Improving the methodology for non-invasive autonomic nervous system recording and its implementation in behavioral research.

RENÉ VAN LIEN

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Preface

Recent years have witnessed an increase in the ambulatory assessment of the effects of psychological and behavioral factors (e.g. personality, stress, exercise) on the cardiovascular system. So far, the cardiovascular parameters measured in naturalistic settings have been largely limited to measures of heart rate (variability) and intermittent cuff-based blood pressure (BP) recordings.

In stress research, large clinical importance is given to separately assessing sympathetic versus parasympathetic nervous system activity (Berntson et al., 1994a; Berntson, Cacioppo, & Quigley, 1991; Berntson, Cacioppo, & Quigley, 1993a; Berntson, Cacioppo, & Quigley, 1994b; Berntson, Cacioppo, Quigley, & Fabro, 1994; Berntson, Norman, Hawkley, & Cacioppo, 2008; Nicholson, Kuper, & Hemingway, 2006). Parameters indexing both these branches of the autonomic nervous system (ANS) can be derived non-invasively from combined recording of the electrocardiogram (ECG) and the impedance cardiogram (ICG). The technology to measure these parameters in a naturalistic setting has been available for some time, and the VU University Ambulatory Monitoring System (VU-AMS) has been a pioneer device in this respect (de Geus, Willemsen, Klaver, & van Doornen, 1995; Willemsen, de Geus, Klaver, van Doornen, & Carroll, 1996). With over 300 devices sold to more than fifty departments worldwide, the VU-AMS has obtained a pole position in the field of ambulatory ANS recording and its application in the field of biobehavioral medicine.

A first major aim of my PhD project was to critically re-examine the validity of the current strategies for ambulatory assessment of parasympathetic nervous system (PNS) and sympathetic nervous system (SNS) activity using the VU-AMS. A second aim was to test new methods to detect SNS activity feasible for ambulatory assessment in naturalistic settings. A third and final aim was to help disseminate the knowledge gathered throughout the development of the VU-AMS device, and in particular to ensure a correct use of VU-AMS hardware and software by the growing community of VU-AMS users.

The organization of this thesis by and large reflects these three aims. In chapter one, a complete overview is given of the invasive and non-invasive assessments of the sympathetic and parasympathetic nervous system currently in use in human participants. The chapter ends by listing those assessment strategies that could potentially be used in an ambulatory recording setting. Current ambulatory studies predominantly use the preejection period (PEP) and respiratory sinus arrhythmia (RSA) as the indices of sympathetic and parasympathetic activity, with co-registration of posture and physical activity by accelerometers to ensure comparison of ANS activity across the same level of physical load. In laboratory situations the PEP and RSA measures seem to perform almost perfectly (de Geus, Kupper, Boomsma, & Snieder, 2007; Houtveen, Groot, & Geus, 2005; Krzeminski et al., 2000; Mezzacappa, Kelsey, & Katkin, 1999; Miyamoto et al., 1983a; Nelesen, Shaw, Ziegler, & Dimsdale, 1999; Newlin & Levenson, 1979; Richter & Gendolla, 2009; Schachinger, Weinbacher, Kiss, Ritz, & Langewitz, 2001; Sherwood, Allen, Obrist, & Langer, 1986; Smith et al., 1989a; Allen & Crowell, 1989; Hatfield et al., 1998; Houtveen et al., 2005; Houtveen, Rietveld, & de Geus, 2002; Kamphuis & Frowein, 1985; Langewitz & Ruddel, 1989; Mulder, 1992; Sakakibara, Takeuchi, & Hayano, 1994; Tulppo, Makikallio, Takala, Seppanen, & Huikuri, 1996), but in ambulatory studies additional challenges exist that have not been fully tackled. Validity of ambulatory RSA, for instance, may be compromised in participants with very low heart rates during nighttime recordings due to ceiling effects in the acetylcholinergic neurotransmission (Goldberger, Ahmed, Parker, & Kadish, 1994; Goldberger, Challapalli, Tung, Parker, & Kadish, 2001; Goldberger, Kim, Ahmed, & Kadish, 1996). Chapter two will deal with this issue and suggest solutions when relating ambulatory RSA to regular exercise behavior with its known bradycardiac effect.

Reliability of ambulatory PEP scoring is very sensitive to the selection of the correct landmarks in the ICG, particularly the B-point, and in the ECG, particularly the Q-onset. Chapter three will test whether the ISTI, based on the more easily detected Z-point, can be used to replace the PEP and whether estimation of the Q-onset based on a fixed interval from the ECG R-wave yields valid results. Detection of the B-point and Q-onset currently always needs to be verified by laborious visual inspection. Furthermore, validity of PEP may be compromised by changes in preload and afterload which are not yet optimally detected in many current ambulatory designs. Chapters four, five, and six test whether adding salivary alpha-amylase (sAA) or the T-wave amplitude could help deal with these imperfections and possibly develop a new and more robust multivariate ambulatory index of the SNS activity.

Chapter seven reports on the gradual build-up of ambulatory assessment expertise in the consecutive VU-AMS hardware and software versions over the past 20 years which was strongly fueled by the interaction with its international user community. To further enhance this productive interaction, I developed a series of tutorials, workshops and instruction video to help disseminate correct and optimal use of the VU-AMS in the growing community of VU-AMS users. The chapter briefly reports on these tools for dissemination, and because web-based instruction video's have a central role in the dissemination strategy, main distribution of this thesis was done through digital information carriers.

The thesis ends with a short summary of the main findings and a projection of expected (and needed) future developments in the ambulatory assessment of the ANS in the behavioral sciences.

CHAPTER 1

Measuring autonomic nervous system activity

René van Lien, Eco J.C. de Geus, Melanie Neijts, and Gonneke Willemsen

This chapter is a rewrite of the published chapter:

Eco J.C. de Geus, René van Lien, Melanie Neijts, and Gonneke Willemsen (2013). Genetics of Autonomic Nervous System Activity. In: Canli, T. (ed), *The Oxford Handbook of Molecular Psychology*, Oxford University Press: London.

Abstract

Cardiovascular disease (CVD) is one of the main causes of death in Westernized countries. The etiology of CVD is complex, with many different factors (demographical, lifestyle, psychological, and genetic) contributing to an increased risk of CVD development (Brotman, Golden, & Wittstein, 2007; Brotman, Walker, Lauer, & O'Brien, 2005). The physiological risk factors that form the final common pathway to CVD include the metabolic syndrome, with hypertension, hyperlipidemia, hyperglycemia, and android obesity as core features (Bayturan et al., 2010); inflammation (Danesh et al., 2008); coagulation/fibrinolysis imbalance (Libby & Theroux, 2005); reduced heart rate variability (Dekker et al., 2000; Dekker et al., 1997); and increased heart rate (Fox et al., 2007). Strikingly, activity of the autonomic nervous system (ANS) is associated with all of these physiological risk factors (Charkoudian & Rabbitts, 2009; Lambert & Lambert, 2011; Malpas, 2010; Straub, Wiest, Strauch, Harle, & Scholmerich, 2006; Task Force of the European Society of Cardiology the North American Society of Pacing, 1996; Tracey, 2009; von Kanel, Mills, Fainman, & Dimsdale, 2001). Because the ANS is very sensitive to psychosocial stress, it plays a key role in almost all models in biobehavioral medicine that try to account for the well-known role of social (Karasek et al., 1988; Rosengren et al., 2004; Siegrist, Peter, Junge, Cremer, & Seidel, 1990) and psychological (Nicholson et al., 2006) sources of chronic stress in hypertension, diabetes, and cardiac disease.

There are large individual differences in the activity of the ANS in the basal resting state (Berntson et al., 1994a; Berntson et al., 1994b; Grossman & Kollai, 1993; Light, Kothandapani, & Allen, 1998; Salomon, Matthews, & Allen, 2000). These differences in ANS activity are further amplified in response to brief laboratory stressors (de Geus et al., 2007; Houtveen et al., 2002; Lucini, Norbiato, Clerici, & Pagani, 2002; Wang et al., 2009) as well as prolonged psychosocial stress (Riese, van Doornen, Houtman, & de Geus, 2000; Vrijkotte, van Doornen, & de Geus, 2004). This chapter reviews the available measures to capture differences in ANS activity at rest and during stress. We first present a short overview of the ANS and a detailed review of the measurement strategies used to study its activity. We close by discussing the ANS measures that are both sufficiently valid and applicable for ambulatory assessment in population-based samples that are sufficiently large to allow epidemiological studies and genetic analyses.

The Autonomic Nervous System

The term “autonomic nervous system” was coined by Langley in 1898. Based on anatomical and functional criteria, he divided the ANS into three separate branches: the sympathetic nervous system (SNS) including the adrenal medulla, the parasympathetic nervous system (PNS), and the enteric nervous system, a collection of neurons embedded within the walls of the entire gastrointestinal tract that control gastrointestinal motility and secretions. In more recent use of the term, the enteric system is discarded and ANS is usually synonymous with the sympathetic and parasympathetic branches.

The sympathetic branch is best known for its key role in the “fight-or-flight” response. Activity of the SNS causes, among other things, an increase in heart rate, contractility, blood pressure, breathing rate, bronchodilation, sweat production, epinephrine secretion, and a redistribution of blood flow favoring the muscles. The PNS, on the other hand, promotes the maintenance of the body by acquiring energy from food and getting rid of wastes. The PNS is therefore often labeled as the “rest and digest” branch of the ANS. Its activity causes slowing of the

heart, constriction of the pupils, stimulation of the gut and salivary glands, and other responses that help restore energy. Many organs are innervated by both the sympathetic and the parasympathetic branches of the ANS, and an increase in the activity of these branches typically exerts opposing actions. However, some organs are not dually innervated (e.g. sweat glands) and, even for dually innervated organs, the autonomic branches may have synergistic rather than opposing effects (e.g. salivary glands).

The main function of the ANS is coordinating bodily functions to ensure homeostasis and performing adaptive responses when faced with changes in the external and internal environment, such as those due to physical activity, posture change, food consumption, or hemorrhage. In addition, the ANS is capable of substantial heterostatic action; it can prepare the body for anticipated threats to homeostasis even in the absence of actual changes in bodily activity. The best known example is the anticipatory response that prepares the body for physical activity in response to a vast range of stressors that can be purely symbolic in nature and are often not followed by actual physical activity (fight-or-flight) or changes in internal environment (e.g. through blood loss or infection). This response is called the physiological stress response.

In humans, subjective experience of stress can be sufficient to trigger the physiological stress response. Subjective experience of stress typically occurs when there is an imbalance between perceived threats/demands and perceived abilities/resources. In the brain, the perception of internal (thoughts) or external (environmental events) threats by neocortical areas leads to the activation of limbic areas, in particular the amygdala (Lovallo, 2005). The amygdala, in turn, projects to paraventricular and other hypothalamic nuclei as well as to a network of neurons in the rostral ventrolateral medulla (RVLM) and the nucleus of the solitary tract (NTS) that initiates changes in the activity of sympathetic neurons in the intermediolateral (IML) column and in activity of the parasympathetic neurons in the n. ambiguus.

Parasympathetic Nervous System Activity

The vagus nerve (CN X) carries preganglionic fibers of the PNS to the heart and lungs (as well as to other organs) and is the primary source of parasympathetic innervation of these organs. Many efferent fibers in the vagus originate in the n. ambiguus. Other preganglionic fibers of the PNS leave from the cell bodies of the motor nuclei of cranial nerves (CN) III, VII, IX, and X in the brainstem and from the second, third, and fourth sacral segments of the spinal cord. The preganglionic axons terminate in parasympathetic ganglia, which lie within or very close to the organs innervated by the short postganglionic neurons. The preganglionic neurons employ acetylcholine (ACh) as the primary neurotransmitter, which binds to a nicotinic receptor subtype on the postganglionic neurons in the ganglia. Postganglionic parasympathetic fibers also employ ACh as a primary neurotransmitter, but the receptor subtypes on the target organ are commonly muscarinic. For instance, the parasympathetic postganglionic receptors in the sinoatrial (SA) node of the heart are type 2 muscarinic (M2) and their activation reduces heart rate.

Sympathetic Nervous System Activity

The preganglionic fibers from neurons in the IML column leave the central nervous system from the thoracic and lumbar regions of the spinal cord. They synapse onto a chain of sympathetic ganglia that lie close to the spinal cord, known as the sympathetic trunk. The preganglionic neurons from the IML column to the sympathetic ganglia employ ACh as the primary neurotransmitter. The

postganglionic neurons from the sympathetic ganglia to the organs employ norepinephrine as the primary neurotransmitter, which can act on α1-adrenergic (e.g. in arterioles) or β1- and β2-adrenergic receptors (e.g. on the heart). Stimulation of the α1-adrenergic receptors causes vasoconstriction by acting on the smooth muscles in the medial layer of the blood vessels. Stimulation of the cardiac β-adrenergic receptors by norepinephrine released from the cardiac sympathetic nerves (nn. accelerantes) increases the pacemaker frequency of the SA node (i.e., heart rate), as well as contractility of the ventricles. Together, vasoconstriction and increased cardiac performance account for the increase in blood pressure seen during increased sympathetic activity.

A first exception to the use of norepinephrine as the final effector in the SNS is found in the sympathetic innervation of eccrine sweat glands, which is cholinergic rather than adrenergic. A second exception is a set of preganglionic neurons that end in a special ganglion, namely the adrenal medulla. On activation by preganglionic neurons, the adrenal medulla releases a small amount of norepinephrine into the bloodstream, but most of the released norepinephrine is converted to epinephrine, which is excreted in much larger amounts than norepinephrine (5:1). Circulating epinephrine preferentially binds to β2-receptors in the vessels and on the heart, causing vasodilatation (mostly in muscle tissue) and increases in heart rate and contractility.

Measurements of Autonomic Nervous System Activity

Many studies of the ANS have focused on the fight-or-flight response, which is often characterized by reciprocal increases in SNS activity and decreases in PNS activity. Such a pattern gives rise to increases in heart rate and blood pressure, and heart rate and blood pressure reactivity are still among the most used variables to indicate changes in ANS activity. Laboratory studies generally involve the measurement of heart rate and blood pressure during one or more rest periods and during mental and physical challenges, with each period often lasting no more than 5–15 minutes. Such studies provide valuable information on the mechanisms underlying ANS responses to stress and have been instrumental in establishing the existence of stable individual differences in the physiological stress response.

Notwithstanding that much has been learned from studies focusing on heart rate and blood pressure as indicators of ANS activity, a disadvantage of these variables is that they represent an unknown mix of sympathetic and parasympathetic effects. It has been shown that the classical reciprocal pattern of sympathetic activation with parasympathetic deactivation describes only a limited part of the total autonomic space (Berntson et al., 1991). Different patterns of co-activation, reciprocal activation and co-inhibition are found across individuals performing the same task or within individuals performing different tasks. For example, dental phobia patients engaged in a stressful mental arithmetic task showed an increase in their SNS activity with decreased PNS activity; but, when exposed to phobic stimuli, the same participants showed increased SNS activity with increased PNS activity (Bosch, de Geus, Veerman, & Amerongen, 2000). Most importantly, health outcomes of sympathetic hyperreactivity need not be the same as those of parasympathetic hyperreactivity. Hyperactivity of the SNS has been mostly associated with an increased risk for hypertension, the metabolic syndrome, and left ventricular failure (Brotman et al., 2007; Esler, 2010; Esler et al., 2008; Esler, Lambert, & Schlaich, 2010; Lambert, Schlaich, Lambert, Dawood, & Esler, 2010), whereas loss of PNS activity causes a reduction in the electrical stability of the heart (Schwartz et al., 2003; Vanoli et al., 1991) and may play a key role in the proinflammatory state (Rosas-Ballina & Tracey, 2009; Tracey, 2009).

Because heart rate and blood pressure do not reveal the underlying pattern of ANS activity, studies in the past two decades began indexing sympathetic and parasympathetic activity separately. Next, we review the various measures of SNS and PNS activity currently in use (see Table 1 for an overview).

Table 1. *Measurement strategies of autonomic nervous system activity in humans.*

Technique	Invasiveness	Principle	Measure	References
PARASYMPATHETIC				
Parasympathetic microneurography	Very High	Direct measurement of action potentials in the parasympathetic nerves converted to bursts with a fixed time constant integrator	Vagal (burst count/time)	(Cerati & Schwartz, 1991; Jewett , 1964; Kunze, 1972)
Microdialysis	Very High	Measurement of acetylcholine (ACh) concentrations in the dialysate samples, for instance in the sino-atrial (SA) node, using high-performance liquid chromatography	[Ach]	(Shimizu et al., 2009)
Pharmacological blockade	Moderate	When the heart rate measured in the unblocked state is subtracted from the heart rate measured during full muscarinic blockade, the change in heart rate (ΔHR) yields a measure of vagal activity.	ΔHR (bpm)	(Berntson et al., 1994a; Berntson et al., 1991; Cacioppo et al., 1994)
Respiratory Sinus Arrhythmia	Noninvasive	Heart rate variability due to respiratory gating of tonic vagal effects on the SA node scales with the height of that activity	RMSSD (ms) pvRSA (ms) HF (ms^2)	(Akselrod et al., 1981; Cerutti, Bianchi, & Mainardi, 2001; Katona & Jih, 1975; Sztajzel, 2004)
Heart Rate Variability (TP, ULF, VLF, LF)	Noninvasive	Same principle as RSA above, but lower	SDNN (ms)/TP (ms^2) SDANN/ULF	(Task Force of the European Society of

		frequencies in heart rate variability are additionally influenced by modulation of the sympathetic effects on the SA node.	(ms^2) VLF (ms^2) LF (ms^2)	Cardiology the North American Society of Pacing, 1996)
Baroreflex sensitivity (BRS)	High (if intra-arterial BP) Noninvasive (if Finapres)	The BRS is computed as the mean slope of the regression line relating beat-to-beat changes in SBP to changes in IBI. As the sympathetic contribution to fast changes in the IBI is minimal, this reflects mainly cardiac vagal activity.	BRS (ms/mmHg)	(Di Rienzo, Parati, Radaelli, & Castiglioni, 2009; La Rovere, Pinna, & Raczak, 2008)
SYMPATHETIC				
Sympathetic microneurography	High	Direct measurement of action potentials by a tungsten microelectrode inserted into the fascicle of a sympathetic nerve in the skin or muscle (m. peroneus). The voltage is rectified and integrated with a fixed time constant (100 ms).	MSNA (burst count/time) SSNA (burst count/time)	(Hagbarth & Vallbo, 1968; Wallin, 1984; Wallin, 2004)
Regional norepinephrine spillover	Very High	During constant-rate infusion of radiolabeled NE , and with regional catheterization, the organ-specific rate of spillover of NE to plasma can be determined based on the degree of dilution of infused radioactive-labeled NE by	Total NE spillover (ng/min) Organ NE spillover (ng/min)	(Eisenhofer, 2005; Esler et al., 1988; Esler & Kaye, 2000b; Grassi & Esler, 1999)

		endogenous-released NE.		
Pharmacological blockade	Moderate	When the response measured in the unblocked state is subtracted from the response measured during blockade, this yields a measure of sympathetic activity to the organ. For instance, (1) the heart rate decrease (ΔHR) that is induced by blockade of β-adrenergic receptors, (2) the blood pressure increase that is induced by blockade of α-adrenergic receptors.	ΔHR (bpm) ΔDBP (bpm)	(Berntson et al., 1994a; Cacioppo et al., 1994; Julius, Pascual, & London, 1971)
Plasma catecholamines	Low (if venous) High (if arterial)	Measurements of concentrations of NE or E in arterial or venous blood using high-performance liquid chromatography. Caveat: intraneuronal vesicular storage/leakage and synaptic reuptake contribute in unknown ways to plasma NE concentrations and (changes in) tissue clearance strongly co-determine the level of both catecholamines.	[NE] pg/mL [E] pg/mL	(Esler et al., 1990; Goldstein, Eisenhofer, & Kopin, 2003; Hjemdahl, 1990)
Urinary catecholamines	Noninvasive	Measurement of (24-h) urinary excretion of NE or E (relative to creatinine). Caveat: same as for plasma levels, and the kidneys	[NE] (pg/mg creatinine) [E] (pg/mg creatinine)	(Esler et al., 1990; Goldstein et al., 2003; Hjemdahl, 1990)

		themselves also produce NE.		
Skin conductance	Noninvasive	Sympathetic activity of skin nerves increases the activity of the sweat glands, which in turn yields a measurable change in the conductance of an applied current across the skin.	SCL (µS) nsSCRs (counts/time)	(Boucsein, 1992; Dawson, Schell, & Filion, 2000; Fowles, 1986)
Salivary α-amylase activity	Noninvasive	NE release from sympathetic nerve terminals near adrenoceptors in the acinar cells of the saliva glands cause an immediate increase in the protein-to-fluid ratio of many salivary proteins, including α-amylase. Caveat: PNS activity also increases α-amylase secretion.	sAA (U/mL)	(Nater & Rohleder, 2009)
LF/HF ratio	Noninvasive	The LF/HF ratio of spectral power of the heart rate in the lower frequencies centered around 0.1 Hz (LF) divided by the power in the higher frequencies centered around the respiratory frequency (HF). Also expressed as LF/(LF+HF) = LFnu. Caveat: validity is very controversial.	LF/HF (dimensionless) LFnu (dimensionless)	(Pagani & Malliani, 2000)
Ejection Fraction	High (if using contrast MRI), Moderate (if using MRI) Low (if using echocardiography)	The ejection fraction (EF) is the ratio between stroke volume and enddiastolic volume which can be derived	EF (%)	(Sherwood et al., 1990)

		from (contrast) MRI or echocardiography. Increased sympathetic activity leads to increased cardiac contractility that causes a higher EF.		
ECG T-Wave amplitude	Noninvasive	The amplitude difference between an isoelectric baseline (e.g. P-Q interval) of the ECG and the peak of the T-wave.	TWA (mV)	(Heslegrave & Furedy, 1979; Malm, Frigstad, Sagberg, Larsson, & Skjaerpe, 2004)
Systolic Time Intervals	Noninvasive	From the thorax impedance cardiogram, the preejection period is derived as the time interval between the onset of ventricular depolarization and the opening of the semilunar valves. This interval shortens with increased sympathetic drive to the left ventricle. Caveat: posture should be controlled.	PEP (ms)	(Sherwood et al., 1990)

Measuring Parasympathetic Activity

The ideal way to assess parasympathetic activity is the direct measurement of action potentials in the parasympathetic nerves (Cerati et al., 1991; Jewett, 1964; Kunze, 1972). For cardiac vagal activity, an alternative was developed that assesses the changes in (ACh) concentration in the SA node by microdialysis (Shimizu et al., 2009). Unfortunately, both these "golden standard" measures are too invasive to be used in research with humans. A theoretical alternative would be to measure the spillover of ACh to plasma by venipuncture, but this is not feasible because of the rapid and extensive clearance of the transmitter in the synaptic space by acetylcholinesterase.

Human studies of parasympathetic activity have therefore focused on the effects of parasympathetic activity on the innervated organs rather than on activity per se. For instance, to assess cardiac parasympathetic activity to the SA node, the heart rate change can be measured in response to pharmacological blockade of the muscarinic receptors (Berntson et al., 1994a; Cacioppo

et al., 1994; Martinmaki, Rusko, Kooistra, Kettunen, & Saalasti, 2006). High doses of M2-antagonists, like atropine, effectively remove all parasympathetic effects on the heart. When the heart rate measured in the unblocked state is subtracted from the heart rate measured during full blockade, participants with higher parasympathetic activity will show a larger increase in heart rate during blockade than will participants with lower activity. The increase in heart rate is not a perfect measure of parasympathetic activity, since the SNS and PNS are known to interact (Mizuno et al., 2008). High levels of SNS activity will act to reduce the release of ACh through the action of the α2-autoreceptors on the terminal buttons of vagal axons. However, these interactive effects can be dealt with using a dual blockade strategy (Berntson et al., 1994a; Berntson et al., 1994b; Cacioppo et al., 1994).

A disadvantage of pharmacological blockade studies is that they are confined to well-controlled (hospital) settings and are not readily amenable to be used in larger samples or in recordings in naturalistic settings. Fortunately, reliable noninvasive estimation of parasympathetic cardiac effects is possible by measuring time- or frequency-domain indices of heart rate variability in the respiratory frequency range, also called respiratory sinus arrhythmia (RSA). Respiratory sinus arrhythmia is the difference in heart rate during the inspiration and expiration phases of the respiratory cycle. Respiratory sinus arrhythmia is generated when tonic firing of motor neurons in the n. ambiguus and sympathetic nuclei is modulated by phasic inhibition and excitation coupled to the respiratory cycle. This modulation is caused by connections between the nuclei that control the respiratory generator in the pre-Bötzinger and Bötzinger complexes and the vagal and sympathetic motor neurons, which lie in close proximity in the brainstem (Rekling & Feldman, 1998). This respiration–ANS coupling yields an oscillatory pattern in the release of norepinephrine and ACh in the SA node, such that ACh levels increase during expiration and decrease during inspiration, whereas norepinephrine shows the reverse pattern of increases during inspiration and decreases during expiration. The effect of this respiratory "gating" (Eckberg, 2003) is that the heart rate increases during inspiration and decreases during expiration. This has an advantageous effect on the efficiency of oxygen exchange in the lungs, at least in the resting state (Yasuma & Hayano, 2004).

Fortuitously, the effect of the respiratory-related changes in vagal activity on heart rate variability is much more prominent than the effect of the respiratory-related changes in sympathetic activity. This is due to the differential filter characteristics of the muscarinergic and adrenergic receptors (Berntson, Cacioppo, & Quigley, 1993b). The muscarinergic receptor rapidly opens potassium channels after ACh release, and closure of calcium channels with parallel changes in the hyperpolarization of the SA node cells occur within hundreds of milliseconds. The β-receptors, that first need to activate protein kinases before channels for sodium and calcium are opened, act much slower and influence the speed of depolarization of the SA cells only on the scale of seconds. This causes the high-frequency changes due to respiration to be filtered from the sympathetic effects on the heart. In keeping, RSA shows relatively little sensitivity to sympathetic blockade but is affected in a dose–response way by muscarinergic blockers in humans (Berntson et al., 1994a; Cacioppo et al., 1994; Martinmaki et al., 2006) or vagal cooling in animals (Katona et al., 1975). This has led to the use of RSA as a proxy for vagal cardiac activity (Berntson et al., 1997), although it is acknowledged that large changes in sensitivity of chemoreceptor and baroreceptor reflexes (Berntson et al., 1997; Berntson et al., 1993b; Houtveen et al., 2002) or respiratory behavior (Grossman et al., 1993; Grossman, Wilhelm, & Spoerle, 2004; Ritz & Dahme, 2006) are important confounders.

Respiratory sinus arrhythmia can be derived from the interbeat interval (IBI) time series obtained from the R waves in the electrocardiogram (ECG) by taking the root mean square of differences (RMSSD) between successive IBIs (Sztajzel, 2004; Task Force of the European Society of

Cardiology the North American Society of Pacing, 1996). When the respiratory signal is co-registered with the ECG, RSA can also be derived by peak-valley estimation (pvRSA). Estimates of pvRSA are obtained by subtracting the shortest IBI during heart rate acceleration in the inspiration phase from the longest IBI during heart rate deceleration in the expiration phase (Katona et al., 1975). Respiratory sinus arrhythmia can also be derived in the frequency domain by Fourier (Akselrod et al., 1981) or wavelet analysis (Houtveen & Molenaar, 2001; Pichot et al., 1999; Wiklund, Akay, & Niklasson, 1997) or by autoregressive model estimation (Cerutti et al., 2001). These analyses describe the periodic oscillations of the heart rate signal decomposed at different frequencies and amplitudes and provide information on the amount of their relative contribution to the variance (also termed power) in the heart rate. Power in the respiratory frequency range of 0.15–0.40 Hz (HF power) can be used to index RSA.

In standardized laboratory recordings, as well as in ambulatory settings, the different time- and frequency-domain measures of RSA (e.g. RMSSD, HF power, pvRSA) were highly correlated, with all r's being greater than 0.80 (Grossman, van Beek, & Wientjes, 1990; Hayano et al., 1991b; Houtveen et al., 2001; Penttila et al., 2001), and this high intercorrelation of the various RSA measures proved stable across a wide range of values for respiration and heart rates (Goedhart, Kupper, Willemsen, Boomsma, & de Geus, 2006). An important feature of the RSA measures is that they can be reliably measured under naturalistic conditions with the use of ambulatory monitoring (de Geus et al., 1995; Wilhelm, Roth, & Sackner, 2003). For the average 24-hour levels of RMSSD and HF power, high test–retest correlations ($.63 < r < .90$) were found after 3 to 65 days in both healthy individuals and cardiac patients (Bigger, Jr. et al., 1992; Hohnloser, Klingenheben, Zabel, Schroder, & Just, 1992; Kleiger et al., 1991; Sinnreich, Kark, Friedlander, Sapoznikov, & Luria, 1998; Stein, Rich, Rottman, & Kleiger, 1995). Good long-term temporal stability ($.58 < r < .76$) for 24-hour levels of pvRSA, HF, and RMSSD has been shown over a period of 7 months (Pitzalis et al., 1996) to 3.4 years (Goedhart, van der Sluis, Houtveen, Willemsen, & de Geus, 2007)

Respiratory sinus arrhythmia is only one component of the total variability in the heart rate. Other heart rate variability measures include the power in the low-frequency (LF, 0.04–0.15 Hz), very-low-frequency (VLF, 0.003–0.04 Hz), and ultra-low-frequency (ULF, < 0.003 Hz) bands (Sztajzel, 2004; Task Force of the European Society of Cardiology the North American Society of Pacing, 1996). Together with RSA, these measures have received a lot of attention in medicine because lowered levels of heart rate variability predict adverse cardiovascular events, including atrial fibrillation, myocardial infarction, congestive heart failure, and coronary artery disease (Bigger et al., 1990; Bigger, Jr. et al., 1992; Bigger, Fleiss, Rolnitzky, & Steinman, 1993; Dekker et al., 1997; Hayano et al., 1991a; Kleiger, Miller, Bigger, & Moss, 1987; Lombardi et al., 1987; Nolan et al., 1998; Saul et al., 1988; Singer et al., 1988; Tsuji et al., 1996; Vikman et al., 2003). Heart rate variability in the LF band arises from so-called Mayer waves, which are periodic oscillations in arterial blood pressure around the 0.1 Hz frequency (Julien, 2006). Through the action of the baroreceptors, these periodic changes in blood pressure are met by parallel changes in vagal activity and sympathetic vascular tone to keep blood pressure constant. This gives rise to a "10-s rhythm" in the heart rate that can be detected by spectral analysis as the power in the LF band. Heart rate variability in the ULF and VLF bands has been hypothesized to reflect circadian rhythms in bradykinin, renin, or angiotensin release, or thermoregulatory effects on peripheral vasomotor tone. However, in ambulatory recordings, the ULF, which is highly correlated to the standard deviation of the average heart rate across all 5-minute segments of an entire recording (SDANN), seems to arise predominantly from changes in behavior from passive to physically active and vice versa, particularly around the transitions to and from sleep

(Roach, Wilson, Ritchie, & Sheldon, 2004). This may also be true of the standard deviation of the heart rate (SDNN), which reflects all of these same components. The SDNN can also be indexed by the total power (TP).

A final measure that has been used to index cardiac vagal activity, particularly in the context of increased risk for cardiac disease, is the sensitivity of the baroreflex (BRS) (La Rovere, Bigger, Marcus, Mortara, & Schwartz, 1998; La Rovere et al., 2003). The baroreflex loop counteracts deviations in blood pressure from an ongoing setpoint by changing sympathetic outflow to the blood vessels to affect peripheral resistance and by changing sympathetic and vagal activity to the SA node to affect cardiac output (Di Rienzo et al., 2009; La Rovere et al., 2008). Deviation of blood pressure from the setpoint is detected by stretch receptors (baroreceptors), mainly located on the wall of the aorta and the carotid arteries. These receptors interface with the sympathetic and parasympathetic motor neurons of the n. ambiguus and the n. tractus solitarius to generate the required ANS responses. To allow blood pressure to increase in emergency situations, the sensitivity of the baroreflex is kept variable; substantial within-participant variation in the BRS over time is found, as well as large between-participant differences (DiRienzo, Parati, Radaelli, & Castiglioni, 2009). Classical approaches to measure BRS have manipulated baroreceptor firing through pharmacological agents that affect blood pressure and by lower body negative pressure, forced expiration against resistance (the Valsalva maneuver), or a neck suction/pressure cuff over the carotid baroreceptors. Changes in mean arterial blood pressure are then regressed on the changes in heart rate to obtain the cardiac BRS, which is defined as the change in heart rate induced by a fixed rise in blood pressure. These methods assess the integrated effects of baroreflex-induced changes in sympathetic vascular activity and sympathetic and vagal cardiac activity.

To selectively index the cardiac vagal component of the baroreflex loop, two methods are available. Both need the continuous time series of IBIs as well as the beat-to-beat changes in blood pressure that can be obtained from intra-arterial pressure recordings or noninvasively from the “Finapres” vascular unloading technique to determine finger arterial pressure (Langewouters, Settels, Roelandt, & Wesseling, 1998). In the sequence method, all occurrences of three or more consecutive beats are identified with progressive increases/decreases in systolic blood pressure of at least 1 mm Hg that are followed by progressive increases/decreases in the IBI. The BRS is computed as the mean slope of the regression line relating changes in systolic blood pressure to changes in IBI (Steptoe & Sawada, 1989). In the spectral method, the BRS can be obtained by calculating the strength of the linear coupling (coherence) between the beat-to-beat fluctuations in the systolic blood pressure and the IBI time series in the low-frequency (0.04-0.15 Hz) band (Robbe et al., 1987). Because the effects of cardiac sympathetic activity are again too slow to follow the rapid beat-to-beat changes in blood pressure, changes in the cardiac BRS are mainly thought to reflect changes in cardiac vagal activity. A depressed BRS (<3 ms/mm Hg) was shown to increase the risk for cardiac mortality by 2.8 times, independent of left heart failure and mostly by increasing sudden death (La Rovere et al., 1998).

Measuring Sympathetic Activity

The golden standards to measure SNS activity in humans are the direct microneurographic recording of action potentials from superficial nerves innervating the skeletal muscle (MSNA) or the skin (SSNA) (Hagbarth, Hallin, Wallin, Torebjork, & Hongell, 1972; Hagbarth et al., 1968; Svedenhag, Wallin, Sundlof, & Henriksson, 1984; Wallin, 1984; Wallin, 2004) or the measurement of spillover of

the postganglionic neurotransmitter norepinephrine using radioactive tracers (Eisenhofer, 2005; Esler et al., 1988; Esler & Kaye, 2000a; Kingwell et al., 1994). The advantage of norepinephrine spillover is that it can be measured on an organ-to-organ basis, which allows separate measurement of, for instance, renal, lung, or cardiac sympathetic activity (Eisenhofer, 2005). This is important because the notion of a single emergency SNS response that affects all organs to the same extent has proven untenable. In some circumstances, like exercise, the SNS acts as a "unitary system," but in many other situations it is capable of differentiated regulation of its activity to separate organs to a substantial degree (Hjemdahl, Freyschuss, Juhlin-Dannfelt, & Linde, 1984).

Much less invasive measurements of norepinephrine and/or its metabolites in antecubital venous blood are possible by venipuncture or by assessing the excretion of norepinephrine and/or metabolites in urine. However, concerns have been raised about differences in intraneuronal vesicular storage and leakage, reuptake, extraneuronal clearance, and urinary filtration/secretion that may (severely) distort the relation between actual SNS activity and plasma and urine norepinephrine concentrations (Eisenhofer, Kopin, & Goldstein, 2004; Esler et al., 1990; Goldstein et al., 2003). Only a very small proportion of norepinephrine released from sympathetic nerves reaches the bloodstream, and the final plasma levels depend on an unknown mixture of changes in the rate of release, as well as on the rate of removal by tissues. Plasma epinephrine levels reflect neural outflow to the adrenal medulla rather well (Goldstein et al., 2003), but the substantial clearance of adrenaline by adrenoceptors in the tissues, which further increases during vasoconstriction, causes antecubital plasma levels to be much lower than the arterial levels (Hjemdahl, 1990). Nonetheless, venous epinephrine levels show systematic increases to psychosocial stress that seem to selectively enhance adrenomedullary activation over general sympathetic nerve activation.

As was true for the PNS, most human studies of parasympathetic activity have focused on the effects of sympathetic activity on the innervated organs rather than on activity per se. Sympathetic nervous system effects can be measured using pharmacological blockade of either α-receptors (e.g. phentolamine) or β-receptors (e.g. propranolol) or even specific β1- (e.g. metoprolol) or β2-adrenergic (ICI 118-551) receptors. This has been extensively done for the assessment of cardiac and vascular sympathetic activity. Cardiac sympathetic activity, for instance, is estimated as the difference between heart period in the unblocked state and during complete blockade of cardiac sympathetic effects (Berntson et al., 1994a; Berntson et al., 1994b; Cacioppo et al., 1994; Lewis, Nylander, Gad, & Areskog, 1980; Shi, Stevens, Foresman, Stern, & Raven, 1995).

Skin SNS effects can be noninvasively measured by the activity of the eccrine sweat glands. Acetylcholine release from the preganglionic sympathetic nerves increases the activity of the sweat glands, which in turn increases the conductance of an applied current across the skin (Foster & Weiner, 1970; Fowles, 1986). Because sweat glands are at the highest density in palmar and plantar regions, approximately 400/mm², most researchers measure skin conductance at these sites. Electrodermal activity incorporates both slow tonic shifts in basal skin conductance level (SCL) and more rapid phasic transient events, that is, skin conductance responses (SCRs), which are also referred to as galvanic skin responses (Boucsein, 1992; Dawson et al., 2000; Fowles, 1986). The frequency of the nonspecific SCRs (nsSCRs) is termed electrodermal lability (Mundy-Castle & McKiever, 1953). Both SCL and nsSCRs have been shown to be influenced by emotional stress (Boucsein, 1992; Critchley, 2002; Schell, Dawson, & Filion, 1988). The test–retest reliability coefficients over time periods encompassing 1 day to a year for SCL levels ranged from 0.40 to 0.85 and from 0.40 to 0.76 for nsSCRs (Freixa i Baque, 1982; Iacono et al., 1984; Schell et al., 1988; Schell, Dawson, Nuechterlein, Subotnik, & Ventura, 2002; Vossel & Zimmer, 1990).

The salivary glands are also innervated by the ANS, and salivary α-amylase secretion (sAA) has been suggested to be a noninvasive marker for SNS activity (Nater et al., 2009). Salivary amylase is a digestive enzyme that breaks down insoluble starch into soluble maltose and dextrin. It comprises approximately 30 percent of total protein secretion from the parotid glands, submandibular glands, and sublingual glands, as well as from minor glands in the submucosa underlying the lips, cheeks, and palate (Humphrey & Williamson, 2001). Various studies have revealed that conditions known to evoke sympathetic activation uniformly increase sAA secretion, including stressful academic examination (Bosch, de Geus, Ring, Nieuw Amerongen, & Stowell, 2004; Chatterton, Jr., Vogelsong, Lu, Ellman, & Hudgens, 1996), stressful computer games (Skosnik, Chatterton, Jr., Swisher, & Park, 2000; Takai et al., 2004), watching a stressful video (Bosch et al., 2003b), mental arithmetic test (Noto, Sato, Kudo, Kurata, & Hirota, 2005), cold pressor test (van Stegeren, Wolf, & Kindt, 2008), and the Trier Social Stress Test (Nater et al., 2006; Nater et al., 2005; Rohleder, Nater, Wolf, Ehlert, & Kirschbaum, 2004). Moreover, administration of the β-adrenergic antagonists reduces sAA concentration in unstimulated whole mouth saliva (Nederfors & Dahlof, 1992) and attenuates the stress-induced increases in sAA concentration (Van Stegeren, Rohleder, Everaerd, & Wolf, 2006).

Unfortunately, most studies used an inadequate methodology that was validated for cortisol research but is not suitable for sAA measurements (Bosch, Veerman, de Geus, & Proctor, 2011). Even more problematic is that the salivary glands are innervated by both branches of the ANS, not just the sympathetic branch (Proctor & Carpenter, 2001). Parasympathetic activity can influence sAA concentrations: (1) via α-amylase release from glands that are solely or mainly parasympathetically innervated (e.g. the palate and sublingual glands); (2) via synergistic sympathetic–parasympathetic interactions whereby parasympathetic activity amplifies sympathetic effects; and (3) via the effects of (parasympathetically-mediated) salivary flow rate (Bosch et al., 2011). In fact, Bosch et al. (Bosch et al., 2003b) showed that a passive-coping stressor that evoked parasympathetic activation (viewing a surgical video), as measured by increases in salivary flow and RSA, also evoked a strong sAA release, which was much larger than the release during a stressor that elicited a dominant cardiac sympathetic activation (a time-paced memory task). The important role of the PNS in sAA and protein secretion invalidates the use of sAA secretion as an exclusive read-out of sympathetic activity.

Pagani and coworkers have advanced the notion that a single ratio, the power of the heart period time series in the LF band divided by the power in the HF band, may capture changes in the ratio of sympathetic to vagus nerve traffic to the heart (Malliani et al., 1991; Montano et al., 1994; Pagani et al., 1997; Pagani et al., 1986; Pagani et al., 2000). The idea behind the LF/HF ratio is that, during sympathetic activation, LF and HF power both decrease as does the TP, but the decrease in LF power relative to the decrease in total power is less strong than that in HF power, whereas the reverse occurs during vagal activation, where the increase in LF power relative to the increase in total power is less strong than that in HF power. Expressing the spectral components in absolute units prevents the appreciation of this fractional redistribution of the power across the LF and HF bands. This information is regained when LF and HF are expressed as a ratio, or when LF and HF power are measured in normalized units (LFnu), which represent the relative value of each power component in proportion to the TP minus the VLF/ULF components (Burr, 2007).

The predictive power of LF power for CVD is beyond question (Bigger et al., 1993; Dekker et al., 2000; Tsuji et al., 1996). However, the use of the normalized LF power as a potential measure of cardiac sympathetic control is the subject of controversy (Eckberg, 1997; Goedhart, Willemsen,

Houtveen, Boomsma, & de Geus, 2008b). The strongest concern about the validity of the LF/HF ratio is provided by those studies that directly compare it against invasive measures of sympathetic activity, such as peroneal muscle nerve activity or cardiac norepinephrine spillover. Although some studies did report a correlation of these measures with the LF/HF ratio (Pagani et al., 1997), most studies did not find this correlation across a range of clinical contexts (Grassi et al., 1999; Kingwell et al., 1994).

Together with electrodermal activity, measurement of cardiac contractility is currently the preferred noninvasive method to measure SNS activity. Contractility is influenced only by the sympathetic branch of the ANS because there is an abundance of functional β-adrenergic receptors on the human ventricle but no ACh receptors. Activation of the β-receptors exerts inotropic effects on the cardiac muscle through the opening of calcium channels in the membrane, as well as of the T tubules of the muscle fibers. The calcium influx increases contractile force and contraction speed of the ventricle. This increased contractility is reflected in a larger ejection fraction of the left ventricle. The ejection fraction reflects the ratio between the stroke volume and the end diastolic volume. The ejection fraction can be obtained from recordings of end diastolic and end systolic volumes (the difference equals the stroke volume) by echocardiography or (contrast) magnetic resonance imaging (Malm et al., 2004).

Contractility is also reflected in a more rapid start of the ejection phase after the onset of ventricular depolarization, a time interval referred to as the preejection period (PEP). The PEP can be measured by using impedance cardiography, in which a HF alternating current is introduced across the thorax by electrodes at the level of the neck and belly (Sherwood et al., 1990). Electrodes at the level of the top and bottom of the sternum measure the changes in the impedance of the enclosed thorax column (dZ). The first derivative of the pulsatile changes in transthoracic impedance (dZ/dt) is called the impedance cardiogram (ICG), and it reflects the momentary changes in aortic blood flow during the systolic phase. From the combined ECG and ICG, the PEP can be derived as the time interval between the onset of ventricular depolarization (QRST-onset) and the opening of the semilunar valves (sharp upstroke in the dZ/dt). Within-participant changes in PEP reliably index changes in β-adrenergic drive to the left ventricle, as was shown in laboratory studies that employed manipulations known to increase cardiac sympathetic activity such as epinephrine infusion, amyl nitrite inhalation, mental stress, and exercise. These manipulations systematically decrease PEP (de Geus et al., 2007; Houtveen et al., 2005; Krzeminski et al., 2000; Mezzacappa et al., 1999; Miyamoto et al., 1983a; Nelesen et al., 1999; Newlin et al., 1979; Richter et al., 2009; Schachinger et al., 2001; Sherwood et al., 1986; Smith et al., 1989a). In addition, pharmacological blockade of cardiac sympathetic effects results in the expected prolongation of the PEP (Berntson et al., 1994a; Cacioppo et al., 1994; Harris, Schoenfeld, & Weissler, 1967; Schachinger et al., 2001; Winzer et al., 1999), whereas PEP is hardly affected by blockade of cardiac vagal effects (Berntson et al., 1994a; Cacioppo et al., 1994; Martinsson, Larsson, & Hjemdahl, 1987).

Since the PEP can be reliably obtained noninvasively from only 5–7 spot electrodes, a number of ambulatory devices are available that allow recording of the PEP in naturalistic settings (Cybulski, 2000; Martinsson et al., 1987; Nakonezny et al., 2001; Sherwood, McFetridge, & Hutcheson, 1998; Willemsen et al., 1996). The only caveat in using PEP as an index of changes in cardiac sympathetic activity is its sensitivity to preload and afterload effects. Cardiac contractility can increase independently from sympathetic effects when the stretch of the myocardial muscle fibers increases through the Frank-Starling mechanism. Thus, increases in end diastolic volume (preload) can shorten the PEP in the absence of increased sympathetic activity, leading to the erroneous suggestion of

increased cardiac sympathetic effects. The reverse problem occurs when the pressure in the aorta is increased (afterload) in the presence of unchanged sympathetic activity. Because it takes longer for the aortic valves to open, the PEP becomes longer, erroneously suggesting decreased cardiac sympathetic effects. Postural changes have a major effect on preload and afterload and indeed lead to paradoxical responses of the PEP. Head-up tilting from supine to upright systematically prolongs the PEP (Frey & Kenney, 1979; Lewis, Rittgers, Forester, & Boudoulas, 1977; Ovadia, Gear, Thoele, & Marcus, 1995), and longer PEPs have also been demonstrated when participants go from supine to sitting to standing (Houtveen et al., 2005; Sherwood & Turner, 1993; Waldstein, Neumann, & Merrill, 1998). Clearly, posture needs to be taken into account when using PEP as a measure of sympathetic activity.

When posture is controlled, the PEP has been shown to be a stable individual characteristic. In the laboratory, test–retest correlations between 0.45 and 0.88 have been found for baseline and stress-task levels of PEP across retest intervals ranging from 28 days to 3 years (Burleson et al., 2003; Matthews, Salomon, Kenyon, & Allen, 2002; Willemsen et al., 1998). For ambulatory 24-hour PEP, high stability ($.67 < r < .93$) has been found across a few days (Vrijkotte et al., 2004), as well as across a much longer period of 2–5 years (Goedhart et al., 2006). It is important to note that the between-participant differences in PEP reflect the extent to which participants differ in the degree of sympathetic effects on their cardiac contractility. These effects are likely to be highly correlated with differences in sympathetic activity, but the correlation is not perfect. Inotropic responses to norepinephrine and circulating epinephrine will be modulated by individual differences in the effectiveness of the cardiac β1- and β2-adrenergic receptors. Density, affinity, and distribution of these receptors may show large individual differences (Liggett, 1995; Liggett et al., 2006). These individual differences in receptor status may, for instance, lead to a paradoxically long PEP in patients with high levels of cardiac sympathetic nerve activity who have very low ventricular β-receptor densities. In healthy participants, however, a high interindividual correlation (0.82) was found between PEP levels and cardiac sympathetic effects as assessed through dual blockade (Cacioppo et al., 1994).

Although the PEP provides a reliable non-invasive index of SNS activity, algorithm detection and even visual detection of its crucial landmarks can be very difficult or even impossible due to noise in the ICG signal caused by movement (Berntson, Lozano, Chen, & Cacioppo, 2004; Lozano et al., 2007; Willemsen et al., 1996). The initial systolic time interval (ISTI) has recently been proposed as an easier to measure alternative to the PEP. Changes in cardiac contractility may not only be reflected in the time it takes the left ventricle to build up sufficient force to open the aortic valve (B-point) but also in the time it takes to reach peak ventricular ejection or maximal aortic diameter (dZ/dt-min peak or C-point), thus extending the PEP (ventricular depolarization plus isovolumetric contraction) with the rapid part of the ejection phase. The ISTI is therefore defined as the interval between the R-wave peak and the clear dZ/dt-min peak. The latter can be detected with much more fidelity than the B-point, which is often a (subtle) inflection defined by a zero-order crossing in the second derivative of the ICG rather than a true minimum. Two groups have independently confirmed that the ISTI is a significant predictor of both the R-wave peak to B-point (RB) interval as well as the PEP (Berntson et al., 2004; Lozano et al., 2007; Meijer, Boesveldt, Elbertse, & Berendse, 2007; Meijer, Boesveldt, Elbertse, & Berendse, 2008). Thus adequate estimation of sympathetic changes in contractility could be achieved by the detection of the two most salient features in the ECG and ICG, the R-wave peak and the dZ/dt-min peak respectively. Additional research, measuring PEP and ISTI

simultaneously and preferably in a blockade design, is needed to further support the validity of the ISTI as an alternative non-invasive SNS measure.

Another, seemingly somewhat forgotten, alternative to measure cardiac SNS activity is the amplitude of the T-wave in the ECG (TWA). The T-wave is the asymmetrical wave in the ECG that comes after the QRS-complex and typically lasts approximately 150 msec. It reflects ventricular repolarization (Abildskov, Burgess, Urie, Lux, & Wyatt, 1977; Burgess, 1979; Haarmark et al., 2010; Lozano et al., 2007; Randall & Hasson, 1977) in which the sympathetic nerves play an important role (Abildskov, 1985; Lozano et al., 2007). Decreases in TWA and even TWA inversion was seen to occur after local stimulation of the stellate sympathetic ganglia in dogs (Anitchkov & Vedeneyeva, 1961; Yanowitz, Preston, & Abildskov, 1966), intracoronary infusion of adrenaline in dogs (Barger , Herd, & Liebowitz, 1961), subcutaneous or intramuscular administration of adrenaline in man (Hartwell et al., 1942; Katz, Hamburger & Lev, 1931; Levine, Ernstene & Jacobson, 1930) and in reaction to pharmacological manipulation (isoproterenol) in man (Contrada et al., 1989; Contrada, Dimsdale, Levy, & Weiss, 1991; Guazzi et al., 1975). Importantly, these functional TWA decreases could be reversed by beta-blockade with propanolol (Contrada et al., 1989; Fukudo et al., 1992; Furberg, 1967; Furberg, 1968; Guazzi et al., 1975; Noskowicz & Chrzanowski, 1968; Rau, 1991). Additionally, TWA has been shown to be a useful indicator of cardiac SNS activity during laboratory testing of sympathetic stress reactivity (Furedy, Heslegrave, & Scher, 1984; Furedy & Shulhan, 1986; Furedy, Szabo, & Peronnet, 1996; Guazzi et al., 1975; Heslegrave et al., 1979; Matyas & King, 1976; Scher, Furedy, & Heslegrave, 1984).

In spite of these findings, TWA has not often been used, in part due to the "competition" from the more popular PEP that entered the literature at about the same time. However, a unresolved threat to the validity of TWA as a "pure" measure of SNS activity is the apparent contribution of vagal activity to decreases in TWA (Annila, Yli-Hankala, & Lindgren, 1993; Contrada, 1989; Contrada et al., 1989; Contrada et al., 1991; Dauchot & Gravenstein, 1971; Schwartz & Weiss, 1983; Weiss, Del, Reichek, & Engelman, 1980), although Gauzi and colleagues (Guazzi et al., 1975) found no changes in TWA in response to decreases in PNS activity by administration of atropine and Kline and colleagues (Kline, Ginsburg, & Johnston, 1998) did not find a significant correlation between TWA and RSA. Additional research, measuring TWA, RSA and PEP simultaneously, and preferably in a blockade design, is needed to further support the validity of the TWA as a non-invasive SNS measure.

ANS measurement in ambulatory monitoring studies

The above review showed that there are a number of invasive measures that have good content and criterion validity for measuring SNS and PNS activity. However, these invasive measures are suitable only for the investigation of individual differences in ANS responses to very standardized and controlled laboratory conditions, many requiring the equipment (or nearby safety precautions) of medical hospital facilities. Unfortunately, individual differences in ANS responses to standardized laboratory/hospital conditions, particularly conditions that should induce psychosocial stress, do not seem to transfer to individual differences in ANS responses to actual real-life stressors because the association between laboratory and ambulatory measurements is moderate at best (Gerin, Rosofsky, Pieper, & Pickering, 1994; Kamarck & Lovallo, 2003; van Doornen, Knol, Willemsen, & de Geus, 1994). It is likely that the psychological and physiological processes induced by laboratory stress are only a poor reflection of the actual processes in everyday real-life stress situations. Perhaps as a

consequence, the predictive value of ANS responses to laboratory challenges for future CVD is low, with the response to a challenge hardly contributing to the prediction of disease when basal levels have been taken into account (Barnett, Spence, Manuck, & Jennings, 1997; Carroll et al., 1998; Coresh, Klag, Mead, Liang, & Whelton, 1992).

As an alternative to bringing "everyday situations to the laboratory," researchers have increasingly tried to bring the "laboratory to everyday situations." This is done by using miniaturized versions of the recording equipment for non-invasive ANS measures that allow prolonged ambulatory monitoring in naturalistic settings (Fahrenberg, Myrtek, Pawlik, & Perrez, 2007; Houtveen & de Geus, 2009; Wilhelm & Grossman, 2010). The expectation is that ambulatory measurement of physiological levels and reactivity in the natural environment will lead to better prediction of morbidity and mortality. For blood pressure, the added value of the ambulatory approach has already been demonstrated (Mallion, Baguet, Siche, Tremel, & de Gaudemaris, 1999; Palatini & Julius, 2004; Verdecchia, 2000; Verdecchia, Schillaci, Reboldi, Franklin, & Porcellati, 2001).

An important question then becomes which of the non-invasive measures for PNS and SNS activity can be optimally employed for ambulatory monitoring. A major demand is that the measure is non-invasive. Secondly, we want the measure to be a relatively "pure" indicator of a single ANS branch only, so changes in the measure either reflecting predominantly PNS activity or predominantly SNS activity. Three additional considerations are (1) established validity, (2) feasibility, both in terms of technical implementation and participant-tolerance, and (3) cost, both in terms of equipment/consumables and labor for data cleaning. The latter becomes increasingly important if we want to study PNS and SNS activity in samples that are sufficiently large to allow epidemiological research and genetic analyses. Using the non-invasive measures listed in table 1 we select an optimal choice for ambulatory monitoring of PNS and SNS activity in terms of the balance between established validity and feasibility. To a minor extent we will also consider cost.

For PNS activity, we can choose from RSA, other HRV measures, or BRS. As HRV measures other than RSA reflect both SNS and PNS activity, our options rapidly reduce to the choice between RSA and BRS. Although changes in BRS are theoretically and empirically solidly linked to vagal activity, the non-invasive registration of BRS requires a beat to beat registration of blood pressure through a finger cuff measuring either pressure or photoplethysmographic changes in the fingertips. This (pulsating) cuff attached to the finger strongly hampers the daily routine of participants, and data collection and reduction is both expensive and very costly. Ambulatory recording of PNS activity should therefore preferably turn to RSA measures.

Two RSA measures, HF and RMSSD are highly feasible for prolonged recording across real life settings and are well-tolerated by participants as they only require a type II lead ECG recording, which can be obtained from chest bands or a minimum of three surface electrodes. The power in the high frequency band has a more clear-cut theoretical link to vagal activity, but empirically RMSSD proved highly correlated to HF across a wide range of conditions and its scoring is less labor-intensive and does not require a correct interpolation of missing IBIs (Goedhart et al., 2007). In principle, this makes RMSSD preferable to the third RSA measure, pvRSA, which requires the additional recording of the respiration signal, which increases both participant and experimenter burden. Yet, it has the clear advantage of allowing correction of pvRSA for both between-participant differences and within-participant changes in respiratory behavior which has been advocated as necessary for its validity as a proxy for (changes in) vagal tone by a variety of authors (Houtveen, Groot, & de Geus, 2006; Ritz et al., 2006). As ambulatory respiratory behavior can be easily captured from recordings of the ICG, a

signal paramount to what we will recommend for SNS activity below, we conclude that RMSSD and pvRSA are the best options for ambulatory monitoring of PNS activity.

For SNS activity, we can choose from urinary catecholamines, sAA, LF/HF ratio, SCL, TWA, and PEP. Urinary catecholamines is still a good measure of sympathetic activity, even with the caveats in validity mentioned before, but they only provide an aggregate measure over prolonged periods of time (e.g. an entire 24-hour period). They further lack precision in that the SNS activity cannot be linked to specific psychological or physiological stressor, although comparisons of workday versus resting day are still meaningful. Urine collection is tolerated rather well but logistics and costs of biochemical analyses make it challenging to use this method on larger scale samples.

Taken the current state of knowledge, perhaps the least attractive of these measures is sAA, in spite of its rapidly gaining popularity. Strong concerns have been voiced regarding its validity (Bosch et al., 2011) particularly when using the Salivette collection method. The alternative method of collection by passive 'drooling' has not been tested in an ambulatory setting, but a priori user-friendliness and tolerance does not seem very high. Additionally, although the literature is still inconclusive at present, it may also be strongly dependent on vagal influences, which refutes sAA as a relatively pure index of SNS activity.

Although highly feasible and cost-effective –it only requires a three lead ECG recording- the LF/HF ratio was discarded as a pure SNS measure due to an unsound theoretical underpinning and a complete lack of empirical criterion validity. In contrast, electrodermal measures like SCL and the nsSCL have a very good theoretical basis and criterion validity, but here it is feasibility in ambulatory monitoring that proves challenging. The typical electrode placement at the palm of the hand restricts individuals in their normal daily routine during 24-hour recordings. Technical challenges compound this problem as movement of the hand affect signal quality and changes in temperature make it hard to interpret SCL outside the laboratory. Alternative electrode placements at the soles of the feet are even less practical and electrodes at the sternum were shown to be relativity unresponsive to psychological stimuli (Freedman, 1989).

Currently, the PEP is the measure of choice for ambulatory recording of SNS activity as its validity is relatively undisputed and ICG measurements using four spot electrodes have been shown to be well tolerated (Kupper, Willemsen, Boomsma, & de Geus, 2006; Vrijkotte et al., 2004). In spite of validity and practical feasibility, two clear downsides of the PEP are that it requires laborious manual inspection despite data reduction strategies such as large scale ensemble averaging (Riese et al., 2003) and that the PEP is sensitive to confounding by preload and afterload effects that require careful co-registration of posture and physical activity as well as the appropriate activity/posture stratified analyses. An alternative ambulatory measure of SNS activity would be most welcome.

The TWA remains untested as such an alternative. TWA relies only on the ECG signal. If valid as an SNS index, a simple ECG recording, yielding TWA and RMSSD, could provide a new way to index both ANS branches with minimal burden and costs. Pending that, combined ECG and ICG recordings from 5 to 7 spot electrodes, yielding PEP and pvRSA, remains the preferred way to assess SNS and PNS activity in ambulatory recordings.

CHAPTER 2

Underestimation of cardiac vagal control in regular exercisers by 24-hour heart rate variability recordings

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Abstract

Objective: To examine whether ceiling effects at long inter beat intervals (IBIs) cause an underestimation of cardiac vagal control in regular exercisers by time and frequency-domain measures of respiratory sinus arrhythmia (RSA). Methods: 24-hour ECG and respiration recordings were performed in 26 regularly exercising participants, actively engaged in aerobic training for the past year, and enrolled in supervised training in the six weeks pre-study, and in 26 age- and sex-matched non-exercisers. Sleep and waking levels of cardiac vagal control were estimated by RSA obtained through the peak–valley method, by the standard deviation of the IBIs, the root mean square of successive IBIs, and the high frequency IBI spectral power. Results: In 11 of the exercisers the IBI–RSA relationship was characterized by a quadratic relationship. This reflected a ceiling effect at very long IBI values attained by regular exercisers, particularly during the nighttime recording. Irrespective of this ceiling effect, RSA as well as other heart rate variability (HRV) measures was still significantly larger in the exercisers with a quadratic IBI–RSA relationship than in non-exercisers or exercisers with a linear IBI–RSA relationship. Conclusions: We conclude that a subgroup of regular exercisers is characterized by a low heart rate paired to high levels of cardiac vagal control. In these exercisers, vagal control is underestimated from HRV measures in ambulatory recordings. Inspection of the IBI–RSA relationship should be routinely added when HRV measures are used to index cardiac vagal control.

