001$), and forearm vascular resistance ($p < 0.001$) than normal controls. FBF was lower in exercise-trained HF patients than in normal controls ($p = 0.002$). No significant differences were found in FBF between untrained HF patients and normal controls.

During exercise, heart rate and mean arterial pressure increased significantly and similarly in all groups (Table 2). FBF during exercise increased progressively in all groups, but this parameter was significantly lower in HF patients (group effect $p = 0.01$, Table 2). Sympathetic burst frequency during exercise increased significantly in all groups (Fig. 1A). However, MSNA values were higher at baseline and remained significantly higher during exercise in HF patients compared to normal controls (group effect $p = 0.003$, Fig. 1A). There were no significant differences in forearm vascular resistance during exercise between HF patients and normal controls (Fig. 1C).

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Table 1

Baseline physiological characteristics in exercise-trained and untrained heart failure patients.

	Exercise-trained HF patients (n = 19)	Untrained HF patients (n = 16)	Normal controls (n = 17)
Age (years)	56 ± 2.01	51 ± 1.9	48 ± 1.09*
Sex (M/F)	13/6	11/5	10/7
Weight (kg)	56 ± 2.30	62 ± 3.24	78 ± 2.70**
BMI (kg/m ²)	24 ± 0.70	23 ± 0.76	25 ± 0.58**
HR (beat/min)	71 ± 2.76	75 ± 3.74	65 ± 1
SBP (mmHg)	122 ± 4	119 ± 5	132 ± 2
DBP (mmHg)	79 ± 1	79 ± 2	69 ± 13*,**
MAP (mmHg)	93 ± 3.55	96 ± 3.13	99 ± 2
LVEF (%)	30 ± 1	31 ± 2	68 ± 4*,**
Peak VO ₂ (mL/kg/min)	14 ± 1	15 ± 1	26 ± 1*,**
HF aetiology			
Chagasic	5	5	
Idiopathic	7	8	
Ischaemic	4		
Hypertensive	3	3	
Medications			
ACEI/ARB	18	16	
Digoxin	18	13	
Diuretics	16	12	
Beta-adrenergic blocker	11	7	
Spirinolactone	15	9	
MSNA (bursts/min)	44 ± 3	45 ± 4	27 ± 2*,**
MSNA (bursts/100HB)	73 ± 5	74 ± 6	46 ± 3*,**
FBF (mL/min/100 mL)	1.67 ± 0.11	2.04 ± 0.13	2.40 ± 0.17*
FVR (units)	58 ± 3	50 ± 4	45 ± 3*,**

Values are mean ± SE. HF = heart failure; BMI = body mass index; HR = heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial pressure; EF = ejection fraction; VO₂ = oxygen uptake; ACEI = angiotensin-converting enzyme inhibitor; ARB = angiotensin II receptor blockers; MSNA = muscle sympathetic nerve activity; HB = heart beats; FBF = forearm blood flow; and FVR = forearm vascular resistance.

* $p < 0.05$ vs. exercised-trained patients.

** $p < 0.05$ vs. untrained patients.

Exercise training significantly increased peak VO₂ in HF patients ($p < 0.001$). No significant changes were observed in untrained HF patients. Exercise training significantly reduced resting and exercise MSNA in HF patients. Sympathetic burst frequency values throughout experimental protocol were significantly lower in exercise-trained HF patients compared to untrained HF patients, and now similar to normal controls (Fig. 1B).

Exercise training significantly increased FBF at rest and during handgrip exercise in HF patients. Thus, FBF was no longer different in exercise-trained HF patients and normal controls and significantly greater than in untrained HF (Table 2). Exercise training significantly reduced forearm vascular resistance throughout the experimental protocol in HF patients. Thus, forearm vascular resistance was no longer different in exercise-trained HF patients and normal controls and significantly lower than untrained HF (Fig. 1D). Exercise training provoked no changes in heart rate and mean blood pressure in HF patients (Table 2).

Previous studies have demonstrated that increasing levels of sympathetic nerve activity, which is observed at rest and during exercise, are associated with increasing severity of left ventricular dysfunction [3]. In humans with HF, we have reported that exercise training decreases resting MSNA [7]. The present study extends the knowledge that exercise training reduces sympathetic nerve activity towards normal levels in HF patients not only at rest, but also during handgrip exercise. Moreover, we report that these changes are consequence of the reduction of resting MSNA, since the response to exercise (delta change) is unchanged in any of the groups.

Table 2

Absolute values of PAM, HR and FBF at baseline and during isometric exercise at 30% of maximal voluntary contraction (MVC) post/pre-exercise or sedentary period.