Introduction

Regular vigorous exercise is associated with many favorable physiological adaptations, including a lower resting heart rate. Dual blockade studies point to a lower intrinsic heart rate as the most replicated source of this resting bradycardia in exercisers (Bouchard et al., 1999; Bouchard, Boulay, Simoneau, Lortie, & L.Perusse, 1988; Goldberger et al., 2001; Katona, McLean, Dighton, & Guz, 1982; Kingwell, Dart, Jennings, & Korner, 1992; Lewis et al., 1980; Smith, Hudson, Graitzer, & Raven, 1989b; Uusitalo, Tahvanainen, Uusitalo, & Rusko, 1996) and this is supported by findings in animals (Lin & Horvath, 1972; Negrao, Moreira, Brum, Denadai, & Krieger, 1992). In addition to lowered intrinsic heart rate, exercisers have long been hypothesized to possess a stronger vagal control over the heart rhythm (Ekblom, Kilbom, & Soltysiak, 1973). Animal studies and studies in cardiac patients generally support an effect of exercise on cardiac vagal control (Billman, 2002; Goldsmith, Bloomfield, & Rosenwinkel, 2000; Gutin et al., 2005; Mueller, 2007) but in healthy human participants, the evidence for an exercise-induced shift in vagal control is more controversial (Goedhart, de Vries, Kreft, Bakkker, & de Geus, 2008a).

Cardiac vagal control is most often quantified by time- or frequency domain indices of heart rate variability (HRV) in the respiratory frequency range, also called respiratory sinus arrhythmia (RSA) (Berntson et al., 1997; Martinmaki et al., 2006; Nunan et al., 2010; Task Force of the European Society of Cardiology the North American Society of Pacing, 1996). A number of cross-sectional studies reported higher RSA in regular exercisers (Buchheit et al., 2005; Dixon, Kamath, Mccartney, & Fallen, 1992; Goldsmith, Bigger, Steinman, & Fleiss, 1992; Martinmaki et al., 2006; Shin, Minamitani, Onishi, Yamazaki, & Lee, 1997) but not all studies support this difference (Goedhart et al., 2008a; Hatfield et al., 1998) and some even report the opposite finding of lower RSA in exercisers compared to non-exercisers (Sacknoff, Gleim, Stachenfeld, & Coplan, 1994). Notably, various randomized controlled training studies that assigned untrained participants to a non-exercise control manipulation or a standardized exercise training program have failed to find a specific training-induced increase in RSA (Boutcher & Stein, 1995; de Geus et al., 1996; Loimaala, Huikuri, Oja, Pasanen, & Vuori, 2000; Uusitalo, Laitinen, Vaisanen, Lansimies, & Rauramaa, 2004).

Here we hypothesize that a specific methodological problem in assessing RSA in well-trained exercisers may have led to an underestimation of the beneficial effects of regular exercise on cardiac vagal control in previous studies, both cross-sectional and longitudinal. We note that in a meta-analysis of training studies by Sandercock et al. (Sandercock, Bromley, & Brodie, 2005) a significant training-induced increase in RSA was seen during short-term laboratory recordings at rest, but *not* in 24-hour ambulatory recordings. Furthermore, some of the ambulatory studies suggested that training effects on HRV may be confined to the daytime but are absent in the whole recording or nighttime levels (de Geus, van Doornen, de Visser, & Orlebeke, 1990a; Schuit et al., 1999). An obvious difference between (laboratory) daytime and ambulatory nighttime recordings is the absolute level of heart rate attained at night. As indicated as early as 1993 by Malik and Camm (Malik & Camm, 1993) RSA may not be a reliable index of cardiac vagal control in participants with a low heart rate.

RSA is formally defined as the difference between the shortest inter beat interval (IBI) during inspiration and the longest IBI during expiration (Grossman, 1983; Katona et al., 1975) and the main physiological rationale to use it as an index of cardiac vagal control is that neural vagal activity is selectively inhibited during inspiration but not during expiration. However, at very high vagal activity, a ceiling effect may prevent lengthening of the IBI during expiration more than during inspiration (Malik et

al., 1993). High vagal activity causes a large occupancy of the available muscarinergic receptors on the sinoatrial (SA) node, and at this level of saturation any further increases in acetylcholine may no longer linearly increase the IBI as it would at low to moderate levels of cardiac vagal activity (illustrated in figure 1).

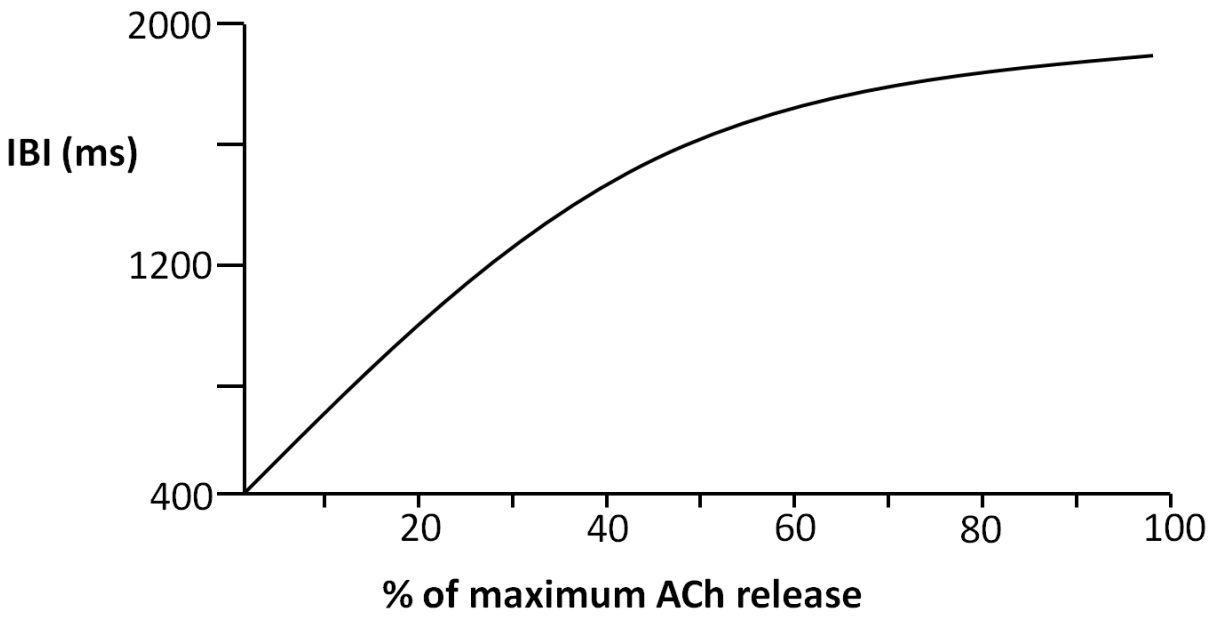

Figure 1. *Predicted relationship between the mean IBI and the release of Acetylcholine [ACh] in the sinoatrial (SA) node. This assumes a 1) near-linear relationship between fractional saturation of the cardiac muscarinergic receptors and the slowing of SA pacemaker cell depolarization, and 2) an asymptotic relationship between [ACh] and the fractional saturation of the cardiac muscarinergic receptors, known as the binding isotherm (Baudiere, Monferini, Giraldo, Ladinsky, & Bali, 1987). Formal models that take into account phasic and tonic aspects cardiac vagal activity arrive at similar predictions (Pyetan, Toledo, Zoran, & Akselrod, 2003).*

This ceiling effect is expected to cause a quadratic relationship between IBI and RSA. A quadratic shape of the IBI – RSA relationship has indeed been found in studies inducing very high vagal activity by phenylephrine and nitroprusside infusion (Goldberger et al., 2001). The quadratic shape of the IBI – RSA relationship will cause an underestimation of cardiac vagal control by RSA in participants with low resting heart rates.

Because regular exercisers often have lower resting heart rates than non-exercisers we hypothesize 1) that exercisers more often have a quadratic IBI –RSA curve, 2) that RSA underestimates cardiac vagal control in these exercisers, and 3) that this underestimation is aggravated during 24-hour ambulatory monitoring that includes evening and night time recordings when vagal activity is high.

Methods

Participants

The 52 participants came from two studies described in detail elsewhere (Goedhart et al., 2008a; Kupper et al., 2005a). Briefly, 26 regularly exercising participants (15 males, 11 females) with a mean age of 38.0 years (SD = 12.2 years) were recruited from several ministries in The Hague and a police station in Amsterdam. During study sign-up it was made explicit that participants had to be actively engaged in leisure time aerobic training for at least 30 consecutive minutes a day, three days a week for the past year. During actual recruitment this was again confirmed by personal interview. To further ensure that they had been exposed to comparable high levels of vigorous exercise prior to ambulatory recording, they underwent a 6 week program of supervised training at the same fitness centre. During this six week period they trained at least 3 times a week, for at least one hour at a minimal intensity of 70 % of the maximal heart rate (measured with a Polar A5 heart rate monitor), which had been established during an all-out test on a bicycle ergo meter (10 min warm-up at 130 bpm followed by two bouts of 60 sec bicycling at an increasing resistance until exhaustion).

Twenty-six sex- and age matched sedentary participants with a mean age of 39.8 (SD =9.8) were recruited from a family study in which 780 participants, who had participated in a longitudinal study with biennial surveys on health and health behaviors, underwent 24-h ambulatory recording of RSA (Kupper et al., 2005a). To ensure that we selected persistent sedentary participants, they had to have indicated not to engage in any work-related or leisure time based regular exercise both in the months before ambulatory recording as well as in at least two past surveys.

All participants were without a history of hypertension or cardiovascular disease and used no cardioactive medication (e.g. beta-blockers or antidepressants). Ambulatory recording protocols were approved by the Medical Ethics Committee of the VU University and the detraining study was approved by the Ethics Committee of the faculty of Human Movement Sciences. All participants gave written consent before entering the study.

Study Protocol

Participants from both studies underwent an identical protocol of ambulatory monitoring using the VU University Ambulatory Monitoring System (VU-AMS). The VU-AMS continuously records the electrocardiogram (ECG) and the impedance cardiogram (ICG) (Goedhart et al., 2007) and produced an audible alarm approximately every 30 minutes (± 10 minutes randomized) to prompt the participant to fill out an activity diary. Using the activity diary entries in combination with a visual display of the output of an inbuilt vertical accelerometer (measuring movement), the entire 24-h recording was divided into fixed periods. These periods were coded for posture (supine, sitting, standing, walking, bicycling), activity (e.g. desk work, dinner, meetings, watching TV), and physical load (no load, light, intermediate and heavy).

Minimum duration of periods was 5 minutes and maximum duration was 1 hour. If periods with similar activity and posture lasted more than 1 hour (e.g. during sleep), they were divided into multiple periods of maximally 1 hour. All periods were classified into three main ambulatory conditions: 1) lying asleep, 2) sitting during the day, or 3) mild physical activity (standing/walking) based on the dominant posture/activity reported in that period; the exact timing of changes in posture/activity was verified using the accelerometer signal from the ambulatory device. For the exercisers the 24-hour recordings took place at least one day after a training session; both exercisers

and non-exercisers were instructed to keep physical activity at a minimal level during the ambulatory recording day.

IBI and HRV measures

The ECG and changes in the thorax impedance (dZ) were recorded continuously using six disposable, pregelled Ag/AgCl electrodes as described detailed elsewhere (Goedhart et al., 2008a; Kupper et al., 2005a). The IBI time series was obtained from the ECG by an online automated R-wave peak detector, where IBI is the interval in milliseconds between two adjacent R waves of the ECG. Artefact pre-processing was performed on the IBI data. When the IBI deviated more than 3SD from the moving mean of a particular period it was automatically identified as an artefact and accepted or overruled by visual inspection. Since artefacts cannot simply be deleted because the continuity of time would be lost for frequency analysis, spuriously short IBIs were summed and missing beats were 'created' by splitting spuriously long IBIs.

In the time domain we computed the mean heart rate across the ambulatory conditions as 60000/IBI. To assess RSA we used the 'peak-valley' method (de Geus et al., 1995; Goldberger et al., 1994; Goldberger et al., 2001; Grossman et al., 1990; Grossman & Wientjes, 1986). In this method, RSA is scored from the combined respiration and IBI time series by detecting the shortest IBI during inspiration and the longest IBI during expiration on a breath-to-breath basis. From the dZ we obtained a continuous respiration signal in which we scored the onset of inspiration and expiration on a breath to breath basis according to the procedures detailed elsewhere (de Geus et al., 1995; Houtveen et al., 2006). Per breath, estimates of peak-valley RSA were obtained by subtracting the shortest IBI in the inspirational phase (which was made to include 1500 milliseconds from the following expiration to account for phase shifts) from the longest IBI in the expirational phase (including 1500 milliseconds from the following expiratory pause/inspirational phase). The shortest IBI in inspiration had to be part of an acceleration in heart rate and the longest IBI in expiration had to be part of a deceleration in heart rate. This means that the selected IBI's not only had to be within the correct part of the respiratory cycle, but also be part of a clear downward or upward slope in the IBI time series. Automatic scoring of RSA was checked by visual inspection of the respiratory signal from the entire recording. Breathing cycles that showed irregularities like gasps, breath holding, coughing etc., were not considered valid and were removed from further processing. In the remaining data the shortest and longest breaths that deviated more than 3SD from the mean were automatically removed from the entire recording before averaging RSA across all remaining breaths to a single mean value for each of the labeled periods. We discarded on average 14.5% of all automatically scored breaths.

In a small percentage of the valid breaths no respiratory phase-related acceleration or deceleration was found or the shortest beat in inspiration was longer than the longest beat during expiration. It is common practice to set RSA to zero in such breaths, assuming that cardiac vagal control truly is low here. This variable is labeled "RSAzero" in our results. This procedure might, however, bias estimation of RSA to lower values in participants with a quadratic IBI-RSA relationship, which we expect to be more prevalent in the group of exercisers, because the ceiling effect may cause a larger percentage of breaths with no valid shortest or longest IBI. We will therefore apply an additional strategy that averages RSA only across breaths that have a valid shortest and longest IBI, and a positive RSA value (variable is labeled "RSA" in the results). Finally, various other measures of HRV often used in the literature were computed from the corrected IBI time series. In the time domain we computed the

standard deviation of the IBIs (SDNN) and the root mean square of successive IBI differences (RMSSD). The latter was defined as:

$$RMSSD = \sqrt{\frac{1}{n-1}\sum_{i=2}^{i=n}\left(IBI_i - IBI_{(i-1)}\right)}$$

In the frequency domain, total spectral power (TP) , very low frequency (VLF), low frequency (LF) and high frequency (HF) power were extracted from the IBI time series by Wavelet decomposition (see (Houtveen et al., 2001) for more information regarding this procedure). Total power was computed as the variance in the .0078125 - .5 Hz window, the VLF power as the variance in the .0078125 - .0625 Hz window, LF power as the variance in the .0625 - .125 Hz window, and HF as the variance in the .125 - .5 Hz window.

To be able to detect potentially confounding group differences in respiratory behavior mean respiration rate (RR) was derived for each participant across the three ambulatory conditions.

Data analysis

As outlined above, the entire 24-h recording was divided into fixed periods of lying asleep, sitting during the day, or mild physical activity. To determine the shape of the relationship between IBI and RSA/RSAzero we further subdivided these periods into bins no longer than 10 minutes. The mean IBI and RSA/RSAzero were determined per bin and the correlation across these mean IBI and RSA/RSAzero means were depicted in a separate scatter plot for each of the participants in the study. Two examples for the IBI-RSA relationship are shown in figure 2 (full set of scatter plots available upon request from the first author). Significance of the regression weights (β1 and β2) in the linear and quadratic terms was tested by the SPSS CURVEFIT procedure. To be classified as quadratic, the β2 parameters had to be significantly different from zero, the quadratic solution had to explain > 20% of the variance in RSA, and the quadratic solution had to improve on the linear solution by at least 10% additional explained variance. Two raters, blinded to exercise status, then verified this algorithmic classification of the scatter plots by visual inspection. Virtual identical classification was obtained when RSAzero was used in the scatter plots rather than RSA. For brevity, we will only use the classification based on the IBI-RSA relationship throughout.

Based on the shape of their IBI-RSA scatter plots, the exercisers and non-exercisers were divided into subgroups with a linear shape of the IBI-RSA relationship and a quadratic shape of the IBI-RSA relationship. To test whether exercisers more often had a quadratic shape than non-exercisers we used a χ^2 test. To test the hypothesis that the different shape of the IBI-RSA relationship in exercisers would underestimate RSA we used a mixed model ANOVA with sex, exercise and shape status as between-participant factors. To test the hypothesis that the potential underestimation of RSA would be larger at night, ambulatory condition (sleep, sitting, mild physical activity) was added as a within-participant factor. Because the distribution of the HRV measures was skewed a logarithmic transform was used for the analyses. Tables and figures present original values for the time domain measures and log-transformed values for the frequency domain measures.

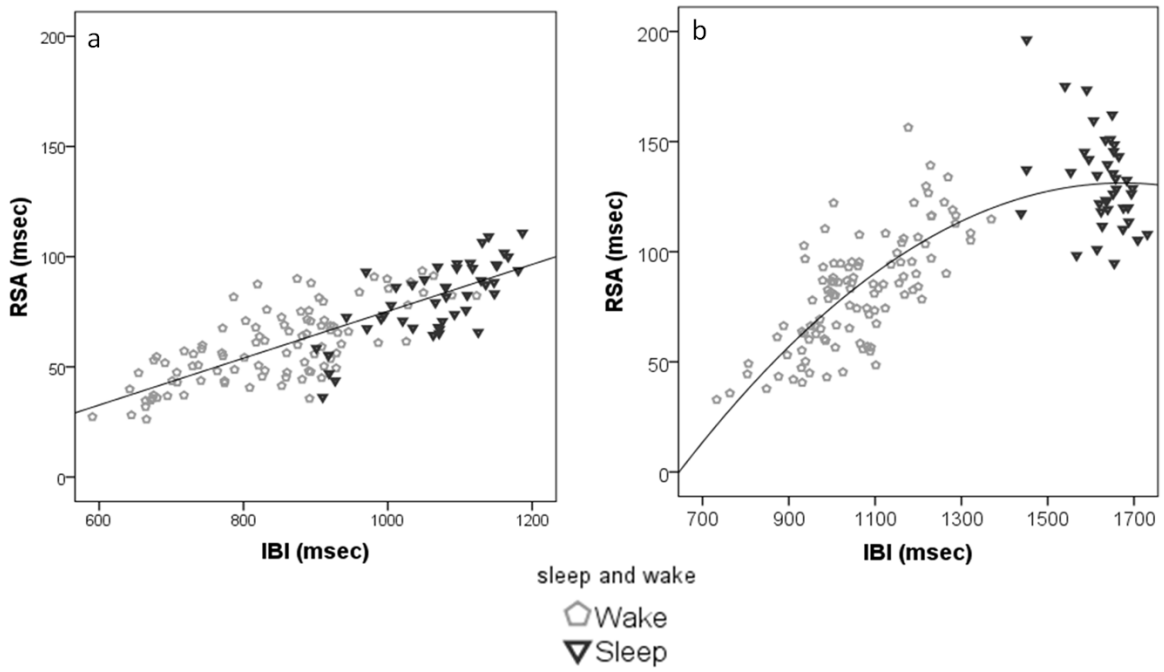

Figure 2. *Scatter plots of the mean IBI and RSA across 10 minute bins of a 24h ambulatory for recording two different individuals (a, b). The IBI-RSA relationship for individual a (N = 167 bins) was judged linear, the IBI-RSA relationship for individual b (N= 127 bins) was judged quadratic. Curves represent the best fitting linear or quadratic function. Data collected during sleep are triangle shaped; data collected during the awake period pentagon shaped.*

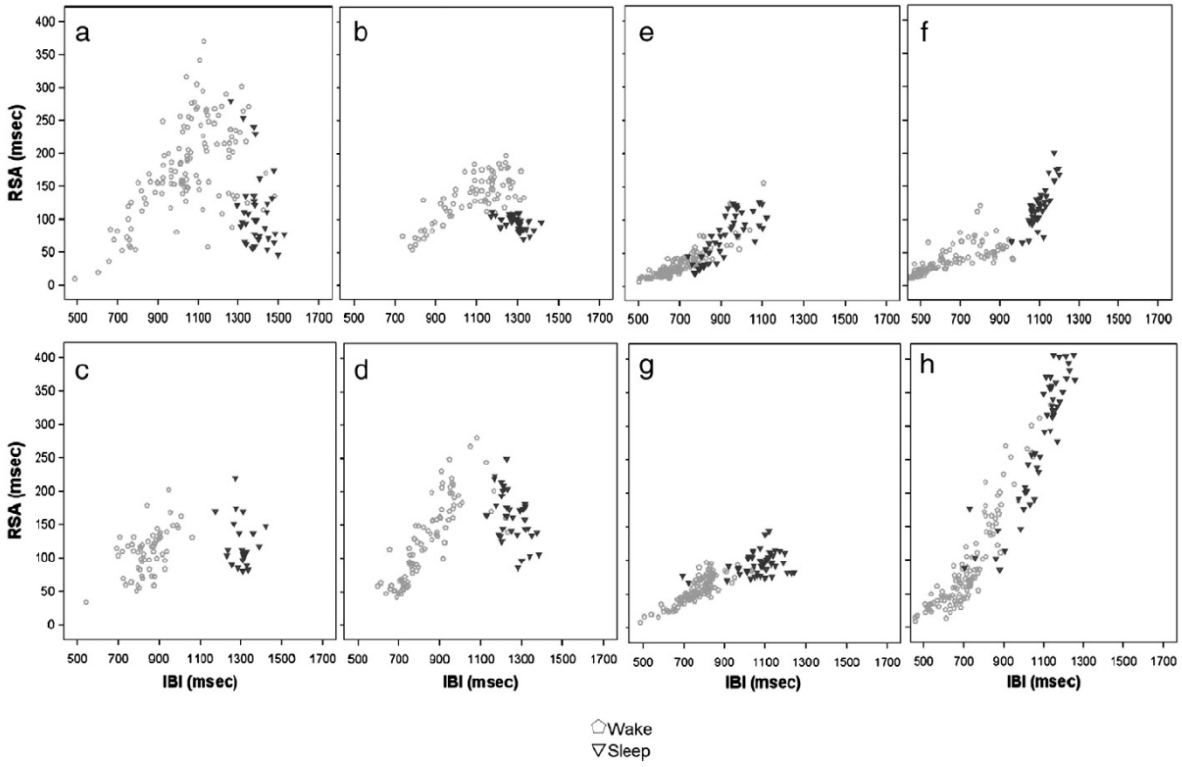

Figure 3: *Representative scatter plots of IBI and RSA for exercisers (a, b, c and d) and non-exercisers (e, f, g and h). The IBI and RSA values were averaged across 10 minute bins throughout the 24-hour recording period. Data collected during sleep are triangle shaped, data collected during the awake period are pentagon shaped.*

Results

In the group of exercisers 11 participants (7 females) showed a quadratic relationship between IBI and RSA and 15 participants (8 females) showed a predominantly linear IBI-RSA relationship. In the group of non-exercisers only 2 out of the 26 participants showed a quadratic IBI-RSA relationship (both males). This group difference is significant (χ^2 = 9.57, p = 0.002). Typical examples from both groups are shown in figure 3.

Based on the observed IBI-RSA scatter plots, the exercisers were subdivided into a group with a linear shape of the IBI-RSA relationship and a quadratic shape of the IBI-RSA relationship. A third group was formed by all non-exercisers (including two non-exercisers with a quadratic shape). No main effects of sex were found and no interactions involving group by sex. Table 1, therefore, depicts the HRV measures as a function of ambulatory condition (night-time sleep, awake sitting, and awake physical active) and exercise/shape status collapsed across males and females. A main effect of condition was found on IBI (F(2, 49)=259.55, p=.000), SDNN(F(2, 49)=64.41, p= .000), RMSSD (F(2, 48)=10.62, p<.0001), RSA (F(2, 49)= 16.95, p=.000) , RSAzero (F(2, 49)= 22.47, p=.000), TP (F(2, 48)=59.93, p=.000), HF power (F(2, 48)= 4.97, p =.011, LF power (F(2, 48)= 5.02, p= .010, VLF power (F(2, 48)= 55.87, p=.000), and RR (F(2, 49)= 18.62, =.000). IBI, RMSSD, RSA, RSAzero, and HF power were highest during sleep, intermediate during sitting, and lowest during mild physical activity. TP, SDNN, and VLF decreased from sleep to awake sitting but higher levels were again found during physical activity. The increase in TP and SDNN during physically active conditions is likely caused by the increase in VLF that, in turn, is due to the heterogenetic nature of this condition, which included periods of standing alternated with periods of walking or sitting. Respiration rate was lower during sleep than during the awake time. Mean 24-hour RSA did not correlate significantly with mean RR (r = .06).

A significant main effect was found of exercise/shape status on IBI (F(2, 49)=14.75, p<.0001), SDNN (F(2,49)= 11.44, p=.000), RMSSD (F(2, 48)=5.36, p=.008), RSA (F(2, 49)=6.41, p=.003), RSAzero (F(2, 49)=4.71, p=.013), TP (F(2, 48)= 8.89, p=.001), HF power (F(2, 48)=3.68, p=.033, LF power (F(2, 48)=6.46, p=.003), and VLF power (F(2, 48)=8.50, p=.001) but not on RR. Post-hoc testing of the effect of exercise/shape revealed that it derived mainly from the difference between the exercisers with a quadratic IBI-RSA relationship and the other two groups. Exercisers with a quadratic IBI-RSA relationship had longer IBIs and significant higher levels of SDNN, RMSSD, RSA, RSAzero, TP, HF, LF, and VLF compared to non-exercisers (p's <0.01) and also significant higher levels of SDNN, RSA, RSAzero, TP, HF, LF, and VLF compared to exercisers with a linear IBI-RSA relationship. The exercisers with a linear IBI-RSA relationship had a longer IBI but showed otherwise comparable levels of RSA, RSAzero and the other HRV measures to those of the non-exercisers.

The effect of exercise/shape status was different during the awake and nighttime recordings. A significant interaction between exercise/shape and ambulatory condition was found on IBI (F(4,49)=6.46, p= .000), SDNN (F(4,49)= 2.56, p= .047), RSA (F(4,49)= 2.49, p= .05), RSAzero (F(4,49)=2.64, p= .045), and HF power (F(4,48)=3.01, p = .027). Post hoc analysis revealed a significant difference between non-exercisers, exercisers with a quadratic IBI-RSA relation and exercisers with a linear IBI-RSA relation during the sitting or physical active conditions for all variables but failed to reach significance during sleep for RMSSD, RSA, RSAzero, HF power and LF power. The interactive effect is illustrated for RSA in figure 4.

Table 1. *Means and standard deviations for inter beat interval (IBI), Standard deviation of normal-to-normal RR interval (SDNN), root mean square of successive differences (RMSSD), respiratory sinus arrhythmia (RSA), RSA with missing breaths set to zero (RSAzero), Total IBI Power (TP), High Frequency IBI power (HF), Low frequency IBI power (LF), very low frequency IBI power (VLF) and Respiration Rate (RR) by exercise/shape status and ambulatory condition.*

		Sleep		**Sitting**		**Mild Activity**			
		Mean	SD	Mean	SD	Mean	SD	N	Effects
IBI (ms)	Non-Exercisers	927.4	94.9	756.5	73.1	667.8	74.4	26	
	Linear Exercisers	989.6	145.8	842.9	105.7	746.1	85.5	15	
	Quadratic Exercisers	1235.9	247.2	945.8	165.6	818.9	125.9	11	a,b,c
SDNN (ms)	Non-Exercisers	93.3	32.8	66.0	14.6	76.9	15.1	26	
	Linear Exercisers	91.3	36.8	70.3	30.7	86.9	29.7	15	
	Quadratic Exercisers	137.1	50.2	111.5	44.2	136.9	50.9	11	a,b,c
RMSSD(ms)	Non-Exercisers	57.7	41.3	36.9	17.1	33.2	12.2	26	
	Linear Exercisers	64.9	47.1	50.5	35.4	46.4	32.6	15	
	Quadratic Exercisers	94.8	52.8	79.4	52.0	71.5	43.0	11	a,b
RSA (ms)	Non-Exercisers	75.1	48.7	53.6	18.2	45.1	13.2	26	
	Linear Exercisers	73.4	51.6	56.1	32.5	47.4	25.7	15	
	Quadratic Exercisers	96.7	41.9	107.0	57.4	92.7	51.0	11	a,b,c
RSAzero(ms)	Non-Exercisers	66.9	41.5	43.7	17.1	34.6	11.5	26	
	Linear Exercisers	63.7	48.6	45.8	28.2	36.8	20.6	15	
	Quadratic Exercisers	77.5	33.9	83.4	41.6	70.8	37.6	11	a,b,c
logTP	Non-Exercisers	8.7	.6	8.2	.4	8.6	.4	26	
	Linear Exercisers	8.7	.8	8.2	.9	8.7	.7	15	
	Quadratic Exercisers	9.4	.7	9.1	.7	9.7	.7	11	a,b
logHF	Non-Exercisers	6.3	.9	5.8	.8	5.7	.7	26	
	Linear Exercisers	6.1	1.3	5.8	1.2	5.7	1.1	15	
	Quadratic Exercisers	6.7	1.1	6.9	1.1	6.9	1.0	11	a,b,c
logLF	Non-Exercisers	6.4	.9	6.3	.7	6.2	.64	26	
	Linear Exercisers	6.2	1.3	6.2	1.1	6.1	1.1	15	
	Quadratic Exercisers	7.2	.9	7.4	.7	7.3	.6	11	a,b
logVLF	Non-Exercisers	7.2	.6	7.1	.5	7.3	.5	26	
	Linear Exercisers	7.8	.8	7.1	.8	7.3	.9	15	
	Quadratic Exercisers	8.5	.7	8.0	.7	8.3	.7	11	a,b
RR (br/min)	Non-Exercisers	15.6	1.6	17.6	1.3	17.7	.8	26	
	Linear Exercisers	16.2	2.0	17.6	1.0	17.6	.7	15	
	Quadratic Exercisers	16.8	2.2	17.8	1.2	17.7	.9	11	a

a = Significant main effect of ambulatory condition ($p < .05$).
b = Significant effect of exercise/shape status ($p < .05$).
c = Significant interaction between ambulatory condition and exercise/shape status ($p < .05$).

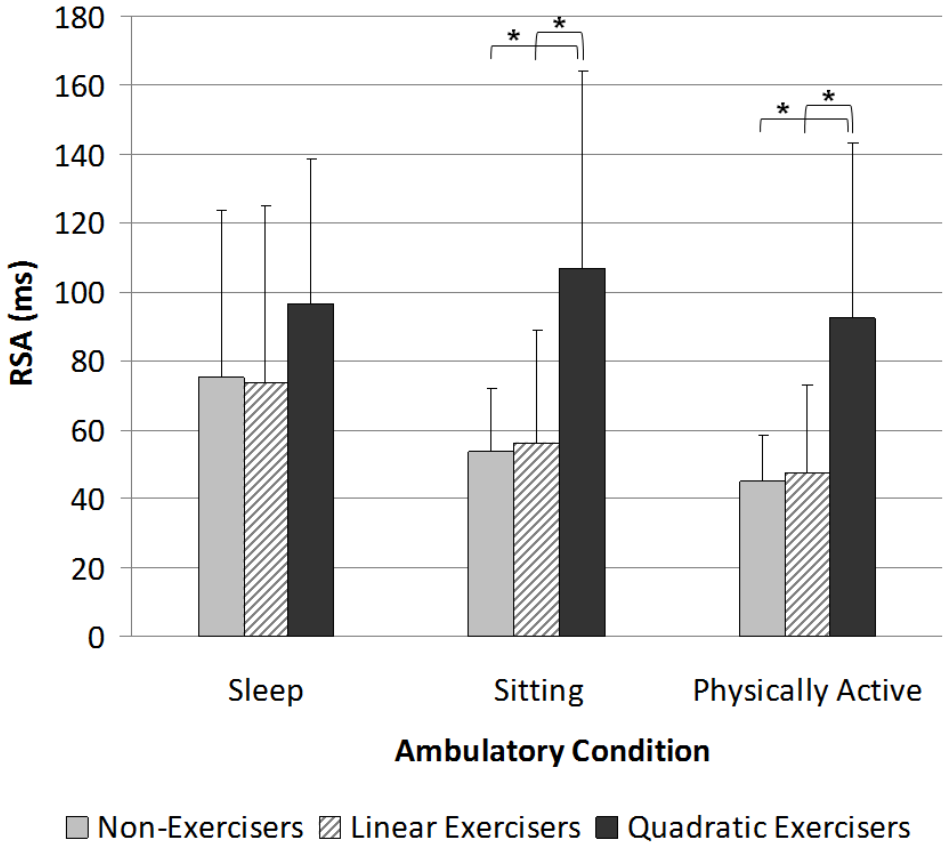

Figure 4: *RSA as a function of ambulatory condition in the non-exercisers and exercisers with a linear or quadratic shape of the IBI-RSA relationship. * = significant group differences at p < .05*

Analyses that do not distinguish between a quadratic and linear IBI-RSA relationship.

The above analyses suggested that in cross-sectional comparisons between exercisers and non-exercisers, higher RSA levels in the exercisers derive entirely from exercisers with a quadratic IBI-RSA relationship, and that grouping the exercisers with quadratic and linear shapes might lead to different conclusions. To test this, we repeated the analyses without the distinction between the exercisers with a linear and a quadratic IBI-RSA relationship. In this analysis IBI ($F(1, 50)=16.3$, $p= .000$), SDNN($F(1, 50)= 6.28$, $p=.016$), and RMSSD ($F(1, 49)= 5.78$, $p=.020$) were still significantly higher in the total group of exercisers when compared to the non-exercisers, but no significant difference was found for the four measures that are most commonly used in the field: RSA ($F(1, 50)=3.22$, $p= .078$), RSAzero ($F(1, 50)=2.36$, $p= .13$), LF power ($F(1, 50)=1.74$, $p= .192$) and HF power ($F(1, 50)=1.47$, $p= .230$).

Discussion

Prospective studies have shown that regular vigorous exercise in leisure time (e.g. sports, jogging, aerobics) is associated with a reduced risk for myocardial infarction and sudden death (Powell, Thompson, Caspersen, & Kendrick, 1987; Williams, 2001). An exercise-induced increase in cardiac vagal control is one of the mechanisms put forward to explain this reduced risk in regular exercisers (Billman, 2002; Frick, Elovainio, & Somer, 1967; Goldsmith et al., 2000; Lewis et al., 1980; Scheuer & Tipton, 1977) but the empirical evidence to support this idea has been mixed. Specifically, a large number of studies using time or frequency domain measures of RSA have not found higher RSA in highly trained regular exercisers (Buchheit et al., 2006; Hatfield et al., 1998; Sacknoff et al., 1994; Scott et al., 2004). Here we show that these previous null findings may reflect a systematic underestimation of cardiac vagal control by RSA measures in a substantial (40%) subset of exercisers in our sample who are characterized by a quadratic relationship between IBI and RSA. During the day, RSA was significantly higher in these quadratic exercisers compared to linear exercisers and non-exercisers, in keeping with a higher vagal control. At night, however, when the IBI-RSA relationship was in the downward arm of the quadratic curve for quadratic exercisers, the difference between the RSA of quadratic exercisers and the RSA of linear exercisers /non-exercisers was greatly attenuated. The quadratic IBI-RSA relationship likely reflects a high level of fractional saturation of sino-atrial muscarinergic receptors, characteristic of high vagal tone. This is in keeping with the much lower resting heart rate in these exercisers. Comparison of the total group of exercisers with the non-exercisers without taking the IBI-RSA relationship into account, would have lead to the conclusion that exercise is not associated with an increase in cardiac vagal control.

The present study shows that regular exercisers constitute a heterogeneous group with regard to cardiac autonomic regulation. There is a group of exercise with a very low heart rate and a quadratic IBI-RSA relationship and a second group with less dramatic bradycardia and a linear IBI-RSA relationship. A first likely explanation for this dichotomy is differences in the amount and duration of regular exercise between our quadratic and linear exercisers, such that the exercisers with a quadratic IBI-RSA relationship are the more vigorous exercisers. This would be in keeping with previous studies showing that moderate exercisers had higher RSA levels than sedentary participants but that vigorous exercisers did *not* have higher levels than moderate exercisers (Melanson, 2000; Sandercock et al., 2005) or even RSA that had returned to the sedentary level (Buchheit et al., 2006; Buchheit, Simon, Piquard, Ehrhart, & Brandenberger, 2004). In the present study large differences in the amounts of regular exercise are unlikely because we selected only individuals in the exercise group that had been engaged in high levels of regular exercise (3 times a week) for at least a year, the intensity and frequency of which had been under our own supervision in the last six weeks preceding testing.

A second explanation is that the dichotomy within the exercisers reflects innate differences in the responses to exercise. There are large individual differences in extent of cardiorespiratory fitness adaptation to comparable levels of regular exercise exposure (de Geus, van Doornen, & Orlebeke, 1993; de Geus, van Doornen, de Visser, & Orlebeke, 1990b) that appear substantially heritable (Bouchard & Rankinen, 2001). Importantly, these heritable differences in trainability include genetic effects on the bradycardiac response to a standardized training program (An et al., 2003; Rice et al., 2002). It is possible, therefore, that the quadratic exercisers simply have a more favorable genetic make-up that makes them respond to regular exercise behavior with a strong increase in cardiac vagal control. However, it is additionally possible that participants with a

quadratic IBI-RSA relationship posses an innate bradycardia and high vagal control that is partly independent of their exercise behavior. Previously, it has been shown that non-exercisers selected for an innate bradycardia also showed high resting HRV (Boutcher, Nugent, McLaren, & Weltman, 1998). In our study, high cardiac vagal tone was not limited to exercisers as demonstrated by the two non-exercisers that also showed a quadratic IBI-RSA relationship in spite of reporting not to have engaged in regular exercise in the past years. The highest mean RSA (287ms) was in fact observed in one of these non-exercisers during sleep (in figure 3, participant in the lower right corner). Twin or family studies that explicitly test the heritability of the shape of the IBI-RSA relationship are needed to resolve this.

Three important limitations of this study must be noted. A first limitation is that we have not confirmed higher vagal activity in quadratic exercisers using pharmacological blockade of the muscarinergic receptors. However, in light of our findings, an unresolved question is whether this golden standard would not also suffer from the ceiling effects depicted in figure 1. A second limitation is that we cannot exclude that the participants in the quadratic and linear groups were different on confounding variables that affect IBI, RSA or their relationship. These include, but are not limited to, the type of regular exercise they had been engaged in before the standardized training program, body composition, smoking behavior and alcohol use, oral contraceptives use, recent life stress, social support, and mental health (i.e. depressive symptomatology).

A third limitation is that this study used cross-sectional data only. It is unclear whether training studies that examined the effects of a vigorous training program on RSA have been similarly affected by ceiling effects. Based on our findings we would expect that the underestimation may not be as severe in training studies, because the heart rate differences between exercisers and non-exercisers in cross-sectional studies is often much larger (10-15 bpm) than the heart rate effects of training (3-5 bpm). Nonetheless, underestimation may still occur in the subset of participants with the largest training-induced decrease in heart rate. In keeping, exercise training studies that did not find a significant training-induced increase in RSA tend to be characterized by significant reductions in heart rate and show low (< 60 bpm) post-training absolute heart rate levels (Boutcher et al., 1995; de Geus et al., 1990a; de Geus et al., 1996; Loimaala et al., 2000; Stein, Ehsani, Domitrovich, Kleiger, & Rottman, 1999; Uusitalo, Laitinen, Vaisanen, Lansimies, & Rauramaa, 2002) whereas training studies that do find an increase in RSA often do not find a significant decrease in resting heart rate, the absolute value of which is in the normal range (around 70 bpm) (Melanson & Freedson, 2001; Sandercock et al., 2005), although there are exceptions (Levy et al., 1998; Schuit et al., 1999). We tentatively conclude that the failure to find a significant increase in RSA in training studies may partly derive from trained individuals with a low post-training heart rate and a quadratic IBI-RSA relationship.

Previous studies using pharmacological manipulation of vagal activity in a laboratory setting had already voiced concerns about the use of RSA as an index of cardiac vagal control under these specific experimental conditions (Goldberger et al., 1994; Goldberger et al., 2001; Levy et al., 1998). The present study shows that these concerns extend to recordings made in normal naturalistic conditions, and suggest that the problem is aggravated in exercisers during nighttime sleep recordings. In about 40% of the regular exercisers cardiac vagal control was underestimated by RSA. This underestimation of vagal control by RSA was not specific to the peak-valley method, since other time domain measures (SDNN, RMSSD) and spectral powers in the low and high frequency range showed an identical pattern. Future studies comparing RSA in exercisers and non-exercisers or studies comparing RSA pre- and post- exercise training should aim to stratify the sample by the shape

of the IBI-RSA relationship. More generally, inspection of the IBI-RSA relationship should be routinely added when using HRV measures as an index of cardiac vagal control.

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CHAPTER 3

Estimated preejection period (PEP) based on the detection of the R-wave and dZ/dt-min peaks does not adequately reflect the actual PEP across a wide range of laboratory and ambulatory conditions.

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Abstract

The current study evaluates the validity of the PEP computed from a fixed value for the Q-wave onset to R-wave peak (QR) interval and an R-wave peak to B-point (RB) interval that is estimated from the R-peak to dZ/dt-min peak (ISTI) interval. Ninety-one participants participated in a 90 minute laboratory experiment in which a variety of often employed physical and mental stressors were presented and 31 further participants participated in a structured 2 hour ambulatory recording in which they partook in natural activities that induced large variation in posture and physical activity. PEP, QR interval, and ISTI were scored and rigorously checked by interactive inspection. Across the very diverse laboratory and ambulatory conditions the QR interval could be approximated by a fixed interval of 40 ms but 95% confidence intervals were large (25.5 to 54.5 ms). Multilevel analysis showed that 79% to 81% of the within and between-participant variation in the RB interval could be predicted by the ISTI with a simple linear regression equation. However, the optimal intercept and slope values in this equation varied significantly across participants and study setting. Bland Altman plots revealed a large discrepancy between the estimated PEP using the R-wave peak and dZ/dt-min peak and the actual PEP based on the Q-wave onset and B-point. We conclude that the PEP estimated from a fixed QR interval and the ISTI could be a useful addition to the psychophysiologist's toolbox, but that it cannot replace the actual PEP to index cardiac sympathetic control.

Introduction

Functional disturbances of the autonomic nervous system have been frequently linked to several diseases (Eckberg, Drabinsky, & Braunwald, 1971; Esler et al., 2000b; Esler, Lambert, & Jennings, 1990; Huikuri et al., 2003; Kleiger et al., 1987; Langewitz, Ruddel, & Schachinger, 1994; Nolan et al., 1998; Nolan et al., 1992; Palatini et al., 2004; Schwartz, La Rovere, & Vanoli, 1992) and hyperactivity of the sympathetic nervous system (SNS) may be an important cause for the detrimental effects of stress on cardiovascular health (Palatini et al., 2004; Schwartz et al., 1992).

Direct recording of action potentials from superficial sympathetic nerves in the muscles and the skin (Wallin et al., 1981; Wallin, Sundlof, & Delius, 1975) or the measurement of organ specific spillover of the post-ganglionic neurotransmitter norepinephrine using radioactive tracers (Esler et al., 1988; Esler et al., 2000b) is extremely valuable for basic research on the sympathetic nervous system. However, when research moves to an epidemiological scale, the expense and invasiveness of these methods becomes prohibitive. Furthermore, these invasive measures restrict research to the confines of a hospital or laboratory setting and are stressful for the participant. This precludes examination of individual differences in sympathetic activity in a natural setting, for instance during sleep or during job-related activities with a substantial mental and emotional load. Nonetheless, it is autonomic control during these naturalistic conditions that may have the largest clinical relevance. It is extremely valuable, therefore, to have non-invasive, unobtrusive measures of sympathetic nervous system activity.

At the moment the preejection period (PEP) is the measure of choice to monitor changes in cardiac sympathetic activity non-invasively. Under conditions of stable preload and afterload, changes in PEP reflect changes in contractility (Newlin et al., 1979) which are influenced by sympathetic but not parasympathetic activity in humans. The extant literature supports changes in PEP as a valid measure of changes in β-adrenergic inotropic drive to the left ventricle. Laboratory studies manipulating β-adrenergic tone in within-participants designs by epinephrine infusion (Houtveen et al., 2005) amyl nitrite inhalation (Mezzacappa et al., 1999; Svedenhag, Martinsson, Ekblom, & Hjemdahl, 1986; Svedenhag, Martinsson, Ekblom, & Hjemdahl, 1991) and adrenoceptor blockade (Nelesen et al., 1999), exercise (Harris et al., 1967; Schachinger et al., 2001; Winzer et al., 1999), emotional stress (Krzeminski et al., 2000; Miyamoto, Nakazono, Hiura, & Abe, 1983b; Smith et al., 1989b) or monetary reward (Berntson et al., 1994a; Newlin et al., 1979; Sherwood et al., 1986) have shown a dose-dependent shortening of the PEP. Between-participant differences in PEP level are stable over time (Richter et al., 2009), show comparable heritability to plasma catecholamine levels (Goedhart et al., 2006; Vrijkotte et al., 2004), and reliably reflect interindividual differences in cardiac sympathetic activity assessed by dual blockade (Kupper et al., 2006; Williams, Puddey, Beilin, & Vandongen, 1993).

PEP can be obtained by simultaneous recording of the thoracic impedance cardiogram (ICG) and electrocardiogram (ECG) (Riese et al., 2003; Willemsen et al., 1996) and is defined as the interval from the onset of left ventricular depolarization, reflected by the Q-wave onset in the ECG, to the opening of the aortic valve, reflected by the B-point in the ICG signal (Labidi, Ehmke, Durnin, Leaverton, & Lauer, 1970; Lozano et al., 2007; Sherwood et al., 1990; Willemsen et al., 1996). Figure 1 displays the ECG and ICG signals with the relevant landmarks. Throughout, the term 'actual' PEP is used to refer to the interval between the ECG Q-wave onset and the ICG B-point. To improve signal quality, PEP is usually scored from the ICG waveform after ensemble averaging over multiple beats, time locked to the R-wave peak. This improves automated detection of the crucial landmarks in the

ECG and in the ICG but even after ensemble averaging substantial errors in positioning of the Q-wave onset and B-point remain (Berntson et al., 2004; Labidi et al., 1970; Lozano et al., 2007; Willemsen et al., 1996). For this reason, visual inspection of the automatically detected Q-wave onset and B-point is needed and, to ensure sufficient reliability, scoring is often repeated by multiple raters. The latter visual inspection can be time-consuming and presents an obstacle to the assessment of PEP in epidemiological studies with thousands of participants or in ambulatory studies collecting data across extended periods of time. In addition, when signal quality of the ICG is compromised, for instance, during unsupervised activities in ambulatory recordings, reliable visual scoring of the B-point is very hard, even when employing multiple raters, leading to the exclusion of a substantial portion of the participants.

Two practical solutions have been proposed to sidestep the difficult detection of the Q-wave onset. The first is to score the more easily detected R-wave onset and add a fixed value for Q-wave duration of 15 ms (Berntson et al., 2004). The R-wave onset was used by Berntson et al. (Berntson et al., 2004) in 30 healthy participants, of which 10 showed no clear Q-wave in a lead II axis ECG derivation. In these participants scoring of Q-wave onset defaulted to the R-wave onset, and it was shown that using the R-wave onset for *all* participants significantly reduced the error variance in the individual differences in the PEP. This suggests that a PEP based on the R-wave onset was more reliable than the actual PEP based on the Q-wave onset, although this was established under resting conditions only. The second solution is to extend this reasoning, and use the R-wave peak instead of the R-wave onset as it is an even more sharply defined landmark in the ECG. This makes the further assumption that the R-wave onset to R-wave peak interval is also reasonably constant. Current practice is to estimate the Q-wave onset by subtracting a fixed value of 48 ms from the time of the R-wave peak (Brydon et al., 2008; Willemsen et al., 1996). To our knowledge the validity of this practice has not been verified.

To assist in the detection of the B-point in the ICG the physiological connection between the timing of the B-point and the dZ/dt-min peak can be exploited. The dZ/dt-min peak (in the literature variously called C-point or Z-point) is a maximum defined by a zero-order crossing in the first derivative of the ICG and can be detected with much more fidelity than the B-point, which is often a (subtle) inflection defined by a zero-order crossing in the second derivative of the ICG rather than a true minimum. Changes in cardiac contractility, the main concept that PEP aims to asses, are reflected in the time it takes the left ventricle to build up sufficient force to open the aortic valve (B-point) but also in the time it takes to reach peak ventricular ejection (dZ/dt-min peak) . Theoretically, the timing of these events should be highly correlated. Empirically, two groups have independently confirmed that the interval between the R-wave peak and the dZ/dt-min peak or the initial systolic time interval (ISTI, see figure 1) is a significant predictor of both the R-wave peak to B-point (RB) interval as well as the actual PEP (Berntson et al., 2004; Lozano et al., 2007; Meijer et al., 2007; Meijer et al., 2008). Meijer et al. (Meijer et al., 2007) found high correlations between ISTI and PEP in the supine position at rest and after light exercise in both old and young healthy adults and a group of older Parkinson patients (Meijer et al., 2007; Meijer et al., 2008; Meijer, Smorenberg, Lust, Verdaasdonk, & Groeneveld, 2010). Lozano et al. (Lozano et al., 2007) used the ISTI in quadratic curve fitting to estimate the RB interval. In 26 young adults their equation ($RB = -31.59 + 1.233 * ISTI + 0.0032 * ISTI^2$) accounted for over 90% of the variance in the actual RB interval during rest, a mental arithmetic task and a speech preparation task in three separate samples.

Taken together, the above suggests that adequate estimation of the PEP could be achieved by the detection of the two most salient features in the ECG or ICG, the R-wave peak and the dZ/dt-

min peak respectively. The estimated PEP is then defined as the sum of a fixed QR interval and an RB interval derived from the ISTI by regression. As this sidesteps the difficult detection of the Q-wave onset and the B-point, PEP estimation could be achieved in a near-complete automated fashion relying less on laborious visual inspection, even in noisy or degraded signals. In spite of these obvious advantages, use of an estimated PEP instead of the actual PEP has not become commonplace in the recent literature. A fixed Q-wave onset to R-wave peak (QR) interval of 48 ms is sometimes employed, but the ISTI has not been used to estimate the RB interval in spite of the encouraging results with this measure (Berntson et al., 2004; Lozano et al., 2007; Meijer et al., 2007; Meijer et al., 2008). Possibly the evidence from the studies published so far is considered to be insufficient to consider estimated PEP as a valid alternative to the actual PEP, as these studies had relatively small samples, were completely confined to a laboratory setting, and used only a few of the many conditions generally employed in psychophysiological studies.

To address these concerns, the current paper reports on two further studies that assessed the estimated and actual PEP scored by multiple raters in participants undergoing a wide variety of controlled experimental stress manipulations in a laboratory setting, and in participants that underwent a supervised ambulatory protocol containing activities that resemble natural daily activities but that induced a large variance in posture and physical activity. First, it was explored whether the QR interval can be approximated by a fixed interval even in very diverse laboratory and ambulatory conditions. Next, multilevel analysis was used to derive an equation to estimate the RB interval from the ISTI and it was tested whether a set of fixed regression coefficients applied to all participants in both laboratory and ambulatory recordings. Finally, Bland Altmann plots were used to test whether the estimated PEP from the R-wave and dZ/dt-min peaks and fixed QR interval adequately predicts the actual PEP across a large set of participants and a wide range of conditions.

Methods

Participants

In total, 91 undergraduate students (20 male, 71 female) with a mean age of 21.7 years (SD = 3.2) and a mean body mass index (BMI) of 22.2 (SD = 2.9) participated in the laboratory study. The participants in the ambulatory study were 31 undergraduate students (11 male, 20 female) having a mean age of 22.0 (SD = 1.9) and a mean BMI of 23.4 (SD = 4.3). Participants to both studies did not report any psychiatric diseases or cardiovascular problems and none were using cardioactive medication (e.g. antihypertensives) or any other kind of medication. They gave their written consent prior to participation. All participants were volunteers and received study credits or a 10 € gift voucher for their participation.

General Procedures

Laboratory study

The participants were asked to refrain from smoking and alcohol- or caffeine-containing beverages the evening before the test day and on the morning of laboratory testing. The experimental sessions took place between 9 a.m. and 4 p.m. and lasted approximately 90 minutes. ECG and ICG leads were attached to the participants using 6 pregelled Ag/AgCl spot electrodes (Ultratrace, Cosmed, USA) after which they were seated in front of a 19” monitor in a dimly lighted,

electrically-shielded, sound-attenuated cabin. The experimental session commenced with some general instructions and a brief period of rest in which optimal signal quality was ensured. To elicit variation in SNS activity, various experimental physical and mental stressors were presented in a fixed order as outlined in figure 2. The participant was monitored by a webcam to ensure safety as well as compliance with experimental instructions.

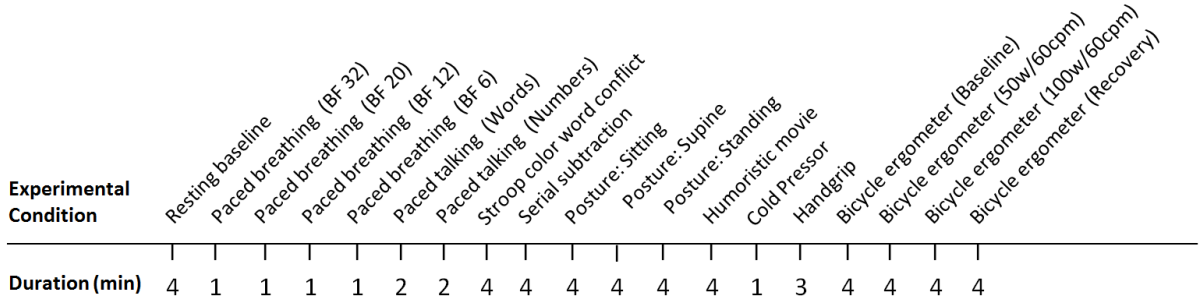

Figure 2. *Experimental protocol of the laboratory study.*

Resting baseline: Participants sat quietly for 4 minutes with their eyes open. The computer screen depicted an empty sandy palm beach or a calm lake in a lush green forest. Paced breathing: Participants were asked to breath in concert with a visual metronome on the computer screen at 32 and 20 cpm, two frequencies that are above the typical spontaneous breathing frequency (14 cpm), at 12 cpm, and at 6 cpm, a rate much lower than the typical spontaneous breathing frequency. Each breathing frequency was maintained for 1 minute. Paced talking: Participants were asked to first read out color words (2 minutes) and then numbers (2 minutes) that were presented on the computer screen in a frequency corresponding to the trial speed in the actual Stroop/Subtraction tasks. Stroop word color conflict: Participants were shown the names of colors printed in conflicting ink colors (e.g. the word "blue" in red ink) on the computer screen and asked to verbally identify the color of the ink rather than the word as fast as possible. Mental load is created by the interference between the discrepant ink color and the color name. The experimenter would correct the participant over the intercom in case of a wrong answer (‘WRONG”). The experimental condition lasted 4 minutes. Serial Subtraction: The participants were presented with a starting number 1248 and were asked to continuously subtract 7 from this number (speaking out loud) until the 4 minute test period ended. Whenever a participant gave the wrong answer, the experimenter stated the correct number from which the participant then had to restart over the intercom (‘WRONG, the correct number was ...’). Posture: Participants were asked to sit down quietly for 4 minutes, followed by lying down quietly for 4 minutes (Supine) and finally to stand quietly for 4 minutes (Standing). The postural transitions were kept below 5 seconds. Humoristic movie: Watching a humoristic movie (4 minutes). The participant got a choice of 4 short humoristic movies. They choose one on a participant of their own liking and sat to watch it for 4 minutes. Cold pressor: The cold pressor test used in this experiment consisted of a 3–5 °C ice bath, composed of tap water and melted ice held in a small plastic container. The container was placed adjacent to the participant on a table. The participant was asked to submerge the dominant hand up to the wrist joint and to hold the fingers in a relaxed position. After exactly 60 sec the hand was removed from the bath. In this condition the experimenter was present in the cabin throughout to ensure compliance. Hand grip: During practice and instruction, maximum grip strength in the dominant hand is established in two separate attempts with a hand grip dynamometer. During the actual hand grip task, participants maintain

isometric contraction at 30% of their maximum voluntary contraction for a period of 3 minutes in sitting position. A dial on the dynamometer indicates deviations of the target force, upon which the experimenter indicated to restore force. In this condition the experimenter was present in the cabin throughout to ensure compliance. Bicycle ergometer: Participants were asked to sit quietly on the bicycle ergometer for 4 minutes before the final exercise condition. Then 4 minutes of biking on the bicycle ergometer at a resistance of 50 Watt with 60 rotations per minute and 4 minutes at 100 Watt with 60 rotations per minute followed. In the second half of the sample, a 4 minute recovery period while still sitting on the bike was added, that followed immediately after biking on the bicycle ergometer at 100 Watt (N = 46 participants).

Ambulatory study

The participants were asked to refrain from smoking and alcohol- or caffeine-containing beverages the evening before the test day and in the morning before coming to the laboratory. All standardized experimental sessions took place approximately 2 hours after wakening and lasted 2 hours. The ECG and ICG leads were attached to the participants using 5 pregelled Ag/AgCl spot electrodes (Ultratrace, Cosmed, USA), after which they were seated in front of a dimly lighted, electrically-shielded, sound-attenuated cabin to measure a resting baseline. Next, a sequence of protocolized normal daily activities in a supervised ambulatory setting was performed in the lab, outdoors, and in the university sports centre. The protocol aimed to create variations in posture and intensity of physical and mental activity in close resemblence to normal daily activies. Figure 3 outlines the experimental protocol for the ambulatory study .

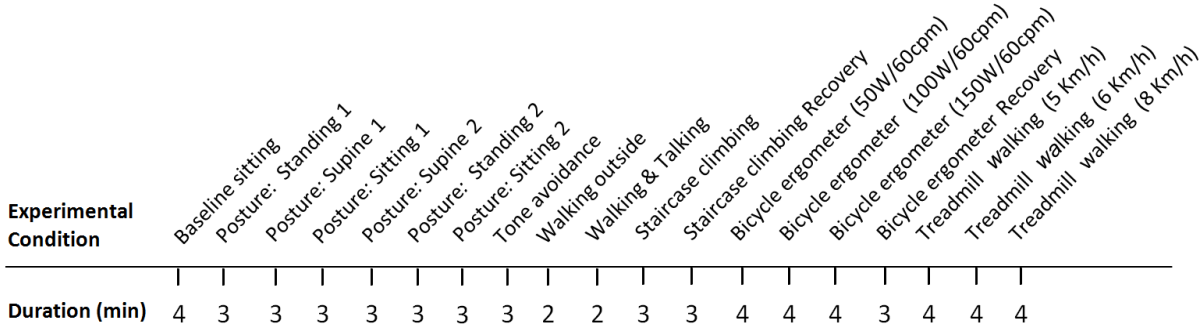

Figure 3. *Experimental protocol of the ambulatory study.*

Resting baseline: 4 minutes of quietly sitting in a chair in a laboratory room. Posture: Several postures were measured in a laboratory room; 3 minutes of standing; 3 minutes in supine position on a stretcher; 3 minutes of sitting in a chair; 3 minutes in supine position on a stretcher; 3 minutes of standing; 3 minutes of sitting in a chair. Tone Avoidance task: The tone avoidance task is four-choice reaction time task where participants have to push the appropriate button within 550 ms to avoid a loud noise (1000 Hz, 85 dB). A cross appears in one of te corners of the screen and the opposite diagonal button on a corner of a sqare keypad has to be pushed. This task is know to elicit strong mental load and was performed in a laboratory room. Standardized physical daily activities: These activities were performed on the way to the VU sports centre where the standardized physical activities were performed; 2 minutes of quietly walking outside; 2 minutes of walking outside while talking; 3 minutes of climbing stairs (7 stores up and down); 3 minutes of quietly sitting as a recovery.

Standardized physical activities: The participants performed the bicylce ergometer test and treadmill test at the VU-sport centre. Three bicycle ergometer tests were done, each for 4 minutes at 60 cycles per minute at 50W, 100W and 150W respectively followed by 3 minutes of sitting quietly as a recovery. Three 4 minute treadmill conditions were done at 5 km/h, 6 km/h and 8 km/h respectively.

In both studies, participants reported to the VU University Amsterdam on the day of testing and were given a brief description of the procedures and an informed consent was signed. A short standardized interview was used to obtain demographic information, smoking behaviour, exercise behaviour, regular medication use, and to verify that they had no current psychiatric complaints (Beck depression scale < 4) or cardiovascular disease. Height, and weight were measured using standard procedures.

Physiological recordings

Laboratory study

The ECG and ICG signals in the laboratory study were continuously recorded at a sample rate of 1000HZ with use of the ECG100C and NICO1000C modules of the BioPac data-acquisition system (BioPac systems INC, Santa Barbara, CA). Cleaning of the skin with alcohol before electrode application ensured that electrode resistance was kept low. The first ECG electrode (V-) was placed slightly below the right collar bone 4 cm to the right of the sternum. The second ECG electrode (V+) was placed at the apex of the heart over the ninth rib on the left lateral margin of the chest approximately at the level of the processus xiphodius. The third ECG electrode (GND) is a ground electrode and was placed on the right side, between the lower two ribs at the right abdomen. The first ICG measuring electrode (V_1) was placed at the top end of the sternum, between the tips of the collar bones. The second ICG measuring electrode was placed at the xiphoid complex of the sternum, where the ribs meet. The two current electrodes were placed on the back: I- on the spine over the cervical vertebra C4, at least 3 cm (1") above the ICG measuring electrode V-, and I+ between thoracic vertebraes T8 and T9 on the spine, at least 3 cm (1") below the ICG measuring electrode V_2. This electrode placement was used in many previous studies that attest to high intra day (Kupper et al., 2006) and day-to-day reliability (Vrijkotte et al., 2004) of the systolic time intervals derived from this placement as well as good temporal stability across a two year period (Goedhart et al., 2006) and the ability to discriminate low and high chronic work stress (Vrijkotte et al., 2004).

Ambulatory Study

The electrocardiogram (ECG) and the impedance cardiogram (ICG) of the ambulatory study were recorded continuously with the 5-lead version of the VU-AMS5fs device (VU University, Amsterdam, www.vu-ams.nl). This device was developed to study autonomic nervous system activity in naturalistic settings (de Geus et al., 1995; Willemsen et al., 1996). Cleaning of the skin with alcohol before electrode application ensured that electrode resistance was kept low. A single dedicated ECG electrode (V+) was placed at the apex of the heart over the ninth rib on the left lateral margin of the chest approximately at the level of the processus xiphodius. The first ICG measuring electrode (V-) was placed at the top end of the sternum, between the tips of the collar bones . This electrode also functions as the first ECG (V-) electrode. The second ICG measuring electrode was placed at the xiphoid complex of the sternum, where the ribs meet. The two current electrodes were placed on the back: I- on the spine over the cervical vertebra C4, at least 3 cm (1") above the ICG measuring

electrode V_1, and I+ between thoracic vertebraes T8 and T9 on the spine, at least 3 cm (1") below the ICG measuring electrode V_2.

Signal Analyses and Data Reduction

The Acknowledge algoritm was used on the laboratory data to detect all relevant landmarks in the ECG including the Q-wave onset and the R-wave peak. The algorithm incorporates the open source OSEA QRS detector and beat classification library provided by EP Limited (http://www.eplimited.com). The QR interval was tested for outliers at the single beat level by using a within-participant criterion of 2 standard deviations, and visual inspection with random sampling was used to confirm proper operation of the Acknowledge algoritm across experimental conditions. The mean QR interval was then computed per condition across all beats with a valid Q-wave onset.

For detection of the Q-wave onset in the ambulatory data, the ECG was imported into the VU-AMS5fs software. For each experimental condition the interbeat interval (IBI, ms) was scored from the R-wave peaks in the ECG. The IBI time series was visually inspected and missed or incorrect R-wave peaks were interactively corrected; bad ECG signal fragments were removed. The mean Q-wave onset for each ambulatory condition was visually scored in the R-wave peak locked ensemble averaged ECG across all valid beats in that condition by two raters. Post-scoring, the raters chose a consensus for the points that did not overlap, and these were retained for the analyses.

The procedures to score the ISTI were identical for both the ambulatory and laboratory study. First, the ICG and ECG signals were imported into the VU-AMS software. After obtaining the corrected IBI time series, interactive visual scoring of the R-wave peak locked ensemble averaged ICG signal from all valid beats was used to mark the B-point and the dZ/dt-min peak in each condition (see figure 1 for an example). The actual PEP was computed as the interval from the Q-wave onset in the ECG to the B-point in the ICG signal. The ISTI was computed as the time interval between the R-wave peak in the ECG and the dZ/dt-min peak in the ICG (Meijer et al., 2007; Meijer et al., 2010). To allow computation of the inter rater reliability, two raters independently scored the B-point and dZ/dt-min peak. Post-scoring, the raters chose a consensus for the points where their judgement did not overlap, and these were retained for the analyses.

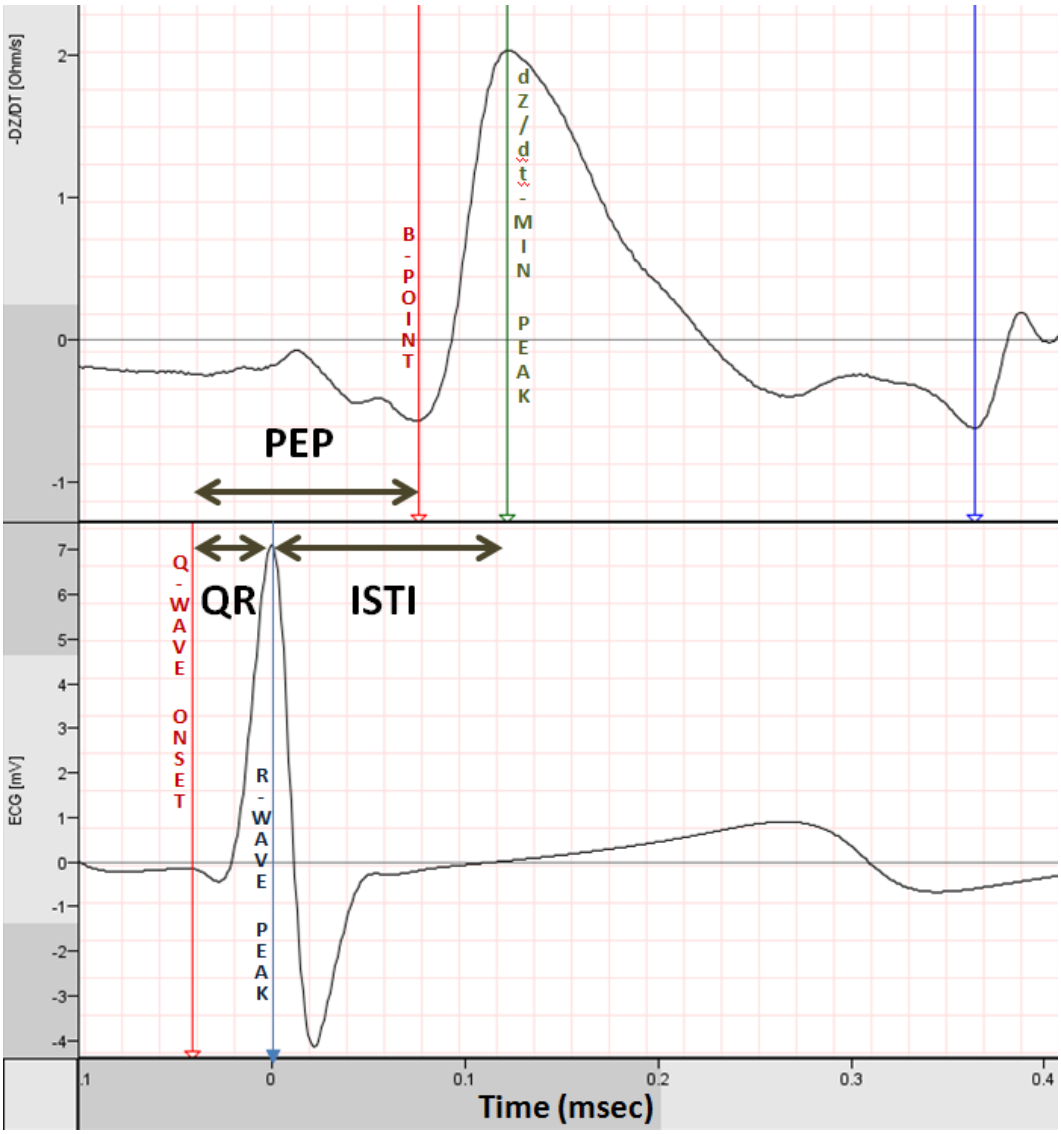

Figure 1. *The impedance cardiogram top) and the electrocardiogram (bottom) with the four landmarks defining the PEP (Q-wave onset to B-point) and the ISTI (R-peak to dZ/dt-min peak).*

Statistical Analyses

For both laboratory and ambulatory studies a mixed ANOVA was used to test for the effect of condition (fixed effect) on IBI, ISTI and the actual PEP to verify succesful manipulation of autonomic tone within participants. Mixed ANOVA was further used to test whether the QR-interval changed over conditions. Post-hoc T-tests were performed for the four variables on *a priori* defined contrasts. In the laboratory the pre-test resting baseline was compared to paced breathing, paced talking, stroop colour conflict, serial subtraction, stress recovery, orthostatic manipulations, humoristic movie, and the handgrip and cold pressor tests, and the ergometer baseline level was compared to the bicycle ergometer (50W and 100) and the bicycle ergometer recovery conditions. In the ambulatory study, the resting baseline condition at the start of the recording was compared to all other conditions.

To test whether the QR interval can be approximated by a fixed interval, a oneway ANOVA compared the QR intervals across study setting (laboratory vs ambulatory) in the resting sitting

baseline condition. Next the grand averaged QR interval across all participants and all laboratory and ambulatory conditions was subtracted from the actual QR interval values observed and the distribution of the difference scores was used to compute the mean error as well as its the 95% confidence intervals.

To compute interrater reliability of the Q-point, B-point and dZ/dt-min peak scoring, intraclass correlation coefficients (ICCs) were computed between rater 1 and rater 2 using a random effects model (absolute agreement) on the values of for the RB interval and the ISTI. This was done separately for each of the conditions across all participants (per-condition inter rater reliability) and for each of the participants across all conditions (per-participant inter rater reliability).

Multilevel analysis was then used to establish the optimal regression equation to predict the RB interval from the ISTI. Multilevel analysis is a general method of analyzing data with a hierarchical or clustered structure (Snijders & Bosker, 1999). Here different conditions were clustered within participants. RB was considered to be a function of ISTI and a random error term:

$$RB_{ij} = B0_j + B1_j * ISTI_{ij} + \varepsilon_{ij} \qquad (1)$$

with i indexing the lower level of repeated samples and j indexing the higher level of the participants. Regression equation (1) defines the relation between RB and ISTI within each of the participants. Coefficient B0j and B1j are the participant-specific intercept and slope which, i.e. $B0_j = g00 + \varepsilon 0_j$ and $B1j = g10 + \varepsilon 1_j$, where g00 and g10 are the fixed mean intercept and slope across all participants, whereas the random coefficients $\varepsilon 0_j$ and $\varepsilon 1_j$ vary across participants. Previous research suggested that a single equation can be used for all participants (Lozano et al., 2007), which means that models with a random slope and intercept should not provide a significant better fit than the more parsimonious model with a fixed intercept and slope. This was explicitly tested by comparing the explained variance and the fit of the model with freely estimated slopes and intercepts to that of the model with fixed values. Explained variance in the RB interval was computed with the following formula suggested by Blackwell et al: (unrestricted error – restricted error) / unrestricted error (Blackwell, de Leon, & Miller, 2006). The deviance fit test, or likelihood ratio test, was used to compare the fit of two regression models. This test is based on the difference between the deviance statistics of the two models, which has a chi-square distribution with degrees of freedom equal to the difference in the number of parameters estimated in the models being compared. If the equation with a random slope/intercept fitted better, sex, age and BMI were added as potential predictors in the level 2 model to see whether these variables could account for the individual variance in the intercept and slope. The multilevel analysis was repeated separately for laboratory and ambulatory conditions, which allowed a comparison of the parameter estimates obtained in different study settings.

Finally, the estimated PEP was computed for each condition for each individual in both studies by summing the grand averaged QR interval to the RB interval estimated from the ISTI using the slope and intercept parameters from the best fitting model in the laboratory study. A Bland-Altman analysis was used to test the absolute agreement between the estimated PEP and the actual PEP across all laboratory and ambulatory conditions.

Results

Effects of the Experimental Manipulations

The means and standard deviations for IBI, PEP, QR interval and ISTI are presented per experimental condition in table 1 (for the laboratory study) and table 2 (for the ambulatory study).

Table 1. *Means and standard deviation for IBI, actual PEP, QR interval and ISTI (all in ms) in the laboratory study. Change scores are given from resting baseline for all experimental conditions, but change scores for bicycle ergometer are compared to bicycle ergometer baseline values. The bicycle recovery was compared to the bicycling at 100W/60cpm condition.*

Experimental conditions	IBI	Δ IBI	PEP	Δ PEP	QR	Δ QR	ISTI	Δ ISTI
	Mean (SD)		Mean (SD)		Mean (SD)		Mean (SD)	
Resting Baseline	845 (111)	NA	107 (18)	NA	38 (7)	NA	127 (17)	NA
Paced breathing (BF32)	803 (114)	-42*	101 (18)	-6*	39 (7)	1*	121 (17)	-6*
Paced breathing (BF20)	805 (112)	-40*	105 (17)	-2*	38 (7)	0	123 (17)	-4*
Paced breathing (BF12)	803 (104	-42*	105 (17)	-2*	38 (7)	0	126 (16)	-1
Paced breathing (BF6)	825 (93)	-20*	107 (18)	0	39 (7)	1*	127 (16)	0
Paced talking (Words)	780 (106)	-65*	109 (18)	2	38 (8)	0	128 (18)	1
Paced talking (Numbers)	757 (97)	-88	107 (21)	0	39 (8)	1*	123 (19)	-4*
Stroop color word conflict	758 (97)	-87*	104 (19)	-3*	39 (7)	1*	121 (19)	-6*
Serial subtraction	748 (101)	-97*	104 (20)	-3*	39 (7)	1*	120 (19)	-7*
Posture: Sitting	859 (108)	14	108 (18)	1	38 (7)	0	128 (16)	1
Posture: Supine	963 (126)	117*	95 (19)	-12*	38 (6)	0	121 (21)	-6
Posture: Standing	723 (83)	-123*	117 (16)	10*	38 (7)	0	138 (16)	11*
Humoristic movie	875 (123)	29*	108 (17)	1	39 (7)	1*	124 (17)	-3
Cold pressor	836 (117)	-9	110 (19)	3	40 (7)	2*	126 (17)	-1
Handgrip	840 (119)	-5	112 (17)	5*	40 (8)	2*	128 (17)	1
Bicycle ergometer (Baseline)	833 (109)	NA	115 (17)	NA	39 (8)	NA	133 (17)	NA
Bicycle ergometer (50W/60cpm)	598 (78)	-235*	77 (20)	-38*	43 (9)	4*	94 (19)	-39*
Bicycle ergometer (100W/60cpm)	504 (80)	-329*	66 (17)	-49*	43 (10)	4*	82 (180)	-51*
Bicycle ergometer (Recovery)	715 (116)	211*	90 (25)	24*	38 (7)	-1*	118 (22)	36*

**= significantly different compared to the appropriate baseline, in Bonferroni-corrected post hoc tests ($p < 0.05/18 = .0028$).*

Table 2. *Means and standard deviations for IBI, actual PEP, QR interval and ISTI (all in ms) in the ambulatory study. Change scores are given from resting baseline for all experimental conditions.*

Experimental conditions	IBI	Δ IBI	PEP	Δ PEP	QR	Δ QR	ISTI	Δ ISTI
	Mean (SD)		Mean (SD)		Mean (SD)		Mean (SD)	
Baseline sitting	784 (98)	NA	112 (17)	NA	43 (6)	NA	118(19)	NA
Posture: Standing1	674 (84)	-110*	117 (16)	5*	41 (6)	-2*	123 (17)	5*
Posture: Supine1	900 (117)	114*	108 (21)	-4	44 (6)	1	120 (19)	2
Posture: Sitting 1	787 (97)	3	113 (17)	1	43 (5)	0	122 (18)	4*
Posture: Supine2	899 (120)	115*	114 (25)	2	44 (6)	1	127 (19)	9*
Posture: Standing 2	691 (79)	-92*	119 (14)	7*	41 (6)	-2	125 (18)	8*
Posture: Sitting 2	808 (105)	24*	117 (13)	5*	44 (6)	1	123 (15)	5*
Tone Avoidance Task	755 (99)	-29*	110 (15)	-2	43 (7)	0	115 (17)	-2
Walking outside	597 (65)	-187*	86 (13)	-36*	43 (7)	0	88 (16)	-30*
Walking & Talking	587 (69)	-197*	85 (11)	-37*	43 (7)	0	87 (13)	-31*
Staircase Climbing	430 (43)	-354*	66 (9)	-46*	41 (6)	-2	61 (14)	-57*
Staircase Climbing Recovery	642 (124)	-142*	80 (16)	-32*	42 (6)	-1	82 (15)	-36*
Bicycle ergometer (50W/60cpm)	557 (124)	-227*	88 (18)	-24*	43 (7)	0	90 (17)	-28*
Bicycle ergometer (100W/60cpm)	463 (76)	-321*	73 (14)	-39*	41 (5)	-2	75 (19)	-43*
Bicycle ergometer (150W/60cpm)	397 (48)	-387*	63 (12)	-49*	40 (6)	-3	57 (15)	-61*
Bicycle ergometer recovery	505 (86)	-279*	79 (19)	-33*	40 (5)	-3	77 (23)	-41*
Treadmill walking (5km/h)	511 (83)	-273*	80 (14)	-32*	42 (6)	-1	78 (19)	-40*
Treadmill walking (6km/h)	486 (77)	-298*	76 (13)	-36*	42 (6)	-1	79 (17)	-39*
Treadmill walking (8km/h)	400 (51)	-384*	72 (19)	-40*	41 (5)	-2	67 (26)	-51*

**= significantly different compared to the baseline, in Bonferroni-corrected post hoc tests (p < 0.05/18 =.0028).*

Laboratory study

Mixed ANOVA showed a significant effect of experimental condition on IBI ($F(17, 154.5) = 110$, $p < .001$), PEP ($F(17, 140.2 = 73$, $p < .001$), and ISTI ($F(17, 144.8 = 38$, $p < .001$). As expected IBI, PEP, and ISTI were found to decrease significantly over baseline levels during conditions known to increase cardiac sympathetic activity, i.e. during mental stress invoked by the Stroop color word and serial subtraction conditions and during dynamic exercise on the bicycle ergometer. IBI, PEP, and ISTI also clearly evidenced the well-known decrease in cardiac sympathetic activity during recovery from

exercise. Significant changes in IBI, PEP, and ISTI also suggest an increase in cardiac sympathetic activity during forced breathing at high frequencies and during the reading aloud of numbers and words at a forced speed. Lying down and standing up caused the expected decrease and increase respectively in heart rate, but the ISTI and PEP showed the expected reversed pattern, which is caused by preload (ventricular filling pressure) and afterload (systemic vascular resistance) effects (Houtveen et al., 2005). The cold pressor test, the handgrip test and the funny movie did not lead to major changes in IBI, PEP or ISTI.

The mean QR interval ranged from 37.9 to 42.7 ms across conditions with a median value of 38.7 ms and a mean of 39.1 ms. The standard deviation was substantial and averaged 7.5 ms across all conditions with the largest variance in QR interval seen during exercise.

Ambulatory study

Mixed ANOVA showed a significant effect of experimental condition on IBI ($F(18, 29.7) = 194$, $p < .001$), PEP ($F(18, 27.9) = 103$, $p < .001$) and ISTI ($F(18, 55.2) = 66.7$, $p < .001$). As expected IBI, PEP and ISTI were found to decrease significantly over baseline levels during conditions known to increase cardiac sympathetic activity, i.e. daily physical activities and during dynamic exercise on the bicycle ergometer and treadmill. IBI, PEP and ISTI also clearly evidenced the well-known decrease in cardiac sympathetic activity during recovery from exercise. Lying down and standing up caused the expected decrease and increase respectively in heart rate while PEP and ISTI again showed the expected reversed pattern.

The mean Q-wave onset to R-wave peak interval ranged from 39.7 to 43.8 ms across conditions with a median value of 42.3 ms and an mean of 42.0 ms. As in the laboratory condtions, the standard deviation was substantial and averaged 6.4 ms across all conditions. Overall interrater reliability of the Q-point scoring was .89.

Using a fixed Q-wave onset to R-peak interval

Mixed ANOVA showed a significant effect of experimental condition on the QR interval in the laboratory study ($F(17, 145{,}1) = 2.7$, $p < .001$) but not in the ambulatory study. Baseline values were significantly different across study settings ($F(1, 119) = 8.9$, $p = .003$). Of note, the QR intervals in both settings were at least one standard deviation lower than the often employed fixed value of 48 ms. To obtain the best possible value for a fixed QR interval we computed the weighted average of both studies of $((39.1 * 91) + (42 * 31))/122 = 39.8 \approx 40$ ms. We substracted this from the observed QR intervals in both settings to get an impression of the error made by using a fixed interval. Figure 4 depicts the distribution of differences between the actual QR intervals minus the fixed estimate of 40 ms. The mean error is acceptably close to zero (-0.03 ms) but the 95% confidence intervals (-14.5ms – 14.5ms) are substantial.

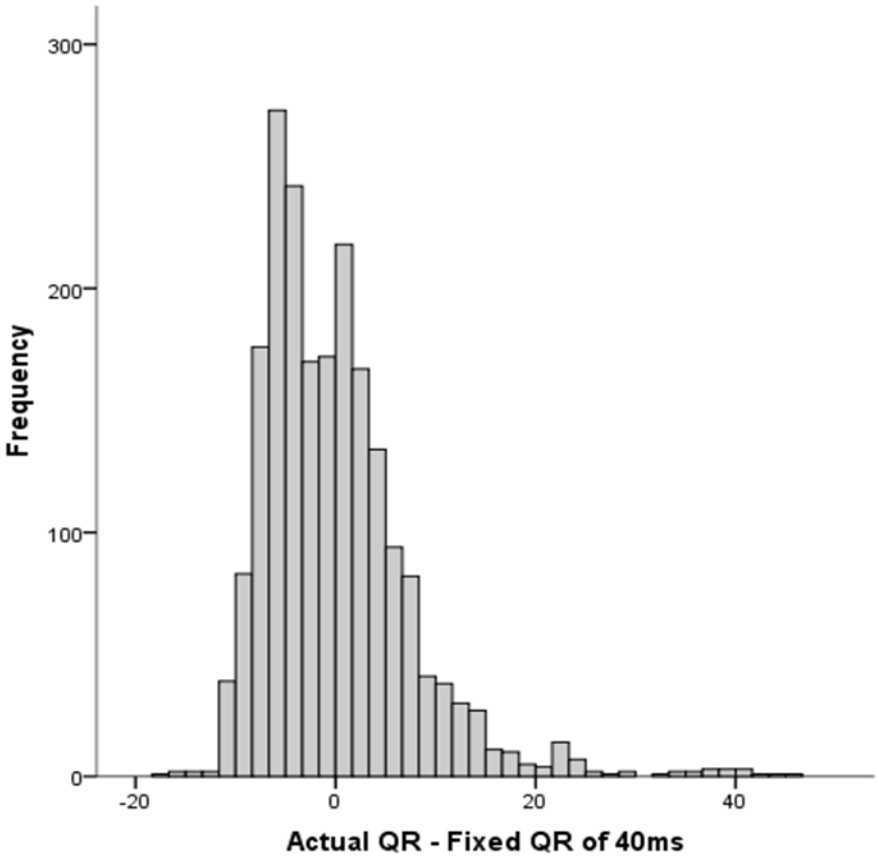

Figure 4. *Distribution of the difference between the measured QR interval and a fixed QR value of 40ms.*

Estimation of the R-wave peak to B-point interval using the ISTI

Mean per-condition interrater reliability for the B-point (using the RB interval, i.e. correlating the RB interval of rater 1 with the RB interval of rater 2 across all participants for each condition) was .92 in the laboratory conditions (from .85 during bicycle ergometer at 100W/60cpm to .99 in bicycle ergometer recovery) and .74 in the ambulatory conditions (from .21 during lying to .99 during the ergometer bike test at 150W/60cpm). For the dZ/dt-min peak (using the ISTI) it was 0.99 in the lab (from .96 during lying to 1.00 in 8 of the other conditions) and .85 (from .48 during walking at 6 Km/h to 1.00 during baseline sitting) in the ambulatory study. The lower per-condition interrater reliability for the B-point compared to dZ/dt-min peak was confirmed in a lower per-participant interrater reliability (e.g. correlating RB/ISTI of rater 1 with RB/ISTI of rater 2 across all conditions for each participant). In the laboratory study, mean per-participant interrater reliability was .92 for the B-point (ranging from .73 to .98) versus 1.00 for the dZ/dt-min peak (ranging from .93 in the 1.00). In the ambulatory study it was .85 for the B-point (ranging from .64 to .98) versus .92 for the dZ/dt-min peak (ranging from .89 to .99).

Table 3 gives the results of the multilevel analysis that was used to examine the relationship between the RB interval and the ISTI. The linear model using ISTI as a predictor of RB with a random intercept and a fixed slope explained 72.2 % of the variance in the RB interval and had a significant better fit than the null model without ISTI, $\chi^2(1) = 2155$, $p < .001$. Allowing individual differences in the regression of ISTI on PEP slightly improved prediction further. This extended linear model with a random intercept and a random slope, *RB = -46.39 + (0.9 * ISTI)*, explained 79 % of the total variance in the RB interval and had a better fit than both previous models, $\chi2(2) = 2276$, $p < .001$ and $\chi2(1) =$

121, p < .001 respectively. Adding a quadratic term improved the model (χ2 (1) = 28, p < .001) but added only .7% to the explained variance. Sex and age and BMI were also added but did not significantly contribute to the individual differences in the slopes or intercepts (model not shown).

Table 3. *Multilevel results for predicting the RB interval from the ISTI in the laboratory study.*

	Null model (without ISTI)		Random intercept and fixed slope for ISTI		Random intercept and random slope for ISTI		Random intercept and random slope with quadratic term.	
		SE		SE		SE		SE
Fixed effects								
Intercept (B0)	64.15	1.47	-43.74	1.82	-46.39	3.32	-82.35	7.44
ISTI (B1)			0.89	0.013	0.90	0.03	1.54	0.12
ISTI squared (B2)							-0.003	0.001
Random effects								
level 1 residual	238.03	8.74	59.21	2.17	50.05	1.9	48.40	1.8
Level 2 residual								
intercept	181.21	28.27	68.64	10.7	771.18	147.72	1085.90	193.8
slope					0.04	0.01	0.06	0.011
Log likelihood	13329.11		11173.93		11053.28		11025.91	
Extra degrees of freedom			1		1		1	
Explained variance (%)			72.2%		79%		79.7%	

When we repeated the multilevel analysis for the ambulatory study (table 4), the linear model with a random intercept and a random slope explained 81 % of the variance in RB and again fitted the data bettter than a model with fixed parameters for all participants (67.4% explained variance), but as can be seen from the standard errors of the estimates, the slope and intercept were significantly different from the laboratory study: *RB = -15.04 + (0.70*ISTI).* This does not bode well for the generalisability of a single set of parameters to estimate the RB interval from the ISTI across different study settings.

Table 4. *Multilevel results for predicting the RB interval from the ISTI in the ambulatory study*

	Null model (without ISTI)		Random intercept and fixed slope for ISTI		Random intercept and random slope for ISTI		Random intercept and random slope with quadratic term.	
		SE		SE		SE		SE
Fixed effects								
Intercept (B0)	52.119	1.59	-14.91	2.07	-15.04	2.25	3.73	5.28
ISTI (B1)			0.69	0.02	0.70	0.03	0.27	.11
ISTI squared (B2)							0.002	0.001
Random effects								
level 1 residual	472.53	19.13	101.35	6.25	89.85	5.7	86.83	5.51
Level 2 residual								
intercept	181.21	28.27	68.64	10.7	771.18	147.72	1085.90	193.8
slope					0.02	0.01	0.02	0.01
Log likelihood	5044		4226		4190		4175	
Extra degrees of freedom			1		1		1	
Explained variance (%)			79%		81%		82%	

Figure 5 depicts the crucial test of whether the estimation of the PEP based on the detection of the R-peak and dZ/dt-min peaks adequately reflects the actual PEP across a wide range of laboratory and ambulatory conditions. Estimated PEP was calculated as the weighted average QR of the laboratory and ambulatory data plus the RB estimated from the regression equation from the best multilevel fit on the laboratory (exploratory) data, with the ambulatory data acting as the confirmatory set. Hence, PEP was estimated as: *40 + (-46.39 + (0.9 * ISTI))*. The mean difference between the actual PEP and the estimated PEP in the laboratory study was 8.3 ms, and in the ambulatory study -3.6 ms. The 95% confidence intervals were very large, ranging from –19.9 to 36.5 ms for the laboratory study and from -25.3 to 17.9 ms in the ambulatory study.

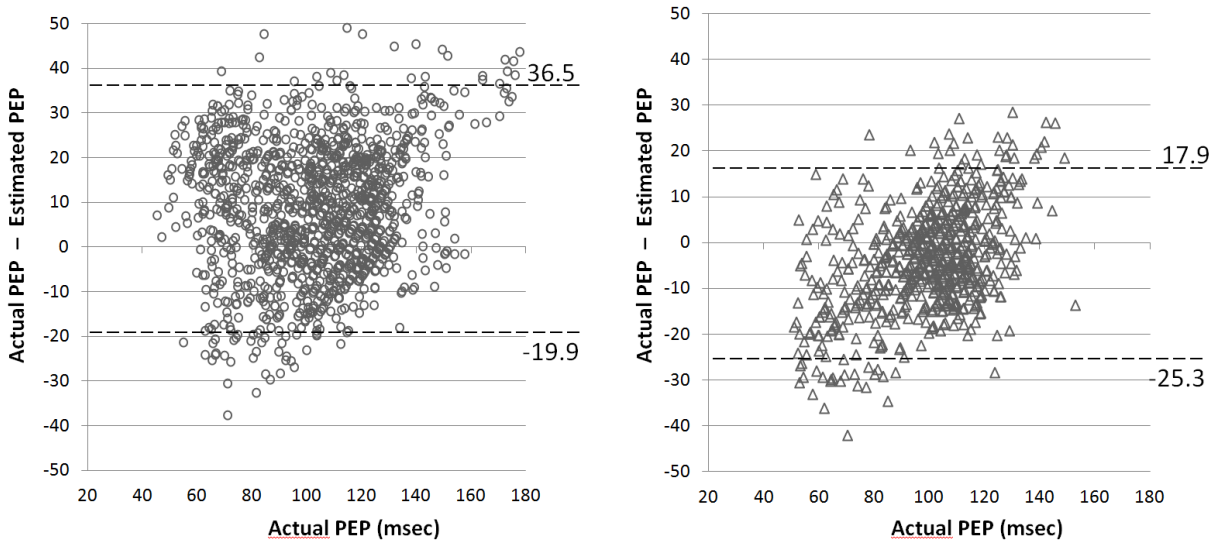

Figure 5. *Bland-Altman plots of the difference between the actual PEP based on the ECG Q-wave onset and the ICG B-point and the estimated PEP based on the ECG R-wave and ICG dZ/dt-min peaks. The difference is plotted as a function of the absolute value of the actual PEP for the laboratory study (left) and the ambulatory study (right). Dotted lines represent the 95% confidence intervals around the mean difference.*

Discussion

Two studies in different samples and settings evaluated the validity of an estimated PEP computed as the sum of a fixed QR interval and an RB interval predicted from the R-wave and dZ/dt-min peaks. These peaks represent the two most clear landmarks in the ECG and ICG respectively and are detected with greater ease and reliability than the Q-wave onset in the ECG and B-point in the ICG that represent the actual physiological events defining the PEP as index of sympathetic control over cardiac contractility. We found substantial discrepancies between the estimated PEP using the R-wave and dZ/dt-min peaks and the actual PEP based on the Q-wave onset and B-point in both study settings. In the laboratory study, at least 84% of the differences between the estimated PEP and the actual PEP exceeded 3.5 ms which was the mean reactivity found to two often used tasks in laboratory stress studies. This is an unacceptable large error of estimation.

About half of the error is due to the use of a fixed QR interval. We found an mean QR interval of 38 ms across a wide range of laboratory conditions. The QR interval in the ambulatory study was

around 42 ms, and the value was significantly larger than in the laboratory. The mean value across both settings was 40 ms which is in good agreement with previous studies. For instance, Goldberger and Bhargava (Goldberger & Bhargava, 1983) reported a QR interval of around 37 ms at rest which decreased by a few ms during exercise. Of note, the QR intervals in both laboratory and ambulatory setting were at least a standard deviation lower than the value of 48 ms often employed to compute PEP. The 48 ms estimate seems to originally derive from a paper that used a 250 Hz sampling of the ECG (Willemsen et al., 1996) which means that precision of the Q-wave detection was only 4 ms.

Apart from the QR interval the estimation of the RB interval from the ISTI also strongly contributed to the difference between estimated and observed PEP. Here, our findings are in clear contrast to previous reports showing the ISTI to be a significant predictor of both the R-wave peak to B-point (RB) interval as well as the actual PEP (Lozano et al., 2007; Meijer et al., 2007; Meijer et al., 2008). Specifically, Lozano et al. (Lozano et al., 2007) found that the equation $RB = -31.59 + 1.233 * ISTI + 0.0032 * ISTI^2$ accounted for 95% of the variance in the actual RB interval during rest, a mental arithmetic task and a speech preparation task in a discovery sample of 26 healthy participants after exclusion of 7 participants without 'a clear B-point upstroke'. The same equation also predicted the RB with high precision in two new samples of 9 adolescents and 15 middle-aged participants, after exclusion of 1 and 4 additional participants respectively based on the difficult B-point. Our results disagree with these earlier findings as different intercepts and slopes were found (even when using the quadratic solution) in both the laboratory ($RB = -84 + 1.58 * ISTI -0.003 * ISTI^2$) and ambulatory ($RB = 3.7 + 0.27 * ISTI + 0.002 * ISTI^2$) setting compared to the previously suggested regression parameters. Also, the explained variance in the observed RB by the estimated RB was only 79% and 81%.

Our study was intended to resemble real data collections as closely as possible making it differ from the earlier study by Lozano (Lozano et al., 2007) in four important respects. First, more participants were included in total (41 vs. 122) creating more room for between-participant variance. Secondly, *no* participants were excluded; we simply scored the B-point as good as we could in all participants. Thirdly, we used multilevel analysis to estimate the regression equation, which takes into account within-participant as well as between-participant variation, whereas Lozano and colleagues based their regression on between-participant variation only. Finally, and potentially most importantly, we used a laboratory and ambulatory setting and used a wider range of conditions in both settings. All conditions were selected as being regularly used in experiments on cardiac autonomic function. We even included conditions that are known to invoke apparently paradoxical changes in the PEP caused by strong afterload and preload effects (e.g. standing and cold pressor test), because the estimated PEP should behave as the actual PEP even in these conditions. With the more complete sampling from the universe of conditions in which PEP is measured in current research, it became more clear that a single regression equation relating ISTI to RB is not working as well as was expected from the first proof-of-principle study (Lozano et al., 2007). This is not unexpected or unprecedented and in no way discredits the original study. However, it does lead us to conclude that a regression equation based on ISTI predicts the RB interval with insufficient precision.

At first sight, these results are not encouraging for researchers involved in large scale data collections either in terms of many participants or in terms of prolonged (e.g. 24 hour) recordings. They show that for valid PEP scoring the detection of the Q-wave onset and B-point remains mandatory. PEP scoring is typically done after ensemble averaging the ICG waveform over all beats in a one-minute period, time locked to the R-peak. Scoring is ideally done by automated algorithms, but current practice is to always visually inspect the correct positioning of the B-point as it is hard to

detect algorithmically with sufficient accuracy in all participants. Two (or more) independent raters ideally score the PEP to reduce subjective rater bias. Multiple raters are particularly required in ambulatory studies, where ICG signal quality can be poor, for instance due to physical activity which creates bimodal ICG waveforms, making it difficult to detect the crucial B-point. This was illustrated by the lower interrater reliability for B-point scoring in the ambulatory (0.86) compared to the laboratory (0.92) setting in the current study. A practical solution is to ask participants not to engage in too many physical activities during their participation in ambulatory recording but this reduces ecological validity, which is a main asset of the ambulatory approach. The requirement of visual inspection by independent raters makes PEP scoring very time consuming and presents a major obstacle in large epidemiological studies or in prolonged ambulatory monitoring studies that generate many hours of data. A current strategy to deal with this is the use of large scale ensemble averaging across longer chunks of time, e.g. 30 minutes (Riese et al., 2003) which decreases work load for the raters and slightly increases reliability of signal scoring but also comes with a reduction in temporal precision (i.e. having one-minute PEP values across the entire 24-hour period). By far the best solution would be to increase the reliability of the automated detection of the Q-wave onset and B-point.

In that regard, our results also contain a clear positive message. The ISTI and a fixed QR interval of 40 ms do present meaningful estimates of the expected location of the Q-wave and B-point scoring. By a priori focusing the detection algoritms in a window around these expected locations should greatly help automated detection. In addition, the ISTI is fairly strongly correlated to the PEP. Of note, the within-participant correlations between ISTI and PEP were significant for *all* 118 participants and in *all* of the 38 different experimental laboratory and ambulatory conditions (data not shown). Changes in cardiac contractility are reflected in the time it takes the left ventricle to build up sufficient force to open the aortic valve (reflected in the B-point) but also in the time to takes to reach peak ventricular ejection (dZ/dt-min peak), which is reflected by the ISTI. Hence the information between PEP and ISTI strongly overlaps empirically and theoretically, and ISTI might itself be considered as a measure of cardiac sympathetic control based on physiological grounds. Clearly, before considering ISTI as an additional indicator of cardiac sympathetic responses, extensive testing against criterion measures or pharmacological validation is needed.

We conclude that for valid PEP scoring the detection of the Q-wave onset and B-point remains mandatory. PEP estimated from the R-wave and dZ/dt-min peaks should not be used to replace the actual PEP, but it could be a useful addition to the psychophysiologist's toolbox because it can help detection of the Q-wave and B-points.

CHAPTER 4

Comparison of within and between subject variation in salivary alpha-amylase and the preejection period

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This chapter wil be merged with chapter 5 and submitted to PNEC.

Abstract

Recently, the secretion of the salivary enzyme alpha-amylase (sAA) has gained interest as a potential non-invasive biomarker for activity of the sympathetic nervous system (SNS). In this study we compared sAA to an established measure of SNS activity, the preejection period (PEP). Twenty-three students between 18 and 35 years old wore an ambulatory recording device (VU-AMS) during one night and one day. Values for PEP were extracted from the impedance- and electrocardiograms recorded during the 30 minutes before a saliva sample was obtained by Salivette cotton rolls to determine sAA. This was repeated 7 times during the daytime recording. Respiratory sinus arrhythmia (RSA) was used to test for a possible interactive effect of the SNS and parasympathetic nervous system (PNS) on sAA. Replicating prior research, a daytime pattern for sAA was found showing an increase in sAA during the day. No significant within or between participant correlation between PEP and sAA was found, even when taking concurrent RSA levels into account. Within and between participant differences in ambulatory measured sAA are not correlated to parallel differences in an established measure of cardiac sympathetic activity.

Introduction

Activity of the sympathetic nervous system (SNS) may be paramount to the detrimental effects of stress on cardiovascular health (Palatini et al., 2004; Schwartz et al., 1992). As a consequence, cardiovascular psychophysiologists need reliable and valid strategies to measure sympathetic nervous system activity in humans. Recently, the secretion of the salivary enzyme alpha-amylase (sAA) has gained interest as a potential non-invasive biomarker for activity of the SNS. An immediate advantage of sAA assessment in large scaled studies is that it can be combined with salivary cortisol assessment, which is an established biomarker of the activity of the hypothalamic-pituitary adrenocortical (HPA) axis. Hence, if sAA truthfully reflected SNS activity, repeated salivary sampling would allow parallel research on the two major stress systems in large-scale samples (Chatterton, Jr. et al., 1996) including those of the sizes needed for genetic epidemiology (Lander & Kruglyak, 1995; The Wellcome Trust Case Control Consortium, 2007). However, before engaging in such efforts, the validity of ambulatory sAA as an index of SNS activity needs to be established beyond reasonable doubt.

Alpha-amylase is synthesized and secreted by the acinar cells of the salivary glands, in particular those of the parotid gland, and makes up about 20% of the total protein in saliva. The theoretical idea that sAA might serve as a non-invasive and easily obtained surrogate marker of SNS activity has been based on the presence of adrenoceptors in the acinar cells of the saliva glands and the increases in the protein-to-fluid ratio of alpha-amylase in response to norepinephrine. Empirical support comes from previous studies that found elevations of sAA after being exposed to stressors known to increase SNS activity, like a stressful academic examination (Bosch et al., 1996), parachute jump (Chatterton, Jr., Vogelsong, Lu, & Hudgens, 1997), stressful computer games (Skosnik et al., 2000; Takai et al., 2004), watching a stressful video (Bosch, de Geus, Veerman, Hoogstraten, & Nieuw Amerongen, 2003a), mental arithmetic test (Noto et al., 2005), and the Trier Social Stress Test (TSST) (Nater et al., 2006; Nater et al., 2005; Rohleder, Wolf, Maldonado, & Kirschbaum, 2006). Reduced sAA was found after watching a relaxing movie (Takai et al., 2004; Takai et al., 2007) although others have reported an increase during relaxation (Morse, Schacterle, Furst, Esposito, & Zaydenburg, 1983). The stress-induced sAA increase can be blocked pharmacologically by the β-adrenergic blocker propanolol (Van Stegeren, Rohleder et al. 2006), and Ehlert et al. (Ehlert, Nater et al. 2005) found a significant increase in sAA after administration of yohimbine which blocks the inhibitory α1-adrenergic autoreceptor.

While the above findings show that, at the level of group comparisons, increases in SNS activity by pharmacological means or through mental and physical stress is indeed accompanied by increases in sAA, they do not show whether between-participant differences in the overall levels of SNS activity are reflected in between-participant differences in sAA or the extent to which changes in SNS activity within an individual are reliably reflected in parallel within-participant differences in sAA. A design is needed that compares between-participant differences and within-participant changes in an established index of SNS activity to parallel between-participant differences and within-participant changes in sAA. For between-participant differences, this comparison has been made using venous catecholamine levels. Chatterton (Chatterton, Jr. et al., 1996) found moderate to good correlations between sAA and plasma epinephrine and norepinephrine (r=.49 and r= .64 respectively) during exercise, but other studies using mental stressors reported no or weaker correlation between plasma catecholamines and sAA (Chatterton, Jr. et al., 1997; Milad, Klock, Moses, & Chatterton, 1998; Morrison, Haas, Shaffner, Garrett, & Fackler, 2003; Rohleder et al., 2004; Skosnik et al., 2000).

These discrepancies do not by necessity reflect poorly on the sAA, as the validity of plasma catecholamines is not perfect. Concerns have been raised about differences in intraneuronal vesicular storage and leakage, re-uptake, extraneuronal clearance that may (severely) distort the relation between actual SNS activity and plasma catecholamine concentrations (Eisenhofer et al., 2004; Esler et al., 1990; Goldstein, 1995; Goldstein, McCarty, Polinsky, & Kopin, 1983; Hjemdahl, Larsson, Johansson, Zetterlund, & Eklund, 1990). If plasma catecholamines are themselves unreliable markers of SNS activity, weak correlations to sAA cannot tell us much about the validity of sAA.

In this study we compare sAA to an alternative measure of SNS activity, the preejection period (PEP) which can be obtained non-invasively by thoracic impedance cardiography. Within-participant changes in PEP index changes in β-adrenergic inotropic drive to the left ventricle as shown in laboratory studies manipulating β-adrenergic tone by epinephrine infusion (Mezzacappa et al., 1999; Schachinger et al., 2001; Svedenhag et al., 1986; Svedenhag et al., 1991), amyl nitrite inhalation (Nelesen et al., 1999) and adrenoceptor blockade (Harris et al., 1967; Schachinger et al., 2001; Winzer et al., 1999). Within participants, the PEP decreases in a dose-dependent way to exercise (Krzeminski et al., 2000; Miyamoto et al., 1983b; Smith et al., 1989b), emotional stress (Berntson et al., 1994a; Newlin et al., 1979; Sherwood et al., 1986) and monetary reward (Richter et al., 2009). Between-participant differences in PEP level are stable over time (Goedhart et al., 2006; Vrijkotte et al., 2004), heritable (Kupper et al., 2006), correlate modestly but significantly to plasma catecholamines (McCubbin, Richardson, Langer, Kizer, & Obrist, 1983) and reliably reflect interindividual differences in cardiac sympathetic activity assessed by dual blockade (Cacioppo et al., 1994). Since the PEP can be obtained by thoracic impedance cardiography (Sherwood et al., 1990) a number of devices are now available for ambulatory recording of the PEP (Cybulski, 2000; Nakonezny et al., 2001; Sherwood et al., 1998; Willemsen et al., 1996). This allowed us to use the ambulatory 'salivary design' typically employed to assess HPA-axis activity, whilst simultaneously recording the PEP values in the period preceding the salivary measurements. To support the sAA as a measure of SNS activity it should show a negative correlation to the PEP such that salivary samples with high sAA should be accompanied by a shorter PEP.

This study also assessed parasympathetic nervous system (PNS) activity using respiratory sinus arrhythmia (RSA) as a proxy of cardiac vagal control. As reviewed in detail elsewhere PNS activity can also increase sAA, and activation of the SNS during co-activation of the PNS can drastically increase the effects of SNS activity on sAA (Garrett, 1987; Rohleder & Nater, 2009). To account for a possible interactive effect of SNS and PNS activity on sAA, we used the concept of autonomic space as detailed in Berntson et al. (Berntson et al., 2008). Specifically, we used PEP and RSA to define a measure that reflects co-activation (Co-AR) of the PNS and SNS. Correlation of the sAA to the RSA and Co-AR was additionally tested.

Methods

Participants

Thirty-three participants (10 males) between 18 and 35 years old (mean = 23 years, SD = 4.3) were recruited and included. All participants were free of any obvious somatic or psychiatric disease, did not use medication, had not travelled through time zones or attended shift work in the prior three weeks and did not smoke or drink excessively (defined by more than 5 drinks or cigarettes a day). Participants were recruited at the VU University Amsterdam campus via the Biological Psychology department's online sign-up system for student participation in experiments.

Procedure

All participants wore an ambulatory recording device (VU University Ambulatory Monitoring System, VU-AMS) for a period of about 30 hours. For twenty-three participants monitoring with the VU-AMS began at 13:00 on the first day and was continued on a second day up till 19:00. For ten participants monitoring started at 16.00 on the first day till 19.00 on the second day. During the second day sAA levels were measured at 7 common time points (see table 1). Participants were instructed to take the first sample immediately upon awakening. A prepaid text message phone service was used to communicate their wake up time to the experimenter. Awakening time was verified and, if appropriate, corrected after visual inspection of the ambulatory recording of heart rate and body movement according to the procedure outlined by Kupper et al. (2005).

Thirty minutes after awakening participants were reminded via text messages to take the second sample. Three samples were taken under the supervision of the researcher during two 1-hour visits to the department for neurocognitive testing, two samples at 10.00 and 18.00 during active testing and at 19:00 at the end of the experimental protocol. The two remaining samples were taken outside of the laboratory (at 12:00 and at 14:00) and for both samples participants were prompted by a text message. The VU-AMS device was removed after the last sample taken in the laboratory (19:00).

Table 1. *Seven common saliva sample moments.*

Recording period	Time of Day	Saliva Sample
Awakening	05:04 – 08:00	Sample 1
Awakening + 30 min	05:34 – 08:30	Sample 2
Morning sample (supervised)	10:00	Sample 3
Afternoon sample 1	12:00	Sample 4
Afternoon sample 2	14:00	Sample 5
Afternoon sample 3 (supervised)	18:00	Sample 6
Evening sample (supervised)	19:00	Sample 7

sAA

Salivary samples were obtained by the standard protocol for salivary cortisol collection (Bartels, de Geus, Kirschbaum, Sluyter, & Boomsma, 2003; Bartels, van den, Sluyter, Boomsma, & de Geus, 2003; Kirschbaum & Hellhammer, 1994) where salivary is collected while participants gently hold a cotton roll (Salivette sampling device, Sarstedt, Nümbrecht, Germany) in their mouth for approximately one minute. Salivettes were stored by the participant in a cool and dark location until transport to the laboratory where they were immediately stored at -20^{o}C. Participants were

requested not to eat (breakfast) until after the second saliva sample and were asked to refrain from eating 30 minutes prior to each saliva sampling.

After thawing for biochemical analysis, samples were centrifuged at 2000 x g at 10°C for 10 min. The sAA assay utilizes the enzymatic action of sAA on the chromagenic molecule, 2-chloro-4-nitrophenyl -D maltotrioside (Fuitest Amyl CNPG3, Biocon, Vöhl-Marienhagen, Germany) that was used as the assay substrate to estimate amylase concentration (Lorentz, Gutschow, & Renner, 1999; Winn-Deen, David, Sigler, & Chavez, 1988). Enzymatic activity of alpha-amylase on this substrate yields 2-chloro-p-nitrophenol, which can be spectrophotometrically measured at 405 nm. The amount of alpha-amylase activity present in the sample is directly proportional to the increase in absorbance at 405 nm. Saliva samples were diluted 1:200 with ddH2O, and 8µl of the diluted saliva was pipetted in duplicates into a 96-well microtiter plate. After adding 320µl substrate solution (CNP-G3), the plate was incubated for 60 seconds at 37°C and mixed at 500rpm in a microtiter plate thermoshaker. After 60 seconds the plate was read kinetically in a plate reader at 405nm and incubated again for exactly 120 seconds at 37°C and 500rpm. After this second incubation the OD was read again at 405nm. The OD change was calculated by subtracting the OD of the first reading from that of the second reading and alpha-amylase activity was expressed in U/ml. (Lorentz et al., 1999; Winn-Deen et al., 1988).

Ambulatory measurement

The VU-AMS device uses a six-electrode configuration to record the ECG, thorax impedance (Z0), changes in thorax impedance (ΔZ) and the first derivative of these changes (dZ/dt) as described in detail elsewhere (Goedhart et al., 2006; Riese et al., 2003). From these signals, the preejection period (PEP), respiratory sinus arrhythmia (RSA), and the inter-beat intervals (IBI) can be extracted. Briefly, PEP was calculated using the R-wave-locked ensemble averaged dZ/dt signal, using all valid beats within 60-second periods. This averaging reduces noise caused by respiration and limb movement related changes in thorax impedance. In these 60-second averages, the onset of a rapid change in dZ (B-point) was manually scored with the VU-AMS interactive software program as described previously (Riese et al., 2003). PEP was computed as the distance between the R-wave and the B-point, and a fixed Q-onset to R-wave period was added (48msec). RSA was obtained through the peak-valley method (Bosch et al., 1996; Grossman, 1983; Katona et al., 1975). Breathing cycles that showed irregularities like gasps, breath holding, coughing etc., were not considered valid and were removed from further processing, as were the shortest and longest breaths that deviated more than 3SD. When no respiratory phase-related acceleration or deceleration was found or when the shortest beat in inspiration was longer than the longest beat during expiration, the breath was assigned an RSA value of zero. Mean respiration rate (RR) and RSA were computed across all valid breaths to a single mean RSA for each of the experimental conditions. The kinetics of SNS effects on cardiac innervation and sAA salivary protein secretion may be quite different. SNS effects on PEP are immediate but sAA might be viewed as a more integrative measure of SNS activity. For a fair comparison PEP levels should be aggregated for a period preceding the time of sAA sampling. The optimal time window for this is unknown so we computed mean PEP, RSA and IBI across various time windows before each of the eight saliva samples were taken. These include windows from 1) 30 minutes before sampling up to 20 minutes before sampling (-30/20), 2) 20 minutes before sampling up to 10 minutes before sampling (-20/10), 3) 10 minutes before sampling up to sampling (-10/0) and 4) sampling moment till 10 minutes after sampling. Within these periods we selected only the

fragments during which the participants were 'sitting with minimal physical activity' (or lying in case of the first pre-awakening sample). To detect physical activity we used the motility signal of the inbuilt vertical accelerometer in the VU-AMS device. Next we assessed the mean PEP during these selected periods. Likewise, a mean RSA and IBI was computed across all breaths falling in the pre-sample sitting/lying fragments during the 4 different periods used. As almost identical results were obtained for all four periods we report in detail only on the 10 minute pre-sample recording period (-10 / 0).

The concept of autonomic space was developed by Berntson et al. (Berntson et al., 2008) to account for a possible interaction between the sympathetic and parasympathetic branches of the autonomic nervous system. They derived two measures of autonomic control from RSA and PEP that reflected reciprocal activation/deactivation versus co-activation/co-inhibition of these branches. Cardiac autonomic regulation (Co-AR) was calculated as the sum of the normalized values of RSA and PEP (formula = zRSA + (-ZPEP)) for each of the 30 minute periods. Co-AR represents the dimension of co-activation/co-inhibition, with low values representing co-inhibition and high values co-activation of the cardiac SNS and PNS branches.

Data analysis

Mixed model analysis of variance was used to test a main effect of time of day on the pre-sample PEP, RSA, and Co-AR levels and the sAA at the time of sampling. We included sex and age as covariates. To examine the between-participant correlations we computed Pearson correlations between sAA and PEP, RSA, and Co-AR for the 7 of the samples. The distribution of the Spearman rank correlations across the 7 samples within each of the 33 participants were used to examine within-participant correlations. In view of multiple testing we adopted a significance level of 0.001, based on an experiment-wise alpha of 0.05 with a Bonferoni correction for the 40 most critical sAA/PEP correlations computed. sAA was mildly skewed so we repeated all analysis with the log-transformed form. Virtually identical results were obtained and we report the analyses on the observed values only.

Results

Table 2 displays the average duration of the sitting periods used to compute the pre-sample PEP, RSA, and Co-AR. The means and standard deviations are given per sample. Mixed model analysis revealed no significant effect of sample time on RSA, PEP, and Co-AR.

Table 2.*Mean (±SD) for IBI, RSA, PEP, and Co-AR in the 10 minute pre-sample recording period .*

Saliva sample	**Time of Day**	**IBI** (ms)	*SD*	**RSA** (ms)	*SD*	**PEP** (ms)	*SD*	**Co-AR**	*SD*
Awakening	05:04 – 08:00	776.71	142.6	70.38	31.4	107.00	21.1	-.06	1.2
Awakening + 30 min	05: 34 –08:30	727.00	172.8	57.96	27.9	101.20	22.5	-.15	1.1
Morning (supervised)	10:00	804.23	96.9	77.25	30.9	101.00	26.4	.13	1.5
Afternoon 1	12:00	815.58	134.9	75.62	30.4	107.10	24.8	.10	1.2
Afternoon 2	14:00	792.40	137.5	66.03	23.9	102.84	23.5	.01	.9
Afternoon 3 (supervised)	18:00	672.32	117.1	54.29	23.4	93.13	21.3	.11	1.1
Evening (supervised)	19:00	786.93	112.1	73.73	29.0	103.31	19.3	.25	1.1

Table 3 and figure 1 display the mean sAA at the various sampling times. Mixed model analysis revealed a significant effect of sample time on sAA ($F(6, 32) = 3.32$, $p < .008$). Post hoc analysis revealed a gradual increase in sAA throughout the day.

Table 3. *Mean (±SD) Daytime variation in sAA.*

Saliva sample	**Time of Day**	**sAA** (Unit/ml)	SD
Awakening	05:04 – 08:00	28.85	±23.87
Awakening + 30 min	05: 34 –08:30	27.38	±35.31
Morning sample (supervised)	10:00	43.60	±40.40
Afternoon sample 1	12:00	37.24	±39.75
Afternoon sample 2	14:00	43.38	±45.82
Afternoon sample 3 (supervised)	18:00	68.58	±65.44
Evening sample (supervised)	19:00	62.85	±70.20

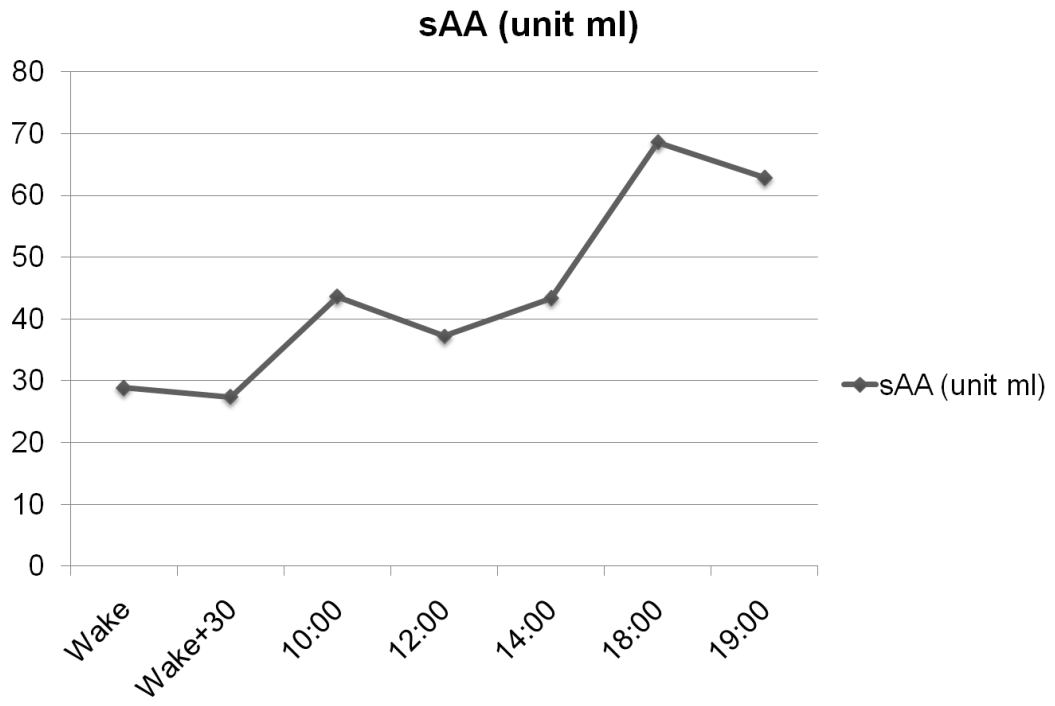

Figure 1. *Daytime increase in sAA (U/ml).*

Correlational analysis

The scatterplots between PEP and sAA and RSA and sAA are given in Figure 2. To examine the between-participant component of the covariance in our measures we first calculated Pearson correlations across the 33 participants, separately for each of the 7 samples (see table 4). No significant correlations between sAA and PEP were detected (range -.18 to .22), and sAA also did not correlate to RSA (range -.22 to .19) or Co-AR (range -.20 to .36).

Table 4. *Between-participant correlations of sAA with IBI, RSA, PEP, and Co-AR (10-minute pre-sample recording period).*

Recording period	**Time of Day**	**IBI-sAA**	p	**RSA-sAA**	p	**PEP- sAA**	p	**CAR- sAA**	p
Awakening	05:04 – 08:00	0.33	.09	0.19	.36	-0.05	.82	0.24	.23
Awakening + 30 min	05: 34 –08:30	-0.12	.58	-0.03	.86	0.22	.25	-0.14	.47
Morning (supervised)	10:00	-0.16	.39	-0.22	.25	0.03	.88	-0.20	.27
Afternoon 1	12:00	0.07	.72	0.05	.78	-0.07	.72	-0.01	.98
Afternoon 2	14:00	0.16	.38	0.09	.65	-0.18	.33	0.04	.83
Afternoon 3 (supervised)	18:00	-0.19	.32	0.03	.87	0.05	.78	-0.12	.53
Evening (supervised)	19:00	-0.01	.94	0.02	.94	0.10	.61	0.36	.05

In addition, no significant within-participant relation between sAA and PEP was seen, and, as indicated, this did not reflect the choice of the time period around saliva sampling (see table 5). For the -10 /0 time period, mean Spearman rank correlation between PEP and sAA was -0.09. Only three individuals showed a significant PEP–sAA correlation in the expected direction.