		Baseline	Exercise (30% MVC)		
			HG 1	HG 2	HG 3
MAP (mmHg)					
Untrained	Pre	96 ± 3.13	99 ± 3.60*	102 ± 3.98*	106 ± 4.83*
HF	Post	94 ± 2.81	97 ± 2.91*	100 ± 3.67*	104 ± 3.39*
Exercise-trained HF	Pre	99 ± 3.55	99 ± 3.85*	100 ± 3.45*	103 ± 3.65*
	Post	91 ± 2.84	94 ± 3.15*	100 ± 3.54*	104 ± 3.77*
Normal control	Pre	99 ± 2	106 ± 3*	112 ± 3*	116 ± 4*
HR (beats/min)					
Untrained	Pre	75 ± 3.74	78 ± 3.28*	81 ± 3.11*	83 ± 3.30*
HF	Post	73 ± 3.33	76 ± 3.50*	79 ± 3.57*	80 ± 3.60*
Exercise-trained HF	Pre	71 ± 2.76	74 ± 2.86*	78 ± 3.09*	79 ± 3.35*
	Post	66 ± 2.94	72 ± 3.16*	73 ± 3.10*	75 ± 3.12*
Normal control	Pre	65 ± 1	69 ± 2*	73 ± 2*	74 ± 3*
FBF (mL/min/100 mL)					
Untrained	Pre	2.04 ± 0.13	2.13 ± 0.12*	2.27 ± 0.13*	2.38 ± 0.15*
HF	Post	1.73 ± 0.14	2.00 ± 0.16*	2.20 ± 0.20*	2.22 ± 0.17*
Exercise-trained HF	Pre	1.67 ± 0.11*	2.13 ± 0.13*,**	2.26 ± 0.16*,**	2.41 ± 0.21*,**
	Post	2.44 ± 0.20	2.86 ± 0.22*	3.16 ± 0.26*	3.24 ± 0.25*
Normal control	Pre	2.40 ± 0.17	2.67 ± 0.22*	2.89 ± 0.23*	3.05 ± 0.25*

Values are mean ± SE. HG = handgrip; MAP = mean arterial pressure; HF = heart failure; and FBF = forearm blood flow.

* $p < 0.05$ vs. baseline.

** $p < 0.05$ vs. normal control.

The mechanisms underlying this reduction in sympathetic nerve activity produced by exercise training are not fully understood, but previous studies in animal models of HF suggest that exercise training improves arterial baroreflex control and cardiopulmonary reflex control [10] and reduces arterial chemoreflex hypersensitivity. Reflex control of MSNA during exercise by muscle metaboreceptors and muscle mechanoreceptors is abnormal in HF [3,11], and therefore normalization of these reflex pathways is another potential mechanism of the lower levels of MSNA following training. However, since no differences in responses to exercise were found, it seems unlikely that these receptors were involved in the reduction of MSNA during exercise in exercise-trained HF patients.

In the present study, we found that FBF during handgrip exercise was substantially increased in exercise-trained HF patients. This increase in muscle blood flow during exercise produced by exercise training can be attributed to reduction in sympathetic nerve activity [12], and/or improvement in endothelial function [13,14]. Thus, potentially both reduced MSNA and improved endothelial function play a role in the augmented FBF during exercise in exercise-trained HF patients in the present study.

In conclusions, the improvement in neurovascular control during exercise is attributable to a reduction in resting MSNA and an increase in muscle blood flow, and not to reflex changes during exercise. Therefore, further studies highlighting the mechanisms behind these findings are needed.

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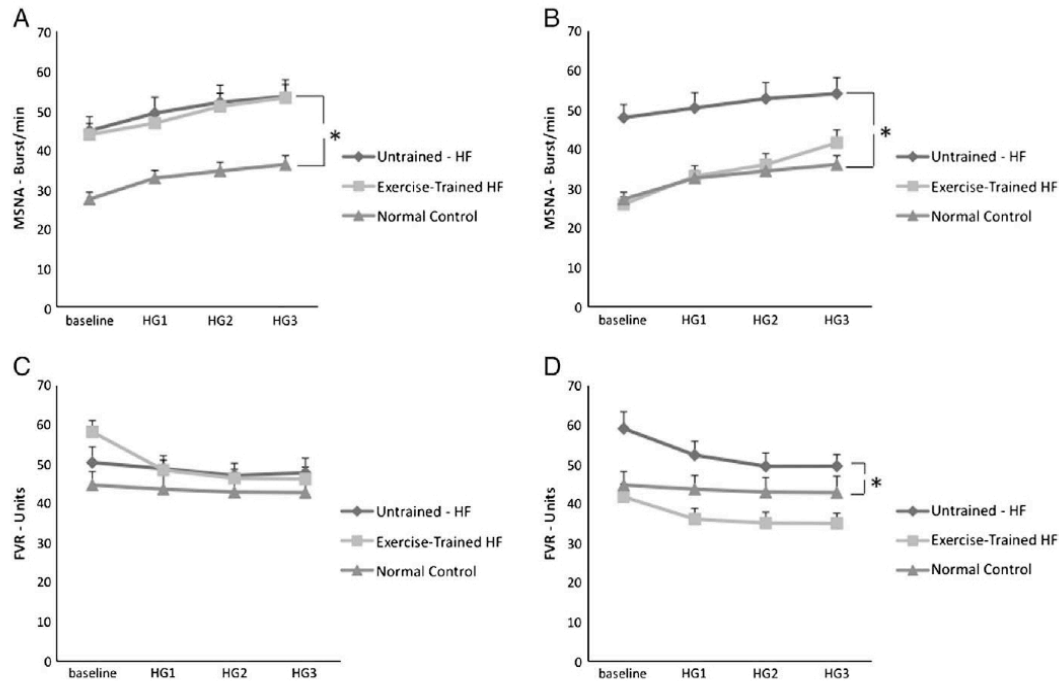


Fig. 1. Muscle sympathetic burst and forearm vascular resistance at baseline and during 30% maximal exercise voluntary contraction in untrained heart failure patients, exercise-trained heart failure patients and normal controls. Note that muscle sympathetic burst frequency (A) is increased heart failure patients and exercise training significantly decreases muscle sympathetic burst frequency (B) in heart failure patients. The forearm vascular resistance (C) was no longer different in exercise-trained HF patients and normal controls and significantly greater than in untrained HF. Exercise training significantly reduced forearm vascular resistance throughout the experimental protocol in HF patients (D). * $p < 0.05$ vs. normal control.

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