Table 5. *Mean, maximum and minimum within-participant correlations of sAA with IBI, RSA, PEP and Co-AR for various time windows around sAA sampling.*

	Timeframe	-30/-20	-20/-10	-10/0	0/10
IBI-sAA	Mean	-.10	-.06	-.02	-.01
	Maximum	-.79	-.73	-.69	-.83
	Minimum	.67	.81	.71	.75
	# Sig. Negative*	1	2	1	3
RSA-sAA	Mean	-.09	-.03	.01	.02
	Maximum	0.61	.88	.93	.84
	Minimum	-0.82	-.58	-.87	-.86
	# Sig. Negative*	1	1	1	4
PEP-sAA	Mean	-.05	-.18	-.04	-.09
	Maximum	-.91	-.98	-.88	-.68
	Minimum	.88	.86	.87	.73
	# Sig. Negative*	2	5	3	0
Co-AR-sAA	Mean	.00	.10	.02	.00
	Maximum	.57	.69	.86	.68
	Minimum	-.95	-.66	-.64	-.81
	# Sig. Negative*	0	1	2	4

** =p < 0.05*

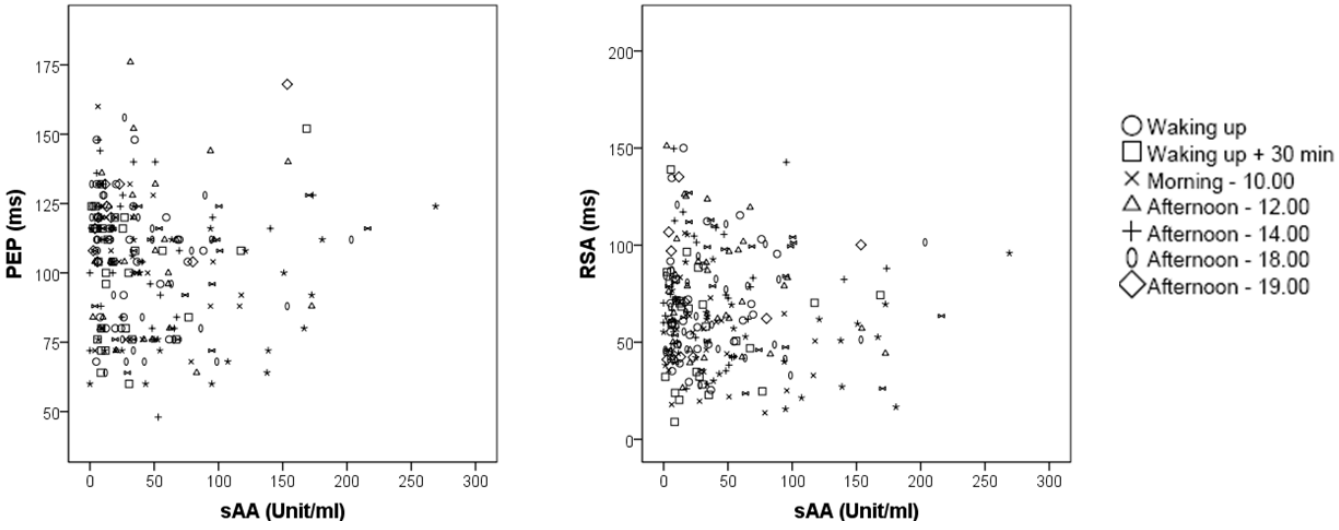

Figure 2. *Scatter plots of sAA with PEP and RSA.*

Area under the curve

In keeping with a recent study that showed the potential relevance of overall daytime sAA levels (Wolf, Nicholls, & Chen, 2008) we also computed the Area Under the Curve (AUC) and incremental AUC (AUCi) of the diurnal sAA curve using the trapezoid formula as described by Pruessner (Pruessner, Hellhammer, Pruessner, & Lupien, 2003). In parallel, we computed the overall daytime averages for PEP, RSA, IBI, and Co-AR. The sAA AUC variables are less dependent on between-participant differences in the basal values in sAA. Table 6 illustrates that no evidence was found for a relationship between sAA and PEP, RSA, or Co-AR.

Table 6. *Pearson correlations between the AUCi and AUCg of sAA with all-day PEP, IBI, RSA, and Co-AR .*

	IBI		**RSA**		**PEP**		**Co-AR**	
	r	p	r	p	r	p	r	p
AUCi	.10	.58	-.03	.87	-.02	.89	-.11	.53
AUCg	.05	.79	-.07	.71	-.08	.67	-.13	.48

Discussion

Enzymatic AA activity in salivary samples obtained by the Salivette device has gained a lot of interest as a potential non-invasive biomarker for activity of the SNS (Nater et al., 2009; Rohleder et al., 2009). If sAA truthfully reflects SNS activity, repeated salivary sampling for determination of cortisol and sAA would allow parallel research on the two major stress systems, the HPA-axis and the SNS, in large-scaled epidemiological research. To support sAA as a measure of SNS activity we tested its correlation to the PEP, which is an established marker of cardiac sympathetic control (Cacioppo et al., 1994; Harris et al., 1967; Mezzacappa et al., 1999; Miyamoto et al., 1983b; Nelesen et al., 1999; Newlin et al., 1979; Schachinger et al., 2001; Sherwood et al., 1986; Smith et al., 1989b; Svedenhag et al., 1986; Winzer et al., 1999). Our results failed to show the expected negative correlation between PEP and the sAA, either within- or between-participants.

The absence of a significant relation between PEP and sAA might have reflected confounding effects of within- or between-participant differences in PNS activity. The PNS mainly controls fluid secretion by the salivary glands, whereas sympathetic activity mostly regulates salivary protein secretion, including secretion of sAA (Bosch, Ring, de Geus, Veerman, & Amerongen, 2002; Garrett, 1987). As reviewed in detail elsewhere (Bosch et al., 2002; Garrett, 1987; Rohleder et al., 2009) an additional effect of the PNS activity is that it synergistically increases SNS-mediated protein secretion. In keeping, we have previously shown that sAA steeply increases during a laboratory stressor that evokes sympathetic-parasympathetic co-activation (viewing a surgical video), whereas only a marginal increase was seen during a stressor that evoked sympathetic activation in conjunction with a vagal withdrawal (a time-paced memory test) (Bosch et al., 2003a). Similarly, El-Sheikh et al. (El-Sheikh, 2005) found that children with higher vagal tone, as indexed by RSA, during stressful mirror-star tracing had higher sAA.

To account for confounding by PNS activity we repeated our analyses using either RSA, a measure that reflects PNS activation, and Co-AR, a measure that reflects SNS-PNS co-activation,

which was adopted from Berntson et al (Berntson et al., 2008). Results from these additional analyses suggest that differences in PNS activity do not explain the absence of a PEP-sAA correlation.

The absence of a significant relation between PEP and sAA also cannot easily be attributed to the procedures specific to this study. We used a salivary sampling procedure that has become standard in sAA research (Nater et al., 2009; Rohleder et al., 2009), under which conditions we fully replicated the circadian rhythm in sAA reported previously, i.e. the sAA showed a significant and progressive increase across the test day (Nater, Rohleder, Schlotz, Ehlert, & Kirschbaum, 2007). Low levels of sAA upon awakening and an increase of sAA during the day has been shown in rats before (Bellavia, Sanz, Chiarenza, Sereno, & Vermouth, 1990; Dawes, 1974), and was later confirmed in humans (Artino et al., 1998; Jenzano, Brown, & Mauriello, 1987; Nater et al., 2007; Rantonen & Meurman, 2000; Wolf et al., 2008). If sAA reflects SNS activity this would suggest that SNS activity is low in the morning and increases towards the evening. This is opposite from what is found using other measures of SNS activity like PEP and plasma catecholamines that show high SNS activity in the morning and a decline during the day (Gold et al., 2005; Karas et al., 2005; Turton & Deegan, 1974); (Burgess, Trinder, Kim, & Luke, 1997; Holmes, Burgess, & Dawson, 2002; McCubbin et al., 1983; van Eekelen, Houtveen, & Kerkhof, 2004).

Clearly, the lack of an association between sAA and PEP in part reflects the fact that autonomic activity at the level of the heart does not fully mirror autonomic activity in other organs (Folkow, 2000). However, the diffuse and highly interconnected anatomy of the SNS is also not consistent with a complete organ response specificity and the correlation between the two measures should have captured some of this generalized SNS activity. In view of the substantial literature supporting PEP as a measure of SNS activity (Berntson et al., 2008; Harris et al., 1967; Krzeminski et al., 2000; Mezzacappa et al., 1999; Miyamoto et al., 1983b; Nelesen et al., 1999; Newlin et al., 1979; Schachinger et al., 2001; Sherwood et al., 1986; Smith et al., 1989b; Svedenhag et al., 1991; Winzer et al., 1999) we believe that sAA, as assessed by utilizing the current protocol, may have some shortcomings.

A first shortcoming is that salivary fluid secretion and protein secretion are partially independently regulated processes, so that sAA concentrations may vary due to between- and within-participant variation in fluid secretion. This study, as the vast majority of sAA studies, did not measure salivary fluid secretion. According to Rohleder et al. (Rohleder et al., 2006) this should not be a problem, but results by Bosch et al. (Bosch et al., 2003a) provide reasons for concern, showing that a substantial part of sAA can be attributed to flow rate changes. A second shortcoming is that saliva secretion may have been induced by mild mechanical stimulation (gentle moving the salivette around in the mouth and possibly chewing). Although this method of saliva collection is common practice in sAA research (Rohleder et al., 2009), it may be problematic because stimulation of mechano-receptors in the mouth, e.g. by chewing, induces local autonomic reflex activity that influences glandular function independent of central sympathetic regulation (Garrett, 1987).

A related issue is that saliva composition can change upon stimulation of saliva flow. Here the problem is not a diluting effect of oral fluid, discussed above, but rather the combination of the facts that saliva glands vary in their response to stimulation and contain differing concentrations of AA. For example, under passive conditions (i.e., without mechanical or gustatory stimulation), most saliva derives from the submandibular glands and only one-fifth derives from the parotid gland, which is very rich in AA (Humphrey et al., 2001; Schenkels, Veerman, & Nieuw Amerongen, 1995). However, during stimulation (e.g. chewing) the contributions of individual glands changes dramatically, whereby half of the total saliva derives from the parotid glands. Importantly, parotid

gland secretions have a 4- to 10-fold higher AA concentration than those of the submandibular glands (Veerman, van den Keybus, Vissink, & Nieuw Amerongen, 1996). In sum, the Salivette collection protocol, which was used in the current study, involves mechanical stimulation that may invoke localized autonomic reflex activity and variability in terms of glandular sources of sAA, which both may mask shared variance in cardiac and glandular SNS regulation.

The above shortcomings may compromise the validity of sAA as a biomarker of SNS activity and could explain the absence of a correlation between PEP-sAA. We should add, however, that the small number of saliva samples per participant (7) implied limited statistical power to detect significant within-participants associations. We deliberately choose this sampling scheme because it reflects a typical HPA-axis study and we aimed to determine if additional sAA assessments would be feasible under such a scenario. Less realistic, but more dense sampling would have yielded better power. Even so, inspection of the analyses presented in Figure 2 argues against anything but a very weak relation between PEP and sAA to begin with.

In conclusion, within- and between-participant differences in ambulatory-assessed sAA are not correlated to parallel differences in an established measure

CHAPTER 5

The role of sympathetic and parasympathetic activation in sAA secretion during exercise

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This chapter wil be merged with chapter 4 and submitted to PNEC.

Abstract

Salivary alpha amylase (sAA) secretion has gained interest as a potential non-invasive biomarker for activity of the sympathetic nervous system (SNS). However, the parasympathetic nervous system (PNS) can also affect sAA secretion through its synergistic enhancement of SNS effects. In the current study 28 participants underwent exercise testing with continuous recording of cardiac SNS and PNS activity. Saliva was collected before and after exercise using the passive drooling method. Exercise-induced changes in enzymatic sAA activity, the most often used sAA measure, were assessed as well as changes in the actual sAA protein concentration and the ratio of sAA protein to total salivary protein. sAA activity and sAA protein concentration were converted to reflect true changes in sAA output by multiplying by flow rate. SNS reactivity was measured as decreased preejection period (PEP) and PNS reactivity as decreased respiratory sinus arrhythmia (RSA). Participants that paired exercise-induced cardiac SNS activation to a relatively small loss of PNS activation showed the strongest increases in enzymatic sAA activity ($r_{RSA\text{-}sAA\ activity}$ = .36), but changes in the true sAA output were not significantly related to SNS or PNS reactivity. Overall, the findings indicate that neither true sAA output nor its easily obtained proxy, enzymatic sAA activity, should be used as a selective indicator of SNS activation.

Introduction

Salivary alpha amylase (sAA) is a digestive enzyme that is synthesized and secreted by the acinar cells (i.e., the main secretory cells) of the salivary glands, in particular those of the two parotid glands. A main function of sAA is to break down insoluble starch into soluble maltose and dextrin, and the salivary concentration of this enzyme can be measured relatively cheap and easy by assessing this enzymatic activity. Enzymatic sAA activity, expressed as Units per milliliter (U/ml), is widely used as a proxy for the amount of sAA protein produced per unit saliva (mg/ml), although the correlation between amylase concentration and activity is only moderate (r = 0.61, (Goedhart et al., 2007; Mandel, Peyrot des, Plank, Alarcon, & Breslin, 2010; Schwartz et al., 1992). This is likely due to the fact that the protein is secreted in several isoforms which differ in enzymatic activity.

Between-individual variation in sAA secretion is strongly determined by individual copy number variation of the *AMY1* gene on chromosome 1p21, with a reported range of anywhere from 2 to 15 diploid copies (Mandel et al., 2010). In the absence of eating/chewing, within-individual variation in sAA secretion is largely caused by changes in the activity of the autonomic nervous system (ANS). Over the past 15 years, enzymatic sAA activity has gained interest as a potential non-invasive biomarker for activity of the sympathetic nervous system (SNS) specifically, which is backed by various compelling findings (Nater et al., 2009; Rohleder et al., 2009). For example, significant elevations in enzymatic sAA activity have been found after exposure to stressors known to increase SNS activity, like a stressful academic examination (Bosch et al., 1996) parachute jump (Chatterton, Jr. et al., 1997) stressful computer games (Chatterton, Jr. et al., 1997; Skosnik et al., 2000; Takai et al., 2004), watching a stressful video (Bosch et al., 2003a) effortful arithmetic or memory test (Bosch et al., 2003a; Noto et al., 2005), and the Trier Social Stress Test (TSST) (Nater et al., 2006; Nater et al., 2005; Rohleder et al., 2006). Moreover, the stress-induced enzymatic sAA activity increase can be blocked pharmacologically by the β-adrenergic blocker propanolol (Van Stegeren et al., 2006), and a significant increase in enzymatic sAA activity was found after administration of yohimbine, which increases sympathetic drive by blocking the inhibitory α1-adrenergic autoreceptor (Ehlert, Erni, Hebisch, & Nater, 2006).

An enhancing effect of SNS activity on sAA secretion is also biologically plausible. The cellular vesicles containing sAA, and other salivary proteins, are released upon sympathetic activation and the amount of amylase that is secreted per unit of time is directly related to the extent of sympathetic activity (Proctor & Carpenter, 2007). However, to equate changes in salivary sAA concentration or enzymatic activity with changes in SNS activity remains contentious because the parasympathetic glandular nerves potently affect sAA secretion. First, changes in salivary sAA concentration/activity reflect the combined effect of changes in salivary fluid secretion which are considered to be largely regulated by parasympathetic nerves, and changes in protein secretion which are largely sympathetic. Changes in actual sAA output, which take into account changes in flow rate, yield a more meaningful measure of changes in secretion. Second, amylase-rich glands such as the palatine glands are almost exclusively innervated by parasympathetic nerves and protein release from these glands would be without sympathetic involvement (Proctor et al., 2007; Veerman et al., 1996). The third and potentially most important factor is that the sympathetic and parasympathetic nerves interactively modulate sAA secretion. For example, SNS activation in the presence of parasympathetic co-activation synergistically enhances sAA secretion (Asking, 1985). This autonomic synergism is termed 'augmented secretion'. Taken together, the interpretation of enzymatic sAA activity as a 'pure' marker of sympathetic activation remains uncertain (Bosch et al., 2011).

The current study examined the specific role of sympathetic and parasympathetic activation in sAA secretion during exposure to a manipulation that elicits a clear pattern of autonomic activation (exercise). We used passive drooling to collect saliva which avoids many of the problems identified with the more often employed Salivette method (Bosch et al., 2011; Nater et al., 2009). Furthermore, in light of the preceding discussion, a number of novel methodological approaches were implemented. First, in addition to determining the association of sAA reactivity with established measures of cardiac parasympathetic and sympathetic reactivity (respiratory sinus arrhythmia and preejection period, respectively) we also determined possible effects of their co-activation, quantified by cardiac autonomic regulation (CAR) (Berntson et al., 1994a; Berntson et al., 2008). Participants with large exercise-induced sympathetic activation paired to small parasympathetic inactivation, i.e. a state of relative high co-activation, were predicted to yield high sAA secretion, whereas participants with small exercise-induced sympathetic activation paired to a relatively strong parasympathetic inactivation, i.e. a state of relative low co-activation, were predicted to yield low sAA secretion. Second, since the assumption of increased enzymatic sAA activity as a proxy measure for increases in the amount of sAA protein has thus far remained untested, we sought to determine if analyses using sAA protein measured by ELISA would yield similar results as the standard enzymatic method. Third, we tested the effects on sAA output which adjusts for confounding effects of changes in salivary flow rate. Fourth, to determine the specificity of sAA secretion compared to other salivary protein secretion, we also tested the exercise effects on relative changes in sAA protein to total salivary protein.

Methods

Participants

In total 28 undergraduates (6 male, 23 female) with an average age of 27 years (SD=9) and an average body mass index (BMI) of 23 (SD=3) volunteered to participate in the laboratory study. Participants did not report any psychiatric diseases or cardiovascular problems and none were using prescribed medication. The Medical Ethical Committee of the VU University Medical Centre Amsterdam approved of the study protocol and all participants provided written consent prior to participation and received study credits or a €10 gift voucher for participating.

General Procedures

Participants were asked to refrain from smoking and alcohol- or caffeine-containing beverages the evening before the test day and in the morning before coming to the laboratory. The experimental sessions took place between 10 a.m. and 4 p.m. and lasted approximately 90 minutes. After the participants had given informed consent, a short standardized interview was taken to obtain demographic information, health behaviors (smoking, exercise, alcohol), medication use, and to verify that they had no current anxious or depressive complaints or were receiving medication or treatment for cardiovascular disease. Height and weight were measured using standard procedures. Electrodes and ECG and ICG leads for cardiac assessments were attached to the participants, after which they were seated on an ergometer bicycle in a dimly lighted, electrically-shielded, sound-attenuated room. The experimental session commenced with general task instructions, saliva sampling practice, and a brief period of rest in which optimal signal quality was ensured. A first saliva sample was obtained at the end of a 4 minute baseline while seated on the bicycle. Then 4 minutes

of biking ensued at a resistance of 100 Watt at 60 rotations per minute. A second saliva sample was obtained immediately after the exercise task.

Saliva sampling

Saliva was obtained by the spitting method (Navazesh, 1993), directly following baseline and exercise. At the start of each saliva collection participants were asked to void the mouth by swallowing and subsequently let saliva accumulate at the floor of the mouth, without oral facial movements, and to expectorate each 30 seconds for 2 minutes in pre-weighed vials. Saliva volume was determined by weighing the vials before and after the saliva collection, assuming the density of saliva to be 1.0 g/ml, (Chicharro, Lucia, Perez, Vaquero, & Urena, 1998). Saliva secretion rate was expressed in ml/min. The vials were kept on ice until the end of the experiment, after which they were vortexed for one minute and centrifuged at 10.000xg for 10 minutes to remove buccal celles and oral micro organisms. The clear supernatant was divided over several aliquots and stored at -20^{o} C until analysis.

sAA determination

sAA enyzymatic activity (U/ml) was determined using The EnzChek® Ultra Amylase Assay by Molecular Probes adapted for a 96-well format™. This enzymatic assay uses a fluochrome-labeled starch derivate, which, upon enzymatic break down by amylase, releases its fluorescent probe. The accompanying increase in fluorescence over time is a measure of the amylase activity. The assay was performed according the instructions of the manufacturer. In short, 50 µl of 1: 20,000 diluted saliva was added to the wells of a 96-wells microtiterplate. Then 50 µl of a 10-fold diluted substrate solution was added and ithe ncrease in fluorescence was monitored at 2 min intervals during 30 minutes in a Fluostar Galaxy microplate fluorimeter (BMFG Labtechnologies, Offenburg, Germany). As reference was used a standard amylase solution (Meridian Life Sciences Inc, Memphis USA) at excitation and emission wavelenghts of 505 and 512 nm, respectively. The intra assay CV was 6% and the between assay CV was 11%.

sAA protein concentration was determined by a direct ELISA. Diluted saliva (1:10,000) was added in duplicate to a 96-well micro-titer plate (Microlon, Greiner bio-one), and incubated overnight at 4^{o} C. After rinsing with phosphate-buffered saline containing 0.1% (v/v) Tween-20 (PBS-T), the plates were incubated at 37°C with a monclonal antibody directed against amylase (clone G-8, Santa Cruz), diluted 1:3,000 in PBS-T supplemented with 2% BSA (w/v), at 100 ul per well. After 2 hours the plates were rinsed 3 times with PBS-T and incubated for 1 hour with an HRP-labelled rabbit anti-mouse polyclonal antibody (P-260, DakoCytomation), in PBS-T supplemented with 1% BSA. After 3 times rinsing with PBS-T the enzymatic colour reaction was initiated using OPD and H2O2, and the plates were read at 450nm using an ELISA reader. The concentration of sAA protein is expressed as a percentage of the amount of sAA protein found in a sample of standard pooled saliva.

Concentration of salivary protein (mg/ml) was measured by the bicinchoninic acid method (Pierce® BCA Protein Assay (Rockford, UK), which provides an accurate determination of total protein concentration in saliva (Bosch et al., 1996). Bovine serum albumin (BSA) was used as a standard (2 mg/ml standard solution in 0.9% NaCl). Samples of the same person were assayed in one assay to eliminate interassay error of measurement. Within-analyses CV=4.5%, between analyses CV=7% (Bosch et al., 1996).

sAA output (u/min) was calculated by multiplying sAA activity (u/ml) with flow rate (ml/min). In the same way sAA protein output (protein in %pooled/min) was calculated. Specific output of amylase was calculated as sAA protein divided by total salivary protein

Cardiac autonomic reactivity

Changes in the preejection period (PEP) are regarded as the gold standard of cardiac SNS reactivity and can be obtained non-invasively by thoracic impedance cardiography (Berntson et al., 1994a; Mezzacappa et al., 1999; Richter et al., 2009; Schachinger et al., 2001; Sherwood et al., 1986). Likewise, respiratory sinus arrhythmia (RSA) is the preferred measure of cardiac parasympathetic reactivity (de Geus et al., 1995; Frazer et al., 2007). RSA was quantified using the peak-valley method (Grossman, 1983; Katona et al., 1975; van Lien et al., 2011).

The ECG and ICG signals in the laboratory study were continuously recorded at a sample rate of 1000HZ with use of the ECG100C and NICO1000C modules of the BioPac data-acquisition system (BioPac systems INC, SAAnta Barbara, CA). Cleaning of the skin with alcohol before electrode application ensured that electrode resistance was kept low. Cardiac signals were recorded using a 6 Ag/Cl electrodes (Ultratrace, Cosmed, USA). ICG and ECG signals were imported into the VU-AMS Data Management and Analysis software (downloadable from www.vu-ams.nl) to score the inter beat interval IBI (ms), PEP (ms) and RSA (ms). For each experimental condition the interbeat interval IBI was scored from the R-wave peaks in the ECG. The IBI time series was visually inspected and missed or incorrect R-peaks were interactively corrected while bad ECG signal fragments were removed before averaging to obtain an experimental condition mean for the IBI (van Lien et al., 2011).

PEP was calculated using the R-wave-locked ensemble averaged ICG signal, using all valid beats within the basline and exercise condition. PEP was defined as the interval from the onset of electrical activity in the ECG (Q-onset) to the B-point in the ICG signal, and automated scoring of both points was confirmed by visual inspection of all ensemble averages. RSA was obtained through the peak-valley method (Bosch et al., 1996; Grossman, 1983; Katona et al., 1975). Breathing cycles that showed irregularities like gasps, breath holding, coughing etc., were not considered valid and were removed from further processing, as were the shortest and longest breaths that deviated more than 3SD. When no respiratory phase-related acceleration or deceleration was found or when the shortest beat in inspiration was longer than the longest beat during expiration, the breath was assigned an RSA value of zero. Mean respiration rate (RR) and RSA were computed across all valid breaths to a single mean RSA for each of the experimental conditions.

Cardiac autonomic regulation

We calculated the CAR as a measure of co-activation/co-inhibition of the cardiac sympathetic and parasympathetic branches of the autonomic nervous system (Berntson et al., 2008). CAR reactivity was derived as the sum of the normalized values of RSA reactivity and PEP reactivity (formula: $\Delta CAR = Z\Delta RSA + (-Z\Delta PEP)$), with low values representing co-inhibition and high values co-activation of SNS and PNS.

Statistical Analyses

Pre-planned paired t-tests contrasted the ergometer baseline level to the bicycle ergometer test (100W at 60 cpm). Reactivity scores reflecting this contrast were created for IBI, PEP, RSA, and the sAA measures and Pearson correlations were calculated to explore the association between the reactivity of the various sAA measures amongst themselves and between cardiac autonomic and sAA reactivity. Significance level was liberally set to 0.05 for all correlations.

Results

sAA and cardiac autonomic reactivity to exercise

Table 1 shows the mean levels of salivary and cardiovascular parameters during baseline and the bicycle ergometer test as well as their reactivity scores to exercise. Exercise increased sympathetic activity (lower PEP, $t(27)=14.27$, $p<.001$) and decreased parasympathetic activity (lower RSA, $t(27)=7.8$, $p<.001$), which led to a substantial decrease in IBI ($t(27)=18.1$, $p<.001$). Exercise also increased enzymatic sAA activity ($t(27)=-2.58$, $p=.016$), total salivary protein concentration($t(27)=-6.29$, $p<.001$), and flow rate ($t(27)=-3.28$, $p=.003$) but increases in sAA protein concentration did not reach significance ($t(26)=-1.89$, $p = .07$). When flow rate was used to create sAA output measures, the effects of exercise remained significant for sAA secretion based on enzymatic activity ($t(27)= -2.254$, $p= .033$) whereas sAA secretion based on the increase in protein concentration relative to the pooled standard again showed a trend only ($t(26)=-1.84$, $p = .077$). The specific amylase protein concentration (sAA divided by total salivary protein) did not reach significance reflecting the parallel increase in total salivary protein induced by exercise.

Table 1. *Means, standard deviations of IBI, RSA, PEP, flow rate, total salivary protein, sAA activity , sAA protein, total salivary protein output, sAA output based on activity, sAA protein output, and sAA protein/total salivary protein ratio during the bicycle ergometer test.*

	Bicycle Ergometer Baseline	**Bicycle Ergometer Exercise**	**Bicycle Ergometer Reactivity**
IBI (msec)	866 (132)	537 (92)	*-339
RSA (msec)	66 (25)	18 (19)	*-47
PEP (msec)	122 (17)	72 (22)	*-50
Flow rate (ml/min)	.41 (.25)	.46 (.27)	*.05
Total salivary protein (mg/ml)	1.39 (4.34)	1.72 (4.83)	*3.33
sAA activity (U/ml)	97.7 (61.8)	113.8 (74.3)	*16.1
sAA protein (% of pooled sample)	67 (58)	88 (70)	21
Total salivary protein output (mg/min)	.58 (.39)	.77 (.45)	*.18
sAA output based on activity (U/min)	45.6 (44.5)	52.8 (47.0)	*7.2
sAA protein output (% of pooled sample/min)	33 (39)	40 (36)	7
sAA protein / total salivary protein ratio	46 (34)	47 (30)	1

**= significantly different compared to the baseline (p < 0.05).*

Enzymatic sAA activity compared to sAA output

Table 2 shows the full pattern of correlations of changes in enzymatic sAA activity, the most often used proxy of sAA secretion, to the exercise induced changes in all other sAA measures assessed. Reactivity of enzymatic sAA activity and sAA protein concentration were significantly but modestly correlated (r=0.60). Exercise induced changes in flow rate were not significantly related to changes in enzymatic sAA activity which caused the correlation between enzymatic sAA activity and sAA output measures to be modest only. Finally, changes in sAA enzymatic activity were significantly correlated to changes in the ratio of sAA protein to total salivary protein.

Table 2. *Spearman rank correlations between the sAA activity reactivity and the other sAA measures to the exercise test.*

	Δ sAA activity
Δ sAA protein	*.60
Δ Flow rate	-.32
Δ sAA output based on activity	*.68
Δ sAA protein output	*.59
Δ Total salivary protein output	*.44
Δ sAA protein / Δ total salivary protein ratio	*.51

** =significant at p<.05.*

The association between autonomic and sAA reactivity to the exercise test.

Although the exercise stressor generally decreased both PEP and RSA, there were substantial individual differences in the patterning of ΔPEP and ΔRSA reactivity (illustrated in figure 1). Table 3 shows the correlation between these cardiac ANS reactivity scores and the concurrent changes in the salivary measures. Changes in enzymatic sAA activity correlated positively with changes in RSA, such that smaller increases in enzymatic sAA activity were associated with larger decrements in RSA ($r_{RSA\text{-}sAA}$=.36, p=.032), suggesting that parasympathetic withdrawal attenuates the exercise-induced increase in enzymatic sAA activity. Changes in enzymatic sAA activity were not correlated with PEP reactivity. When flow rate was taken into account, neither the sAA output measure based on enzymatic activity nor the reactivity of the sAA output measure based on the ELISA were significantly correlated to either PEP or RSA reactivity. Changes in flow rate, however, were very sensitive to changes in SNS activation ($R_{PEP\text{-}flowrate}$ = .46, p=0.01) with participants showing the largest increase in SNS activation having the largest increase in flow rate.

The CAR parameter combines the effects of SNS and PNS activation and, based on the concept of augmented secretion, participants with high CAR values that show an above-average increase in SNS activity with a below-average decrease in PNS should be expected to have the highest sAA output. We found, however, no significant correlation between sAA output measures and CAR. The three parameters which did show a significant correlation with CAR were enzymatic sAA activity ($r_{CAR\text{-}sAA}$= .32, p=.049), sAA protein ($r_{CAR\text{-}sAA}$= .36, p=.032) and the specific amylase concentration (sAA protein concentration/ total salivary protein concentration, $r_{CAR\text{-}ratio}$ = .36, p=.032).

Table 3. *Correlations of IBI, PEP, RSA and CAR reactivity with parallel reactivity of sAA activity, sAA protein, flow rate, sAA secretion based on activity and protein, and sAA protein / total salivary protein ratio.*

	Δ sAA activity	Δ sAA protein	Δ Flow rate	Δ sAA output (based on activity)	Δ sAA output (based on protein)	Δ sAA protein / Δtotal salivary protein ratio
Δ IBI	-.11	-.08	-.06	-.05	-.10	-.10
Δ PEP	-.02	-.19	*.46	.28	-.02	-.13
Δ RSA	*.36	.24	.01	.18	.20	.30
Δ CAR	*.32	*.36	*-.38	-.08	.18	*.36

** =significant at p<.05.*

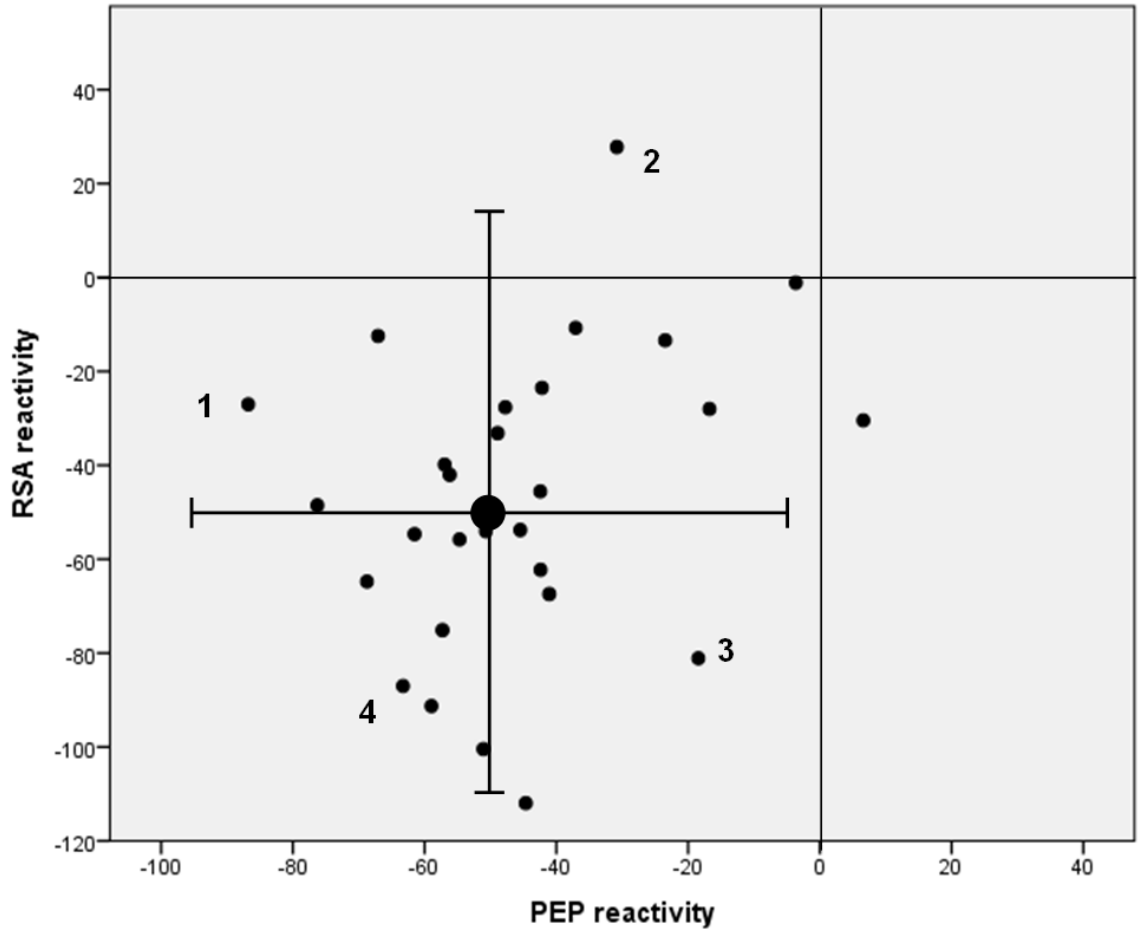

Figure 1. *Scatter plot of RSA and PEP reactivity to the bicycle ergometer test. Circles are the bivariate reactivity scores for the 28 participants. The large filled circle is the group average with 2 SD bars for both RSA and PEP. Four participants are highlighted for illustrative purposes. Participant 1 has above average cardiac sympathetic reactivity but below average parasympathetic reactivity. Participant 2 has below average cardiac sympathetic reactivity and below average parasympathetic reactivity. Participant 3 has below average cardiac sympathetic reactivity but above average parasympathetic reactivity. Participant 4 has above average cardiac sympathetic reactivity and above average parasympathetic reactivity.*

Discussion

The present study attempted to elucidate the role of sympathetic and parasympathetic activation, as well as their interaction, on the increase in enzymatic sAA activity induced by moderate intensity bicycle exercise. The results confirmed an effect of exercise on enzymatic sAA activity, and this effect remained intact when the increase in enzymatic sAA activity was multiplied by flow rate to obtain an indication of the actual increase in sAA secretion.

Exercise led to a significant increase in SNS activity, as evidenced by a decrease in the PEP, and a decrease in PNS activity, as evidenced by a decrease in RSA. Importantly, both cardiac autonomic and salivary responses to exercise showed substantial individual differences and these were used to probe the relative contribution of the autonomic branches to changes in sAA secretion. We found a significant association between the reactivity of enzymatic sAA activity and cardiac vagal reactivity such that a larger vagal withdrawal during exercise predicted a smaller exercise-induced increase in enzymatic sAA activity. In fact, visual inspection of the scatter plots showed that individuals with the largest vagal withdrawal during exercise even showed a net *decrease* in enzymatic sAA activity.

In contrast to our expectation, which we based on the previous use of sAA as an indicator of SNS activity (Allwood, Handwerger, Kivlighan, Granger, & Stroud, 2011; Gordis, Granger, Susman, & Trickett, 2008; Vaughn, Bradley, Byrd-Craven, & Kennison, 2010), the association between increases in cardiac SNS activity and increases in enzymatic sAA activity was not significant. Only when the increase in SNS activity was combined with the decrease in PNS activity, using the CAR measure, enzymatic sAA activity changes showed a significant correlation in the expected direction such that the activation of SNS with a relatively small deactivation of the PNS yielded the largest increase in enzymatic sAA activity. The pattern of the data clearly suggests, however, that the PNS contribution to the CAR measure was the main driver of this effect.

Although enzymatic sAA activity is an easily obtainable proxy for sAA secretion, it is now well recognized that it may not accurately measure concentration and that, in addition, flow rate should be taken into account to improve its correlation to true sAA secretion (Bosch et al., 2011; Nater et al., 2009; Rohleder et al., 2009). For instance, Mandel et al. (Mandel et al., 2010) showed that enzymatic sAA activity does not perfectly capture sAA protein concentration at rest (r=0.61) and we replicate this finding (r =0.60) for the changes in enzymatic sAA activity and sAA protein concentration during exercise. The imperfect association between changes in sAA amount obtained by using an ELISA based method and the more often used enzymatic sAA activity may be explained by the fact that saliva contains multiple sAA iso-forms that differ in enzymatic activity and which may be affected differentially by exercise.

Flow rate was also confirmed as an important confounder of the relationship between enzymatic sAA activity and sAA secretion during exercise. At a fixed level of secretion an increase in salivary fluid production would decrease sAA activity per volume of saliva, underestimating actual secretion. As flow rate appeared to be very sensitive to the degree of SNS activation during exercise it will attenuate any relationship between enzymatic sAA activity amd and actual sAA secretion. This can be resolved by quantifying actual sAA output. In this study we did so by multiplying the changes in enzymatic sAA activity and sAA protein concentration by flow rate. The resulting output measures theoretically best capture the phenomenon of interest: the changes in SNS and PNS activity to the secretory cells in the salivary glands. Exercise induced changes in these output measures did not

show a correlation to cardiac SNS reactivity. Furthermore, in contrast to enzymatic sAA activity, our secretion measure no longer showed a correlation to cardiac PNS reactivity.

Because changes in flow rate would affect sAA protein and total protein concentration to the same degree, an alternative measure of sAA secretion is the ratio of sAA protein to total protein. This measure additionally takes into account the potential specificity of increases in sAA compared to other salivary proteins. When the increases in sAA protein were expressed as a ratio of the total increase in salivary protein, the pattern of correlations to RSA and CAR seen for enzymatic sAA activity was recaptured. In the presence of increased SNS activity, a relatively small deactivation of the PNS during exercise yields the largest increase in sAA to total protein ratio. The sAA to total salivary protein ratio could therefore be a useful index of SNS and PNS co-activation.

Our results are consistent with experimental studies using psychological stressors characterized as “passive coping” tasks (Bosch et al., 2003a; Sanchez-Navarro, Maldonado, Martinez-Selva, Enguix, & Ortiz, 2012). Such tasks are known to engage different brain circuits than active coping tasks (Keay & Bandler, 2001) and increase rather than decrease PNS activity (Obrist et al., 1974). In keeping with a role for PNS activity, these tasks tend to cause augmented sAA output. For example, Bosch et al. (2003) found that secretory responses during a passive coping task, eliciting little change in PEP but an increase in RSA (a surgical video), were markedly higher than during an active coping task that elicited a decrease in PEP and a decrease in RSA. Sanchez-Navarro et al. (Sanchez-Navarro et al., 2012) found a marked increase in sAA activity after viewing unpleasant pictures like human mutilations which most likely elicit passive coping with PNS activation, but no or only a modest sAA response after pictures of human attack or erotic pictures which are likely to give rise to the more reciprocal SNS activation/PNS deactivation seen in active coping.

Conclusion

Analogous to the regulation of heart rate, and consistent with glandular biology, SNS-mediated sAA secretion is co-determined by concurrent PNS effects on sAA secretion, which may show substantial individual differences that are only partly correlated to individual differences in SNS reactivity. This means that sAA secretion, like heart rate, cannot be used to index either SNS or PNS activity selectively. Importantly, the findings indicate that neither true sAA output nor its easily obtained proxy, enzymatic sAA activity, should be used as a selective indicator of SNS activation.

CHAPTER 6

Ambulatory measurement of the ECG T-wave amplitude

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Abstract

The preejection period (PEP) can be used to index changes in sympathetic nervous system (SNS) activity, even under naturalistic conditions by non-invasive recording of the impedance cardiogram (ICG) and electrocardiogram (ECG) signal. A remaining obstacle is the necessary interactive visual scoring of 24-hour recordings, which has proven laborious. The ECG T-wave amplitude (TWA) could be an alternative ambulatory SNS measure that suffers less from this drawback and is even more easy to record under naturalistic conditions. Here we report on 24-hour ambulatory monitoring of the TWA in a sample of 564 healthy adults. The TWA could be reliably extracted from the ensemble-averaged ECG in over 90% of the participants. It showed a clear decrease in response to a mental stress task and a stepwise decrease from nighttime sleep to daytime sitting to more physically active behaviors during the 24 hour recording, echoing the expected pattern of SNS activity across these various conditions. In addition, within-participant changes in TWA across the standardized stressors were significantly correlated with the PEP (r=.41). TWA and PEP were also significantly correlated across the unstandardized naturalistic conditions in the majority of the participants (mean r=.35), even after partialling out RSA and IBI. The correlation increased when the analyses were repeated in 96 participants with completely non-ambiguous ICG B-point scoring. We conclude that the TWA seems sensitive to SNS activity, and that ambulatory co-recording of the TWA and PEP may provide a more comprehensive picture of changes in SNS activity across the day than the PEP alone.

Introduction

Functional disturbances of the autonomic nervous system have been frequently linked to several diseases and hyperactivity of the sympathetic nervous system (SNS) may be an important cause for the detrimental effects of stress on cardiovascular health (Lambert et al., 2010; Parati & Esler, 2012). At the moment the preejection period (PEP) is the measure of choice to monitor changes in SNS activity non-invasively in psychophysiological stress research (Berntson et al., 2004; Kelsey & Guethlein, 1990; Kelsey, Ornduff, & Alpert, 2007; Vrijkotte et al., 2004). PEP can be obtained by simultaneous recording of the thoracic impedance cardiogram (ICG) and electrocardiogram (ECG) (Riese et al., 2003; Sherwood et al., 1990; Willemsen et al., 1996) and is defined as the interval from the onset of left ventricular depolarization, reflected by the Q-wave onset (Qonset) in the ECG to the opening of the aortic valve, reflected by the B-point in the ICG signal (Labidi et al., 1970; Lozano et al., 2007; Sherwood et al., 1990; van Lien, Schutte, Meijer, & de Geus, 2013; Willemsen et al., 1996).

The literature supports changes in PEP as a valid index of SNS induced changes in contractility of the left ventricle (Berntson et al., 1994a; Goedhart et al., 2006; Harris et al., 1967; Houtveen et al., 2005; Krzeminski et al., 2000; Kupper et al., 2006; Mezzacappa et al., 1999; Miyamoto et al., 1983a; Nelesen et al., 1999; Newlin et al., 1979; Richter et al., 2009; Schachinger et al., 2001; Sherwood et al., 1986; Smith et al., 1989b; Svedenhag et al., 1986; Vrijkotte et al., 2004; Williams et al., 1993; Winzer et al., 1999). A large advantage of the PEP is that it can be measured outside the confines of a hospital or laboratory setting by using ambulatory monitoring devices (Nakonezny et al., 2001; Sherwood et al., 1998; Wilhelm et al., 2003; Willemsen et al., 1996). This allows examination of individual differences in sympathetic activity in a natural setting, for instance during sleep or during job-related activities with a substantial mental load. These naturalistic conditions may have the largest clinical relevance (Kubiak & Stone, 2012; Trull & Ebner-Priemer, 2013).

However, when ambulatory research moves to an epidemiological scale collecting data in thousands of participants across extended periods of time, the practical feasibility of PEP measurement becomes an issue. Though automated scoring of the PEP has been made more efficient over the years by for example implementing large scale ensemble averaging (Riese et al., 2003) it still remains a time consuming process requiring visual inspection by multiple raters to ensure sufficient reliability of identification of the relevant landmarks (Berntson et al., 2004; Lozano et al., 2007; van Lien et al., 2013). Taken the laborious visual scoring required it would be extremely valuable to have alternative non-invasive measures of cardiac SNS activity that could be assessed through ambulatory monitoring with more ease.

Two such alternative measures are currently in use, the ratio of power in the low to high frequency bands of the heart rate power, (LF/HF ratio, (Pagani et al., 1991; Pagani et al., 1997; Pagani et al., 1986) and salivary alpha-amylase (sAA, (Nater et al., 2009). Unfortunately, the validity of these measures as indices of SNS activity has been strongly questioned. Although its use has become widespread, the LF/HF ratio is theoretically a poor measure of SNS activity (Eckberg, 1997) that does not correlate with other indicators of SNS activity (Goedhart et al., 2008b; Grassi et al., 1999). sAA, particularly when collected by cotton rolls (as is typical), has severe methodological drawbacks and seems to capture parasympathetic activity in addition to sympathetic activity (Bosch et al., 2011). Also, it cannot be sampled with a high resolution during recordings in naturalistic settings or during the nighttime.

In this study we set out to test a third, and seemingly somewhat forgotten, alternative to measure cardiac SNS activity, namely the amplitude of the T-wave in the ECG (TWA). The T-wave is

the asymmetrical wave in the ECG that comes after the QRS-complex and typically lasts approximately 150 msec. It reflects ventricular repolarization (Abildskov et al., 1977; Burgess, 1979; Haarmark et al., 2010; Randall et al., 1977) in which the sympathetic nerves play an important role (Abildskov, 1985). TWA is often defined as the difference between the peak of the T-wave and the isoelectric level during the same heart cycle (Furedy et al., 1984; Kline et al., 1998) where alternative baseline levels are in use, such as the midpoint of the PQ interval (Matyas, 1976) and the isoelectric period between the T-wave offset and the P-wave onset (Contrada et al., 1989). These isoelectric periods represent moments where only a negligible number of fibers in the cardiac conduction system are depolarizing (Goldman 1970).

Decreases in TWA and even TWA inversion were seen to occur after local stimulation of the stellate sympathetic ganglia in (Anitchkov et al., 1961; Yanowitz et al., 1966), intracoronary infusion of epinephrine (Barger et al., 1961), norepinephrine (Russell & Dart, 1986) or the non-selective β-adrenergic agonist isoproteronol (Autenrieth, Surawicz, Kuo, & Arita, 1975) in dogs. In addition, TWA decrease was seen after subcutaneous or intramuscular administration of epinephrine (Hartwell, Burrett, Graybiel, & White, 1942; Katz, Hamburger, & Lev, 1932; Levine, Ernstene, & Jacobson, 1930), and after administration of non-selective (isoproterenol) and cardioselective β-adrenergic agonists (metopolol and alprenolol) in man (Contrada et al., 1989; Contrada et al., 1991; Guazzi et al., 1975; Rau, 1991).

Importantly, such functional TWA decreases could be reversed by β-blockade with propanolol (Contrada et al., 1989; Furberg, 1967; Furberg, 1968; Guazzi et al., 1975; Noskowicz et al., 1968). Additionally, TWA has been shown to be a useful indicator of cardiac SNS activity during laboratory testing of stress reactivity (Furedy et al., 1984; Furedy et al., 1986; Furedy et al., 1996; Heslegrave et al., 1979; Matyas, 1976; Matyas et al., 1976; Scher et al., 1984) since the stress-induced TWA decreases could be blocked by β-adrenergic antagonists (Contrada et al., 1989; Rau, 1991).

The initial enthusiasm for the TWA was tempered when Bunnell (Bunnell, 1980) showed only modest correlation (mean r= .41) between TWA decreases and other, at the time, accepted sympathetic measures (PTT, carotid dP/dt, HR). However, Furedy et al (Furedy & Heslegrave, 1983) rightfully noted that the weak correlations of TWA decreases to criterion measures merely showed that it is not a *perfect* index of SNS activity, and that other SNS indices also did not correlate perfectly amongst themselves. A more serious concern about the TWA is how 'purely' it reflects SNS activity. Increases in SNS activity are often accompanied by increases in heart rate and decreases in vagal activity. Increased heart rate leads to a shortening of the interbeat interval (IBI) which could decrease TWA simply by reducing the rise time of the T-wave. This means that decreases in vagal activity, by increasing heart rate, could also contribute to a reduction in TWA (Schwartz et al., 1983; Weiss et al., 1980).

Dauchot and Gravenstein (Dauchot et al., 1971) and Annila et al. (Annila et al., 1993) indeed found that an acetylcholinergic antagonist (atropine) led to a decrease in the TWA. Contrada et al (Contrada et al., 1989; Contrada et al., 1991) further reported a sudden paradoxical TWA increase during very high doses of isoproterenol infusion and reasoned that (baroreflex-induced) increases in vagal activity might have caused the increase inthe TWA. These findings suggest that changes in cardiac vagal activity also affect the TWA, which would invalidate it as a 'pure' SNS measure. Furedy et al. (1996) have argued that the effects of cardiac vagal activity may partly act by modulating SNS activity through the mechanism of accentuated antagonism (Furedy et al., 1996; Levy, 1977; Levy, Ng, Lipman, & Zieske, 1966; Levy & Zieske, 1969). If TWA reflects ventricular depolarization, it is

unclear how this would work, because accentuated antagonism requires sympathetic and parasysmpathetic nerves to converge on the same neuromuscular synapses, whereas the human ventricle is not innervated by the parasympathetic nervous system. Nonetheless, various studies reported an enhanced decrease of TWA by isoproterenol after atropine infusion compared to isoproterenol alone (Fukudo et al., 1992; Stratton, Pfeifer, & Halter, 1987).

One way to resolve the role of PNS activity in the TWA is to coregister TWA with heart rate variability in the respiratory frequency (RSA) which has been proposed as a proxy for cardiac PNS activity (Berntson et al., 1997; Task Force of the European Society of Cardiology the North American Society of Pacing, 1996). A number of studies reported a TWA decrease in parallel to vagal withdrawal as indexed by a decrease in RSA (Kreibig, Wilhelm, Roth, & Gross, 2007; Palomba, Sarlo, Angrilli, Mini, & Stegagno, 2000; Pan & Li, 2007; Roth et al., 1992). However, these studies employed TWA as an accepted SNS measure and did not correlate TWA to other measures of SNS activity nor directly to RSA. Only Kline et al (Kline et al., 1998) directly correlated RSA and TWA across various stress tasks (Valsava`s maneuver, serial subtraction and cold pressor) in 20 participants and found that after controlling for between-person variance, RSA did not contribute to TWA.

Taken together, the past studies on the TWA are overall suggestive of a role of the SNS in this measure, whereas the additional role of the PNS remains unclear. It is possible that the relative popularity of the PEP rather than poor validity of the TWA has led to its demise in the past two decades. As stated before, the automated scoring of the PEP from the ECG and ICG has a number of practical setbacks and requires visual inspection that can become prohibitive when PEP is used in long term recordings in epidemiology-sized samples. This breathes new life into the attractiveness of the TWA that might not suffer from this setback as it only requires detection of clear landmarks in the ECG. Here we report on 24-hour ambulatory monitoring of the TWA in a sample of 564 healthy adults. First, we assess the feasibility of automated scoring of ECG landmarks required for the TWA using large scale ensemble averaging of the ECG. Large scale ensemble averaging has been successfully applied to the ambulatory impedance cardiogram (ICG) but not yet to the ambulatory ECG. Second, to compare to previous literature, we will score the TWA in an ensemble averaged ECG across a 4 minute resting baseline and 4 minute stress task. These standardized conditions were embedded within the larger 24-hour recording. Third, in the ambulatory recordingwe will test whether the TWA varies in a predictable way within a wake/sleep cycle and across different levels of physical activity. Finally, we will test whether the changes in TWA within a participant across the 24-hour recording are correlated with the changes in PEP. We hypothesize that the TWA will vary in a predictable way between rest and stress conditions and across arousal and physical activity level. We further hypothesize that within-participant changes in TWA will show significant correspondence with changes in PEP, and that these changes survive correction for parallel changes in RSA and IBI. In short, we expect to provide evidence that the TWA can provide meaningful information on SNS activity in ambulatory recordings.

Methods

Participants

Participants were all registered in the Netherlands Twin Register (NTR) and had previously participated in a larger biobank project (Willemsen et al., 2010). A priori reasons for exclusion were participation in an earlier ambulatory recording study (Kupper et al., 2005b; Kupper et al., 2006), heart transplantation, presence of a pacemaker and known ischemic heart disease, congestive heart

failure, or diabetic neuropathy and pregnancy. Ambulatory cardiovascular recordings of 582 participants were collected of which 11 recordings had either a missing or noisy ECG or thorax impedance signal due to equipment failure, and were therefore excluded from the analysis. Seven participants using beta-blockers were excluded from the analysis. The final sample consisted of 277 identical twins (97 men), 234 fraternal twins (96 men), and 53 singleton siblings (21 men) from 297 families. Mean age was 36.9 years (SD 5.4). Zygosity of the twins was determined by DNA typing for 98.6% of the same-sex twin pairs. For the other same-sex pairs, zygosity was based on survey questions on physical similarity and the frequency of confusion of the twins by parents, other family members, and strangers. Agreement between zygosity based on these items and zygosity based on DNA was 96.1% (Willemsen et al., 2013). The Medical Ethical Committee of the VU University Medical Centre Amsterdam approved of the study protocol and all participants gave written consent before entering the study. Participants received a payment of 10 Euros and an annotated review of their ambulatory ECG recording.

Procedure

A detailed description of the general ambulatory monitoring procedure has been provided elsewhere (Kupper et al., 2006). Briefly, all participants were visited in the morning at home or at the work location when this was deemed more convenient. They were fitted with the VU University Ambulatory Monitoring System (VU-AMS) device that records the electrocardiogram (ECG) and the impedance cardiogram (ICG) continuously during a 24-h period (daytime and sleep) through seven disposable, pregelled Ag/AgCl electrodes (de Geus et al., 1995; van Dijk et al., 2013; Willemsen et al., 1996). After visually establishing proper signal quality the recording was started and participants were first interviewed on health, medication, lifestyle and socioeconomic and demographic information after which they filled out a questionnaire on psychological wellbeing. The questionnaire lasted on average 10 minutes and was completed while quietly sitting in a secluded part of the house/work area. The last 4 minutes of this quiet sitting period functioned as a baseline. Next participants were instructed to execute a 2-minute serial subtraction task and a 2-minute Stroop ColorWord conflict task. After a final equipment check the experimenter then departed and participants were left to their daily routine until the next morning when they were asked to remove the VU-AMS device and cables and to mail it all back to the experimenter in a prepared return envelope with a special protective layer. During the daytime and the evening before bedtime participants were asked to give a chronological account of posture, physical activity, physical load, location, and social situation every 60-min period. Participants were asked to refrain from heavy exercise during the recording day.

Physiological recording

The electrocardiogram (ECG) and the impedance cardiogram (ICG) were recorded continuously with the 7-lead version of the VU-AMS device (version 5fs, VU University Amsterdam, www.vu-ams.nl). Cleaning of the skin with alcohol before electrode application ensured that electrode resistance was kept low. ECG electrodes were carefully placed according to a standard protocol to obtain a lead II derivation, which yields the most prominent R-wave peak as well as a clear T-wave amplitude. The first ECG electrode (V-) is placed slightly below the right collar bone 4 cm to the right of the sternum. The second ECG electrode (V+) is placed at the apex of the heart over the ninth rib on the left lateral margin of the chest approximately at the level of the processus

xiphodius. The third ECG electrode (GND) is a ground electrode and is placed on the right side, between the lower two ribs at the right abdomen. The first ICG measuring electrode (V1) is placed at the top end of the sternum, between the tips of the collar bones. The second ICG measuring electrode (V2) is placed at the xiphoid complex of the sternum. The two current electrodes are placed on the back: I- on the spine over the cervical vertebra C4, at least 3 cm (1") above the ICG measuring electrode V-, and I+ between thoracic vertebrates T8 and T9 on the spine, at least 3 cm (1") below the ICG measuring electrode V2.

Data reduction

The ECG and ICG signals were imported into the VU-DAMS software (version 2.3, VU University Amsterdam, www.vu-ams.nl). After automated detection of bad ECG signal fragments (artefacts), R-wave peak detection was done using a modified version of the algorithm by Christov (Christov, 2004). From the R-wave peaks, an interbeat interval (IBI, ms) time series is constructed that was visually displayed for interactive correction of missed or incorrect R-wave peaks. This correction takes on average 2 minutes per 24 hour recording.

Large scale ensemble averaging

Using the activity diary entries in combination with a visual display of an in-built vertical accelerometer signal, the entire 24-h recording was divided into fixed periods coded for posture (e.g. lying, sitting, standing), ongoing activity (e.g. desk work, eating/drinking, meetings, watching TV), physical activity (non, light, medium, and heavy), location (e.g. work, home, outside), and social situation (e.g. alone, with colleagues, with friends). The ECG and ICG signals were ensemble averaged across these periods, time locking both signals to the R-wave peaks. This reduced the data set to, on average, 49 (range: 17-88) ensemble averaged ECG and ICG traces per participant with an average length of 27 minutes (SD= 19). These had less than 0.5% ECG artefacts. An example of a large scale ensemble-averaged ICG and ECG is presented in figure 1. In these ensemble averages, the mean Q-wave onset (Qonset), R-wave onset (Ronset), S-wave peak (Spoint), S-wave offset (Soffset), T-wave peak (Ttop), T-wave offset (Toffset) in the ECG and the B-point, dZ/dt min (C-point), and incisura (X-point) in the ICG were automatically detected and presented for interactive visual scoring. During interactive scoring, undetectable points were deleted which effectively sets them to missing per complex. Interactive ECG and ICG scoring took on average 15 minutes per 24 hour recording, the bulk of which was spent on the ICG. In some participants, Qonset and/or Ronset tended to be systematically missing, which has been reported before (Lozano et al., 2007). In these participants, Qonset and/or Ronset were set to missing. The ICG points, that caused the largest ambiguity, were scored by two raters as recommended (van Lien et al., 2013), and a third rater arbitrated when no consensus could be reached. To remove outliers, Z- scores were calculated for the timing of Qonset, Ronset, Spoint, Soffset, Ttop, Toffset, B-point, C-point and X-point across all participants and conditions and, per point, the top and bottom two percent of Z values of each of these parameters were set to missing.

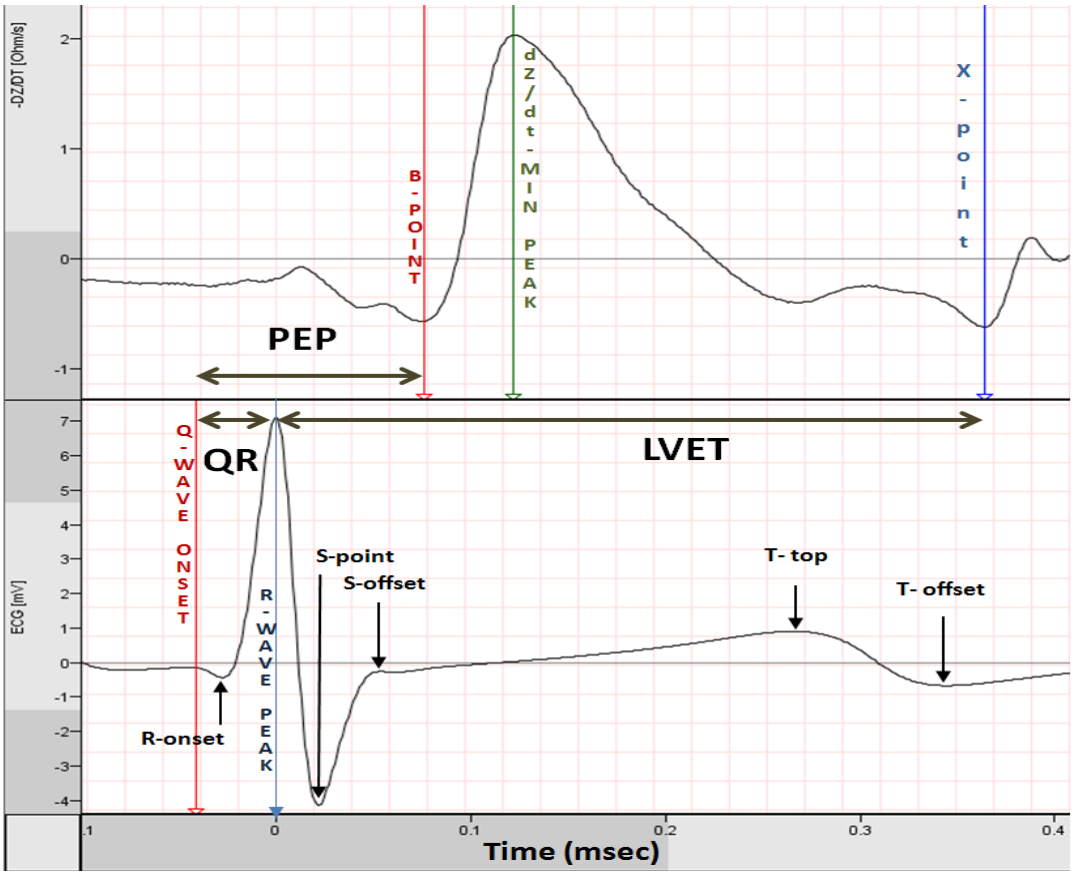


Figure 1. *The impedance cardiogram (top) and the electrocardiogram (bottom) with the four landmarks defining the PEP (Q-wave onset to B-point) and TWA (T-wave top and T-wave offset).*

Cardiac time intervals

From the ECG landmarks indicated in figure 1, we computed the Qonset to R-wave onset (QRonset) interval, the Q-wave onset to R-wave peak (QR), the Ronset to R-wave peak (RonsetR) interval, and the QRS, RS, QT, and ST intervals in milliseconds. From the ICG landmarks we computed the left ventricular ejection time (LVET) as the interval between the B-point and the X-point, and by combining with the ECG, the R-wave peak to B-point (RB) interval and the PEP as the interval from the Q-wave onset in the ECG to the B-point in the ICG signal. As indicated before, a number of participants had no detectable Q-wave onset. For these participants we used the procedure typically used to estimate PEP from the R-wave peak only (Lozano et al., 2007; van Lien et al., 2013) but modified it to exploit the large dataset available. If only Qonset was missing but Ronset was present, PEP was estimated by adding the grand average QRonset interval across 531 participants with valid QRonset (12.6 ms) to the sum of the individual participants' RonsetR + RB intervals. If Ronset was additionally missing we defaulted to the usual estimation of PEP by adding the grand average QR interval across 455 participants with valid QR (43.7 ms) to the individual participants' RB interval.

T-wave amplitude

We computed the TWA (in µV) using different baseline values in the ECG. First we calculated TWA_zero as the difference between the ECG value at the Ttop and the isoelectric level represented by the zero µV line in the ECG. Because ECG signals are prone to drifting due to movement, especially

during ambulatory recordings, the TWA might be under- or overestimated in some participants by using this static baseline. We therefore secondly calculated TWA_Toffset as the difference between the ECG value at the Ttop and the ECG value at the T-offset. We deviated from the usual approach that averages the entire T-offset to P-onset interval with the intention to obtain a stable baseline. We found that at T-offset there is indeed negligible electrical activity, whereas activity is not always low in the entire T-offset to P-onset interval. In addition, we note that the T-offset is more reliably detected than P-onset.

RSA

Combining the ECG with the respiration signal extracted from the thorax impedance signal (dZ), the 'peak–valley' RSA method was used to asses vagal chronotropic effects (de Geus et al., 1995; Grossman et al., 1990; Grossman et al., 1986). In this method, RSA is scored from the combined respiration and IBI time series by detecting the shortest IBI during inspiration and the longest IBI during expiration on a breath-to-breath basis according to the procedures detailed elsewhere (de Geus et al., 1995; Houtveen et al., 2005; van Lien et al., 2011). Breathing cycles that showed irregularities like gasps, breath holding, coughing etc., were considered invalid and were removed from further processing. If no shortest or longest IBI could be detected in inspiration and experiation respectively, the breath was either set to missing or to zero when computing the condition average for RSA. Similar results were found for RSA computed either way and we employed only one (breaths set to missing) in further statistical analyses..

Statistical Analyses

For statistical analyses, the total recording, consisting of the standardized and the ambulatory part, was first converted into six experimental conditions. The first two experimental conditions were the baseline of quietly sitting and the mean of the two stress tasks. The next four conditions came from the ambulatory part of the recording. Because ambulatory activities were not standardized and could differ per participant, all coded periods were aggregated into four comparable conditions that were determined by the posture and the level of physical activity (PA) of the participant: sleep, sitting activities during the day, light physical activity in awake time, moderate physical activity in awake time.

To test content validity of TWA, a mixed model ANOVA with age, sex and BMI as covariates and family as a random factor and baseline versus stress during the experimental part of the recording as the fixed factor was first applied. We expected TWA to be systematically decreased in response to mental stress. Next, a similar ANOVA was applied to the four ambulatory conditions, where we expect the TWA to significantly decrease with stepwise increases in SNS activity from sleep to sitting awake to physical activity.

Criterion validity of TWA in the experimental setting was then tested by computing the correlations between the participants' TWA and PEP reactivity to the stress tasks. To account for the effects of concurrent decreases in RSA and IBI, we also computed the partial correlation between TWA and PEP reactivity, with the effect of IBI, RSA, or joint RSA/IBI reactivity partialled out. Criterion validity of TWA in the naturalistic setting was assessed by computing the within-participant correlations between TWA and PEP across the day on all coded ambulatory periods available for a particular participant. The mean number of coded ambulatory periods was 49. We selected only participants that had complete TWA, PEP, RSA and IBI data in at least 15 coded ambulatory periods

with a minimum of two periods in at least three of the four conditions (sleep, sitting, light and moderate activity). We next plotted the distribution of these within-participant correlations and tested whether they deviate from zero (the expected value if TWA and PEP are not systematically correlated during the recording day).

To take into account parallel changes in vagal activity and heart rate, we recomputed the within-participant TWA-PEP correlations as a partial correlation using the IBI, RSA, or IBI and RSA values during the condition as a covariate. We compared the distributions of the uncorrected and partial TWA-PEP correlations using a test of the difference in correlation coefficients based on the Fisher Z transformation (Preacher, 2002).

Because the TWA-PEP comparison could suffer from poor quality of scoring of the criterion variable (PEP) in some participants, the analyses were repeated in the 96 participants for whom the raters expressed the highest confidence in ICG B-point scoring quality.

Results

Cardiac time intervals from the Large Scale Enembled Averged ECG

The means and standard deviations of the intervals derived from the large scale ensemble averaged ECG are shown per condition in table1. In general the relevant landmarks could be clearly detected in the ensemble averaged ECGs, but as noted before Qonset and Ronset are not always detectable. Overall, the Qonset was missing and estimated in 19.3% of the participants (109 of 564) and Ronset was additionally missing and estimated in 5.8 %, (33 of 564) and this was not different across the ambulatory conditions.

Mixed ANOVA analysis with correction for family relatedness, sex, age and BMI showed a significant main effect of ambulatory condition on most intervals derived from the ensemble averaged ECG; QR ($F(3, 1459)= 32.5$, $P<.001$), RonsetR ($F(3, 1747)=39.7$, $P<.001$), QRS, ($F(3, 1774)=20.6$, $P<.001$), RS ($F(3,1855)= 58.5$, $P<.001$), QT ($F(3, 1719)=1649$, $P<.001$), ST ($F(3, 1674)= 1459$, $P<.001$), RB($F(3, 1893)=132.3$, $P<.001$). Post hoc analyses of the condition effects revealed that the QT, ST, RS and RB intervals showed the expected significant stepwise decrease with increased arousal and physical activity. The effect of condition on QR, RonsetR, QRS, and Qronset was negligible and completely driven by the discrepancy between sleep and physically active periods.

From the covariates considered, sex had the most effect on these intervals. Female participants had shorter Qonset, Ronset, RS and QRS durations and longer ST and QT intervals than males ($P's < 0.001$). The Qonset, Ronset, QRonset and QRS duration was longer in older participants ($p's <0.001$). A greater BMI was significantly associated with shorter Qonset ($r=-.111$, $p<.001$), Ronset ($r=-.148$, $p<.001$), ST ($r=-.074$, $p<.001$).

Table 1. *Means and standard deviations (msec) for the intervals derived from the large scale ensemble averaged ECG during 24-hour ambulatory recording.*

Condition		QR	RonsetR	QRS	Qronset	QT	ST	RS	RB
Sleep	N	*427*	*508*	*508*	*416*	*483*	*469*	*533*	*539*
	Mean (SD)	44(4)	32(3)	93(10)	13(4)	411(21)	318(23)	25(3)	62(14)
Sitting activities	N	*443*	*521*	*529*	*432*	*519*	*504*	*552*	*559*
	Mean (SD)	44(4)	31(3)	[a] 91(9)	13(4)	[a]377(22)	[a] 285(23)	[a] 24(3)	[a] 58(16)
Light PA	N	*430*	*504*	*511*	*417*	*500*	*487*	*535*	*542*
	Mean (SD)	43(4)	31(3)	91(9)	13(4)	[b] 367(21)	[b] 276(22)	24(2)	[b] 55(17)
Moderate PA	N	*443*	*521*	*528*	*433*	*514*	*504*	*547*	*557*
	Mean (SD)	43(4)	31(3)	91(10)	13(4)	[c] 356(18)	[c] 264(19)	24(2)	[c] 49(16)

Sample sizes (N) vary depending on the number of participants in which the particular ECG landmark could be scored or in which the condition was missing.
[a] Significantly different compared to sleep, p <. 05.
[b] Significantly different compared to sitting activities, p <. 05.
[c] Significantly different compared to light physical activity, p <. 05.

Response of the TWA to a standardized stressor

The mean values and standard deviation for IBI, LVET, PEP, RSA, TWA_Toffset, and TWA_Zero during baseline and stress task are presented in table 2. Mixed model analyses with correction for family, age, sex and BMI showed a significant main effect of the stress task on IBI (F(1,777)=180.8, p<.001), LVET (F(1, 722)=17.0 p<.001), PEP (F(1,674)=19.4, p<.001), RSA (F(1, 718)=4.2, p<.005), TWA_Toffset (F(1,639)=28.3, p<.001) and TWA_Zero (F(1,659)=41.8, p<.001). Post-hoc analyses on the effect of the mental stress tasks generally showed the expected effect of our manipulation on the ANS measures. The IBI, LVET, PEP, TWA_Toffset and TWA_Zero decreased significantly during the stress task, although RSA did not.

Table 2. *Means and standard deviations for the variables derived from the ECG and ICG during the standardizd baseline and stress task conditions.*

	Baseline	Stress task	N baseline/ N stress task	Reactivity (Δ)
IBI	834 (123)	769 (113)	524/542	*-65
LVET	284 (34)	277(36)	491/510	*-7
PEP	107 (19)	103 (19)	472/488	*-4
RSA	58 (24)	60 (22)	498/530	*2
TWA_Toffset	1.480 (0.548)	1.374 (0.539)	452/474	*-.106
TWA_Zero	.964 (.32)	.877 (.31)	460/485	*-.087

**= Significantly different during stress compared to baseline, p <.05.*

Response of the TWA to increase levels of arousal and physical activity in a naturalistic setting

The mean values and standard deviation for IBI, LVET, PEP, TWA_Toffset, and TWA_Zeroduring the ambulatory recording are presented in table 3. Mixed ANOVA analysis with correction for family relatedness, sex, age and BMI showed a significant main effect of physical

activity on IBI (F3,1923)= 2138, p<.001), LVET (F(3,1907)=691, p<.001), PEP (3, 1893) = 148, p<.001), RSA (F(3,1874)=192, P<.001), TWA_Toffset (F(3, 1711)= 180, P<.001) and TWA_zero (F(3,1759) = 109, P<.001). The overall correlation between TWA_Toffset and TWA_Zero was .97 (p < .001) but as expected the TWA_Toffset, that uses a dynamic baseline, performed slightly better than the TWA_Zero measure, that uses a static baseline. Post hoc analyses of the ambulatory condition effects revealed the expected significant stepwise ordinal decrease of IBI, LVET, PEP, RSA and TWA_Toffset with increased arousal and physical activity. TWA_Zero also showed a significant stepwise decrease across sitting to increased levels of physical activity but in contrast to TWA_Toffset failed to differentiate between sleep and sitting activities. TWA_Toffset was selected as the single TWA measure to be used in further analyses.

Female participants had lower IBI, LVET, RSA and TWA_Toffset values but a longer PEP (p's<.001). RSA was significantly lower in older participants and a larger BMI was associated with shorter IBI (r = -.077, p< .001) and PEP (r = -.153, p< .<001), lower RSA(r = -.113, p< .001) and TWA_Toffset (r = -.113, p< .001) but a longer LVET (r = .074, p< .001).

Table 3. *Means and standard deviations for the variables derived from the ECG and ICG during the ambulatory conditions.*

	N (Range)	Sleep	Sitting activities	Light PA	Moderate PA
IBI	549 - 564	983 (132)	[a] 815 (101)	[b] 743 (93)	[c] 660 (77)
LVET	545 - 561	315 (28)	[a] 281 (29)	[b] 270 (31)	[c] 258 (50)
PEP	539 - 559	106 (14)	[a] 102 (16)	[b] 99 (16)	[c] 92 (16)
RSA	539- 555	63 (24)	[a] 58(20)	[b] 54 (18)	[c] 44 (14)
TWA_Toffset	492 - 516	1.46 (0.55)	[a] 1.38 (0.52)	[b] 1.19 (0.51)	[c] 1.07 (0.49)
TWA_Zero	504 - 525	0.92 (0.32)	0.90 (0.30)	[b] 0.80 (0.29)	[c] 0.75 (0.29)

[a] *Significantly different compared to sleep, p <. 05.*
[b] *Significantly different compared to sitting activities, p <. 05.*
[c] *Significantly different compared to light physical activity, p <. 05.*

Criterion validity of the TWA during the mental stress task

Significant correlations of the reactivity scores were found for TWA_Toffset with PEP reactivity (r=.406, p<.001) and IBI reactivity (r=.694, p<.001). TWA_Toffset decreases were also significantly correlated with RSA reactivity, albeit more modestly (r=.175, p<.001). As expected, IBI reactivity was correlated with both RSA (r=.228, p<.001) and PEP (r=.435, p<.001) reactivity. The average correlation between PEP and TWA_Toffset reactivity remained significant after controlling for RSA reactivity, as shown in the partial correlation coefficient (r_{part} = .417 , p< .001) and after controlling for IBI (r_{part} = .171 , p< .001), and joint IBI/RSA (rpart= .173, p<.001).

Criterion validity of the TWA in a naturalistic setting

Valid levels of TWA_Toffset, PEP, RSA and IBI could be obtained in more than 15 ambulatory conditions in 447 participans. The distribution of the within-participant correlations for TWA_Toffset and PEP for these participants is plotted in the upper panel in figure 2. This panel also gives the correlations between TWA_Toffset and RSA and TWA-Toffset and IBI. The TWA_Toffset showed a mean within-participant correlation of .35 with PEP (p<.001). The number of participants that had a positive within-participant correlation between TWA and PEP meeting a nominal p=0.05 significance

threshold of .11 was 261. In 186 (41.6%) participants no significant within-participant correlation between PEP and TWA was found in the expected direction. The mean within-participant correlation of TWA_Toffset with IBI (.61) and RSA (.38) was also significant (p<.001).

The lower panel in figure 2 shows the partial correlation between PEP and TWA_Toffset after partialling out IBI, RSA and joint IBI/RSA. The mean within-participant correlation between PEP and TWA_Toffset remained significant after controlling for within-participant changes in IBI (r_{part}=.14), RSA (r_{part}=.29) or joint IBI/RSA (r_{part}=.13). The drop in the mean correlation between TWA and PEP before and after taking RSA into account was not significant (p=0.31), but taking IBI (p<.001) or taking both IBI and vagal activity (p<.001) into account significantly reduced the PEP TWA_Toffset correlation.

Optimal ICG signal recording and PEP scoring quality

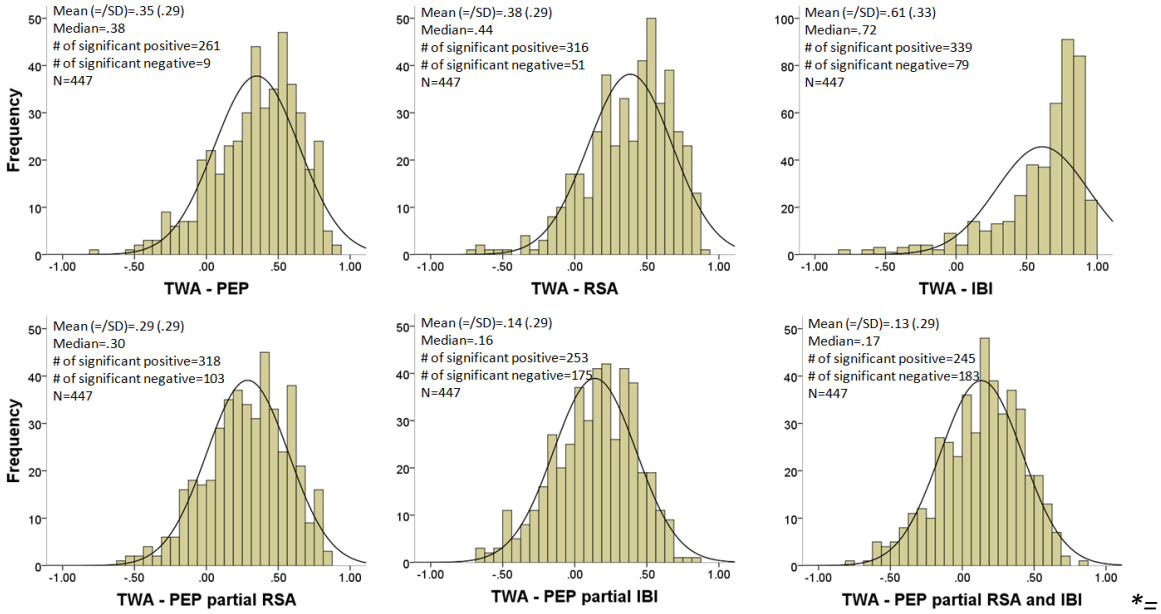
Poor ICG signal quality could have led to low quality PEP scoring and thus an underestimation of the PEP TWA_Toffset correlation. Repeating the analyses in the 96 participants with the highest quality of the ICG B-point scoring yielded higher within-participant TWA_Toffset – PEP correlations although a similar distribution was seen as when including all participants (upper panel in figure 3). The TWA_Toffset showed a mean within-participant correlation of .43 with PEP. The number of participants that had a positive within-participant correlation between TWA_Toffset and PEP meeting a nominal p=0.05 significance threshold of .24 was 76. In 20 participants no significant correlation between PEP and TWA_Toffset was found in the expected direction. The mean within-participant correlation of TWA_Toffset with IBI (.71) and RSA (.43) was also significant (p<.001).

The lower panel in figure 3 shows the partial correlation between PEP and TWA_Toffset after partialling out IBI, RSA and joint IBI/RSA. The mean within-participant correlation between PEP and TWA_Toffset (r=.43) remained significant after controlling for within-participant changes in IBI (r_{part}=.25), RSA (r_{part}=.34) or joint IBI/RSA (r_{part}=.15). The drop in the mean correlation between TWA_Toffset and PEP before and after taking RSA into account was not significant (p=0.47). Taking IBI and joint IBI/ RSA into account again reduced the PEP TWA_Toffset correlation, although this did not reach significance (p's > 0.06).

Discussion

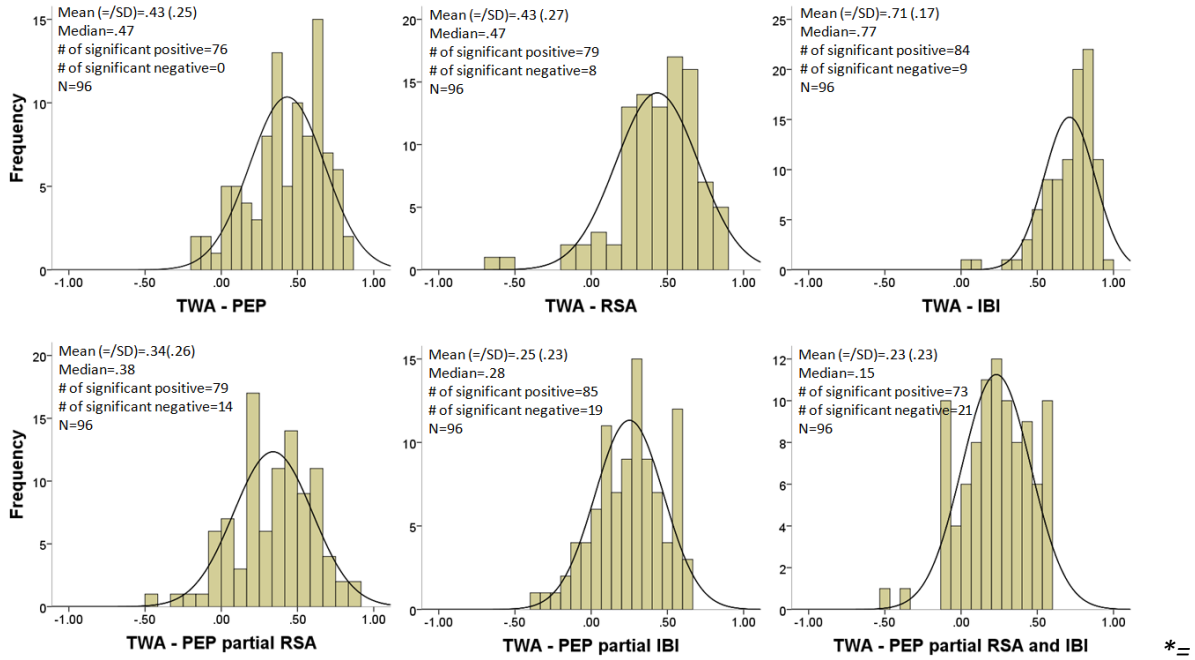
Ambulatory assessment of fluctuations in SNS functioning could greatly help epidemiologists understand the link between psychosocial stress and unfavorable physical and mental health outcomes. The PEP is currently the measure of choice to assess SNS activity in ambulatory studies. The aim of the present study was to test the validity of the ECG T-wave amplitude as an alternative index of cardiac sympathetic nerve system activity. The ambulatory TWA can be derived from three spot electrodes that only present a minimal burden to the participant. If obtained from the ensemble-averaged ECG, the TWA requires less laborious data scoring and may prove more amenable to automation than PEP scoring. PEP scoring requires a detectable Q-onset, which is missing in about 20% of participants, and visual inspection of the ensemble-averaged ICG waveform, which is occasionally ambiguous.

Figure 2. *Distribution of the within-participant correlations of TWA-PEP, TWA-IBI and TWA-RSA in the full sample. TWA-PEP correlations are re-plotted with IBI, RSA and joint IBI/RSA partialled out.*



Significant at p <.05.

Figure 3. *Distribution of the within-participant correlations of TWA-PEP, TWA-IBI and TWA-RSA in the sample only including participants with unambiguous ICG B-point scoring. TWA-PEP correlations are re-plotted with IBI, RSA and joint IBI/RSA partialled out.*



Significant at p <.05.

In our large sample of healthy adult participants, the TWA could be reliably extracted from the ensemble-averaged ECG in over 90% of the participants. It showed a clear decrease in response to a mental stress task in line with earlier research on the TWA (Contrada et al., 1989; Furedy et al.,

1984; Furedy et al., 1996; Heslegrave et al., 1979; Scher, Hartman, Furedy, & Heslegrave, 1986). TWA also showed a stepwise decrease from nighttime sleep to daytime sitting to more physically active behaviors during an ambulatory 24 hour recording, echoing the expected pattern of SNS activity across these various conditions. In addition, within-participant changes in TWA across the standardized as well as the unstandardized naturalistic conditions were correlated with parallel changes in the PEP, which we used as a criterion variable. However, these within-participant correlations were significant in 75% of the participants only, even when selecting the subset of participants with the best quality ICG data. A first conclusion of our data, therefore, is that the ambulatory TWA can be a valuable addition to the epidemiologist's psychophysiology toolbox, but that caution is needed to interpret changes in TWA at the level of a single individual. Below we discuss their validity as a 'pure' index of changes in SNS activity in more detail.

The literature validating the TWA as an SNS index in standardized laboratory conditions precedes the first application of regional cardiac NE spillover (Eisenhofer, Lambie, & Johnson, 1985; Esler et al., 1988) which is probably the only true golden standard of cardiac SNS activity. This is nontrivial, because low or absent correlation to cardiac NE spillover has been the major reason to distrust the LF/HF ratio as an index of SNS activity (Goedhart et al., 2008b). It could be reasonably argued that true validity of the TWA likewise can only be assessed by a comparison of TWA to NE spillover or direct SNS nerve recording (Goedhart et al., 2008b; Grassi et al., 1999). A large amount of pharmacological studies nonetheless bodes well for TWA. There is a clear pattern of decreased TWA with β-adrenergic agonists (Barger et al., 1961; Contrada et al., 1989; Hartwell et al., 1942; Katz et al., 1932; Levine et al., 1930; Russell et al., 1986) and the effect is attenuated or disappears with β-adrenergic antagonists (Contrada et al., 1989; Furberg, 1967; Furberg, 1968; Guazzi et al., 1975; Noskowicz et al., 1968; Rau, 1991), although not all studies have been able to reproduce this pattern (Contrada et al., 1991; Russell et al., 1986; Schwartz, Stone, & Brown, 1976; Taggart et al., 1979).

Admittedly, pharmacological studies have disadvantages in that they engage cardiac and vascular reflex regulation which may prominently include cardiac vagal activity. There has been some debate on whether vagal activity itself might cause a decrease in TWA which would invalidate it as an exclusive SNS index. If the assumption is correct that the TWA reflects ventricular repolarization, the theoretical rationale for such a vagal effect is not strong as the human ventricle is not vagally innervated. Autonomic effects on contractility, for instance, are driven entirely by adrenergic innervation. This provides the basis for using PEP as a "pure" index of cardiac SNS activity. To test for the independent contribution of changes in SNS activity to increases and decreases in TWA our analyses partialled out the parallel changes in RSA. Although RSA was significantly correlated with the TWA, this did not lead to a significant reduction in the within-participant correlation between PEP and TWA reactivity. This suggests that PNS activity is not a confounder of the TWA-PEP correlation. Only when changes in IBI were also partialled out, a decrease in the TWA-PEP correlation was seen. In general, the correlation between changes in TWA and IBI was strong, and it remained significant after partialling out PEP ((r_{part}=.55 in ful set ; r_{part}=.62 in best quality subset). This suggests that the TWA is sensitive to the shortening of the cardiac cycle, independent of SNS activity. However, even after correcting for concurrent changes in IBI a significant relationship between PEP and TWA reactivity was found.

A necessary limitation of this study is the use of changes in the PEP as an index of SNS activity to establish criterion validity of TWA. Although the PEP is the only available measure of cardiac SNS activity in ambulatory recording with established validity, it must also be recognized that the PEP is sensitive to postural or physical activity driven changes in preload that influence contractility

independent of the SNS through the Frank-Starling mechanism (Houtveen et al., 2005). In addition, the PEP is sensitive to changes in mean aortic pressure that can elongate PEP even under conditions of increased SNS activity, as is seen during exposure to cold or static muscle work (de Geus et al., 1993; Krzeminski et al., 2000). Changes in temperature, posture and static or dynamic exercise activities are a necessary element of naturalistic ambulatory monitoring. The PEP itself, therefore, will imperfectly correlate with SNS activity. In ambulatory settings it is the best possible criterion measure to compare to TWA, but certainly not a golden standard.

This limitation is balanced by a number of strengths of this study. First, we had a large sample size allowing us to provide a normative dataset for the main ECG intervals including the QonsetR and RonsetR intervals which we used to improve estimation of the PEP in participants where these landmarks were difficult to score. Secondly, by grace of the large dataset we could repeat our analyses on a high quality ICG data set to avoid ambiguity in the PEP scoring as a potential source of poor cross-measure correlation. Thirdly, although our major aim was to show validity in a naturalistic ambulatory setting, the addition of the standardized stress testing allowed us to also investigate TWA in the same participants independent of confounding posture and activity effects, diurnal variations, temperature fluctuations, ambient noise, ingestive behaviors, etc.

In conclusion, we find support for the usefulness of ensemble-averaged ECG derived TWA in epidemiological research to estimate changes in 24 hour SNS activity. Validity is not sufficiently strong to recommend replacement of the PEP by the TWA; instead recording and analysis of both measures seems prudent and feasible. Prospective follow-up of the physical and mental health of our participants, who are part of a nation-wide longitudinal study, must resolve the clinical value of these ambulatory SNS measures, separately and in combination.

CHAPTER 7

Development and dissemination of the VU-AMS

René van Lien and Eco J.C. de Geus

Abstract

With regard to both psychological and physiological processes, ambulatory measurement is 'worth the trouble' because it provides higher ecological validity and higher predictive validity for clinical outcomes than laboratory studies. This observation has led to the development of the VU University Ambulatory Monitoring System (VU-AMS) that specifically focuses on ambulatory assessment of activity of the autonomic nervous system. In this chapter we review the ongoing development of the VU-AMS hardware and software, with an emphasis on the contributions of technological progress and of the expanding community of VU-AMS researchers worldwide. Technology has increased the extent, robustness and precision of ambulatory recording. The experience, feedback and requests from the user community, however, have been a driving force in its conceptual development. So much so that the wider dissemination of the VU-AMS as a research tool has become a major goal for its developers. This chapter reviews 1) the VU-AMS development, and 2) the technical documentation, tutorial video`s, and hands-on workshops that are used to grow the VU-AMS user community.

Introduction

Ambulatory monitoring is a method of acquiring behavioral and physiological data in subjects who are free to go about their normal daily activities, outside the confines of the laboratory or hospital environment. In the past decades, ambulatory monitoring has evolved from an innovative tool in fundamental research to a widely used method in clinical and applied research settings. Because ambulatory monitoring takes place during everyday life, in the subject's own environment, such measurements have high ecological validity (Bussmann, Ebner-Priemer, & Fahrenberg, 2009; Ebner-Priemer, Trull, & Pawlik Kurt., 2009; Fahrenberg et al., 2007). A huge advantage of ambulatory monitoring over laboratory assessments is that it captures physiological processes that have a prolonged time scale, including circadian rhythms, work-nonwork transitions, and wake-sleep patterns. In addition, it may boost the incremental validity of the psychological constructs compared to a study that measures these only by self-report (Houtveen et al., 2009; Trull & Ebner-Priemer, 2009).

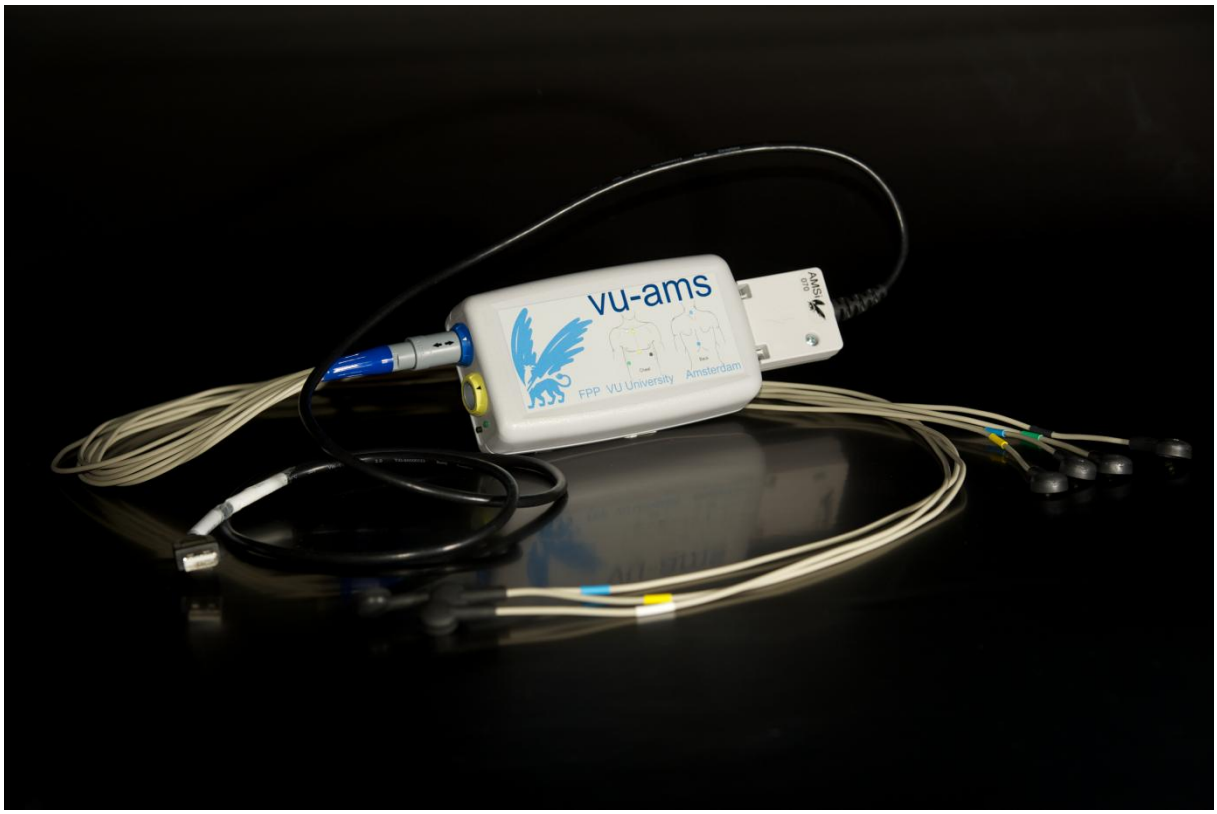
Specific advantages of prolonged ambulatory monitoring over laboratory testing are found in the assessment of individual differences in reactivity to psychosocial stressors. In psychosomatic medicine, negative consequences of such reactivity are expected on cardiovascular health (Ebner-Priemer et al., 2009; Kamarck et al., 2003; Krantz & Manuck, 1984; Linden, Gerin, & Davidson, 2003). These consequences will derive from the responses to frequent exposure to realistic stressors, encountered repeatedly at home or in the work setting. It has been found that the individual differences in the amplitude of cardiovascular stress reactivity to standardized laboratory stressors predict the response to actual real life stressors only moderately at best (Gerin et al., 1994; Kamarck, Schwartz, Janicki, Shiffman, & Raynor, 2003; van Doornen et al., 1994). Measurement of the frequency of stress reactions in real life cannot be done in a laboratory design and such exposure can only be obtained by ambulatory monitoring.

The added value of ambulatory recording has been illustrated most clearly in hypertension research. Measurement of resting baseline blood pressure in a clinic or laboratory setting can suffer from the "white coat effect". In these settings blood pressure is often higher than it would be when blood pressure was measured at home, because subjects tend to feel more anxious in the clinic or laboratory as compared to familiar surroundings. Ambulatory blood pressure measures, that do not suffer from white coat effects, have proved to be much better predictors for cardiovascular morbidity and mortality than laboratory or office measurements (Barry, Moroney, Orlebeke, & de, 1991; Pickering & Devereux, 1987; Verdecchia et al., 1994; Verdecchia et al., 1998; Verdecchia et al., 2001). There is no good reason to assume that a white coat effect would be limited to blood pressure only; more likely, it affects many other physiological measures as well. Directly assessing autonomic function in naturalistic settings, including leisure time at home and sleep, should also circumvent possible white coat effects on autonomic nervous system activity.

To maximally achieve its goals, ambulatory psychophysiological studies require 1) multiple physiological and psychological parameters with the highest possible precision, reliability and validity, and 2) a solid registration of confounding factors that (strongly) influence these signals but have no direct bearing on the psychological state and behavioral events that are of interest to the psychologist. Increases in the number of parameters/confounders that can be ambulatory recorded and improvement in the precision of such recordings have been largely **technology driven**. However, increases in the reliability and validity of ambulatory recording and data analysis strategies have been largely **research community driven**. Both processes, the role of technology and the role of the

user community, are amply demonstrated in the developmental history of the VU University Ambulatory Monitoring System (VU-AMS) (Figure 1.).

Figure 1. *of the VU University Ambulatory Monitoring System (VU-AMS).*



The VU-AMS

The VU-AMS developed out of a close collaboration of the department of Biological Psychology and the Technical Instrumentation department of the Psychology and Education Faculty, which was started in the '80's of the previous century and continues to date. Currently over 375 devices have been produced, about one-third of which are used internally to support research at the VU University itself. The VU-AMS, for instance, plays a central role in the ongoing genetics research on autonomic and cardiovascular stress-reactivity at the department of Biological Psychology and in the Netherlands Twin Register. The other two-thirds of the VU-AMS devices are used world-wide by over 50 research groups to study the effects of acute mental and social stress, ADHD, aggression, anxiety and depressive disorders, poor attachment, circadian rhythms, exercise training, hyperventilation, migraine, sleep, sleep deprivation, sleep disorders, chronic work stress, repeated worrying, and in studies linking the autonomic nervous system to metabolic and immunological risk factors.

The earliest 1984 predecessor of the current *5fs* version of the VU-AMS device, called the HRM[1] (Heart Rate Monitor) was originally developed for Lorenz van Doornen to measure heart rate

[1] The naming of the VU-AMS devices is a mildly confusing story. Reflecting the typical pioneering spirit in academia with its low attention to standardization and documentation, versions of the device have been called

responses to naturalistic stressors that could not be simulated in the laboratory, like a motor-bike driving exam, a PhD thesis defense, or an academic examination (van Doornen, 1988). First large scale application was done by two of van Doornen's PhD students. Irene Houtman studied stress and coping in lecturing (Houtman, 1992) and Eco de Geus examined the effects of aerobic fitness training on ambulatory heart rate which was the first 24-hour use of the HRM (de Geus, 1992). In due time the recorded signals expanded from heart rate only to include skin conductance level (Barry et al., 1991), motility (Klaver, de Geus, & De Vries, 1994), and thorax impedance (de Geus et al., 1995; Willemsen et al., 1996). Increasingly the device focused on the separate measurement of the activity of the sympathetic and parasympathetic nervous system, which is still the main trust of the newest *5fs* version. This monitoring of the two branches of the ANS required the recording of three core signals, the electrocardiogram (ECG), the impedance cardiogram (ICG) and the skin conductance level (SCL). In addition, a continuous indicator of ongoing physical activity, based on accelerometry (Montoye et al., 1983), was added early on.

ECG

In the early versions, ECG processing was limited by technology. The VU-AMS recorded the ECG signal at 250Hz from three electrodes and used a powerful dynamic online R-wave peak detector to compute the interbeat intervals (IBIs) from which mean heart rate values were computed and stored every 30 seconds. The device did not store the full ECG waveform due to limited internal storage space at the time (2Mb). This situation became increasingly undesirable when the value of heart rate variability (HRV) as a clinical predictor became evident (Bigger, Jr. et al., 1989; Bigger, Jr., Fleiss, Rolnitzky, & Steinman, 1992; Bigger, Steinman, Fleiss, & Rolnitzky, 1991; Chandra, 1987; De Maso, Myers, & Sellers, 1992; Feldman et al., 1991; Kleiger et al., 1991; Malik et al., 1991; Mogaard, Sorensen, & Bjerregaard, 1991; Odemuyiwa et al., 1992). Therefore, in addition to the average 30-second heart rate values, the device could be programmed to periodically perform a beat-to-beat registration (BBR), typically for a period of 5 minutes once every 30 minutes (Klaver et al., 1994). These IBI time series from the BBR periods were stored for offline calculations of the HRV using the at the time popular tools for spectral analysis like Ben Mulder's CARSPAN (Mulder & Mulder, 1981). A second measure of HRV, the root mean square of successive differences (RMSSD), was now also computed from the online R-peak detection, and 30 second averages of this value were added to the VU-AMS output.

This intermittent BBR sampling strategy balanced the technological possibilities available with the wish to achieve long recording durations with a light-weight device (in contrast to, for instance, more bulky Holter tape recorders). When technology advanced, newer generations of microprocessors with larger memory capacity consuming less power allowed adaptation of the VU-AMS for *continuous* recording of the IBI time series throughout a 24 hour recording. In addition, sampling frequency was increased to 1000 HZ as a 1 msec precision became the standard for IBI. Even so, a remaining downside of the online R-wave peak detection by the microprocessor was the number of errors in peak detection. These errors lead to incorrect IBIs that especially affect estimations of HRV and occur more frequently in physical active periods which cause most ECG signal distortion. The errors could usually be spotted offline but as raw signals were not stored, visual correction of missed or wrongly placed R-wave peaks was not feasible. It was common practice

HRM, HAM, AMD01-AMD03, AMS3.1-AMS3.6 (with SCL), AMS4.0-4.6 (with ICG) before settling on VU-AMS [version]. The current version is 5fs.

therefore to play safe and remove all signal parts containing physical activity. This caused a substantial loss of data.

For this reason, in the new *5fs* version the solid state memory solution was abandoned in favor of an onboard compact flash card that can store up to 4GB of data. The increased storage capacity allowed for continuous ECG recording, sampled at 1000Hz with a 16 bit resolution, for up to 72 hours. Improved filtering techniques furthered increased the signal-to-noise ratio and automated generation of the IBI time series could be done offline, using improved artefact detection and a more powerful R-peak detection algorithm (Christov, 2004). Short fragments of low quality signal with undetectable R-peaks were still removed but the many valid R-peaks surrounding these fragments were conserved.

Importing the raw ECG signal into *offline* processing software also allowed for more robust visual inspection and manual correction of the IBI time series. In the older software (AMSIBI), many bad R-peaks had to be manually flagged as sharp spikes on a visual rendering of the IBI time series. This required a lot of manual scrolling through the data, especially in long recordings. With the AMSQRS program for the *5fs* data, the full ECG signal was shown with the putative R-wave peaks marked on top of the actual QRS complex. This greatly improved reliability of scoring but another upgrade in the software was needed to facilitate the user in the somewhat daunting task of checking each and every R-wave peak in a 24 hour recording. This led to the development of the Data Analysis and Management Software (DAMS).

The DAMS program was based on JAVA programming and introduced many improved user interface features such as mouse-wheel controlled zooming and a continuity of the timeline when switching between signals. It further greatly reduced the interactive inspection of the R-wave peak detection by integrating a *suspicious beat selector*. The distribution of the IBIs is computed in a running window and all deviant beats (+2SD) are internally flagged. These are plotted on top of the raw signal using yellow (medium suspicious) or red (highly suspicious) color codes to indicate the need for correction. The program rank orders the red and yellow R-peaks in terms of being most deviant and takes the user through this rank ordered list. This way, only the suspicious beats need to be inspected. Compared to serially scrolling through the entire recording, the *suspicious beat selector* greatly sped up interactive inspection of the R-wave peak detection, while retaining a visual review by the user of the ECG and integrity of automated scoring.

The high quality IBI series delivered by DAMS made it possible to integrate a spectral analyses module into its ECG scoring. Previously, spectral analysis on the exported IBI time series was done by external software programs using either Fourier (Mulder et al., 1981) or Wavelet-based (Houtveen et al., 2001) approaches to calculate the total power in the 0.0001 Hz to 0.4 Hz range (TP), the power in the 0.04-0.15 Hz band (LF) and the 0.15-0.40 Hz band (HF). These programs required lots of file conversions and extensive preprocessing to correct the IBI time series, both of which are no longer required now that the Spectral analysis option is integrated into DAMS.

An additional advantage of storing the entire ECG is that the VU-AMS developers could finally honor the repeated request to extract multiple other intervals from the ECG, including the QT interval which is an important risk factor for sudden death (Arking et al., 2011). This inspired a further innovation in ambulatory ECG data processing. Single beat scoring of the many ECG landmarks (Q-onset, R-onset, S-point, S-offset, T-wave top and T-wave offset) is feasible but laborious and also error-prone. As a solution DAMS implemented an ensemble averaging step that created an average ECG waveform from the ECG of individual beats by time-locking them to their

respective R-peaks[2]. As described in chapter 6, a plethora of ECG landmarks can now be efficiently detected and interactively inspected, even in 24-hour data.

Motility

As soon as the first large scale data sets with 24 hour heart rate recordings became available, even in the very first versions of the device that simply compute 30-sec averages, it became clear that daily fluctuation in heart rate was dominated by the homeostatic reflexes in response to fluctuation in energy expenditure and postural change. The main determinants of real life heart rate variance are physical activity level and posture. First and foremost, in the words of van Doornen, "the heart is a pump". Its main function is to safeguard oxygen delivery to the tissues. This required a firm grip on the ongoing energy expenditure of the participants during ambulatory recording and it was realized from the start that self-report of physical activity through diaries might not yield the needed precision. Therefore, a 'motility' indicator was added using a vertical accelerometer that integrated the acceleration across 30-second fragments and added this 30-second motility value to the stored data.

The motility signal became a crucial building block of the 'Labeling' procedure which is possibly the most characteristic conceptual element of the VU-AMS. The Labeling procedure addressed two problems inherent in ambulatory data. First, there is a clear need to reduce the high dimensional data set to more manageable chunks for statistical analysis of main research questions. An example of a research questions would be "do people with high work stress have higher heart rate and is this restricted to the work hours?" To answer this question at the level of single beats is meaningless. Some sort of data reduction is needed in which the average heart rate is computed across specific ambulatory conditions like 'work hours' and 'leisure hours'. Whether more detail is required depends entirely on the research questions, with a further breakdown for instance into 'first work hour', 'second work hour', etc.

Second, in ambulatory recording participants engage in many different activities which only partly overlap with the activities of other participants, and if activities do overlap, they usually are not done at the same moment or for the same duration. A meaningful comparison of heart rate at rest and at work requires averaging the heart rate across instances of comparable activities during work and leisure time that mainly differ in the aspect of interest (work vs. leisure) but are maximally standardized otherwise, at least for posture and physical activity. Examples would be to compare desk work during the work day with watching TV in leisure time in the evening or talking to the boss in a meeting (standing) at work to talking to a spouse while doing the dishes at home.

To support Labeling, ambulatory recorded participants regularly note their activities in an activity diary. Half hour diary entries the list posture and physical activity (e.g. lying, sitting, standing, walking, biking), type of ongoing activity (e.g. desk work, eating/drinking, meetings, watching TV), energy expenditure level (no, light, medium or heavy physical effort), location (e.g. work, home, outside) and social situation (e.g. atone, with colleagues, with friends). This information from the activity diaries is used in combination with a visual display of the inbuilt vertical accelerometer signal to help the researcher interactively divide the entire 24 hour recording into fixed periods. An average of about 40 (typical range 20 to 60) coded periods ('labels') is created per subject with an average duration of 30 min. Sleep is divided in blocks of 1 hour. An average value across these labels is generated for further statistical analysis addressing the main research questions. For instance, mean

[2] This procedure was inspired by a similar approach in the scoring of the impedance cardiogram.

heart rate and RMSSD values across all labels representing (sitting desk work during) work hours were contrasted with mean values during (sitting at) leisure time after work or sitting on a weekend day in groups of subjects with low and high levels of work stress in the first two PhD theses based on VU-AMS ambulatory recordings by Harriëtte Riese and Tanja Vrijkotte (Riese et al., 2000; Vrijkotte, 2001).

As the quality of the motility signal is vital to the labeling procedure, the VU-AMS has striven to maximally adopt the many rapid technological advances in accelerometer design. These advances were driven by the requirement of high quality motion sensors for the controllers of game computers and for the adaptive horizontal/vertical orientation of the screen in a Smartphone. The current VU-AMS hardware uses a tri-axial acceleration sensor (X, Y and Z, -3g/+3g, 1khz, 12 bit)). Modern day tri-axial accelerometers are almost perfectly calibrated, in contrast to older versions, and are sufficiently sensitive to detect postural changes, discriminate between various intensities of walking and bicycling, and can even capture subtle 'movements in place'.

SCL

In a very early version of the device, the HGM1, there was a somewhat odd addition of a module to measure SCL for the computation of galvanic skin response (GSR) in addition to heart rate. The choice for SCL was odd because almost all GSR research at the time was done in laboratory settings. It would have been nice if the addition of SCL to the HGM1 had reflected the visionary idea that all psychophysiological measures should be accessible outside the lab, but in reality it foreshadowed the popularity of the VU-AMS devices for use in the psychophysiological laboratory (Barry et al., 1991). This ‘illegal’ use of the VU-AMS as a handy, lightweight, and portable polygraph for laboratory studies has been not restricted to versions recording SCL. Although clearly intended for prolonged recording in naturalistic settings, most versions of the device have also been used as an alternative for laboratory equipment like Nihon Kohden (http://www.nihonkohden.com/) or Biopac (http://www.biopac.com/) by many users.

In principle, this laboratory use of the VU-AMS is unwise as the need for an unobtrusive and portable device forces many suboptimal choices in the design and materials used in the microprocessor and electronic circuitries. This is most dramatically illustrated by the fact that the VU-AMS is powered by a few Volts from 2 batteries which have a limited life span, rather than the (although transformed) 220 Volts of the electrical power grid that are infinitely at the disposal of laboratory equipment. In practice, the VU-AMS does confer clear advantages in those experiments where the design requires subjects to move around between different setups/rooms, i.e. not confined to a single lab room. In these settings, not having to re-attach the electrodes after each change in location is an advantage. Ambulatory devices also have advantages in standardized settings when studying vulnerable populations in who the overt attention to the physiological recording in a lab setting might induce anxiety. A good example is provided by the NESDA study (Penninx et al., 2008), where anxiety and depression patients underwent a prolonged interview and testing session while wearing the VU-AMS (Licht et al., 2008). The advantage of applying ambulatory monitors outside real life settings is also obvious in experiments with a hybrid design, e.g. combining supervised indoor laboratory with supervised outdoor experimental conditions as was illustrated in chapter 3.

Various attempts have been made to employ the VU-AMS recording of the SCL in a true ambulatory design but the electrodes to the hand palm greatly reduce ecological validity of

recordings and a lot of noise is introduced by temperature and movement. Placement of the SC electrodes on the sternum can be helpful, but only SCRs to hot flashes seem powerful enough to be picked up (Carpenter, Andrykowski, Freedman, & Munn, 1999). It remains to be tested whether hybrid laboratory/ambulatory SCL recordings are more feasible. In anticipation, recent software upgrades in DAMS allow spontaneous or event-related Skin Conductance Responses (SCRs) to be scored and visually inspected. In addition, the VU-AMS has early on allowed the recording of the exact timing of stimulus events through an infrared connection to a computer running for instance E-prime software for stimulus presentation.

ICG

To explain the effects of psychosocial stress on cardiovascular health, a major hypothesis in psychosomatic medicine has been that repeated flight-fight reactions lead to permanent damage in the cardiovascular system (Krantz et al., 1984). Individuals characterized by strong fight-flight reactions to stressors are therefore considered at increased risk. Ever since its first elaborate articulation by Walter Cannon (Cannon, 1932), the SNS has been recognized as the major component of the flight-fight reaction and its cardiovascular effects (Brod, Fencl, Hejl, & Jirka, 1959). As discussed in chapter 2, the preejection period (PEP) became the measure of choice in cardiovascular psychophysiology to index SNS reactivity. It could be measured non-invasively from surface band electrodes that recorded the changes in thorax impedance (dZ) driven by left ventricular ejection of blood into the thoracic area. Integrating the dZ signal over time (dZ/dt) yields the ICG which gives a beat-to-beat indication of the timing of the opening and closing of the aortic valves and the dynamic changes in the speed of ventricular ejection. From the ICG various systolic time intervals can be computed, including the left ventricular ejection time (LVET) and the PEP.

It was noted early on that the ICG signal often contained lots of noise due to movement and respiration. This noise appeared to be handled effectively by ensemble averaging all single ICG waveforms locked to the ECG R-peak over a period of 60 seconds to create a single robust average ICG waveform (Muzi et al., 1985; Sherwood et al., 1990). Systolic time intervals scored in the resulting 60-second ensemble averaged ICG waveform corresponded closely to systolic time intervals scored on single beats ICGs in this same minute (Boomsma, de Vries, & Orlebeke, 1989; Kelsey et al., 1990; Muzi et al., 1985). However, even the improved automated scoring of the more stable and noise–free 60-sec ensemble averages has not abolished the need of interactive visual inspection of the scored landmarks in the ICG. As explained in chapter 3, the ICG waveform shows large variation, even within-subjects, and particularly the location of the B-point can be ambiguous.

Taken the popularity of the PEP as an index of SNS activity, ambulatory assessment with the VU-AMS became hugely more attractive with the introduction of the version 4 devices[3] that allowed the recording of the ensemble-averaged ICG from only 6 spot electrodes. Four of these electrodes were used to replace the band electrodes used with the ICG laboratory devices, because especially the neck collar was considered to obtrusive for 24 hour recordings and spot electrodes had been shown to be a valid alternative to band electrodes (Qu, Zhang, Webster, & Tompkins, 1986). Ensemble averaging of the ICG data was entirely implemented at the hardware level because internal storage capacity was still limited to 2Mb. Instead of storing the continuous ICG signal, it was sampled beat-to-beat for a duration of 512 msec at a sample rate of 250Hz, directly after the occurrence of an

[3] The AMS4.3 and AMS4.6 are still the versions that have been produced the most (~180) although the VU-AMS *5fs* is rapidly gaining ground (~175).

R-peak, and ensemble averaged online by the microprocessor over a fixed period of one minute. These ensemble-averaged ICG waveforms, at least the crucial first 512 msec, were saved to the internal data storage of the device for offline processing.

In the first versions, the ensemble-averaged ICG was only available during the BBR periods. An ICG scoring tool called AMSIMP was added to the software package to allow visual scoring of the 5 blocks of 60-second ensemble-averaged waveforms generated each half hour. Though ICG waveforms were only collected intermittently and minute ensemble averages substantially reduced the amount of scoring needed, it was still a labor intensive process. In the pioneer study by Gonneke Willemsen that validated the ambulatory VU-AMS PEP scoring, data from 21 subjects already resulted in 1332 five minute periods (=6660 complexes) to be scored and visually inspected (Willemsen et al., 1996).

The visual inspection burden further increased when improved versions of the VU-AMS 4.6 allowed the storage of *all* 60-second ensemble averaged ICG waveforms across a 24 hour recording. A typical ambulatory recording day yields up to 1440 in a single subject rendering visual inspection virtually impossible in larger sample sizes. For example, Riese et al. (Riese et al., 2003) described that the 750 subjects in their NETSAD study summed up to 947,250 waveforms to be scored. Concerns about this large user burden led to the introduction of large scale ensemble averaging (LSEA) of the ICG waveforms. LSEA entailed a further averaging all the 60-s ensemble averaged ICG waveforms within the same activity/label to a single waveform. During an hour of sleep e.g., only one LSEA has to be scored in contrast to sixty 60-second ensemble averaged waveforms. Extensive validation of PEP scoring by LSEA was done (Riese et al., 2003) and this procedure is now widely accepted for systolic time interval scoring in large epidemiologic studies.

The introduction of storage on the onboard compact flash card in the new *5fs* version not only allowed continuous storage of the ECG but of the ICG as well. This allowed the VU-AMS developers to improve ICG scoring greatly and address two often voiced concerns by the users, namely that the resolution of the ICG was only 250 Hz and that the online ensemble averaging would also introduce noise by indiscriminately co-averaging the ICG waveforms time-locked to wrongly scored R-wave peaks. The ICG waveform in the *5fs* version is stored continuously at 1000 Hz, that is yielding systolic time intervals with 1 msec resolution, and ICG scoring is done entirely offline. With the improved R-wave peak detection in the continuous ECG, the number of erroneous ICG waveforms entering the ensemble average decreased sharply, and the ICG scoring module of DAMS now additionally removes beats from the LSEA for a particular label that do not conform to the template waveform for that label. DAMS therefore delivers much cleaner LSEA waveforms, in addition to a more user-friendly interface for visual interactive inspection.

A further improvement in PEP scoring came from the addition of scoring landmarks in the ECG. Previously, PEP scoring was based on an estimated Q-wave onset (by subtracting 48 msec from the R-wave peak time). This was considered acceptable because within subject variation in early ventricular depolarization (QR interval) was minimal and the major part of contractile variation is captured by variation in the RB interval. However, we have shown that, although there is minimal within subject variation, between subject variation can be substantial (van Lien et al., 2013). The DAMS, therefore, now allows the scoring of the actual Q-wave onset and defaults to Q-wave onset estimation only if it cannot be reliably detected (~20% of the subjects). Q-wave onset estimation is further improved by use of the R-wave onset rather than the R-wave peak, reducing the absolute estimation error in msec roughly by half (see chapter 6).

Respiration

The addition of the thorax impedance signal to the VU-AMS, which was driven mainly by the desire to score PEP, provided an additional advantage with regard to the measurement of heart rate variability, in particular respiratory sinus arrhythmia (RSA). Researchers in the field had expressed concerns about the use of HRV measures like HF or RMSSD as a measure of cardiac vagal control without appropriate control for respiratory behavior (Eckberg, 2003; Grossman et al., 1993; Grossman et al., 2004; Houtveen et al., 2006; Houtveen et al., 2002; Ritz et al., 2006). In the laboratory this is now usually solved by coregistration of a respiration signal. In a standardized setting this presents little problems as the subject needs to wear one or two (breast, belly) respiration bands, or a nose thermistor. However, such measurement devices give more concern in ambulatory monitoring, where a harness-type construction is needed (Wilhelm et al., 2003). Fortunately, the dZ recordings required for the PEP also contain rich information on the thorax movement related to respiration.

Filtering of the dZ signal (0.01 – 0.5 Hz) delivers a stable respiration signal that can be used to detect the beginning of inspiration and expiration on a breath to breath basis. It can even yield reliable tidal volumes provided appropriate calibration (Houtveen et al., 2006). The breath to breath information on the phases of the respiratory cycle in combination with an IBI time series allows the calculation of peak-valley RSA (pvRSA), which is the difference between the longest and shortest heart period within a single breath and is entirely caused by vagal activity. Although HF and RMSSD correlate very highly to pvRSA they cannot be expressed on a breath to breath basis. In comparison to pvRSA they do not address short term changes in vagal activity and they also do not allow breath-to-breath correlational plots of RSA and IBI which could be essential in detecting the ceiling effects described in chapter 2.

The scoring of pvRSA requires interactive visual inspection in which incorrect respiratory cycles need to be deleted, as well as movement related distortions of the respiration signal. Also, breaths with strongly deviant IBIs need to be removed. A dedicated module called AMSRES was introduced in the version 4 devices to facilitate pvRSA scoring and this module has been further improved in DAMS. The respiration signal and IBI time series (as a cardiotachogram) are plotted side by side with the inspiratory and expiratory intervals and the corresponding shortest and longest IBI linked by color coding. In general, automated detection of pvRSA is extremely robust, thanks to the improved R-wave peak detection, and interactive visual inspection of the respiration scoring can be very fast.

Looking backwards

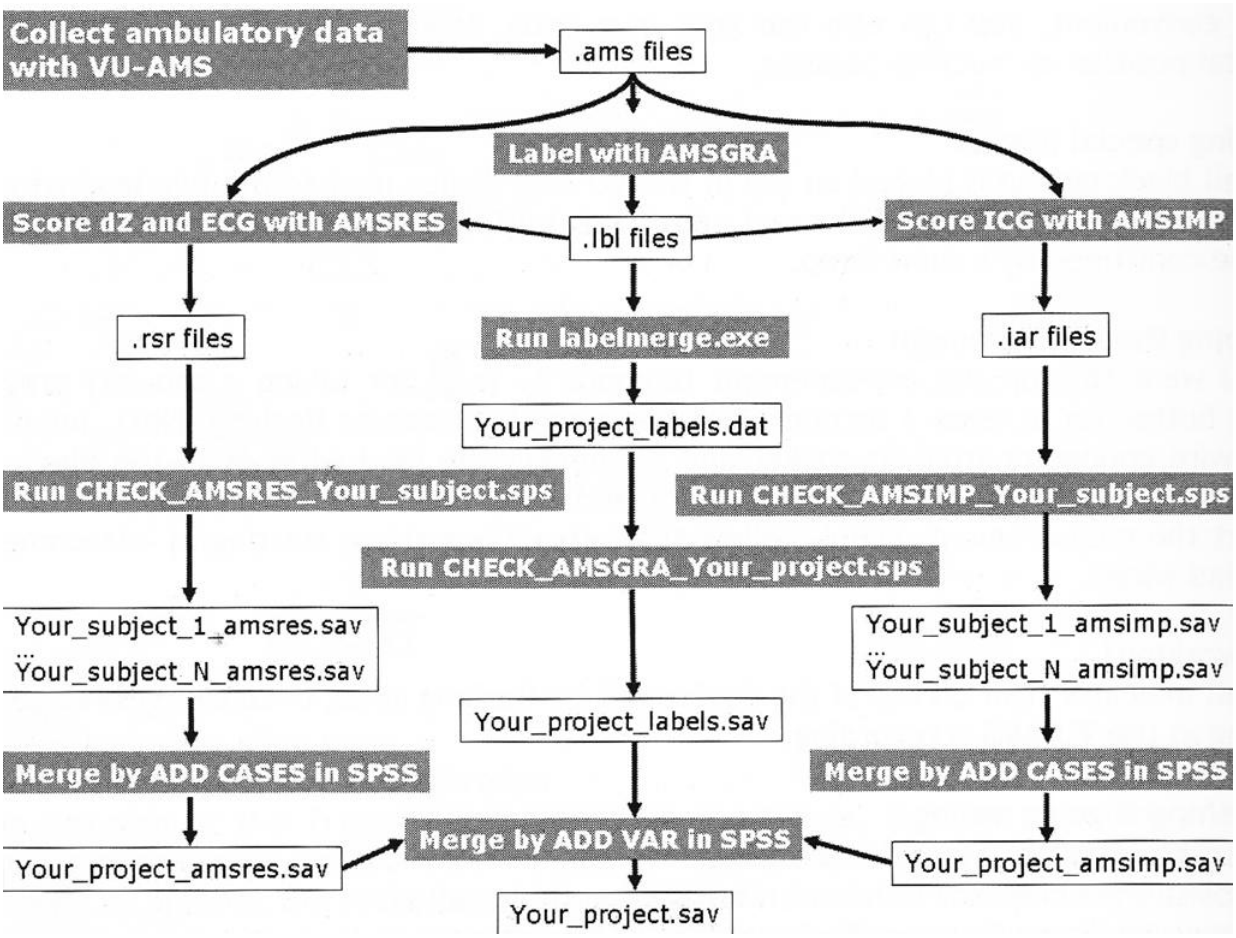
The incremental technological improvements have made the current *5fs* version in many respects a completely different device than the HRM1. This process is irreversible[4]. This progression of the hardware needs to be balanced by the appreciation that various VU-AMS researchers are engaged in a longitudinal data collection. This means that the current ambulatory assessment strategies and measures must remain compatible with the data collected in the past. For a large part

[4] Many of the components used in older versions are in fact no longer commercially available. This is a large concern to the VU-AMS developers who are often confronted with a request to repair an older version, for which the components can no longer be purchased.

this is quite feasible as the core VU-AMS measures are based on time intervals (e.g. PEP, RSA, IBI, RMSSD, respiration rate). Even if precision is (greatly) increased the interpretation of a PEP of 80 msec in 1993 is still the same as the interpretation of a PEP of 80 msec in 2013. In addition to the 'stability' of the recorded variables, a decent amount of backward compatibility has been built into the software. The new DAMS program for instance can read in version 4.6 files and will do its best to apply all improvements in signal scoring to these, admittedly, lower grade signals than produced by version 5fs.

Although major changes were made in the software, core elements like the labeling procedure and interactive visual inspection at a large scale ensemble averaged level have remained intact. In general the pattern in the software development has been from a delegated to an integrated system. Previous versions of the software divided the signal scoring across various modules (AMSGRA, AMSIBI, AMSIMP, AMSRES) requiring lots of file conversions and exchanges (See the flow chart in figure 2). In addition, data reduction to the level of label averages and synchronizing between the output of the various modules was delegated to SPSS scripts, which at some point started to proliferate in earnest and required the VU-AMS developers to additionally function as an SPSS help desk.

Figure 2. *A graphical description of the old VU-AMS 5fs data reduction strategy.*



DAMS did away with these cumbersome procedures and integrated all modules (AMSGRA, QRS, AMSIMP, AMSRES) of the previous VU-AMS software versions. It also terminated a painful

period where a *revert format* module was used to downgrade *5fs* signals to lower resolutions in order to be compatible with the older VU-AMS programs. DAMS now optimally exploits the current hardware and is constantly kept up to date with new improvements by a dedicated VU-AMS programmer. All ECG, ICG, SCL scoring as well as labeling are done in a single program. Tabs representing different parts of the analysis (just like the modules in the previous VU-AMS software) are ordered in a logical flow and guide the user from the import of raw continuous data to quality controlled output. Raw data, IBI series creation, RSA and PEP scoring, spectral power analyses, and SCL analysis are offered in consecutive tabs. The resulting output from all tabs is stored in a single text file with the extension *'.amsdata'* (or an excel sheet) and this output reflects the average values of the relevant variables per labeled period. Very little (SPSS) preprocessing is needed before proceeding to the actual statistical analysis.

Looking forwards

The current version of the VU-AMS device(*5fs*), can record ECG, ICG, dZ, SCL and tri-axial accelerometry simultaneously with very low noise and at a sufficient sampling speed for the time intervals extracted. From these signals almost every non-invasive cardiac ANS measure described in chapter 1 can be obtained by the current version of the DAMS (3.0). Still, further improvements are direly needed and major hardware and software improvements are planned including (1) downsizing the device and reducing the electrodes, which decreases the burden on the participants, (2) using wireless (e.g. Bluetooth) connections to link to the startup-PC and enable online monitoring, (3) reducing the amount of interactive visual inspection in favor of fully automated scoring, (4) integrating the recording with (behavioral) data entered into a tablet or Smartphone.

Subject burden

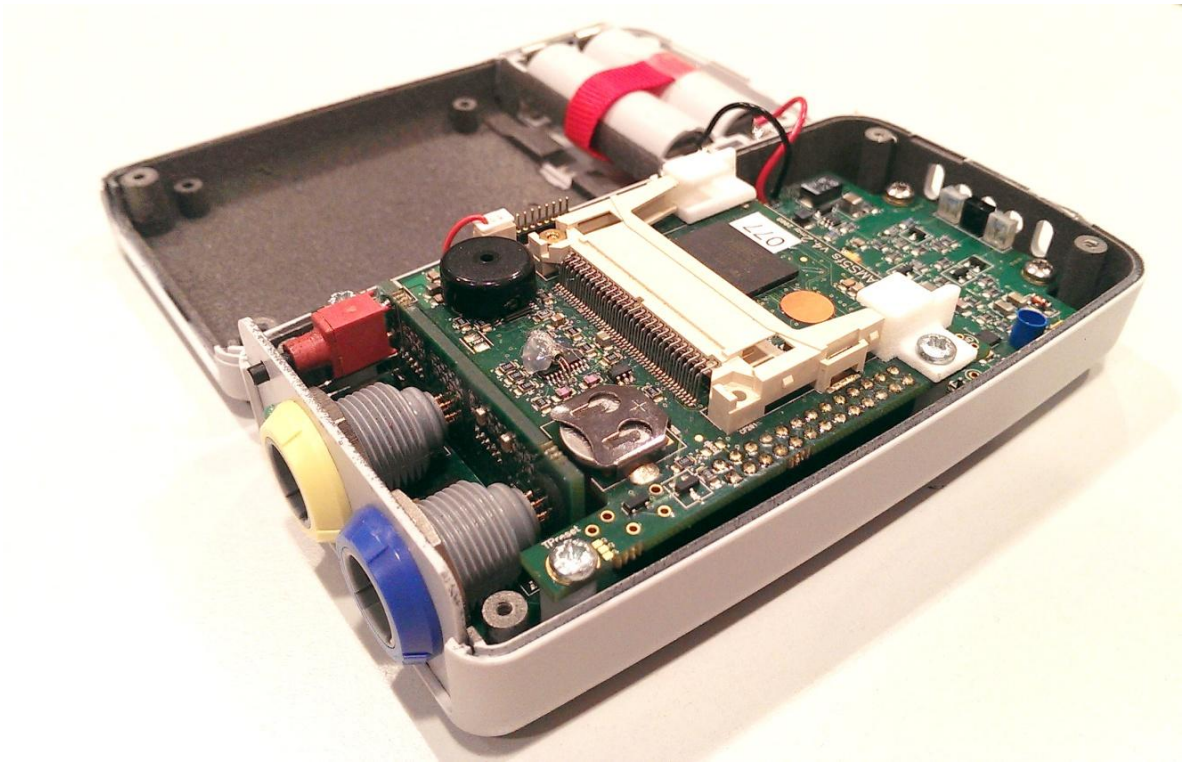
Ongoing technological advances in electric engineering would allow us to further reduce the burden on the participants by creating a VU-AMS of smaller dimensions. The internal technological components of the VU-AMS can by now be replaced with smaller components and placed in a housing of about 1/4 of its present size (figure 3). A smaller size would allow alternative placements of the device on the body, e.g. at the chest instead of the hip. In addition, success pilots have been completed with a VU-AMS device that records the ECG and ICG recording only 5 electrodes instead of the default 7electrodes. The ECG V- and ground electrode can be combined with the V- and V+ electrode of the ICG respectively, without compromising signal quality[5]. This further reduces subject burden (and also saves on electrode costs).

Major hardware changes in the VU-AMS device might compromise downward compatibility and can inadvertently introduce new unforeseen technical problems. This can be a problem for longitudinal studies that need to replace older with newer devices and expect unchanged functionality. The VU-AMS developers have therefore created two parallel production lines. The first line produces the current stable well-tested *5fs*. New features are implemented in a separate line of experimental versions that are tested in master student projects and, once confidence has increased, in PhD projects at the VU-University Amsterdam. This division between a production line and an

[5] There had already been 6-electrode versions that combined an ECG/ICG electrode. The ICG was extracted by phase locking the measured dZ signal to the phase of the alternate current generator.

experimental line guarantees stability of the quality of the VU-AMS devices for the VU-AMS user community, while not impeding further technological innovation.

Figure 3. *The VU-AMS device could be downsized thanks to new technologies. The figure displays the inner electronics of the current device.*



Bluetooth

Extensive on line signal quality checking is essential for 24 hour recordings. To initiate online signal checking the VU-AMS device was traditionally connected to a start-up PC by a RS232 serial cable through an infrared coupling. This connection is safe as there is no galvanic contact between the participant and the PC. Disadvantages are that the subject's movement is restricted during online verification and that the even minor movement of the infrared connector could lead to a lost connection. Luckily, devices have gone wireless on a massive scale in the past years and the costs of wireless communication devices are now below that of older technologies such as infrared. One of the recent experimental versions of VU-AMS 5fs was equipped with an integrated Bluetooth module to establish a wireless connection between the VU-AMS device and the DAMS interface.

The quality of the ECG and ICG signal can now be monitored wireless in real time at all times during an experiment, even at a fair distance from the computer. This is especially useful in observational (e.g. attachment) research or during exercise research where the average heart rate should be monitored to assure subject safety. Another advantage is that subjects don't get to see their own ECG during signal quality check right before the baseline measurement of your experiment. The Bluetooth connectivity can additionally be used to add markers remotely at moments of interest during observational research. During attachment research for example the

reaction from a mother to the crying of her baby needs to be marked right after the start of the cry. These markers thus need to be placed ad hoc and without intrusion. All together, Bluetooth offers improved freedom of movement during research and opens up new (long distance) monitoring possibilities and might prove useful in combination with secondary devices such as android phones or palmtop computers.

Automated scoring

A major improvement of the signal scoring with DAMS would be to reduce the amount of interactive visual inspection needed in favor of automated scoring. This is particularly necessary for ambulatory PEP scoring as this is still the most time consuming activity for VU-AMS users, even with the LSEA strategy. The recent addition of the LSEA ECG intervals and their relative position to the B-point creates strong opportunities to improve automated scoring. The resulting large normative data sets of chapters 3 and 6 should be used in future algorithms to predict the likely location of the ICG landmark, and use *a priori* weighing of candidate points based on these predictors. The B-point for example is typically found within 120 ms after the R-peak and searching for it in the middle of the T-wave is senseless. The dZ/dt-max is often located close to the S-point, whereas the X-point is close to T-offset. In anticipation of these improved algorithms the ECG and ICG ensemble averaged waveforms are now already simultaneously displayed in the impedance scoring window which users have reported to greatly aid the confidence during interactive visual PEP scoring.

Labeling is the second most time consuming step in ambulatory data analysis. This was true in the older software (AMSGRA) and still is true in the current DAMS version, although the manual placement of the labels has been made increasingly user-friendly. An improvement direly needed is the automated scoring of energy expenditure from the accelerometer and others have already shown this to be feasible (Bouten, Westerterp, Verduin, & Janssen, 1994; Bussmann, Hartgerink, van der Woude, & Stam, 2000; Crouter, Antczak, Hudak, DellaValle, & Haas, 2006; Eston, Rowlands, & Ingledew, 1998; Westerterp, 1999). A more detailed analysis of the postural changes and physical activity patterns is also possible but this requires more sophisticated approaches (Freedson, Lyden, Kozey-Keadle, & Staudenmayer, 2011; Liu, Gao, & Freedson, 2012; Staudenmayer, Pober, Crouter, Bassett, & Freedson, 2009) including non-linear regression, Hidden Markov Models, artificial neural networks, or support vector machines.

The absolute values of the motility signals might be used to automatically indicate quiet, light, moderate and intense activity periods in the recording. The information in the separate axes of the tri-axial accelerometer on the other hand offer more detail and might be used to detect and label posture changes throughout the day by means of regression. Accurate body position detection traditionally would require a second or even a third motility sensor placed on different parts of the body and would be a last resource as it would increase subject burden. Adding other measures to the equation, such as HR and RMSSD, might prove to be a less obtrusive solution to reliably detect body position in fully automated fashion.

Integrating the recording with a tablet or smartphone

Ambulatory monitoring requires reliable recording of physiological data with optimal quality, but also requires adequate behavior sampling strategies during the day to make psychophysiological inferences. Ambulatory assessment has up until recently depended on paper diaries by participants but are now rapidly replaced by digital diaries, with clear advantages. Paper diaries require labor

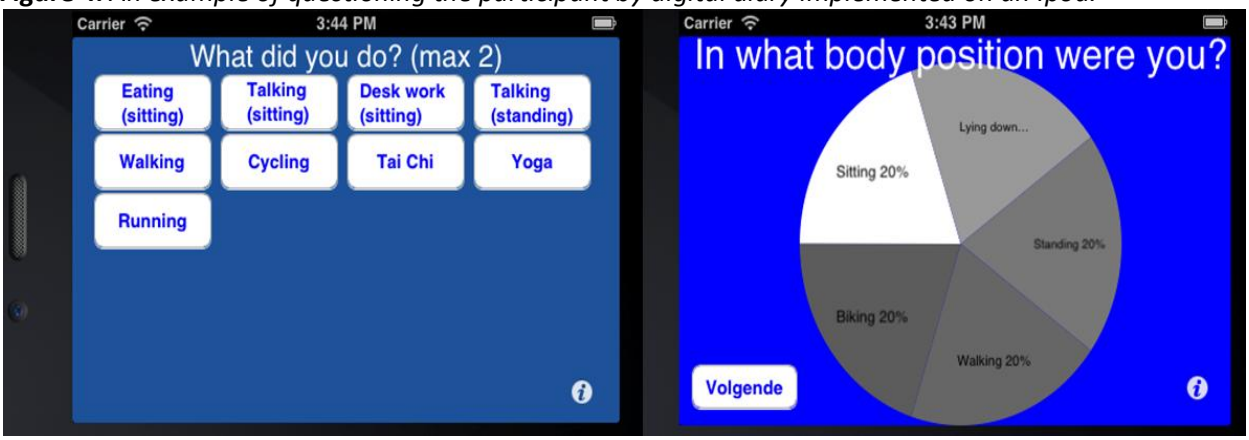
intensive translation and interpretation from paper into the labeling procedure of the DAMS program. This is not always easy as participants often describe activities incompletely, in the wrong order, or not all. Additionally researchers are often challenged by incomprehensible handwriting of a certain number of participants which may lead to coding errors. Digital diaries can be programmed to prompt the subject to respond to certain questions across the day. This requires less time and less effort for the participant and completely removes coding errors (figure 4 gives an example of a digital dairy). DAMS should have an easy tool to import the digital diary information and perform initial labeling of activities in a completely automated fashion. In combination with automated posture and movement detection, labeling would be virtually automatic at that point and merely require visual inspection.

Perhaps the main technological opportunity in this regard is provided by the implementation of digital diaries on a Smartphone (Hicks et al., 2010; Runyan et al., 2013; Stumpp, Anastasopoulou, & Hey, 2010; Trull et al., 2013) which removes the need for participants to carry additional electronic devices: smart phone penetrance is over 60% in the Netherlands. The latest generation Smartphones are essentially mini-computers with a wide range of standard integrated sensors including an accelerometer, gyroscope, magnetic sensor, digital compass, proximity sensor, ambient light sensor and Global Positioning System (GPS). Information from these sensors might be utilized by dedicated research software applications in combination with information from the VU-AMS device. The kinematic sensors (triaccelerometer, gyroscope and magnetic sensor) could be utilized to recognize physical activity with even higher precision (Yi He and Ye Li, 2013).

The GPS for is already utilized in many commercially available android applications. A good example of such application is MyTracks (http://www.google.com/mobile/mytracks/), which records travel route, speed, distance and even altitude over prolonged periods. The GPS information can potentially be used to differentiate between types of transportation (active vs. passive) in combination with the motility senor. It is even possible to remotely observe relatively fine-scale movement or migratory patterns in people to infer some indication of location and social situation. Social and geographical interactions are diverse and differ substantially per subject and indexing by GPS would at least offer a more detailed picture than the traditional paper diaries.

Smartphones do not only offer an opportunity to collect a variety of participant data and they also allow for a more flexible and interactive study design. For instance, certain questions may pop-up on the Smartphone after a sensor of either the Smartphone or a connected heart rate monitor has detected a certain (physiological) event of interest.

Figure 4. *An example of questioning the participant by digital diary implemented on an Ipod.*



Dissemination

As should be clear from the above, the VU-AMS user community has been a driving force in the innovation of VU-AMS. Table 1 lists some examples of how user requests shaped the development of the ICG/ECG variant of the VU-AMS.

Table 1: *Examples of VU-AMS development in response to user requests.*

Requests from users	**New hardware or software development**
HRV is recognized to be a clinical marker and a selective marker of vagal activity. Can the VU-AMS measure more than average HR?	Addition of the BBR option that collects all IBIs during a 5 minute period each half hour of ambulatory recording. 30-second average of RMSSD was added.
Can you detect when electrodes come off in unsupervised recordings and warn the participants?	Addition of alarm beeping if the ECG signal failed – participants received extra electrodes and a graphic overview of how to attach the electrodes.
How do we know the device is still recording?	A blinking light was added to indicate VU-AMS status.
During 24 hour recording HR appears to be clearly dominated by physical activity. How can we separate psychology from homeostasis?	Addition of parallel movement recording ('motility') by vertical accelerometry to allow correction or stratification by physical activity in the analyses.
Can we have the user indicate specific events happening in the unsupervised settings (e.g. hyperventilation, panic attack)?	Addition of an event marker button.
The large amount of data difficult to grasp, Can the signals be visualized?	Creation of a graphical interface to show the heart rate and motility signals of the entire 24 hour recording
Can we have Momentary Assessment of mood, social situation, job strain, posture and activities (following similar developments in ambulatory blood pressure monitoring and salivary cortisol sampling)?	Addition of random prompting of the subject to fill out diary by a beeping signal – disabled at night.
VU-AMS is also used in laboratory or in hybrid designs – part supervised laboratory, part unsupervised. Can we import stimulus event	Addition of an interface with E-prime and other stimulus-presentation software to record stimulus-event times in synchrony with physiological signals.

times into the recordings?	
Conisdering the large dimensionality of ambulatory data, how can we reduce this massive dataset to manageable yet meaningful chunks?	Development of the VU-AMS 'Labeling' strategy, combining both diary and motility information to break up the recording in labeled periods reflecting stable posture and physical activity and a single type of daily activity.
There is a need for a specific measure of SNS activity. Can we measure PEP by ambulatory recording?	Development of thorax impedance monitoring for PEP scoring (versions 4.0 to 4.6).
Can we monitor the signal at startup to ensure correct ICG electrode placement?	Addition of online monitoring of the IBI time series and dZ/dt signal (later expanded to continuous ECG, dZ, and ICG display).
Paul Grossman's peak valley RSA needs a breath to breath respiratory signal. Could we measure this?	The impedance signal is also used to extract respiratory parameters, and compute pvRSA.
Interactive visual scoring of each one-minute ensemble average is very laborious. Is there a way to reduce the scoring effort?	Large Scale Ensemble Averaging was implemented in the graphic ICG scoring interface –while preserving the individual 60-sec ensemble-averages for 'underwater' control.
The ICG points are often ambiguous and ICG waveforms are degraded during noisy ECG; also 4 msec PEP precision is no longer accepted by journals. Can the PEP scoring be improved?	A major change to continuous recording of the ECG and ICG on flash cards allowed for 1000 Hz sampling and offline scoring with much more control over the parts of the signal that were used for PEP scoring.
The motility signal is not always clearly discriminating between activities – calibration is needed across devices. Can you do better?	Implementation of high quality triaxial accelerometers and switch to 1000 Hz recording of X, Y, Z accelerations.
It is hard to organize the data with all the various scoring modules. Can this be integrated?	DAMS integrates all signal recording and signal processing steps into a single package.
The software is slow on longer recordings. Can it be sped up and made more always user-friendly?	Large improvements in user friendliness and computational efficiency in DAMS.
The ECG contains many other interesting intervals including the QT. Can the full ECG be stored and scored?	Full storage ECG was realized and a efficient LSEA scoring of ECG landmarks in 24-hour data implemented.

Could online monitoring be done without a fixed connection – for instance for online demonstration purposes or exercise monitoring?	Creation of a set of experimental 5fs versions with a Bluetooth connection.
Can we import signals from other parallel ambulatory devices for validation purposes?	DAMS was expanded by the option to import an external signal that can be displayed next to the VU-AMS signals.

The primary VU-AMS users originated from the network of scientific collaborators maintained by the department of Biological Psychology at the VU University. When the visibility of the VU-AMS increased through scientific publications this led to an increased demand and a rapid expansion of its use beyond this personal acquaintance network, both in the Netherlands and abroad. Although the experience, feedback and requests from the user community became increasingly recognized as a major driver of conceptual and technological development, they also presented a serious challenge. A first challenge was that ambulatory monitoring of the RSA and PEP was now done by researchers not deeply trained in the psychophysiology, which sometimes threatened to compromise the correct collection and/or interpretation of these parameters in ambulatory recordings. A second challenge was that training and explanation of the hardware and software could no longer be done 'in the flesh' and a large demand grew in the user community for detailed product information, detailed manuals and tutorials, and systematic studies on reliability and validity of the VU-AMS. Fortunately, this was paired to an increased demand for the device which is sold with a margin of profit. This profit has been completely reinvested in further technical development of the VU-AMS hardware and software. Since 2004, when the first dedicated VU-AMS PhD ('VU-AMS AIO') started, VU-AMS income is increasingly used to also address these new challenges.

PhD projects are started with the explicit aim of further scientific validation of the system and the PhD students in these projects have the additional mission of supporting the appropriate use of the VU-AMS in behavioral and biomedical research. This dissemination of ambulatory assessment became an important scientific aim in itself, an idea further fueled by the birth and rapid growth of the Society for Ambulatory Assessment (http://www.ambulatory-assessment.org). The main goals of this dissemination are to stimulate its widespread use in research questions where laboratory studies might yield incomplete answers and to educate researchers with a broad range of backgrounds in the correct use of the VU-AMS for ambulatory recording. Both goals were addressed by creating a VU-AMS website (www.vu-ams.nl) containing written technical documentation and e-teaching materials, including online tutorial videos. In addition, the frequency of customized hands-on workshops was intensified and long-distance assistance to remote research groups was set up.

Written documentation

Already in 1993 a first official AMS manual was released (de Geus, de Vries, Klaver 1993) and these were amended over time, or supplements were added. A first major update followed in 1998 (de Geus, de Vries, & Klaver, 1993; de Groot, de Geus, & de Vries, 1998) and the most recent major update was released in 2012 (van Lien, den Hartog, & de Geus, 2013). This version can be found in appendix 1, and provides step-by-step instructions on the operational use of the *5fs* and DAMS.

These instructions cover the appropriate electrode attachment, starting or stopping recordings and online signal checking. They outline the workflow of data processing with the VU-DAMS program from continuous raw data to quality controlled output on e.g. PEP, RSA and HR averaged per label. The manuals also specify the hardware and software used by the VU-AMS which is needed for the methods section of papers. Most importantly perhaps, they should assist the users in interpreting the psychophysiological results in a correct way.

Video Tutorials

The manual is an important basic document to have around, especially when using miscellaneous features, but some aspects are easier understood when visualized. Visualization greatly facilitates the understanding and efficient reproduction of experimental techniques, thereby addressing two of the biggest challenges faced by today's life science research community: 1) low transparency and poor reproducibility of biological experiments and 2) time and labor-intensive nature of learning new experimental techniques. Written word and static picture-based traditional print journals are no longer sufficient to accurately transmit the intricacies of modern research. It can take weeks or even months to learn, perfect, and apply new experimental techniques. Thus, much time in the laboratory is spent learning techniques and procedures.

The use of video can overcome much of these problems. Tutorial videos provide a more detailed and intuitive documentation of the methods used in ambulatory assessment, particularly when these methods become more dynamic and complex. Video documentation is therefore increasingly accepted in the scientific community as an official publication. Recently the world`s first peer reviewed, PubMed-indexed video journal called Journal of Visualized experiments (JoVE) was started. JoVE takes advantage of video technology to capture and transmit the multiple techniques of life science research and opens a new frontier in scientific publication by promoting efficiency and performance of life science research. Video allows visualization of the temporal components in an experimental design and the dynamic interactions with a participant during electrode attachment. As an example we recorded the experimental procedure during a study using the VU-AMS to asses autonomic nervous system functioning in 3097 children, aged between 5 and 7 years in many different locations (e.g. school, sports centre, science museum). The video was published in JoVE (van Dijk et al., 2013) and can be found under:
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3667644/. The accompanying document describing the methodology used in the video can be found in appendix 2.

In line with the philosophy outlined above, we produced a further series of dedicated tutorial videos to educate on the most basic features of the AMS hardware and DAMS software. We used Camtasia (www.techsmith.com) to record the computer screens encountered while running DAMS, and to add text, audio and animations. These edited videos are shared through the internet by placing them on e.g. *Youtube*, and provide a much appreciated support tool to train researchers as well as their research assistants. The videos take relatively little time to produce and have been watched over 1100 times since their release. The full set of VU-AMS tutorial videos can be found on the VU-AMS website:

Recording with the VU-AMS video (22:15 min, viewed over 331 times in 1 year).

R-peak detection video (10:17 min, viewed over 492 times in 1 year)

Data labeling video: experiment (11:46 min, viewed over 215 times in 1 year)

Data labeling video: naturalistic setting (5:43 min, viewed over 63 times in 1 year)

Hands-on education

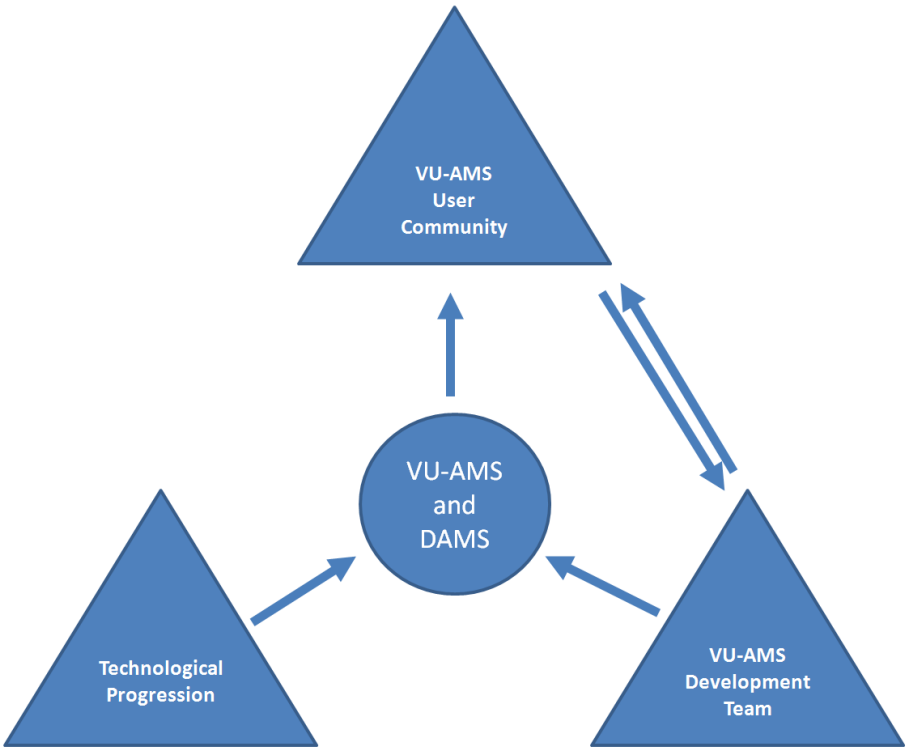
In appreciation of the importance of a correct use of the VU-AMS, one of us (van Lien) gave over 34 hands-on workshops to groups of 4 to 6 people. The workshops takes about 5 hours and teaches the user to record an ambulatory measurement, to process the data efficiently and to understand the major caveats in using the VU-AMS in various ambulatory designs. The workshops come in many flavors, but a few elements usually return, including doing an actual recording on workshop participants (or its staff), processing of illustrative pre-recorded example recordings varying from perfect to very bad quality, and peer comparisons of signal scoring. The workshop materials are made available for additional practice, and can be propagated by the workshop participants to their students or research assistants. A workshop example is shown in appendix 3.

Long distance education

Distance can of course prevent the participation in the hands-on workshops (although there is substantial international attendance). In this case, remote counseling can be helpful. Teamviewer (www.teamviewer.com) for example is an excellent software package that allows two users in different locations to share one computer screen. It offers an opportunity for interactive PEP scoring to reach e.g. consensus on deviant cases in multi-rater comparison of the scoring of the B-point. Before such a remote-counseling, the (large) recording files can be send over through services on the internet like WeTransfer (www.WeTransfer.com) or DropBox (www.DropBox.com), although this should be restricted to encrypted files or files that contain no identifying information in the subject ID or the labels. To support remote counseling, short customized videos are sometimes created.

Most recently, the VU-AMS website opened an user forum where any sort of question concerning hardware and software can be posted. The VU-AMS developers answer these questions on the forum whenever possible to eventually build a knowledgebase around technical, practical, and conceptual issues in ambulatory monitoring. Users can then find their way around such issues without direct interference from the VU-AMS development team and add their own experience and expertise to the knowledgebase.

Figure 5. *The triangle that is the driving force behind the VU-AMS device and DAMs program.*



Conclusion

Throughout its existence, the VU-AMS has adopted newly available technology to implement the new ways of data collection, study design, and data reduction. The new features in the AMS hardware and software in turn led to new research questions in the user community which posed further demands on the system. The interweaving between the VU-AMS development team, technological progress, and the user community remains the driving force behind the VU-AMS device and DAMS program (see figure 5). In this, the VU-AMS is a poster child for the field of ambulatory monitoring in general, where research innovation and technological progress fuel each other constantly.

CHAPTER 8

Summary & conclusions

Improving the methodology for non-invasive autonomic nervous system recording and its implementation in behavioral research.

The aim of my PhD project was three-fold. First, to critically re-examine the validity of the current strategies for ambulatory assessment of parasympathetic nervous system (PNS) and sympathetic nervous system (SNS) activity. The second aim was to test the feasibility and validity of new ambulatory measures of SNS activity. The third aim was to actively disseminate the knowledge gathered throughout the development of the VU-AMS device, and in particular to ensure a correct use of VU-AMS hardware and software by the growing community of VU-AMS users. In this final chapter, I briefly summarize the outcomes of the studies I conducted on current and new indices of ANS activity for use in ambulatory monitoring. These studies used data from four new experiments conducted at the VU University and two existing data sets from the Netherlands Twin Register. I conclude with some concrete implications of my research, and possible next steps to increase the use of ambulatory monitoring of autonomic nervous system function in the behavioral sciences.

Current available indices of SNS and PNS activity

Chapter one critically reviewed the currently available indices for the measurement of PNS and SNS activity and the feasibility of measurement of these parameters in daily life. The last is important, as individual differences in autonomic nervous system (ANS) responses to laboratory challenges do not generalize very well to real life situations, and this may be reflected in the low predictive value of laboratory ANS reactivity to future cardiovascular disease. Invasive measures, even though they have good content and criterion validity for measuring SNS and PNS activity (microneurography, microdialysis, pharmacological blockade, regional spillover and plasma catecholamines), thus need to be discarded as they cannot be measured in real-life without severely hindering normal life function. Looking at the non-invasive indices available, it was concluded that when conducting ambulatory monitoring the best index of PNS activity is Respiratory Sinus Arrhytmia (RSA). Both HF and RMSSD measures of RSA can be easily obtained, while pvRSA becomes available when respiration is also measured. For SNS activity in real life, the preejection period (PEP) was seen as the measure of choice. However, due to the labor-intensive scoring procedures and the sensitivity to preload and afterload effect, an alternative measure of SNS activity would be welcome.

In chapters two and three, the focus was on the limitations of these two ambulatory measures of choice, RSA and PEP, with the aim of providing the means to detect or even overcome these limitations. Chapter two focused on the possible underestimation of RSA in participants with low heart rates, due to ceiling effects in the acetylcholinergic neurotransmission. Such effects would be most pronounced during sleep. The 24-hour ECG and respiration recordings were examined in 26 regularly exercising participants who enrolled in a 6-week supervised training program and in 26 age- and sex matched non-exercisers. The IBI-RSA relationship was examined by visual inspection of the IBI-RSA plot for each individual. A subgroup of exercisers showed an IBI-RSA relationship that was characterized by a quadratic profile, compared to the expected linear profile, indicating an underestimation of vagal control in this group. Indeed, only when accounting for the ceiling effect a beneficial effect of exercise on cardiac vagal control was apparent. Inspection of the IBI-RSA relationship is thus mandatory when using HRV measures to index vagal control.

Chapter three focused on the detection of the specific points in the impedance signal which are used to estimate PEP. PEP is defined as the interval from the onset of the left ventricular depolarization, reflected by the Q-wave onset in the ECG, to the opening of the aortic valve, reflected

by the B-point in the ICG signal. However, the position of the Q-wave onset and the B-point are not always easily detected and automatically scored points need to be visually detected. This is not only labor-intensive but also makes the PEP estimate more error-prone. Using data obtained during several different postures and physical activity conditions from two studies conducted in different settings (laboratory versus ambulatory), I evaluated two alternatives to the detection of the Q-wave onset and B-point: computing PEP from a fixed value for the Q-wave onset to R-wave peak (QR) interval and from an R-wave peak to B-point interval that is estimated from the R-peak to dZ/dt-min peak (ISTI) interval. The evaluation of the QR interval and ISTI provided meaningful estimates of the expected location of Q-wave and B-point. Also, ISTI by itself may provide an additional measure of cardiac contractility, reflecting the time it takes to reach peak ventricular ejection. However, as discrepancies between estimated PEP and actual PEP were large, the detection of the Q-wave onset and the B-point remains highly advisable.

Evaluation of two alternative measures of SNS activity

In the chapters four to six, two alternative indices of SNS activity were studied: salivary alpha-amylase (sAA) and the T-wave amplitude (Twave). Chapters four and five focused on the enzyme sAA, which can be easily obtained from saliva and has gained interest as a potential non-invasive biomarker for SNS activity. In a first study, presented in chapter four, ECG and ICG signals were registered during 24 hours to obtain PEP and RSA, and participants provided 7 saliva samples throughout the day by gently chewing on Salivette cotton rolls. In contrast to what is known about diurnal patterns in SNS activity, sAA increased throughout the day and there was no significant association between PEP and sAA, not even when correcting for RSA. To exclude the possibility that the economic but suboptimal Salivette sampling method for sAA had caused the negative findings, a second study was conducted in which saliva was more carefully collected using the passive drooling method before and after cycling, a task certain to elicit SNS activation. In addition to sAA activity, which is the most commonly used sAA measure, actual sAA protein concentration and the ratio of sAA protein to salivary protein were also determined. The sAA responses to exercise were compared to changes in PEP and RSA in response to exercise. As expected, cycling increased SNS activity and decreased PNS activity, as indicated by a decreasing PEP and decreasing RSA. SAA activity and concentration also increased in response to the task. Participants who showed a combination of SNS activation and a small loss of PNS activation in response to the exercise task did show the strongest increase in SAA activity, but PEP and RSA changes were not related to sAA output. Based on the results of these two studies presented in chapters four and five, the use of sAA output or sAA activity as proxy of SNS activation is not recommended.

In chapter six the use of the ECG T-wave amplitude (TWA) as an indicator of SNS activity was determined. Using a large sample of 24 hour data it was shown that large scale ensemble averaging of the ECG is feasible and allows meaningful scoring of the major ECG landmarks. The TWA, for instance, could be reliably determined in over 90% of the participants and showed a clear increase in response to a mental task. Also, during 24 hour monitoring TWA decreased stepwise from nighttime sleep to daytime sitting to physical activity. As such TWA does seem to covary with expected changes in SNS activation. Within subjects, TWA also correlated with PEP, with an average correlation of .35 after partialling out RSA and IBI. This suggests that TWA cannot replace PEP as indicator of SNS activity but that the inclusion of both TWA and PEP may provide a more comprehensive picture of SNS activation.

The VU-AMS; development and dissemination

The studies on ambulatory monitoring of ANS activity made use of the VU University Ambulatory Monitoring System (VU-AMS) that was specifically developed for this purpose at the VU University, through collaboration between ICT/electronics specialists and psychology researchers. Chapter seven describes the history of the development of the VU-AMS, and shows how technological innovation linked to user input improved signal recording, signal quality, data processing and general usability of the VU-AMS. Improvements of the VU-AMS in turn lead to new applications and study designs that generated again more requests for further development, creating a positive feedback loop. It is in the triangle of the VU-AMS development team, technological progress and user community that ambulatory monitoring with the VU-AMS will continue to progress and this model seems very suitable for the development of ambulatory monitoring in general. Chapter 7, together with the appendices, also review the various tools created to support the dissemination of the correct application of the VU-AMS in behavioral research.

Main conclusions

- At the moment RSA and PEP are the indices of choice for measuring PNS and SNS activity in daily life.
- In individuals with lower heart rates, such as regular exercisers, vagal control may be underestimated by RSA, particularly during sleep. Inspection of IBI-RSA plots is mandatory in these subjects to detect underestimation of RSA due to ceiling effects
- Although the QR interval and ISTI provide meaningful estimates of the expected location of Q-wave and B-point, the detection of the Q-wave onset and the B-point remains highly advisable to obtain the actual PEP.
- ISTI by itself may provide an additional measure of cardiac contractility, reflecting the time it takes to reach peak ventricular ejection.
- The current evidence does not support the use of sAA activity or SAA output as an index of sympathetic nervous system activity.
- The TWA seems sensitive to SNS activity, but should not be seen as a replacement for the measurement of PEP. The joint measurement of T wave amplitude and PEP may provide a more complete picture of SNS activity.
- To further progress of ambulatory monitoring, a continuous and close interaction between behavioral researchers and the VU-AMS development team is essential.

Suggestions for further research

Based on the results presented in this study TWA and ISTI emerged as possible indicators of SNS activity to be used in addition to PEP. To provide more insight in the validity of TWA or ISTI as SNS indices, studies are now needed that compare changes in TWA, ISTI (but also PEP itself) to the other measurements of cardiac contractility for instance the ejection fraction obtained by echocardiography. In fact it is nigh time to do a large study that compares the various other indices extracted from thorax impedance recording that were not yet discussed in this thesis, including heather index and stroke volume, to similar measures extracted from echocardiographic recordings.

At the moment we have the tools to measure ANS activity in daily life, and a fair number of studies have studied ANS activity in relation to current health (depressed versus non-depressed) or

current situation (high versus low work stress). However, the move from the laboratory to real life monitoring was given in by the low predictive value of laboratory assessments for future disease development, for which low lab-real life generalizability was seen as one of the reasons. It is essential that longitudinal studies are now conducted to determine the importance of ANS activity in daily life for future disease development.

The rapid development within the communication technology opens up new possibilities for ambulatory studies. For instance, the precise determination of time and location has become possible, as well as repeated prompting for mood indications via mobile phones. The integration of this information with the continuously recording of ANS activity is expected in the near future and will be another step forward in understanding the influence of everyday life on our biology.

Samenvatting

Verbetering van de methodologie voor non-invasieve autonome zenuwstelsel metingen en het gebruik in gedragsonderzoek.

Het doel van mijn promotieonderzoek was drieledig. Ten eerste wilde ik de huidige strategieën voor het ambulant meten van parasympathische zenuwstelsel (PNS) en sympathische zenuwstelsel (SNS) activiteit verbeteren. Een tweede doel was om de haalbaarheid en validiteit van nieuwe ambulante meetmethoden van SNS activiteit te onderzoeken. Het derde doel was het actief verspreiden van de kennis die is opgedaan tijdens de ontwikkeling van het VU-AMS apparaat. In het bijzonder wilde ik daarbij een correct gebruik garanderen van de VU-AMS hardware en DAMS software door de groeiende gemeenschap van VU-AMS gebruikers. In deze samenvatting staan kort de uitkomsten weergegeven van de studies die ik heb uitgevoerd op de huidige en nieuwe indices van ANS activiteit voor ambulante metingen. Deze studies gebruikten gegevens van vier nieuwe experimenten door mij uitgevoerd aan de Vrije Universiteit, en twee sets van gegevens door collega's verzameld in het Nederlands Tweeling Register (NTR). Ik sluit af met de concrete implicaties van mijn onderzoek, en de mogelijk te nemen stappen om het gebruik van ambulante metingen van autonome zenuwstelsel activiteit in de gedragswetenschappen te verbeteren.

Huidige beschikbare indices van SNS en PNS activiteit

In hoofdstuk 1 werd een kritisch overzicht gegeven van de beschikbare indices voor het meten van PNS en SNS activiteit en de haalbaarheid van het meten van deze parameters in het dagelijks leven. Het laatste is van belang omdat individuele verschillen in autonome zenuwstelsel (ANS) reacties op laboratorium stressors niet erg goed te generaliseren zijn naar ANS reacties in levensechte situaties. Dit is weerspiegeld in de lage voorspellende waarde van laboratorium ANS reactiviteit op toekomstige hart- en vaatziekten. Invasieve bepalingen van de SNS en PNS activiteit (microneurografie, microdialyse, farmacologische blokkade, regionale spillover en plasma catecholamines) zijn vaak betrouwbaar en valide maar kunnen niet gemeten worden buiten een gecontroleerde laboratoriumomgeving en zijn dus niet geschikt om ANS activiteit te meten in de dagelijkse woon- en werkomgeving. Met betrekking tot de beschikbare niet-invasieve meetmethoden werd geconcludeerd dat de respiratoire sinus arrythmia (RSA) de beste maat is van PNS activiteit in het dagelijks leven. Zowel HF als RMSSD, beide maten van RSA, kunnen gemakkelijk worden verkregen uit het elektrocardiogram (ECG), en met toevoeging van een ademhalingssignaal kan ook RSA met de piek-dal methode worden gemeten. Voor het ambulant meten van SNS activiteit in het dagelijks leven, wordt de preejectie periode (PEP) als best bruikbare maat gezien omdat deze heel goed de sympathische effecten op de contractiliteit van de hartspier weergeeft. Echter, vanwege de arbeidsintensieve scoring van de PEP en de gevoeligheid van de contractiliteit voor preload en afterload effecten zou een alternatieve niet-invasieve maat van SNS activiteit zeer welkom zijn.

In de hoofdstukken 2 en 3 lag de focus op de tekortkomingen van de twee beste ambulante maten, RSA en PEP, en hoe deze zoveel mogelijk kunnen worden ingeperkt of zelfs vermeden in het onderzoek. Hoofdstuk 2 richtte zich op de mogelijke onderschatting van RSA in mensen met een lage hartslag door plafondeffecten in de acetylcholinerge neurotransmissie. Dergelijke effecten zijn het meest aanwezig tijdens de slaap. Er werden 24-uurs ECG en ademhaling registraties gemaakt in 26 sporters die gedurende 6 weken een vast trainingsprogramma hadden gevolgd, maar ook in 26 niet-sporters van overeenkomende leeftijd en geslacht. Het plafondeffect op de RSA werd onderzocht door voor elk individu de vorm van de relatie tussen de Inter Beat Intervallen (IBI) en de RSA visueel

te inspecteren. Een subgroep van sporters vertoonde een IBI-RSA relatie die werd gekenmerkt door een kwadratisch profiel, in contrast tot het verwachte lineaire profiel. Het kwadratische profiel duidde op een sterke onderschatting van de vagale controle in deze subgroep. Deze onderschatting leidde tot misleidende resultaten: het leek er op dat er geen gunstig effect van lichaamsbeweging op de cardiale vagale controle bestond. Wanneer rekening werd gehouden met het plafondeffect bleek dat gunstige effect wel degelijk te bestaan. Inspectie van de IBI-RSA relatie, en het uitsluiten van kwadratische verbanden in personen en/of condities waar een lage hartslag wordt gezien is dus noodzakelijk wanneer RSA wordt gebruikt als maat voor cardiale vagale controle.

In hoofdstuk 3 lag de focus op de detectie van specifieke punten in het impedantiecardiogram (ICG) en het ECG die gebruikt worden voor het meten van de PEP. PEP is gedefinieerd als het interval vanaf het begin van de linker ventriculaire depolarisatie, gekenmerkt door het begin van de Q-golf in het ECG, tot aan de opening van de aortaklep, gereflecteerd in het B-punt van het ICG. De positie van de start van de Q-golf en B-punt zijn niet altijd gemakkelijk te detecteren en alle gescoorde punten moeten visueel worden geïnspecteerd en, met enige regelmaat, handmatig gecorrigeerd. Dit is niet alleen arbeidsintensief maar maakt de PEP schatting ook foutgevoelig. Met behulp van gegevens die zijn verkregen uit twee studies, uitgevoerd in verschillende omgevingen (laboratorium versus ambulant) en met metingen tijdens verschillende houdingen en fysieke activiteit, evalueerden we een alternatief voor de PEP waarbij geen detectie van de Q-golf en B-punt nodig is. Hierbij wordt de PEP berekend met een vaste waarde voor de start van de Q-golf tot R-top (QR) interval en een interval (RB) dat wordt geschat op basis van de R-top en de dZ/dt- piek (ISTI). Dee laatste ISTI (inter systolic time interval) geeft informatie over de tijd die het kost om de pieksnelheid van de ejectie van bloed uit het linkerventrikel te bereiken en zou op zichzelf ook nog een aanvullende maat van de cardiale SNS activiteit kunnen zijn. Echter, de verschillen tussen de alternatieve PEP en de werkelijke PEP waren erg groot. De arbeidsintensieve detectie van het begin van de Q-golf in het ECG en het B-punt in het ICG blijft dus sterk aanbevolen.

Evaluatie van twee alternatieve maatregelen van SNS activiteit

In de hoofdstukken 4, 5 en 6 werden twee alternatieve maten van SNS activiteit onderzocht: enzymatische activiteit van het speekselenzym alfa-amylase (sAA) en de amplitude van de T-golf in het ECG (TWA). In hoofdstuk 4 en 5 onderzocht ik sAA, een maat die gemakkelijk te verkrijgen is door het kauwen op een wattenrolletje en daarom snel populair is geworden als indicator van SNS activiteit in het dagelijks leven. In een eerste studie, gepresenteerd in hoofdstuk 4, werden ECG en ICG signalen gedurende 24 uur geregistreerd om PEP en RSA te verkrijgen, en werden 7 speekselmonsters verzameld op gezette tijden gedurende de dag met de Salivette wattenrolletjes. Het patroon in sAA klopte niet met het dagelijkse patroon in SNS activiteit. In tegenstelling tot de bekende daling in SNS activiteit van ochtend naar avond vonden we in de loop van de meetdag een stijging in sAA. Er was bovendien geen significant verband tussen PEP en sAA, zelfs niet na corrigeren voor RSA (invloed van het PNS). Om uit te sluiten dat de collectiemethode van speeksel met Salivettes de oorzaak was van de negatieve bevindingen werd een tweede studie uitgevoerd waarin speeksel zorgvuldiger werd verzameld met de ‘passieve kwijl’ methode. sAA werd gemeten voor en na een periode van intensief fietsen, een activiteit waarvan we zeker weten dat ze SNS activiteit uitlokt. Naast sAA, de meest gebruikte maat in het veld die de enzymatische activiteit van amylase weergeeft, werden ook de werkelijke amylase eiwitconcentratie bepaald en de verandering in speekselvolume. Daarmee kon de eigenlijke amylasesecretie worden berekend. De toename in sAA

en amylasesecretie veroorzaakt door het fietsen werd vergeleken met veranderingen in de PEP en RSA. Zoals verwacht verhoogde het fietsen de SNS activiteit en verlaagde het fietsen de PNS activiteit, aangeduid door een afname in de PEP en de RSA. Enzymatische activiteit van amylase en amylaseconcentratie stegen in reactie op het fietsen, maar de amylasesecretie steeg niet. Ook waren de PEP en RSA reactiviteit niet gerelateerd aan de secretie. Op basis van de resultaten van de resultaten van de studies in de hoofdstukken 4 en 5 is het gebruik van enzymatische sAA activiteit als maat van SNS activiteit zeer sterk af te raden.

In hoofdstuk 6 onderzocht ik tenslotte het gebruik van de TWA als mogelijke maat van SNS activiteit. In een relatief grote groep personen werden 24-uurs metingen met het VU-AMS gedaan en werd aangetoond dat de kenmerkende punten in het ECG betrouwbaar gedetecteerd kunnen worden na zogenaamde 'large scale ensemble averaging' van het ECG, waarbij een gemiddeld ECG over periodes van 5 tot 30 minuten wordt berekend. De TWA kon in meer dan 90% van de deelnemers betrouwbaar worden bepaald en nam significant af in reactie op een mentale en fysieke belasting. Zo daalde de TWA stapsgewijs van nachtelijke slaap naar zitten overdag tot lichte en matige lichamelijke activiteit. Daarmee co-varieert de TWA met de verwachte veranderingen in de SNS activiteit. Binnen proefpersonen correleerde de TWA ook met de PEP, met een gemiddelde correlatie van 0,35 na correctie voor de hartfrequentie en de PNS activiteit. Hoewel de TWA de PEP niet kan vervangen als maat van SNS activiteit heeft het toevoegen van de TWA aan de PEP wel meerwaarde. Samen lijken de TWA en PEP een vollediger beeld van SNS activiteit te kunnen geven.

De VU-AMS; ontwikkeling en verspreiding van kennis

De ambulante ANS activiteit in de bovenstaande studies werd gemeten met behulp van het VU Ambulatory Monitoring System (VU-AMS) dat is ontwikkeld aan de Vrije Universiteit door een samenwerking tussen ICT/elektronicaspecialisten en wetenschappelijk onderzoekers binnen de psychologie. Hoofdstuk 7 beschreef de geschiedenis van de ontwikkeling van het VU-AMS en liet zien hoe technologische innovatie, gekoppeld aan de input van de gebruikers, heeft geleid tot verbeterde signaalopname en signaalkwaliteit, snellere dataverwerking en bredere toepasbaarheid van het VU-AMS. Verbeteringen van het VU-AMS op hun beurt leidden tot nieuwe toepassingen die weer verdere ontwikkeling stimuleerden. De interacties in de driehoek tussen het technische team dat de VU-AMS ontwikkelt, de algemene technologische vooruitgangen in ICT en elektronica en de onderzoekers die met het systeem werken zorgen voor een continue ontwikkeling van het VU-AMS. Die interacties zijn gebaat bij een goede uitleg aan de onderzoekers van de correcte toepassingen van het VU-AMS, maar vooral ook van de juiste interpretaties van de VU-AMS gegevens. In hoofdstuk 7 en in de bijlagen werd een overzicht gegeven van de diverse hulpmiddelen die we ontwikkeld hebben om de juiste toepassing van het VU-AMS in gedragsonderzoek breder te verspreiden.

Conclusies

- Op het moment zijn RSA en PEP de beste keus voor het meten van PNS en SNS activiteit in het dagelijks leven.
- Bij personen met een lagere hartslag, zoals regelmatige sporters, kan vagale controle worden onderschat door RSA, vooral tijdens de slaap. Inspectie van IBI-RSA plots is in deze personen nodig om de onderschatting van RSA vanwege plafondeffecten te detecteren.

- Hoewel QR -interval en ISTI een betekenisvolle schatting van de verwachte locatie van het begin van de Q-golf en het B-punt geven, blijft de detectie van het begin van de Q-golf en het B-punt noodzakelijk om de werkelijke PEP te meten.
- De ISTI, de tijd die het kost om de piek in de ventriculaire ejectie te bereiken, zou op zichzelf een aanvullende maat van cardiale SNS activiteit kunnen zijn.
- Het gebruik van sAA of amylasesecretie als een index van SNS activiteit wordt niet voldoende door bewijs ondersteund.
- De TWA lijkt gevoelig voor SNS activiteit, maar moet niet worden gezien als een vervanging voor het meten van de PEP. De gezamenlijke meting van de TWA en PEP kan een completer beeld van SNS activiteit kunnen geven.
- Om het ambulante meten in het dagelijkse leven te bevorderen is een voortdurend samenspel tussen gedragsonderzoekers en het VU-AMS ontwikkelteam essentieel.

Suggesties voor verder onderzoek

Op basis van de resultaten van mijn onderzoek is het meten van de TWA en ISTI een waardevolle aanvulling gebleken op het meten van de PEP. Om meer inzicht te verkrijgen in de validiteit van TWA of ISTI als indicatoren van SNS activiteit zijn nu studies nodig die de TWA en ISTI vergelijken met andere maten van sympathische effecten op de contractiliteit van het hart, bijvoorbeeld de ejectiefractie verkregen met echocardiografie. Daarin zouden ook andere maten die kunnen worden verkregen uit thoraximpedantie metingen zoals de Heather index en het slagvolume kunnen worden vergeleken met hun echocardiografische tegenhangers.

Met de huidige ambulante meettechnieken kan de ANS activiteit nu al uitstekend in het dagelijks leven worden gemeten. Een aantal studies heeft met deze technieken de ANS activiteit buiten het laboratorium bestudeerd in relatie tot de huidige gezondheid (depressief versus niet-depressief) of de huidige werkomstandigheden (hoge versus lage werkstress). Echter, deze studies waren nog vaak cross-sectioneel. De verhuizing van het laboratorium naar het echte leven werd ingegeven door de lage voorspellende waarde van laboratoriumbepalingen voor het ontstaan en beloop van ziekte. Het is dus essentieel dat er nu longitudinale studies worden uitgevoerd om de voorspellende waarde van ambulante gemeten ANS activiteit in het dagelijkse leven voor ziekte te bepalen.

De snelle ontwikkelingen binnen de communicatietechnologie openen nieuwe mogelijkheden voor de haalbaarheid van dergelijke grootschalige ambulante studies. Zo is het nu mogelijk om herhaald de stemming en de sociale situatie op te vragen via smart phones en deze gegevens te integreren met de gelijktijdig opgenomen beweging en ANS activiteit. Het vragen naar de stemming en sociale situatie kan zelfs door momentane veranderingen in ANS activiteit worden ingeleid. Er liggen fikse stappen voorwaarts in het verschiet voor het begrijpen van de invloed van het dagelijks leven op onze biologie.

APPENDIX I

VU-AMS 5fs and Data Analysis Management Software (DAMS) manuals

René van Lien, Jarik den Hartog, and Eco J.C. de Geus

1. Recording with the VU-AMS device.

1.1 Requirements

Two AA batteries:	Use 1.2V rechargeable NiMH batteries or non-rechargeable 1.5V alkaline batteries. Make sure the bottom contact sticks out (in some batteries it is the covered by an outer plastic ring; these won't work properly with the VU-AMS). Leave rechargeable batteries in the charger up till the very last moment.
Compact Flash card:	External memory card. The VU-AMS5fs has been extensively tested with the 1GB 80x Compact Flash card from Transcend (TS1GCF80) and the 2GB Ultra Compact Flash card from SanDisk (SDCFH-002G-U46).
Compact Flash card reader:	Card reader unit to extract the VU-AMS data from the Compact Flash card after recording and to erase the card for a next recording. Any brand or built-in Compact Flash card reader will do.
Electrodes:	Typically, seven electrodes are needed for a single recording. We use the 'Kendall ARBO H98SG' single use ECG electrode with Wet Gel for the ICG and ECG. For skin conductance we use the Biopac TSD203 combined with their isotonic electrode gel (GEL101).
Lead wire connector:	A blue lead wire connector with 7 lead wires is used for the recording of the ECG and thorax impedance. Optionally a second yellow connector for skin conductance recording is needed.
VU-AMS*5fs*:	The ambulatory recording device.
VU-AMS*i* (for RS232 or USB):	An infrared interface cable that either connects to the RS232 serial port of a PC or to an USB port.
Flashcard with latest firmware (optional):	The VU-AMS device comes with the latest firmware installed. From time to time updates will be posted on the VU-AMS website (www.vu-ams.nl). These need to be installed once from an update flash card. Detailed instructions on how to install the update are on the VU-AMS website.
Data Analysis and Management Software (DAMS).	The VU-AMS device is configured using this program (referred to as 'DAMS program') and measurements can be started and stopped with the program. The DAMS program is also used for primary data extraction and for data reduction. It can be downloaded from the VU-AMS website (www.vu-ams.nl).

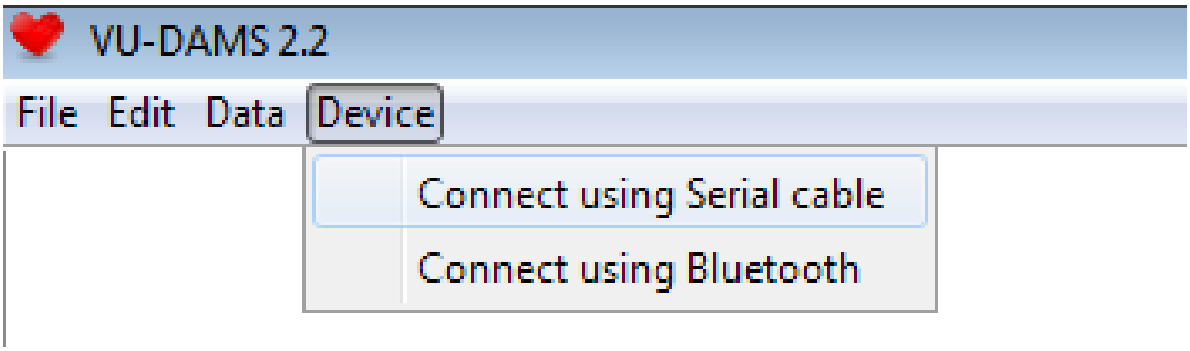
1.2 Preparing the VU-AMS device

Always use an empty Compact Flash card with all previous files removed from the card before each new measurement. Put the flash card bottom up in the VU-AMS and then place two completely charged AA batteries in the battery holder. Battery clips are vulnerable so do this carefully. Successful placement is signaled by a triple beep tone.

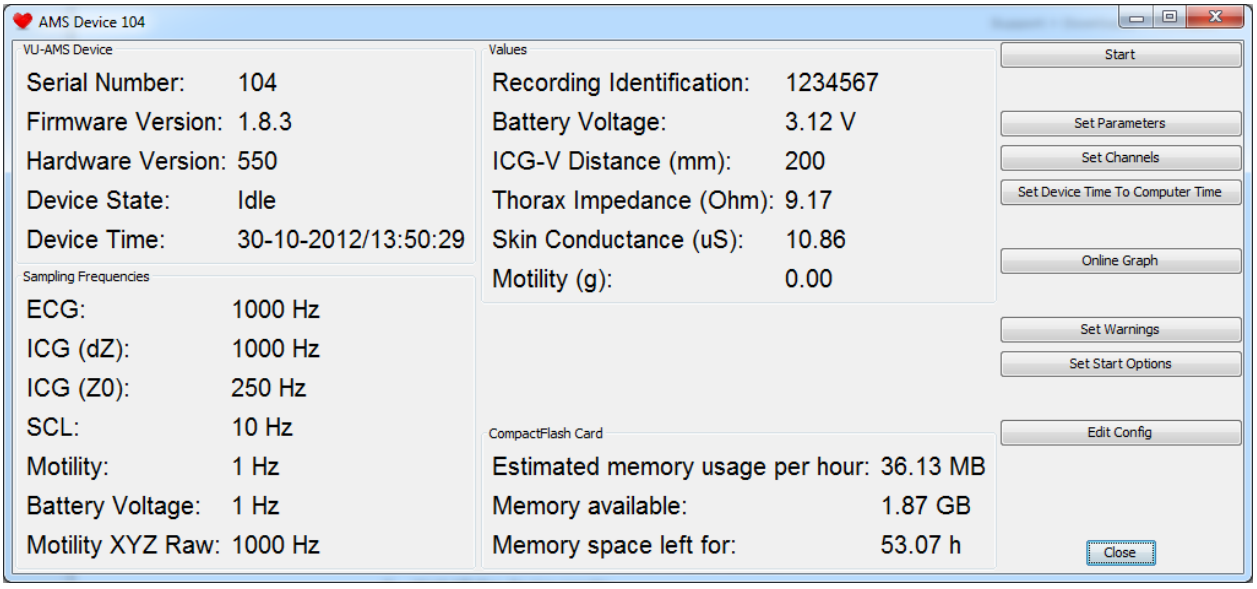
The VU-AMS is now on standby and the green light will flash twice every ten seconds. This indicates the VU-AMS is ready, but not recording. When the VU-AMS is recording the green light will flash once every three seconds.

1.3 Configuring the VU-AMS device for recording

Connect the VU-AMS to the PC with the interface cable. Connect the infrared end of the interface cable to the VU-AMS; the electronic end of the interface cable goes to the serial port or the USB port of the PC. Start the DAMS program and select *Device* tab in the main menu. Choose to *Connect using Serial cable*.



You will now see the configuration screen:



1.3.1 Clock Synchronizing

Before starting, make sure to set date and time of the PC correctly. All dates and times in the VU-AMS data files will be based on the time and date read from the PC at start-up, so it is important to make sure your PC has the correct time and date. Do this by clicking on *Set Device Time To Computer Time*.

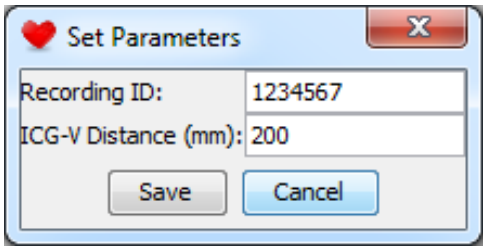
TIP: *Synchronize the watch of the subject/observer to the exact time of the PC used to start up the VU-AMS for optimal time-locked self-report diaries and physiological data. When electronic diaries are used make sure that their clocks are synchronized with the configuration PC too.*

1.3.2 Battery Types

Check battery voltage indication (should be about 3.1 Volt for alkaline and about 2.7 Volt for rechargeable NiMH batteries), and re-check time and date. Though battery voltage gives an indication of battery capacity left, for 24 hour recordings it is safest to start with new alkaline batteries or batteries that were in the charger up till the very last moment.

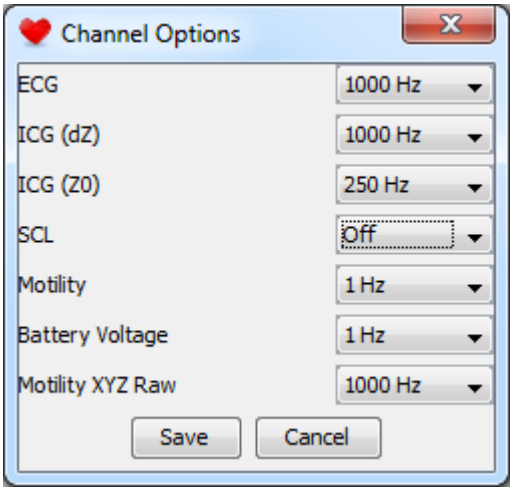
1.3.3 Set Parameters

Click *Set Parameters* and fill in the recording identification field. Also fill in the distance measured in millimeter between the two front ICG electrodes for later stroke volume estimations.



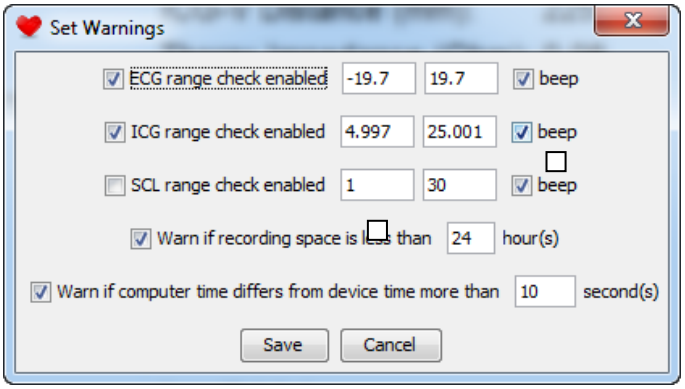
1.3.4 Set Channels

The typical sampling frequencies are as shown in the figure below. By clicking on *Set Channels* you are allowed to set sampling frequencies for the various signals. You can disable signals by setting them to *'Off'* (like we did with the SCL signal). When changing any setting, make sure to save the settings to the device before closing the DAMS program!



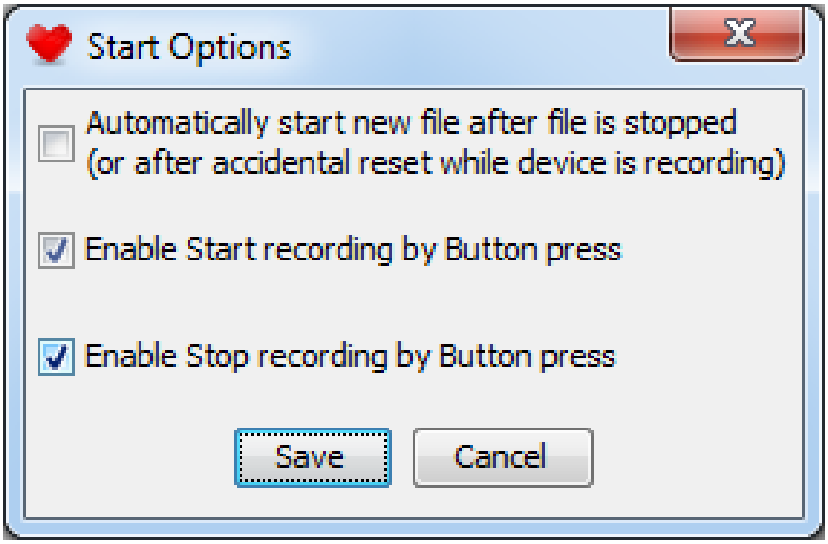
1.3.5 Set Warnings

Here you can activate or deactivate audio warning signals when ICG, ECG or SCL signals exceed their boundary values. You can also activate warning for low recording space and time-sync problems. These beeps are useful in field recordings when subjects are able and sufficiently instructed to reconnect electrodes themselves. Otherwise it is advisable to turn the warning signals off because the beeps will persist as long as the electrodes are not properly reattached.



1.3.6 Set Start and Stop Options

Pressing the button on the VU-AMS device shortly always results in a time marker in the data file. Hence the button can be used as an event marker by the subject during field recordings. You can further program the button on the VU-AMS device to act as a start and/or stop button. If the event marker function is used it is advised to either disable the stop button or to explicitly instruct your subjects to only *shortly* press the black button to mark specific events. Accidentally pressing the button for more than 3 seconds may otherwise stop the recording.

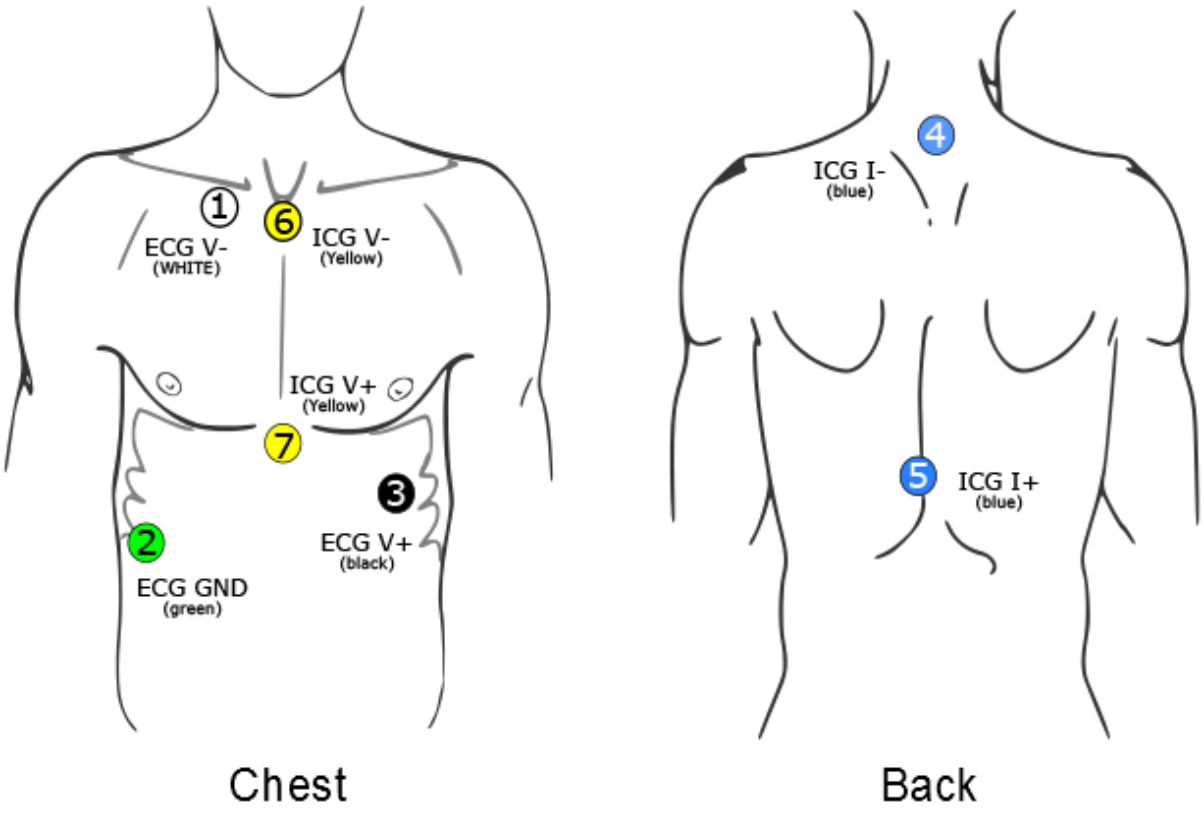


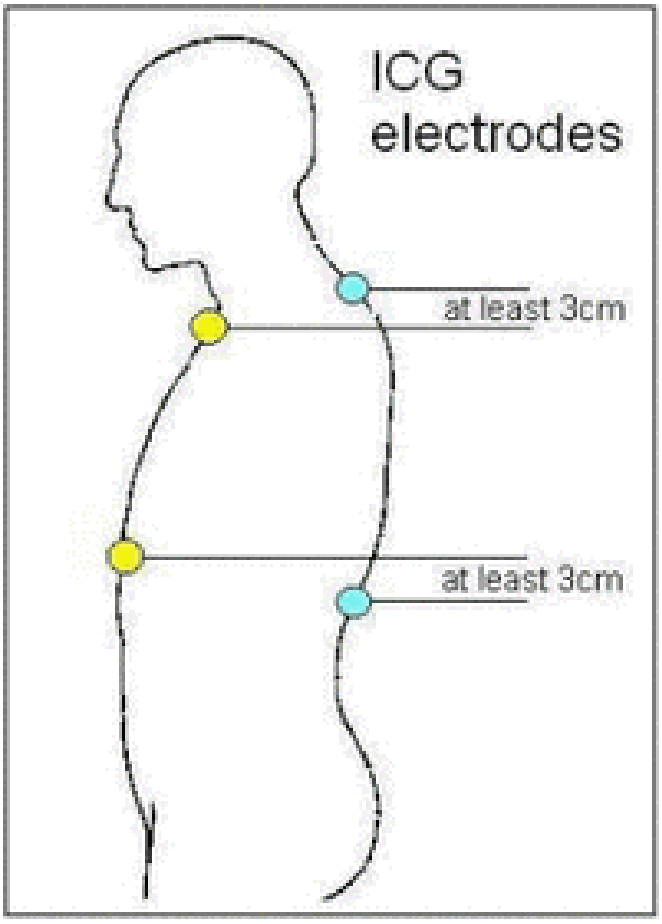
When the top option is selected recording will continue by itself when the battery is replaced (which may be needed during recordings lasting >48 hour). Or even when a subject tries to stop the recording using the black event button. In fact to only way to really stop the recording is using the DAMS program.

1.4 Electrode hook up

1.4.1 Attachment of the ECG/ICG electrodes

Clean the skin at the 7 positions indicated in the figure. Rub the skin firmly with an alcohol soaked tissue or, if alcohol is not available, use a clean dry tissue. Attach an electrode by pressing the sticky plastic brim of the electrode on the skin and subsequently pushing the metal stud at the center of the electrode firmly, to properly spread the contact gel.





ECG:

1 Slightly below the right collar bone 4 cm to the right of the sternum

2 (GND) On the right side, between the lower two ribs

3 At the apex of the heart on the left lateral margin of the chest approximately at the level of the processus xiphodius.

ICG:

Electric current generating electrodes

4 At the back, on the spine, at least 3 cm (1") above electrode 6

5 At the back, on the spine, at least 3 cm (1") below electrode 7

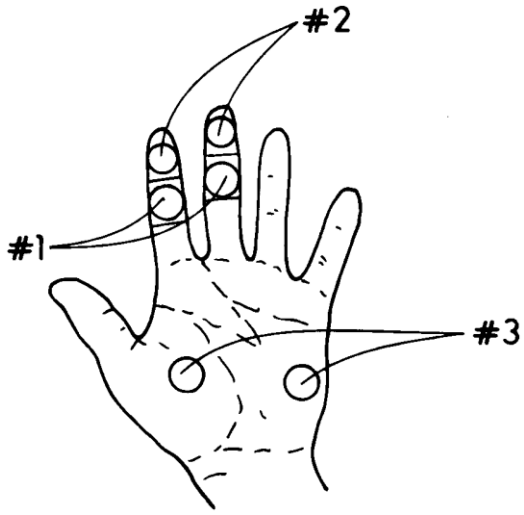
Impedance measuring electrodes

6 At the suprasternal notch above the top of the sternum

7 At the processus xiphodius at the bottom of the sternum

1.4.2. Attachment of SCL electrodes (optional):

Skin conductance can be recorded from the medial phalanges of the index and middle or ring finger or from the thenar and hypothenar eminences of the hand palms. For the phalanges Velcro straps with an electrode holder that is filled with gel is used, whereas for the palms dedicated SCL electrodes can be used or even ECG electrodes as long as they have a gel with a low NaCl content.



As the sweat ducts acts as parallel conductors it is essential to keep the surface area of measurement constant across subjects.

1.4.3. Attachment of the lead wires and lead wire connector

The blue ECG/ICG lead wire connector has to be plugged in the blue socket. Optionally, the yellow SCL lead wire connector is plugged into the yellow socket.

1.4.4. Wearing the device

Put the VU-AMS device in its carrier bag with the lead wire connector facing up. Fasten the device with the strap in the bag and gird it on with the VU-AMS belt (you can also supply your own belts). Make sure the device is attached in a vertical position.

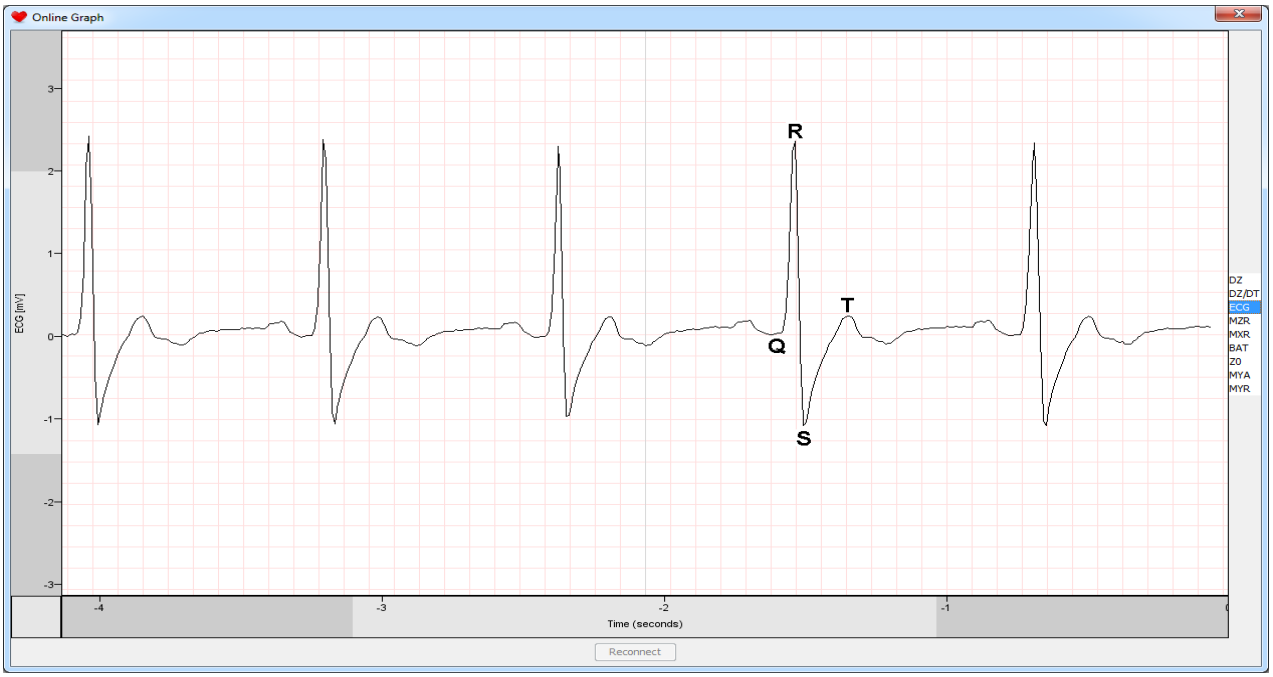
**Please also see the "Recording with VU-AMS" tutorial video for a demonstration: www.vu-ams.nl/support/tutorials/hardware/vu-ams-recording*

1.5 Signal Quality Control

After connecting the ECG/ICG lead wire plug to the VU-AMS device, the *Online Graph* option should be used to display the ECG, Z0, dZ (≈ change in impedance due to respiration and heartbeat) and dZ/dt (= Impedance CardioGram) to check for proper quality of the recorded signals.

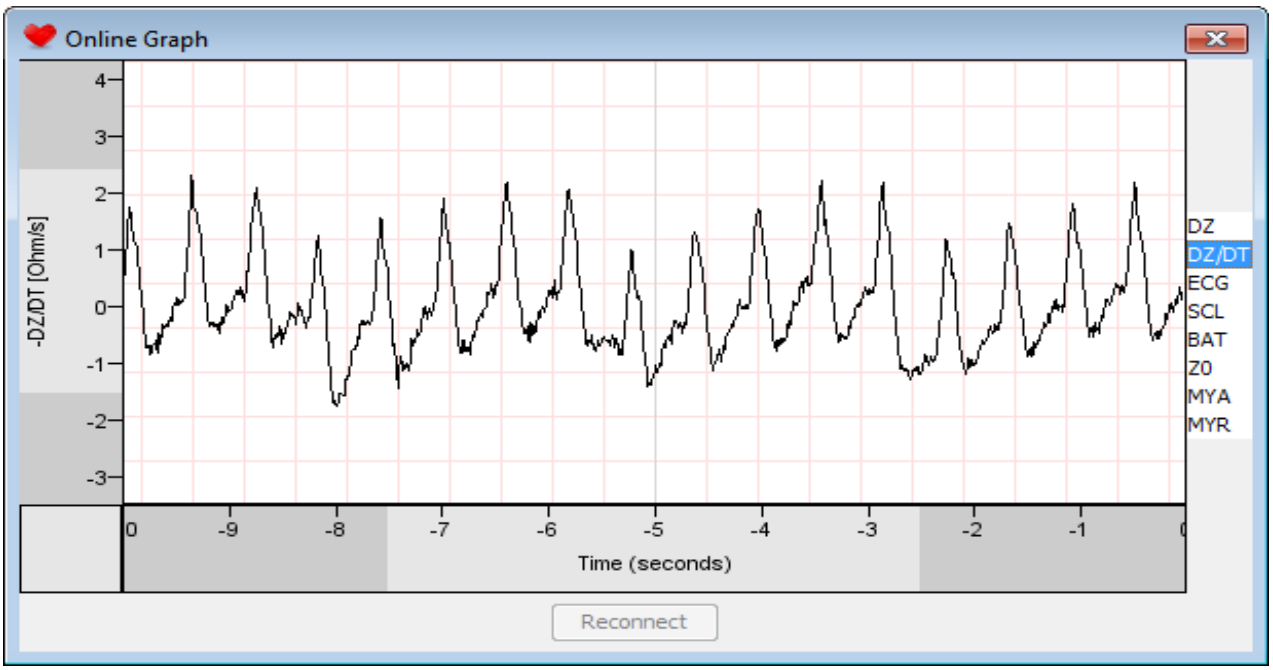
1.5.1 ECG

A clear QRST-complex should be detectable in the ECG. The R-wave should be upward and it should be the peak with the largest (absolute) amplitude in either direction (but upward has the most preference). If either S-wave or T-wave are of comparable magnitude re-attach the black(+) ECG electrode first more laterally then more medially until a satisfactory QRS complex is seen.



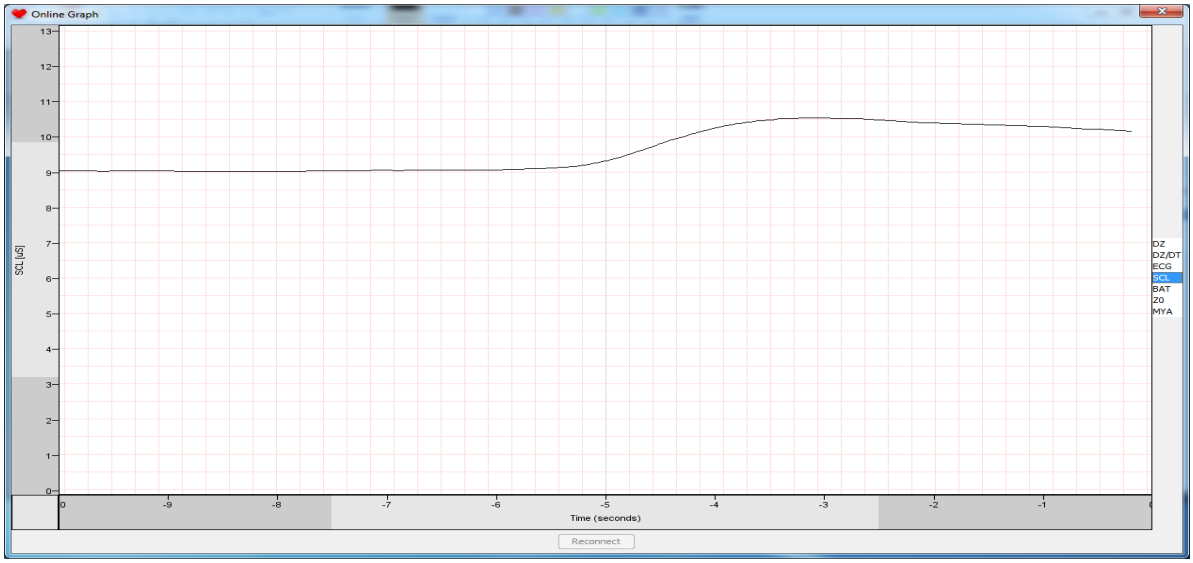
1.5.2. Z0, dZ, dZ/dt

The dZ should be within -0.5 and +0.5 Ohm most of the time. Z0 should always stay within an 8 to 20 Ohm range. The dZ signal should reflect deep breathing clearly. In the ICG the typical upward waveform of the cardiac ejection phase should be clearly detectable. Light movement of the subject should not overly distort it. If these criteria are not met, re-attach the electrodes in the order 7,6,1,3,4,5,2 (see illustration at 1.4 Electrode hook up) until satisfactory signals are obtained. The scrollbar on the Y-axis of the *online graph* can be used to scale the signals (or hit F5 to auto scale).



1.5.3. SCL

The SCL signal should be within a 1 to 12 micro Siemens range. Spontaneous phasic responses should be discernible in most subjects and an orienting response to a sudden unexpected stimulus should also give a phasic increase (e.g. clapping hands behind the back of the subject).



NOTE: In ambulatory paradigms, online signal inspection is your onlyopportunity to re-attach faulty electrodes.

1.6 Starting a measurement

When satisfied, start data recording by pressing *Start* in the configuration screen. A beep will be heard to acknowledge the start of the recording and the green light will start flashing once every three seconds. Close the configuration screen of the DAMS program and disconnect the VU-AMS device from the interface.

1.7 Marking special events

A small black button is placed on top of the VU-AMS device next to the two lead wire plug connectors. To mark a special event during the recording, push this button shortly. Pushing it will be confirmed by a short beep.

1.8 Is the VU-AMS device recording?

A small indicator light on top of the device will be flashing once every three seconds as long as the VU-AMS is recording.

1.9 Stopping a measurement

The measurement by the VU-AMS device can be stopped by:

- Reconnecting the device to the PC and connecting again by serial cable by choosing the appropriate action in the DAMS menu under *Device*. You can now press *Stop*. This is the preferred method. Close the DAMS program and then disconnect the device from the interface cable.

- Pressing the event button for more than three seconds. This requires that the option of stopping with the event button was **enabled** in the *Set Start Options* menu. After this action

the light will flash every 10 seconds to indicate standby mode. The method is preferred when subjects have to stop the recordings themselves at home at a designated time. NB: When the device is returned to you after a day of ambulatory research, make sure to check whether (1) the measurement has been stopped already with the button (the light flashes twice every ten seconds), (2) is still recording (the light flashes every three seconds), or (3) has stopped because of empty batteries (the light does not flash at all).

- Removing the batteries. This is strongly discouraged as it may lead to corrupted data files (0 KB).

Once the VU-AMS has stopped recording, the yellow and/or blue lead wire plug(s) may be disconnected from the VU-AMS device and the lead wires from the electrodes. Now remove the batteries and place the Compact Flash Card in the card reader. Move the *.5FS* data file to a designated directory.

1.10 Saving an .amsdata file

Double click the *.5FS* data file to open the recording. When you close DAMS it will automatically save the data in a new file with the extension *.amsdata.* This file can be opened by using the *Open data* option in the main menu or just double-clicking it. Using *.amsdata* files (once these are created) will make DAMS load the data much faster. Also all manual scoring will be saved in the .amsdata file.

NB: you can batch convert .5FS files to the .amsdata format. Files recorded with the older AMS 4.6 system can be converted too, but no visible ECG signal will be present as only the inter beat intervals were stored with this device and not the ECG signal itself. Click on ***batch convert*** *in the menu and select the folder with all .5FS or .ams raw data files together with the .lbl and .cfg files (they need to be placed in one directory). After conversion the folder will contain an .amsdata file for each of the raw data input files.*

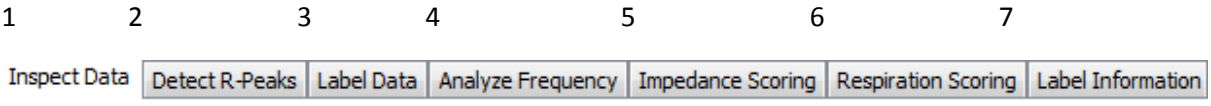
1.11 Merging multiple .5FS files

If the recording has been interrupted by the experimenter or by the subject (e.g. because the participant took a shower or batteries have been replaced), multiple *.5FS* data files with different start times will be generated. There is a possibility to use a standalone tool, *AmsMerge,* that concatenates the *.5FS* files into a single *.5FS* file that spans the entire recording. The AmsMerge program can be found in Start menu >> Programs >> VU-DAMS folder from version 2.2 and up.

2. Data Analysis and Management with the DAMS program

Use the DAMS program to process the VU-AMS data. Double clicking on a *.5FS* file will open the file once the DAMS program is set to be the default program to open it with.

The typical flow of VU-AMS data analysis and management is represented by a series of tabs in the main screen:

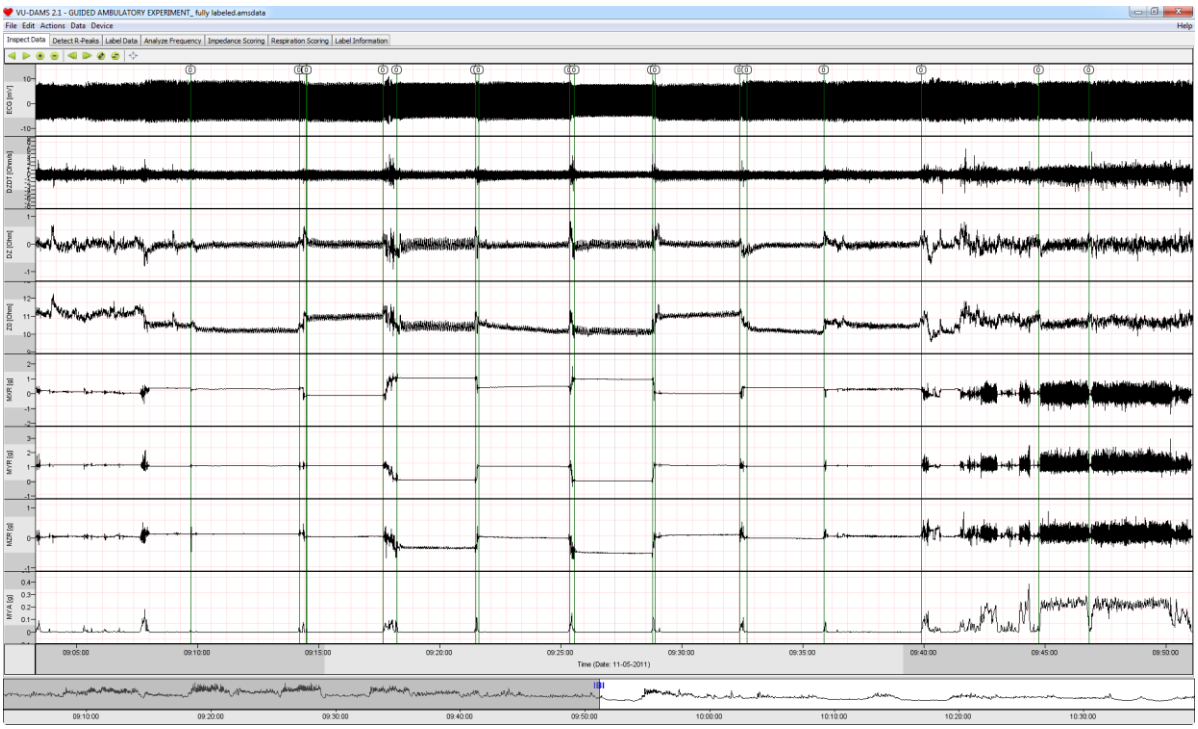


- Inspecting the raw data
- R-peak detection and correction

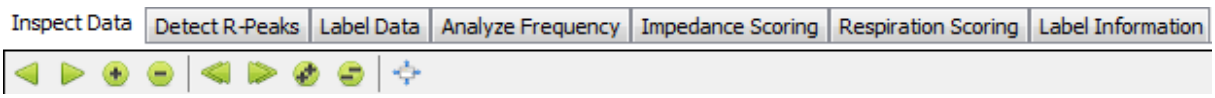
- Labeling your data
- Spectral analysis
- Impedance scoring
- Respiration / RSA scoring
- Exporting the results (label-based)

2.1. Inspect Data

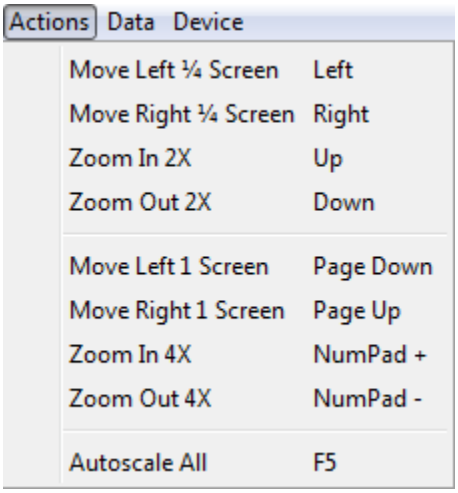
The *Inspect Data* tab simply gives you an overview of your data. All recorded signals are shown as continuous time series. Recording time is at the lower line. Above the recording time, the Inter Beat Interval (IBI) time series is given as extracted from the ECG. The clock time and IBI signal of the entire recording will be presented in all data analysis tabs, and function as orientation point. Zooming can be done by changing the size of the hatched rectangle in the IBI window (select one of the borders of the rectangle and drag with the mouse cursor). Vertical lines represent the times at which the event button was pressed.



All actions for the *Inspect Data* tab are presented in the form of buttons:



These actions and their keyboard shortcuts are also available in the dropdown menu Actions. This setup goes for all tabs in the DAMS program. Only the type of Actions will differ per tab.



The function of these buttons should be self-explanatory. Hovering the mouse cursor over a button will display a short description.

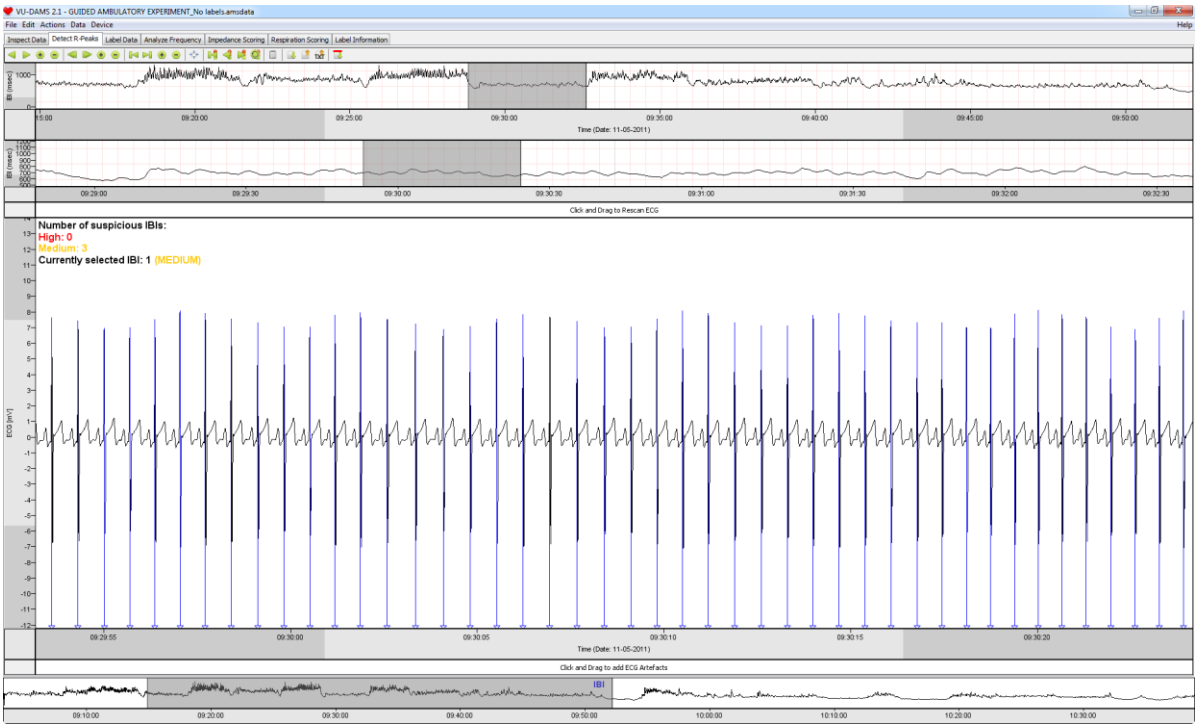
The mouse can also be used to zoom in and out: By using the mouse wheel a shorter or a longer period of the recorded data can be shown. Zooming can also be done by dragging the darker grey rectangle just below the time line. Moving the lighter grey rectangle in the middle will move the data either right or left.

Each of the signals can be autoscaled separately by right clicking on the Y-axis of the signal.

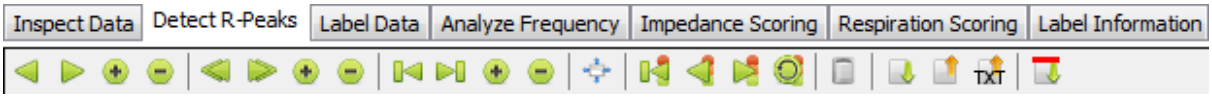
2.2 Detect R-Peaks

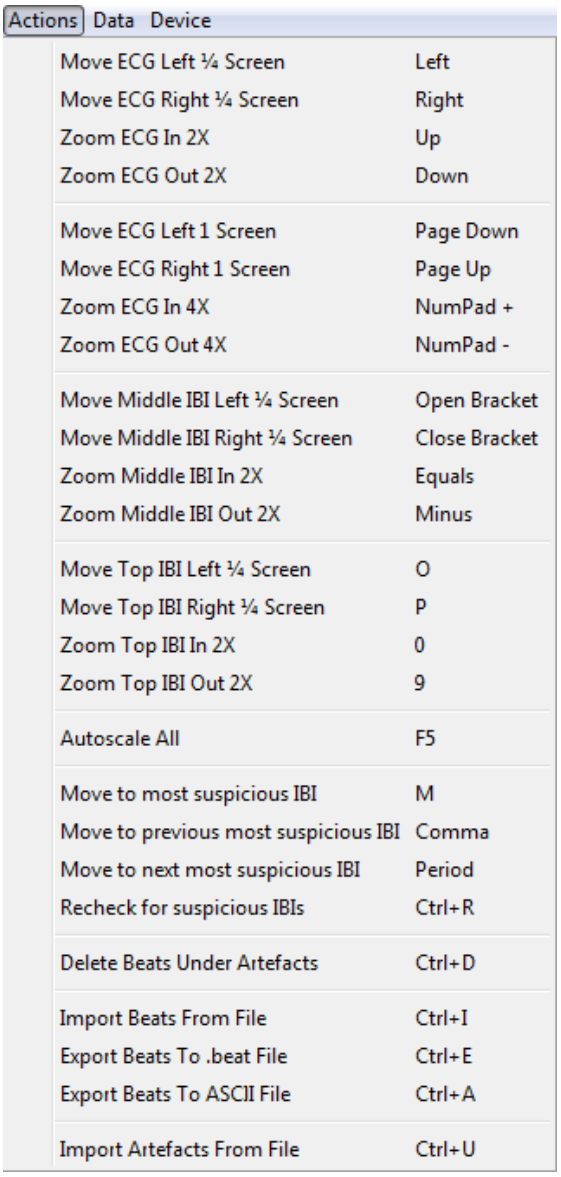
The *Detect R-Peaks* tab will assist you to create an artefact free IBI signal as fast as possible by applying automated artefact and peak detection. The mandatory visual inspection and correction of the resulting IBI signal is made as easy as possible by multiple zoom levels and an automated suspicious beat detector. The default settings of this detector work well on most of the ECG recordings but changing the peak detection settings can be required in case of noisy or strongly deviant ECGs.

After opening either the raw *.5FS* or a saved *.amsdata* file click on the *Detect R-Peaks* tab. The QRS detector software runs three separate automated analyses on the ECG signal. The first automated analysis detects and marks periods with missing data or clipping of the electrocardiographic signal. These periods are called artefacts. A second automated analysis of the QRST waveform detects the occurrence of all R-peaks. The R-peaks are converted to the inter beat intervals time series which is simply the distance in milliseconds between two consecutive R-peaks plotted against time, giving rise to the continuous line seen in the lower and upper top windows. The third automated analysis checks the plausibility of the duration of each IBI in the context of its surrounding inter beat intervals. This feature was created to ease visual inspection and user-driven correction of the IBI time series.



All actions for the *Detect R-Peaks* tab are presented in the form of buttons:





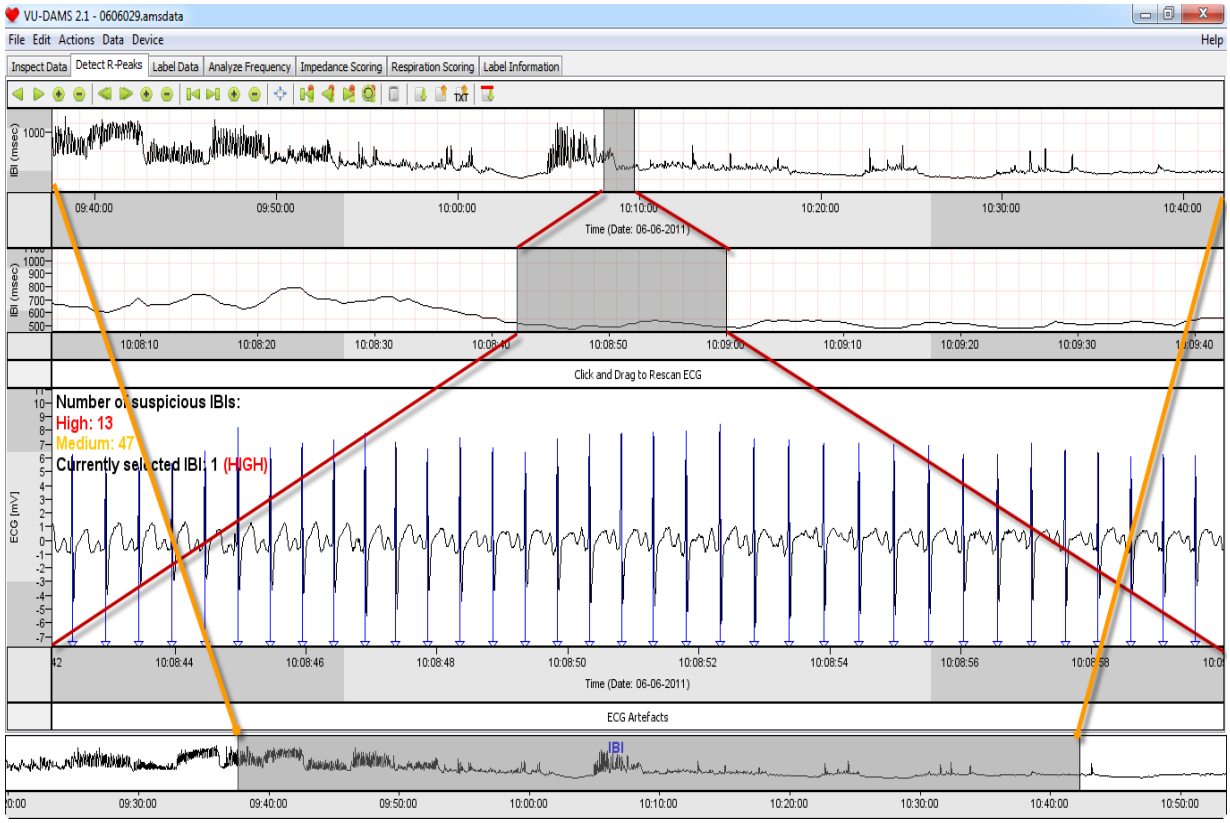
The function of these buttons should be self-explanatory. Like in all other tabs hovering the mouse cursor over a button will display a short description.

2.2.1 Visual inspection and manual correction

Automated artefact labeling reliably detects clipping and signal loss, but detection of noisy ECG is not perfect. Manual selection of bad ECG signal parts may be additionally needed. The three main windows will help you select the parts of the IBI time series that need to be manually labeled as artefacts. The *bottom window* is our overview of the IBI signal of the entire recording that is also present in the other modules (tabs) of the Data Analysis Management Software.

What is marked as a grey bar in the first top window will be displayed in the second middle window. The X-axis of the IBI time series in these windows represents the time at which a beat was recorded. The Y-axis of the IBI time series is the interval duration of that beat in milliseconds. The third lower window displays the actual ECG signal. You can zoom the ECG window in or out by dragging the dark grey area in either of the IBI time series windows. You can also zoom in or out by using the scroll wheel of the mouse on the part of the data you want to see in more detail. By using the mouse wheel

a shorter (scroll forward) or a longer (scroll backward) period of the recorded data can be shown. Selecting a different part of the recording can be done by changing the size of the hatched rectangle in the IBI window at the bottom (select one of the border of the rectangle and drag with the mouse cursor) or by moving the rectangle left (backward in time) or right (forward in time).



2.2.2. Removing artefacts

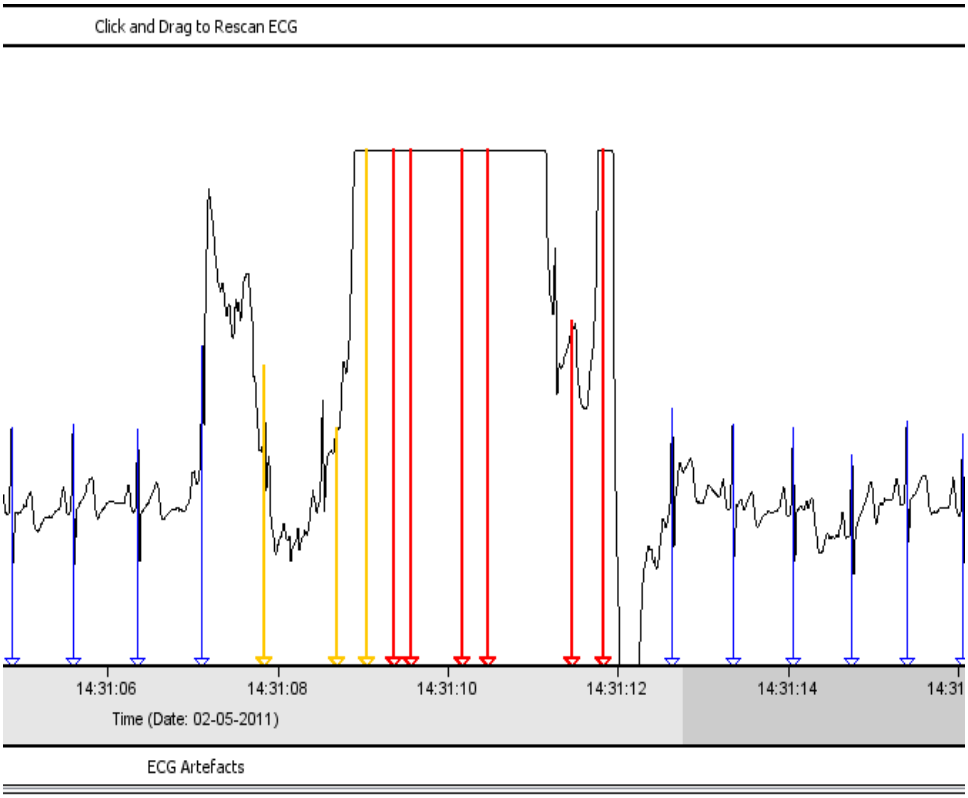
In the *ECG artefacts* Bar all automatically detected artefacts and user-supplied artefacts are labeled by a red bar. These artefacts are deleted from all further data analyses.

In the main window with the ECG signal the detected R-peaks are marked by vertical lines, mostly blue. A blue line means that the beat was considered to be correct according to the automatic beat detector. Potential mistakes in automated beat detection are termed 'suspicious beats' and are flagged by a red or yellow color. Some parts of the data might not be bad enough to be detected by the automated artefact detector and at the same time they are too noisy for the R-peak detector. Hence the R-peak detector will try to make something out of noise and may still score occasional beats as being correct (blue) where they are not. These periods of noisy data will also contain a lot of red and yellow lines which makes them easy to detect.

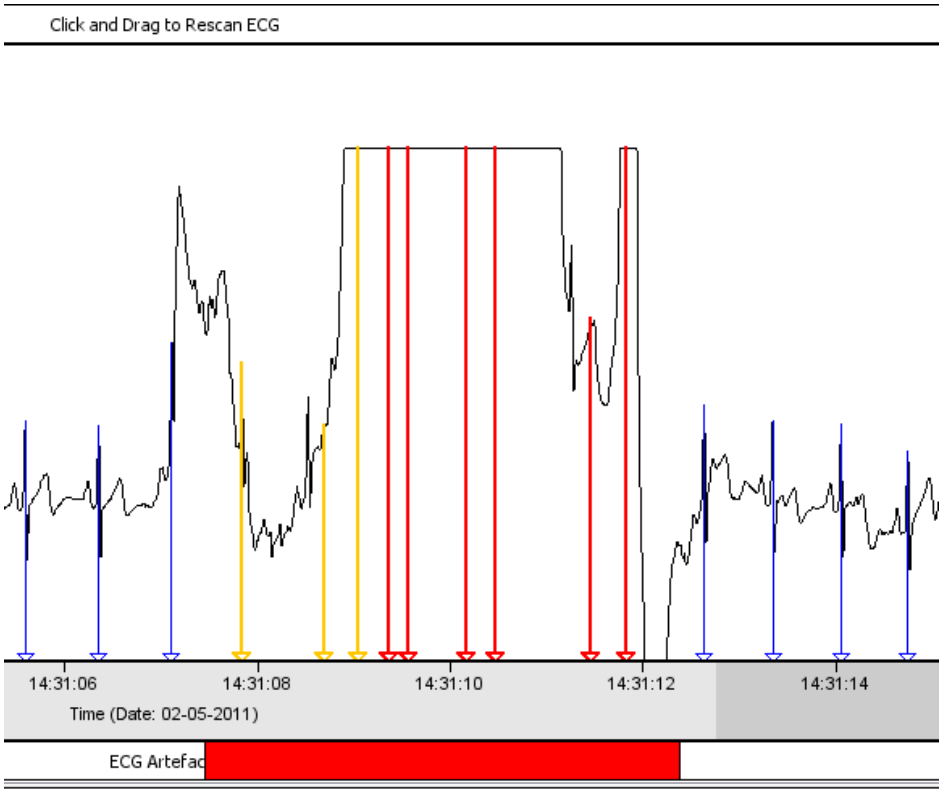
These noisy parts may arise because an electrode became (partly) detached. This is known to happen occasionally in unsupervised ambulatory recordings. To find noisy parts from the IBI signal, search for deviant parts in the bottom IBI time series window. The IBI time series will be highly irregular with sharp peaks/spikes in the IBI time series wherever the detector failed (as it assigns inter beat intervals that are disproportionally long or short). Zoom out so the entire artefact period is visible in your screen to make deleting easier.

To mark the bad ECG as an artefact, click with the left mouse button in the artefact bar and drag it from left to right until it covers the entire period that is to be marked as an artefact.

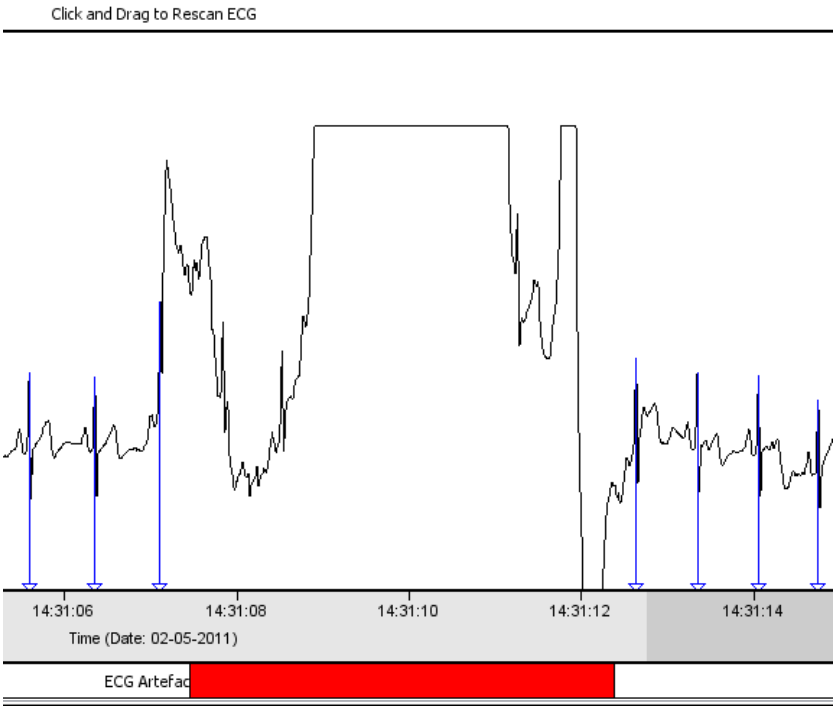
Find the artefact period:



Select the artefact period:



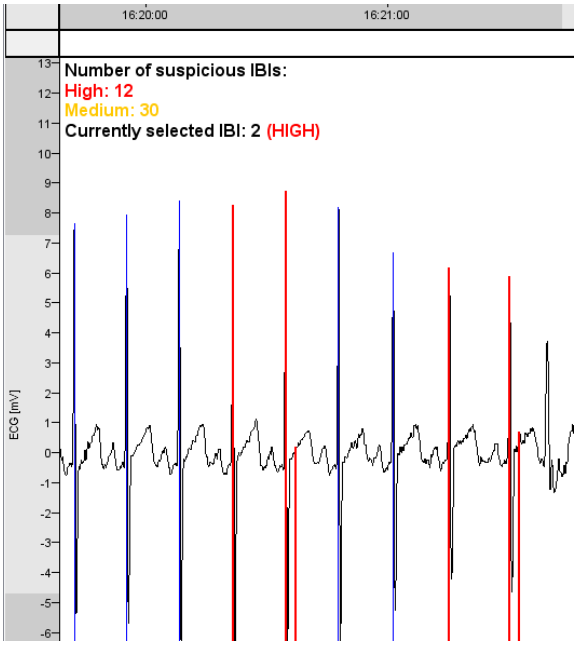
And remove the beats within the artefact period:



You can also wait until all periods with missing data or artefacts have been marked and then use the option *Delete Beats Under Artefact* in the menu bar or use the shortcut Ctrl+D. The IBI signal should look much more smooth now. As stated before, the beats within the periods labeled as artefact will be ignored in all further analyses with the Data Analysis Management Software.

2.2.3 Suspicious beat correction

In the main window`s top left corner the number of suspicious beats are displayed. For highly suspicious beats the R-peaks are marked in red. R-peaks in less suspicious beats are marked in yellow and R-peaks marked by blue lines are considered to be correct.



You can easily browse through all suspicious beats from most to least suspicious by pressing *Dot/right pointer* keyboard key for next, and *comma/left pointer* keyboard key for previous suspicious beat. Or you can simply use the menu buttons at the top of the window. When you are at a suspicious beat you can either delete a beat by right clicking on it, e.g. when a beat was placed in an obvious wrong location in between beats. Or you can add a beat by left clicking on the correct location of the R-peak, e.g. when a beat was completely missed. Notice that the surrounding beats might also change color when adding or deleting a beat. You can also move a beat by placing the left mouse button on the vertical line and then move it left or right. Releasing the mouse button will lock the vertical line to its new location.

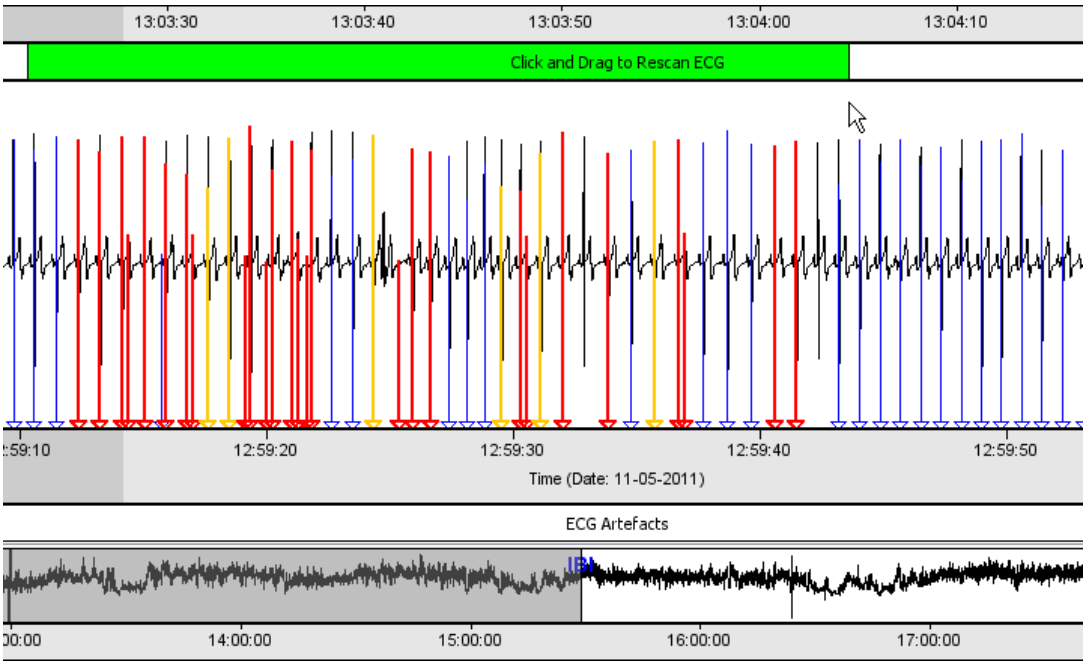
After your corrections (or in fact at any time) you can select the menu button *re-check suspicious IBI`s* to see how many beats are still considered suspicious. Just remember that suspicious is not wrong per se!

It can happen that you can have a perfect IBI time series with no misplaced beats, but that the DAMS program still reports some suspicious beats. This is because strong sinus arrhythmia may throw the beat detector off and extrasystolic beats always will. Clearly, suspicious is not always guilty.

**NOTE: You can also choose to export the inter beat time interval series to a text file for use in different software packages like the CarSpan or Kubios by clicking on the menu buttons 'Export Beats To ASCII File'. Chose the appropriate directory and file name and save the IBI time series as a text file.*

2.2.4 Adjustment of R-peak detection in deviant ECG signals.

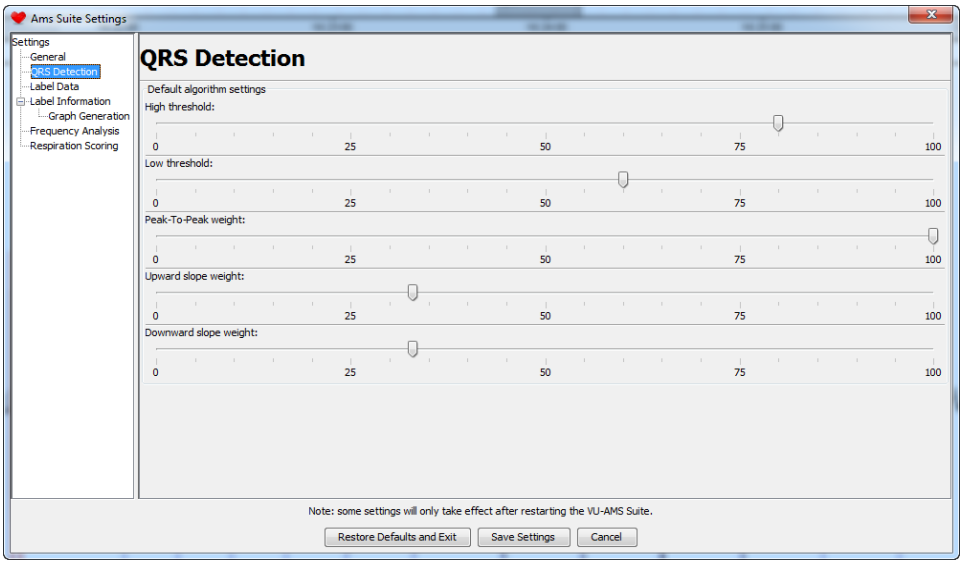
When an ECG signal is of good/reasonable quality but you can clearly see that the automatic detector placed the beats anywhere but on top of the R-peak, you should rescan the complete ECG signal with different settings for the detection algorithms. The default settings for the algorithm upon opening the *.amsdata* file can be changed in the main menu by selecting *Edit* → *Settings* → *QRS Detection*. You can also use the Rescan bar to select only a certain part of the recording. Drag your mouse across the incorrectly scored part of the ECG (minimum rescan length is 10 seconds) in the Rescan bar while holding the left mouse button.

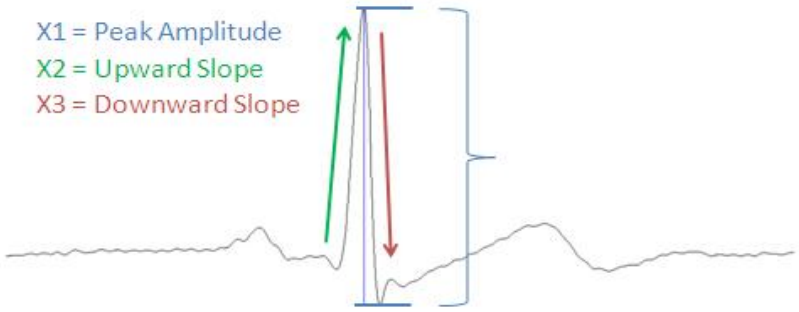


A pop up screen with 5 sliders (the same as in the settings screen) appears. You see a high and a low Threshold slider and three Relative weight sliders that change the weight of the peak amplitude, the downward slope, and the upward slope. The algorithm calculates a Peak score based on the sum of these parameters multiplied by their weights, and then divides this by the sum of the weights. The R-peaks of the selected area will be rescored when clicking on Rescan. This peak score will be used in combination with the threshold sliders/settings, such that all peaks with a score between the low and the high thresholds will be considered an R-peak. Set the sliders as desired and press Rescan to apply the new settings on the selected part of the ECG signal. Adjust the settings until satisfied.

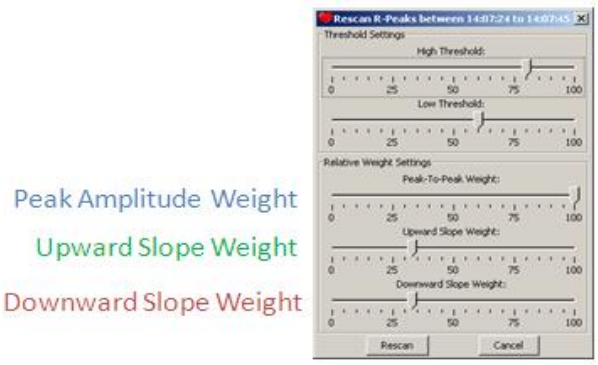
**Please also see the "R-Peak Detection" tutorial video for a demonstration: www.vu-ams.nl/support/tutorials/software/r-peak-detection*

The default values can be changed in the settings screen:





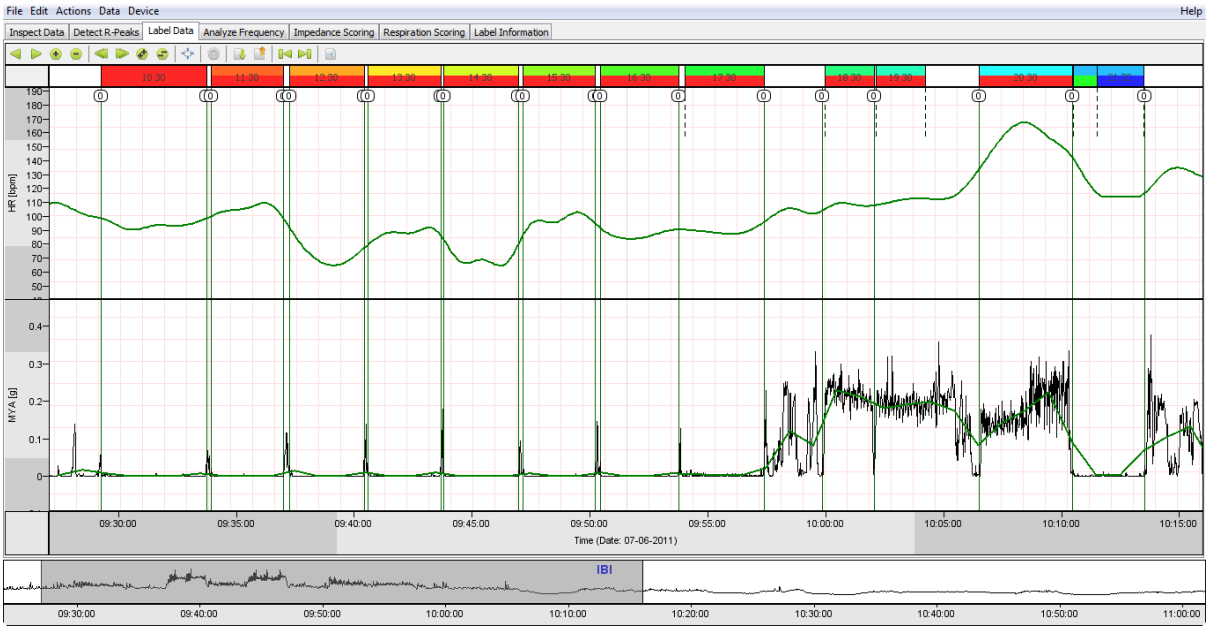
Peak score =((Weight 1 * X1) + (Weight 2 * X2) + (Weight 3 * X3)) / (W1 +W2+W3)



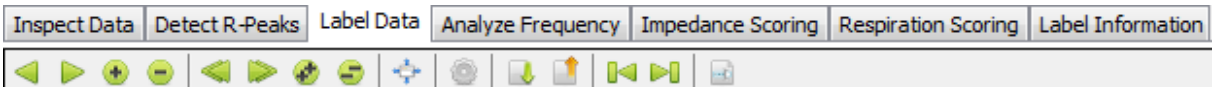
2.3 Labeling your data / dividing your data into separate pieces

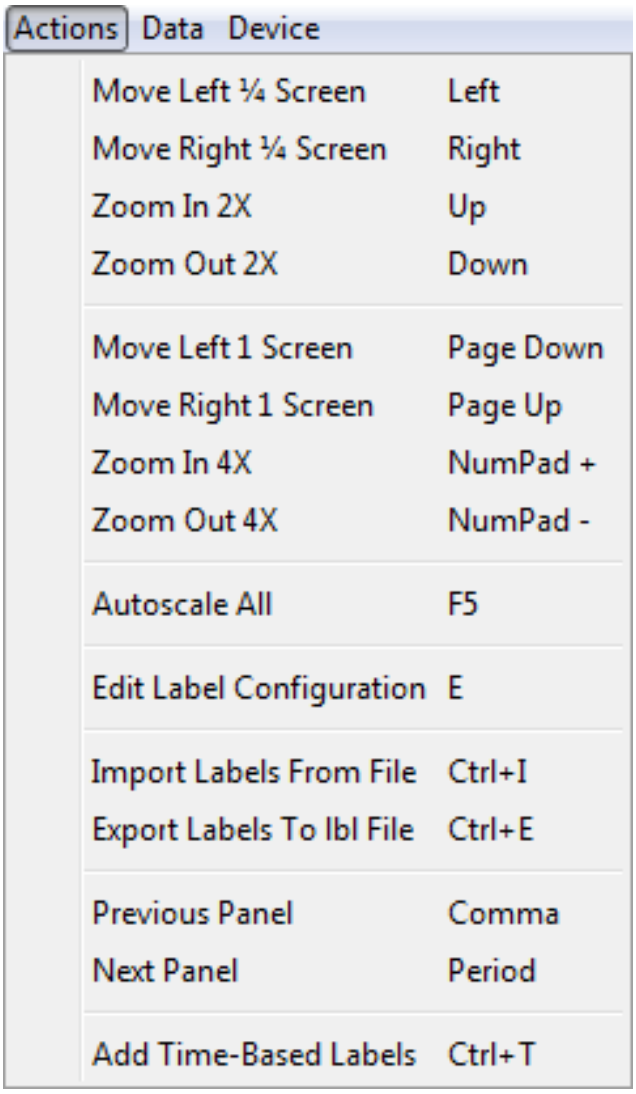
Here you divide the continuous data collected with the VU-AMS device into logical periods for further analysis. We call this labeling. The aim of labeling is to get an average value for each available parameter, such as HR, PEP, and RSA, for each experimental condition or ambulatory activity.

Click on the *Label Data* tab. In the upper window we see the raw IBI time series and/or the smoothed (and hence lightly time lagged) heart rate signal. Because the heart rate signal is dependent on the quality of the IBI time series, make sure the R-peaks have been detected correctly in the *Detect R-Peaks* tab. In the lower window we see the 'motility' signal. This signal is based on the Y axis of the tri-axial accelerometer. The clock time and IBI time series are presented in the two bottom windows, which will help us to quickly locate our current position in the total recording.



All actions for the *Label Data* tab are presented in the form of buttons:

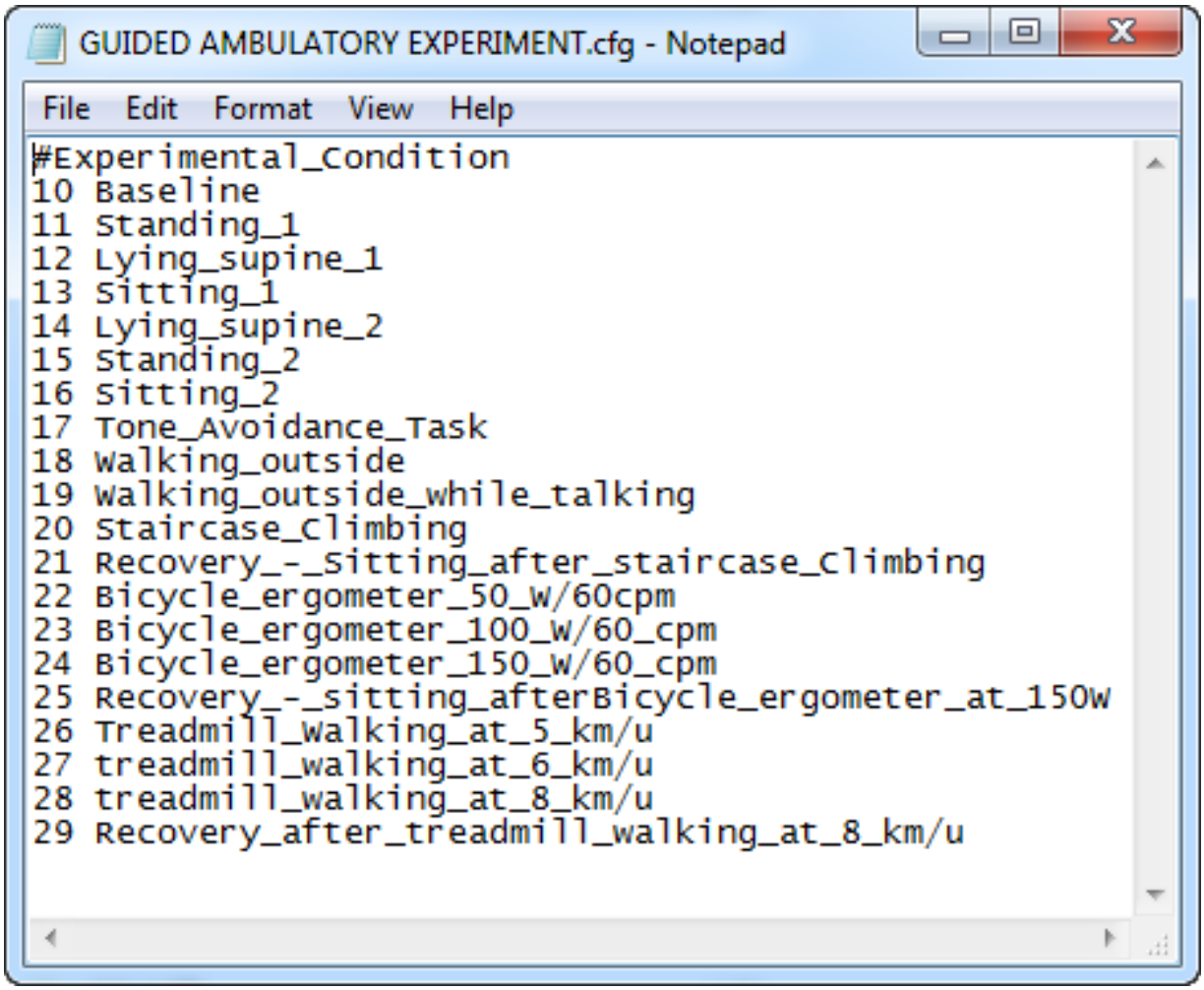




The function of these buttons should be self-explanatory.

2.3.1 Creating a Label Configuration File

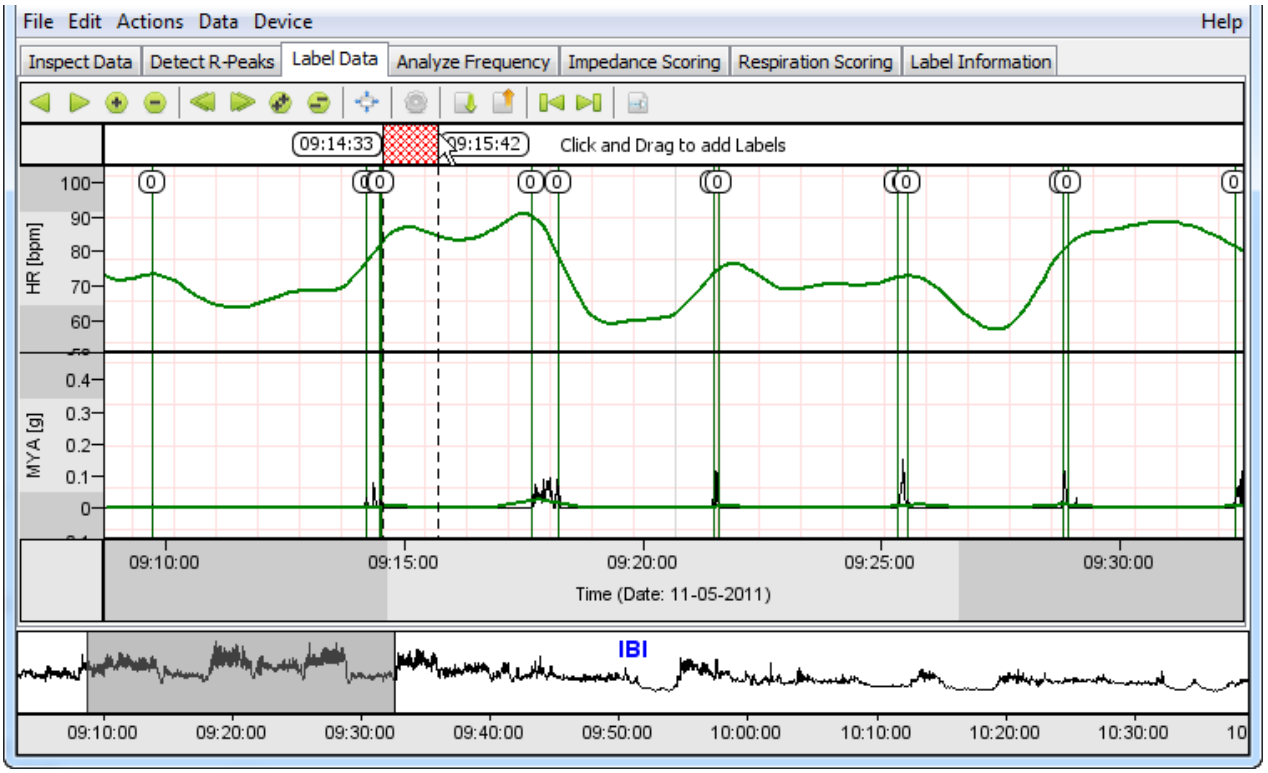
Before we can actually begin labeling the data, we need to create the blueprint for all possible labels first. This blueprint lists all the experimental conditions or ambulatory activities in our experiment. This is done in the label configuration file (.cfg file). The easiest way to create a label configuration file that suits your purpose is by manually creating or editing a text file. A main category is always indicated by a hash key, (#) followed by the name you want for that main category. Then each level of that category, in the example below these were all the experimental conditions, needs to have a unique numerical value followed by a name for each condition. Save it under a logical name with the extension *.cfg*.



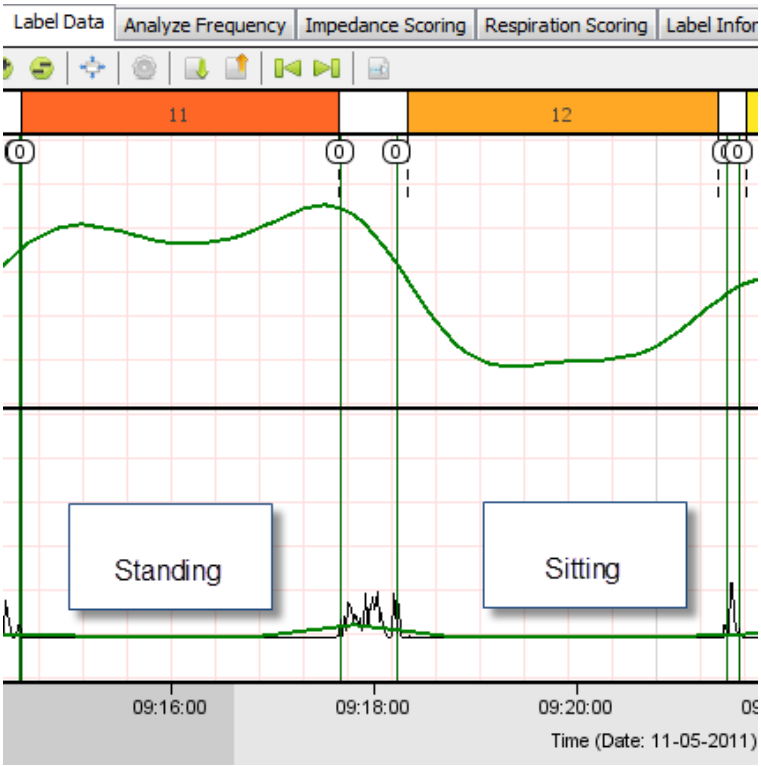
When you are done creating the label configuration file you need to make sure that the DAMS program recognizes this file. To do this ; Go to the main menu → *Actions* → *Edit Label Configuration* → *Import Label Configuration From File* Now select the label configuration file you manually created and click *open*. When the same label configuration file is used for a longer time, like for an entire research project, you could click on the option *Set Label Configuration As Default.*

2.3.2 Labeling your data

Now you can start the actual labeling of your data. If you have notes containing the start and stop times of each condition, you can use the top bar where it says “Click and Drag to add Labels”. When hovering the mouse on the bar you will see an indicator of the time window appear. This will help you place the starting point of your label accurately. Click with the left mouse button in the top bar at your starting point and drag the mouse to the right until the desired end time of the label is reached.



A dashed vertical line will also appear on both ends of the label extending into the motility signal. This may help you remove the transition periods with movement between two experimental conditions from the final labeled data.



Note: The motility signal becomes indispensible when labeling VU-AMS data from self reported activities in diaries as often done in 24-hour ambulatory recordings.

A pop up screen with our previously defined categories will appear upon releasing the mouse button. From our category "Experimental_condition" we choose the level "Baseline" and click *OK*. The pop up window will close now and a colored bar appears representing the new label. You can verify the information in the label by hovering with the mouse on top of it. If you want to edit or delete a label, just right click on it and choose the required action from the popup menu. If you want to change the length of the label click and drag with the mouse on the edges of the label. To reposition a label, click and hold the left mouse button on the label to grab and move it around.

Repeat the labeling process until all relevant periods (i.e. all experimental/ambulatory conditions) are labeled. Each label will be represented by a row in the excel sheet obtained under the *Label Information* tab.

2.3.3 Use of event button marker lines

In case of an experiment you have the option to use the event button of the VU-AMS device to mark the beginning and ending of each condition. Shortly pressing the event button on the VU-AMS device creates a marker in the data at the exact time the button was pressed. These markers are displayed as lines here, and help us to more easily detect the start and stop times of each experimental condition. So, we can use the marker lines or our notes with start and stop times or a combination of both, to make sure we label the correct periods for all conditions.

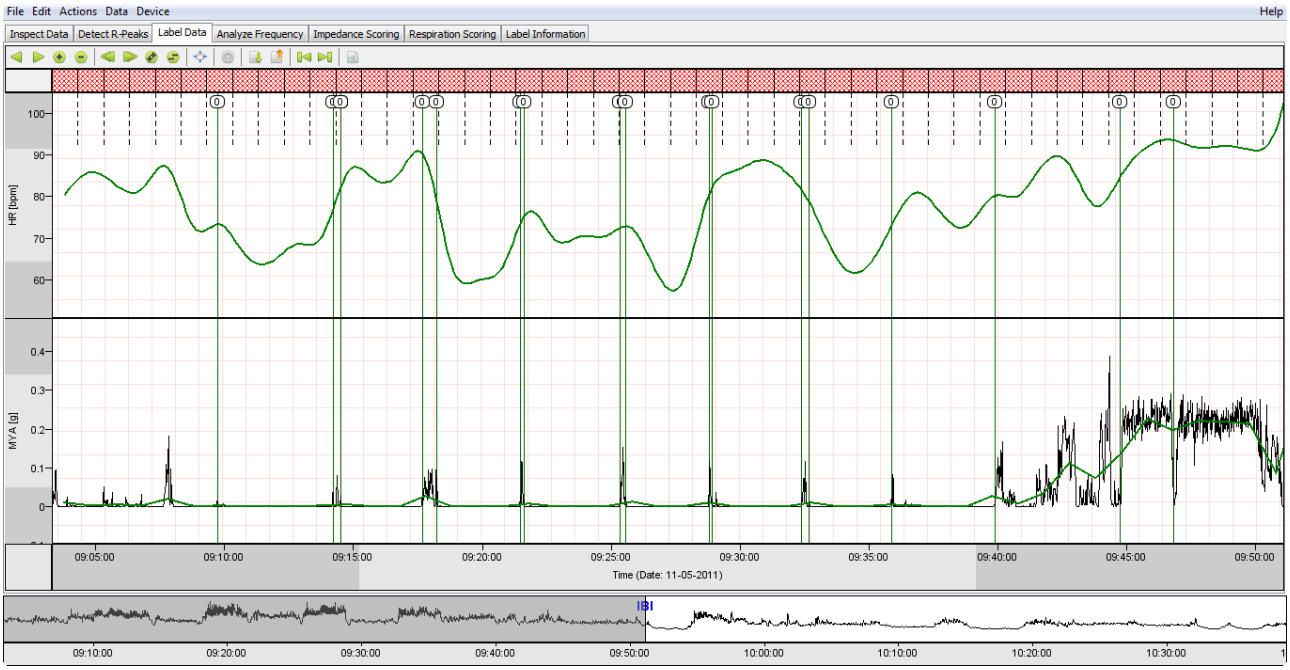
When working with the marker lines generated by the event button of the VU-AMS device you have the possibility to automatically place a label exactly between two marker lines. By clicking with right mouse button on the label bar between the two lines, we get the option to place a label between the lines automatically.

Note: *In laboratory studies that use a stimulus computer, this computer can be used to send markers through the VU-AMSi infrared interface cable. Please refer to VU-AMS website: www.vu-ms.nl/support/downloads/extras*

2.3.4 Time based labels

Instead of predefined category labels representing a specific condition, you can also choose to create time-based labels, where all labels represent a fixed amount of time that can last in duration between 10 seconds and 1 hour. This is especially useful if you want to generate continuous 60 sec ensemble averages of the impedance cardiogram for PEP scoring instead of a large scale ensemble average that spans an entire condition.

To do this use; *Actions* → *Add Time-Based Labels* and type in the amount of seconds you want each label to be. The entire data is now cut up into labels with your pre-defined length.



**Please also see the Data Labeling tutorial videos for a demonstration: www.vu-ams.nl/support/tutorials/software/data-labeling*

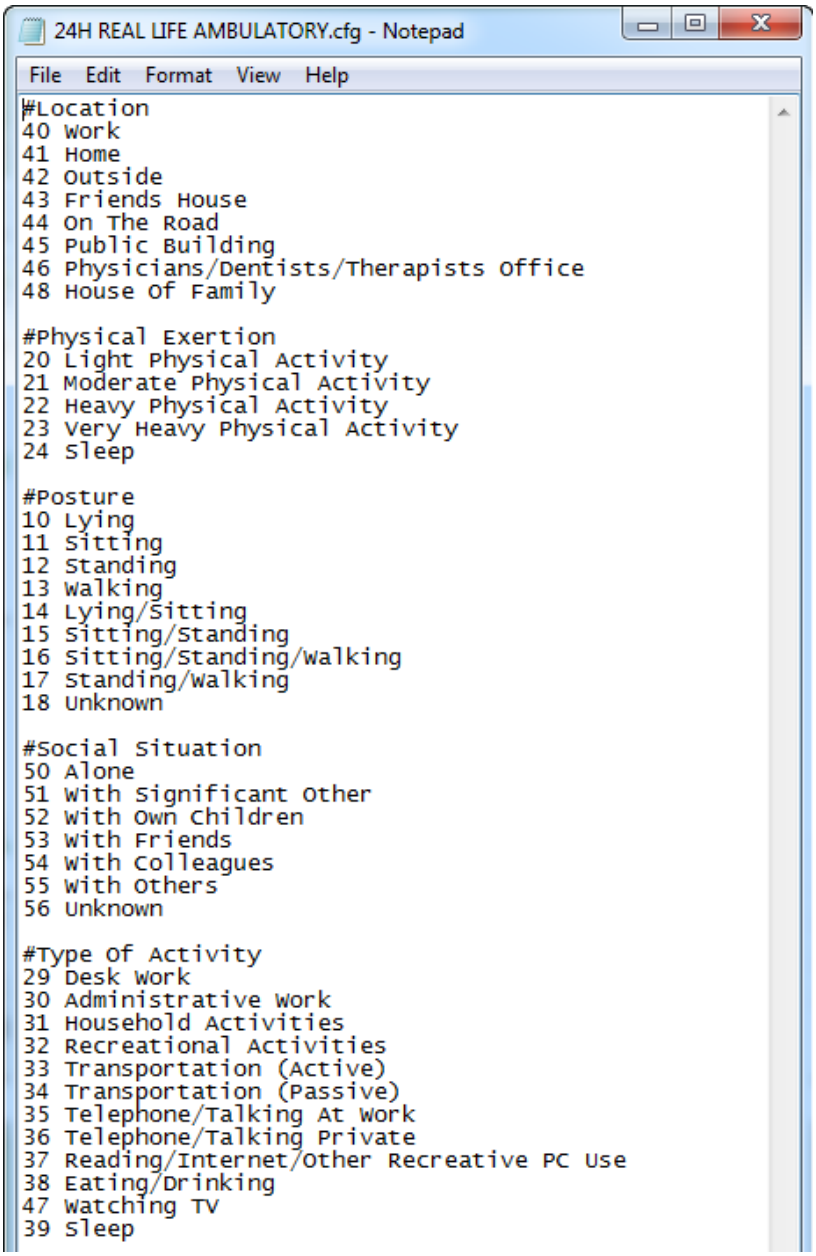
2.3.5 Labeling Naturalistic Recordings

The previous example assumed a supervised setting with fixed experimental conditions of which start and end times were under the experimenter's control. This is of course not the case in ambulatory recordings in naturalistic settings. Still many of the principles apply, as "real life" can also be divided into periods of 'fixed' and frequent occurring activities.

To create a label file that will capture most daily activities of the subjects in your target population is a crucial but feasible step in ambulatory data analysis. As the autonomic nervous system is highly sensitive to changes in posture and physical activity it is first of all very important to obtain information about these aspects of a subject's daily routine. This is usually done by asking all subjects to keep a detailed diary (paper-and-pencil or electronic hand held devices) during the ambulatory measurement day. This diary information about (changes in) posture and physical activity is then used during the labeling procedure. The VU-AMS device also contains a tri-axial accelerometer to support this self-report with objective data. Further categories to be used in the ambulatory labels will entirely depend on the research question and the target population. Here are some examples of categories that could be considered:

Category	**Levels of Category**
Type of activity	Reading, attending a meeting, eating, dancing, PC work, conversing, watching TV, exercising, attending a musical, car driving, ironing, etc.
Posture	Sitting, standing, lying, walking, etc.
Physical activity	Heavy, moderate, light, none
Social situation	Alone, with significant other, with friends, With colleagues, etc.
Location	At home, at the office, travelling, restaurant, etc.
Time of day	Work, leisure time, sleep
Mood state	Angry, friendly/happy, sad, anxious, tired

When you have decided on the type of categories you want to use during labeling you again need to summarize these in a *label configuration file* (default name *label.cfg*). An example for a 24-hour recording project is given in the figure below.

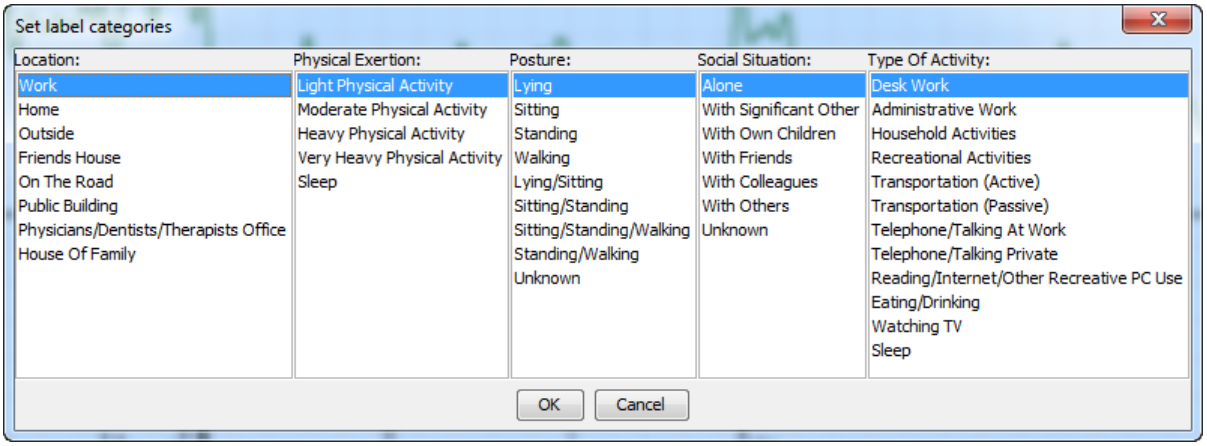


In the label configuration file all ambulatory activities you might want to use as separate conditions in future analyses should be present as all further processing in the DAMS program is tailored to the labels.

When you are done creating the ambulatory label configuration file you need to make sure that the DAMS program recognizes this file. To do this use; *Actions* → *Edit Label Configuration* → *Import Label Configuration From File* Now select the label configuration file you manually created and click *open*. When the same label configuration file is used for a longer time, like for an entire research project, you could click on the option *Set Label Configuration As Default.*

Now you can start the actual labeling of your data. Using the subjects' diary estimate the start and stop times of each activity. The motility signal can be extremely helpful to detect posture transitions and changes in physical activity which are the natural boundaries of changes in real life activities (e.g. when the subject reports "sitting desk work, walking to my car, driving home" three distinct motility patterns will be evident).

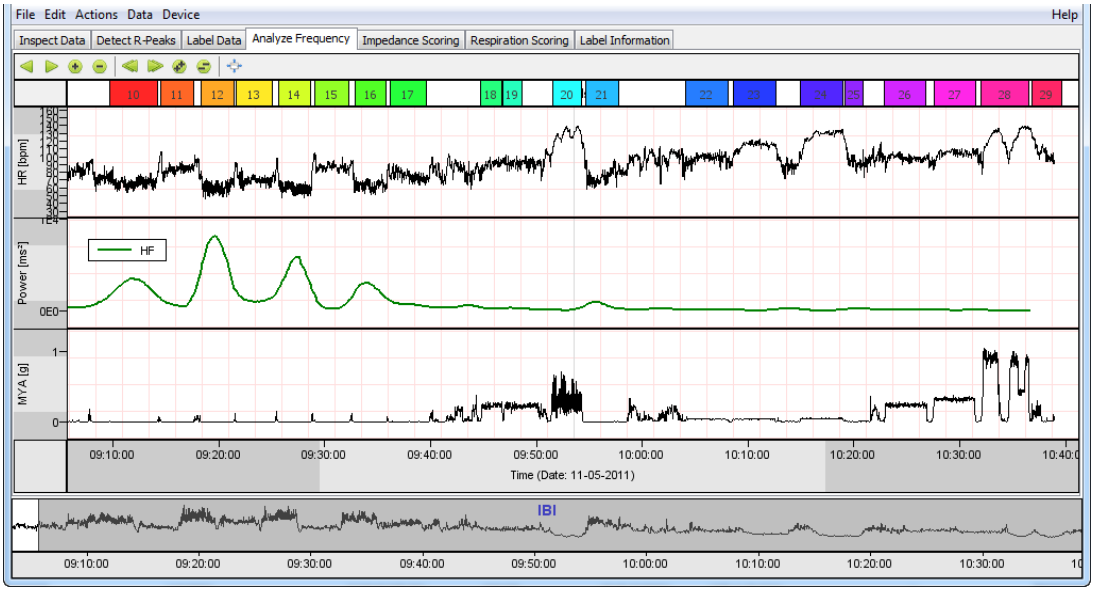
Use the top bar where it says "Click and Drag to add Labels" and click with the left mouse button in the top bar at the starting time and drag the mouse to the right until the desired end time of the label is reached. The popup window that appears after the label is drawn will reflect all categories in the label configuration file:



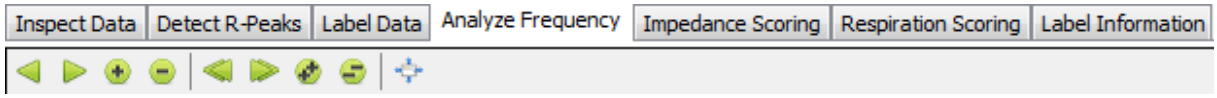
For each labeled period start and end times are given as well as a set of codes and text labels describing the state of subject during that period in terms of location, posture, physical exertion, social situation, type of activity etc..

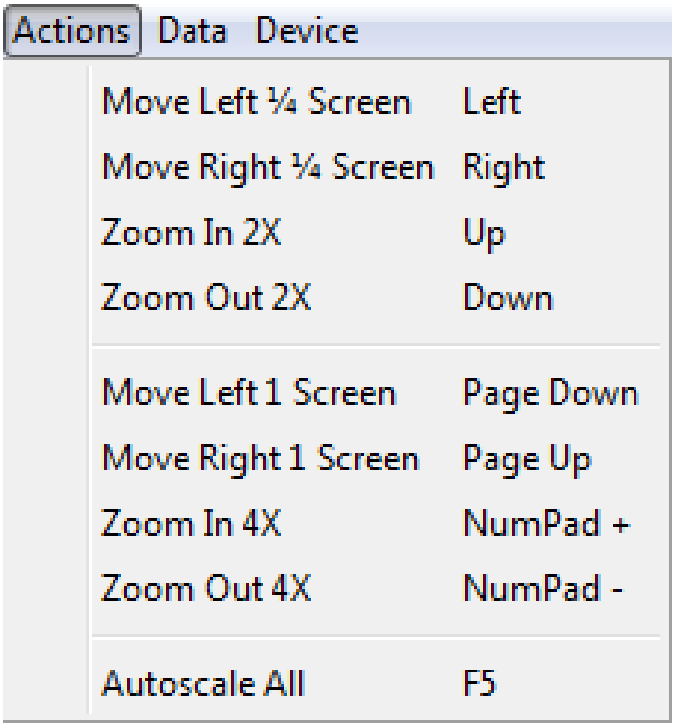
When done, make sure you have labeled all periods that might be considered of interest. There seems to be no urgent need to also label periods that you consider to be "irrelevant" or that are expected to occur in only a few subjects. However, we advise to always label the entire 24 hour recording as completely as possible. With an average length of labeled periods of 20 minutes this would result in about 72 labels per subject.

2.4 Analyze Frequency



All actions for the Analyze Frequency tab are presented in the form of buttons:





The function of these buttons should be self-explanatory.

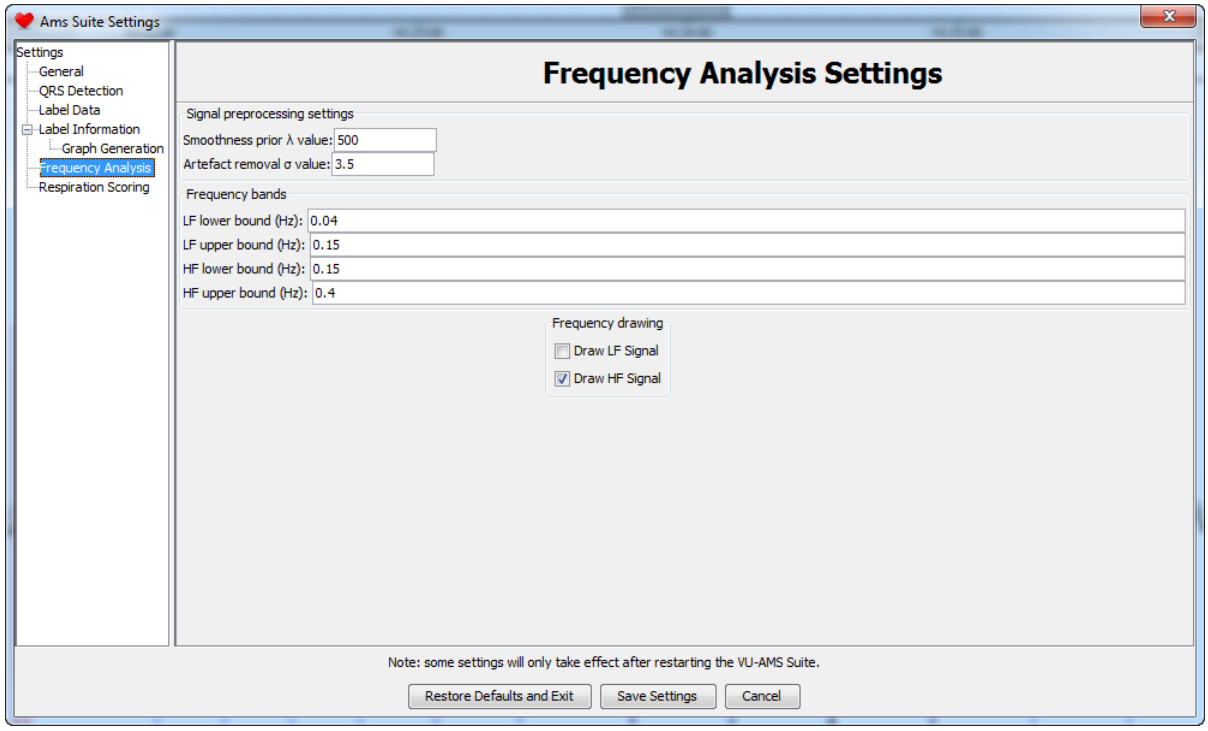
2.4.1. Analyze Frequency explained

Spectral analysis in the Analyze Frequency tab is performed on the corrected IBI time series according to the following method:

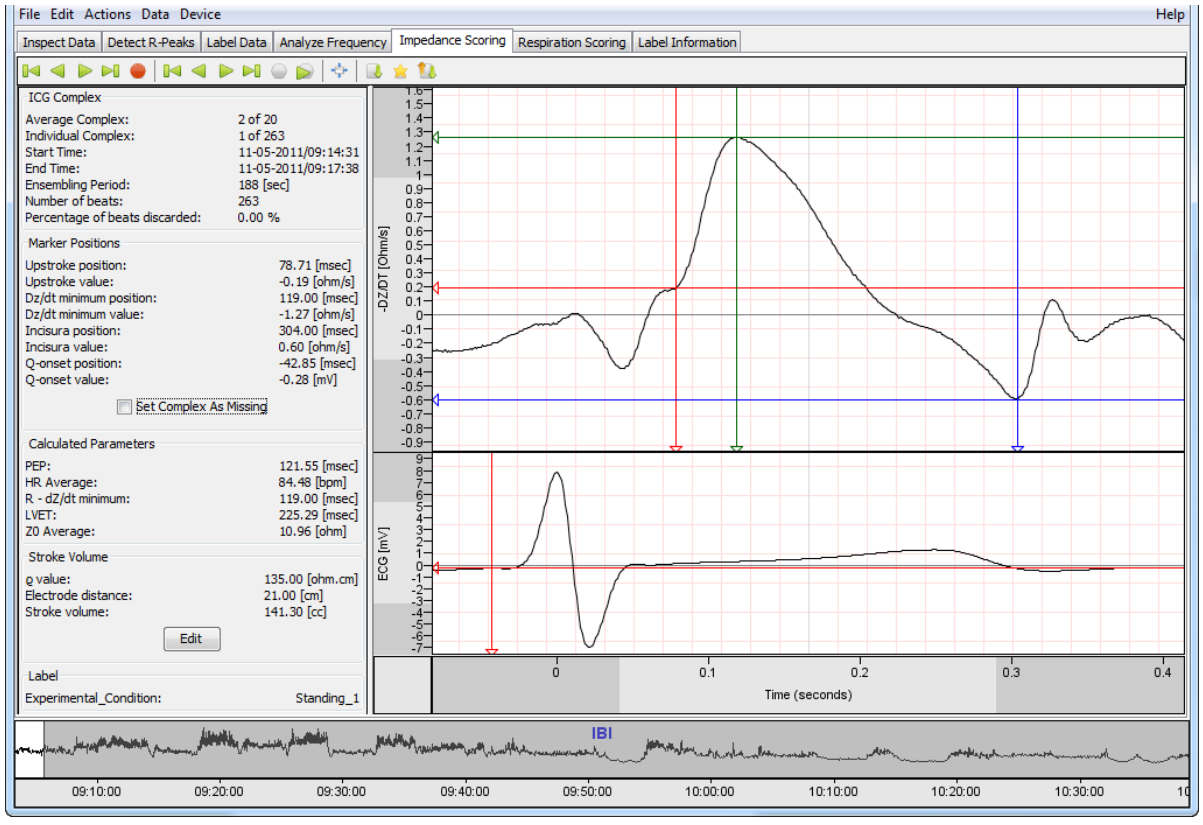
The IBI time series within each label is interpolated with a cubic spline and the resulting function is resampled at 4 Hz. The resampled signal is split into overlapping periods of 256 seconds, each with 1024 data points. The overlap between two consecutive periods is 128 seconds. Periods that have intervals longer than 5 seconds without IBIs are discarded. Missing data from the final period are padded with zero's. Each period of 1024 data points is convoluted with a smoothness prior matrix (An advanced detrending method with application to HRV analysis, Mika P. Tarvainen, Perttu O. Ranta-aho, and Pasi A. Karjalainen) to yield a stationary signal on which a discrete Fourier analysis is performed after additional convolution with a quadratic window. Power values for each of the 1024 data points are then averaged across all available periods in the condition (Welch method). Next the total power in the 0.0001 Hz to 0.4 Hz range is computed (TP) as well as the power in the 0.04-0.15 Hz band (LF) and the 0.15-0.40 Hz band (HF). It is important to note that the IBI time series is first detrended and 'corrected' by interpolation to deal with too short and too long IBIs (e.g. in case of an extrasystolic beat) because slow trends as well as strongly deviant beats can distort the spectrum. Because at least 4 minutes are required to obtain a reliable estimate of the LF power, these values are not supplied for labels with a duration shorter than 4 minutes.

2.4.2 Change Settings

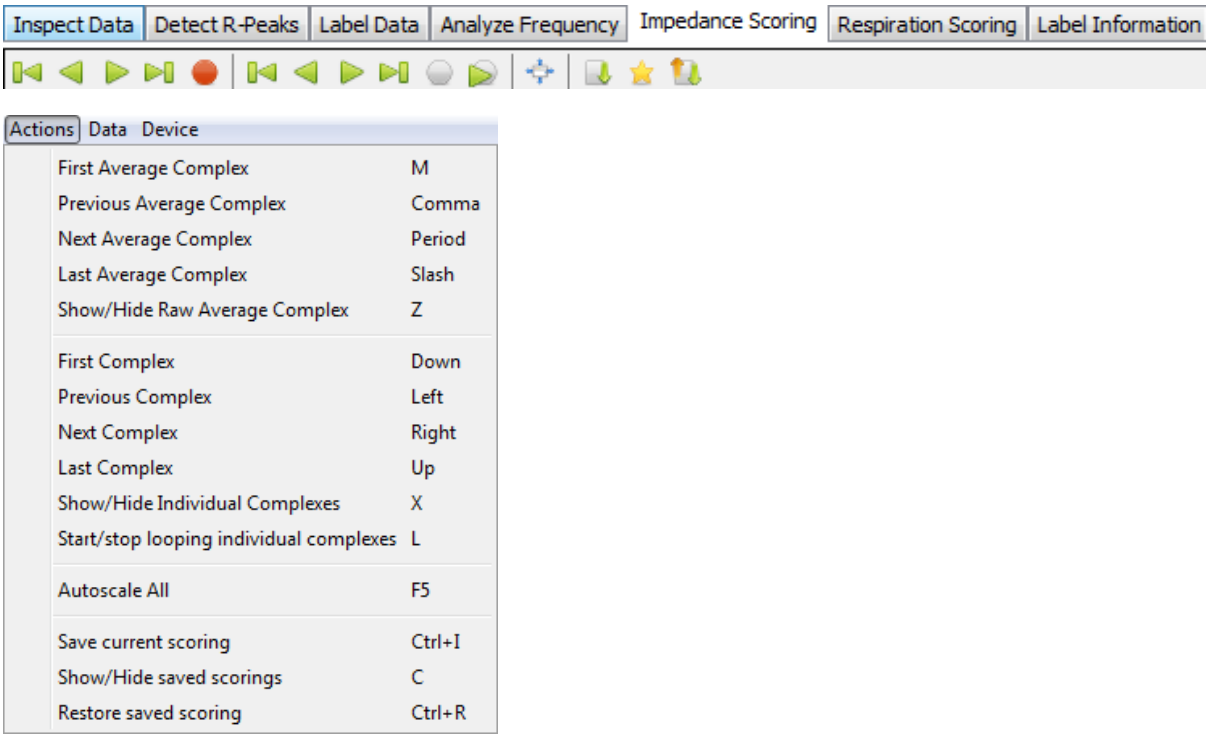
In case you want to change the default settings of the power spectral analysis you can do this in the main menu by selecting *Edit* → *Settings* → *Frequency Analysis*. Settings for preprocessing affect detrending and deviant beat removal ('artefact'). The frequency bands reflect the typical bands now commonly used in the literature, but can be changed if desired.



2.5 Impedance Scoring



All actions for the *Impedance Scoring* tab are presented in the form of buttons (the function of these buttons should be self-explanatory).



2.5.1. Impedance explained

The impedance cardiogram (ICG) is the first derivative of the change in thorax impedance using time as the basis (dZ/dt). This characteristic ICG waveform derives from the change in thorax impedance caused by left ventricular ejection of blood into the descending aorta during the systolic phase of the cardiac cycle. To improve signal quality, the ICG waveform is often obtained by ensemble averaging over beats within in a fixed time period, time locked to the R-wave peak. The typical period for ensemble averaging is one minute. In DAMS we deviate from this practice and instead compute a Large Scale Ensemble Average across the entire label (see Riese et al., 2003 for the rationale).

The most important variables extracted from the ICG are the preejection period (PEP) and the Stroke Volume. The PEP is an index of contractility which is only influenced by sympathetic but not parasympathetic activity in humans making PEP the measure of choice to monitor changes in cardiac sympathetic activity non-invasively. The PEP is defined as the interval from the onset of left ventricular depolarization, reflected by the Q-wave onset in the ECG, to the opening of the aortic valves, reflected by the B-point in the ICG signal (Lozano et al., 2007; Sherwood et al., 1990; Willemsen et al., 1996).

Stroke volume (SV) is the average amount of blood ejected during the cardiac cycle. When multiplied by heart rate this yields the cardiac output (CO), the total amount of blood circulated through the body per minute. SV is computed from the ICG by using the product of the maximal amplitude of the dZ/dt and the ejection time, weighing for blood resistivity, baseline thorax impedance and the total volume enclosed by the measuring electrodes.

NB: The reliability of between-individual differences in Stroke Volume (SV) computed by impedance cardiography remains a heavily debated issue. With ambulatory SV the concerns are even more valid, because movement artefacts and the lack of a phonocardiogram do NOT increase SV reliability. In

addition, spot electrodes pick up only half of the impedance measured by band electrodes, and without correction for this, the Kubicek formula yields supraphysiological SV's. If you use percentual changes in SV strictly in a within subject design all these concerns are greatly reduced.

2.5.2 Automatic detection

The DAMS program runs an automatic scoring algorithm, which tries to detect three specific locations in each ICG waveform (termed 'ICG complex') and one in the ECG:

B-point or upstroke, the opening of the aortic valves, marking the end of the electromechanical systole and the beginning of the left ventricular ejection time. The upstroke occurs somewhere in the middle of the first heart sound. An increase in heart rate is usually accompanied by a shift of the B-point to the left (increased sympathetic activation) and a decrease in heart rate by a shift of the B-point to the right (decreased sympathetic activation). However, heart rate may also change purely by changes in parasympathetic activation, in which case no shift in the B-point may be seen.

dZ/dt min or C-point, the point where the velocity of ejection is at its maximum and impedance at its minimum (in the graph, it is drawn in reverse polarity, so the dZ/dt minimum is shown as a maximum by the program).

X-point or incisura, the closing of the aortic valves, marking the end of left ventricular ejection time (LVET). The X-point corresponds well to the first high frequency component of the second heart sound. As the LVET is typically between 1/3 (low heart rate) and ½ (high heart rate) of the total cardiac cycle time, an increase in heart rate (shorter cardiac cycle) should be accompanied by a shift of the X-point to the left and a decrease in heart rate should be accompanied by a shift of the X-point to the right.

Q-wave onset in the ECG, marking the start of the electromechanical heart cycle. Currently this is set to a default of 48 ms before the ECG R-wave peak.

These 4 points are shown as vertical (movable) lines in the ICG- and ECG graphs.

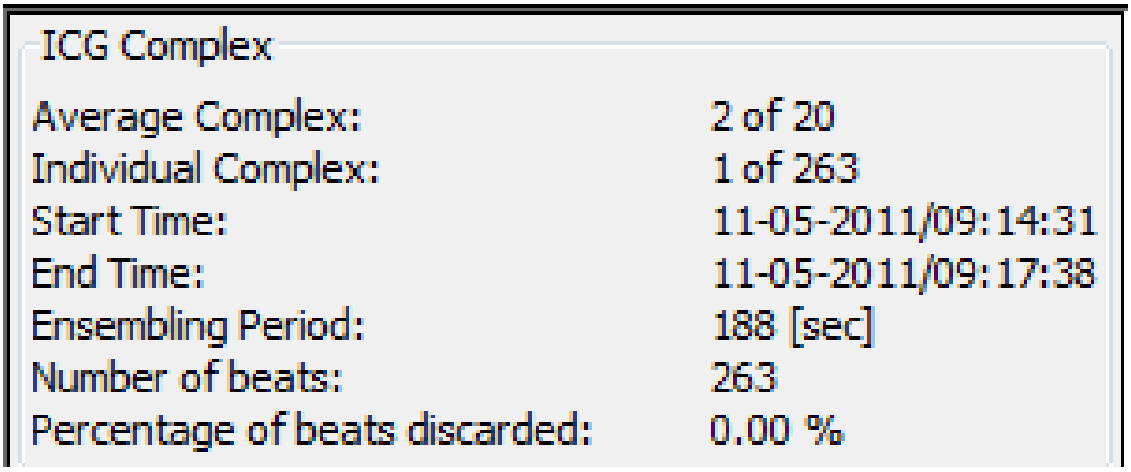
2.5.3 Visual Inspection and manual correction

Ensemble averaging improves automated detection of the crucial landmarks in the ECG and in the ICG but even after ensemble averaging substantial errors in positioning of the B-point remain (Lozano et al., 2007; Willemsen et al., 1996; Berntson et al., 2004). The number of algorithms proposed to score the impedance cardiogram is countless. We have tried quite a few at the Vrije Universiteit. Our current stance is that automatic scoring simply will not work for all signals. We therefore visually inspect every ensemble averaged ICG complex and manually correct the locations of the 3 key time points when needed. Scoring of the Q-point in the ECG also always needs to be inspected and, when necessary, corrected. Manual scoring is inherently subjective but it does lead to reliable and valid results. To safeguard reliability, scoring should be ideally repeated by multiple raters.

We further recommend reading Sherwood et al.'s "Methodological Guidelines for Impedance Cardiography" published in Psychophysiology before starting with scoring and analyzing the impedance cardiogram.

After clicking on the *Impedance Scoring* tab the window is displayed as in the figure on the start of this paragraph. On the right side of the window there is a graphical representation of the current ICG complex. On the left side there is a text box with several frames:

- *ICG complex*



- *Average Complex:* This is the number of the ensemble averaged ICG complex (the second one out of 20 is drawn in this example)

- *Individual Complex:* This is the ICG complex of one valid single beat within the current label (in this example we are at the first beat out of 263 valid beats that are present in the second averaged complex). You can make the individual ICG complexes (light grey line) visible in the back of the ensemble averaged complexes (black line) by toggling the button *Show/Hide Individual Complexes* in the menu bar. This is helpful when the B-point is not completely clear in the ensembled ICG. The individual complexes can sometimes give you a hint on where to score the B-point. You can even let DAMS play the individual complexes in the background of the ensemble averaged complex by toggling the button *Start/stop looping individual complexes*.

- *Start Time:* Start time of the current ensemble averaged complex.

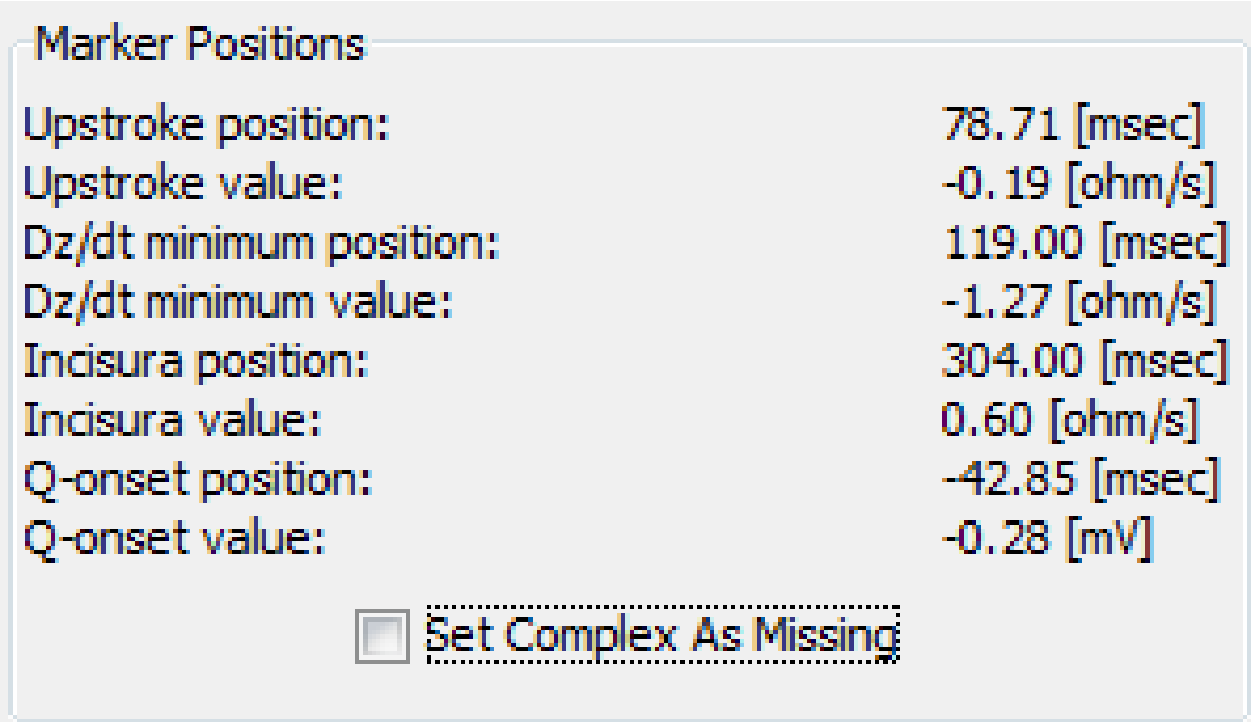
- *Stop Time:* End time of the current ensemble averaged complex.

- *Ensembling Period:* Duration of the label on which the ensemble average ICG complex was based (regardless of number of discarded beats within the label).

- *Number of beats:* The number of valid beats within the label (this is the total amount of beats within the label minus the beats that were discarded by the artefact detector in the R-peak detection tab).

- *Percentage of beats discarded:* The number of beats within the label that are discarded by the impedance scoring algorithm due to bad ICG signal quality. With the button *Show/Hide Raw Averaged Complexes* you can show the original (raw) ensemble averaged signal (red line) that contains all beats within the label regardless of the quality of the ICG on top of the 'clean' ensemble averaged signal (black line) that contains only beats with high quality ICG complexes.

- *Marker positions*

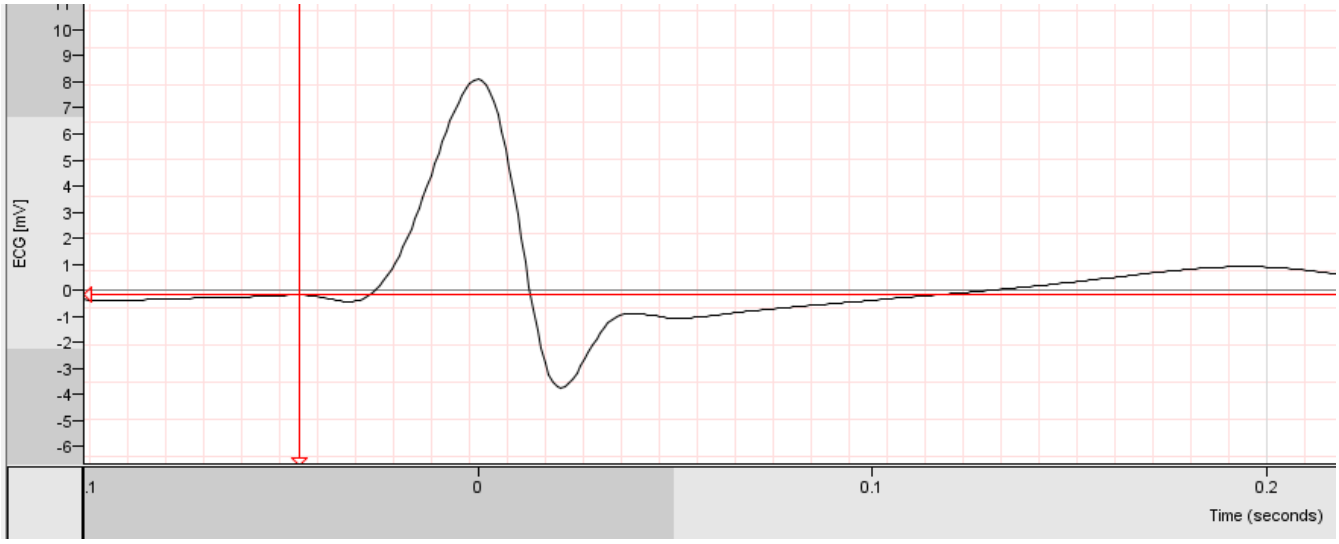


- The distance from the ECG R-peak to all marker lines and the corresponding value of the amplitude of the ICG or ECG at that time point are given in the left panel under the heading 'marker positions':
- *Upstroke position:* B-point, position of the dZ/dt upstroke relative to R-Peak in [msec]
- *Upstroke Value:* dZ/dt amplitude at the B-point in [ohms/sec]
- *dZ/dt minimum position:* C-point, Position of the dZ/dt minimum relative to the R-Peak in [msec]
- *dZ/dt minimum value:* dZ/dt amplitude at the dZ/dt minimum in [ohms/sec]
- *Incisura position:* X-point, Position of the incisura relative to R-Peak in [msec]
- *Incisura value:* dZ/dt amplitude at the X-point in [ohms/sec]
- *Q-onset position:* Position of the Q-wave onset relative to R-peak in [msec] (default =48 msec)
- *Q-onset Value:* ECG amplitude at the Q-wave onset in [mV].
- *Set complex as missing:* The complex is omitted in the output when this box is checked.

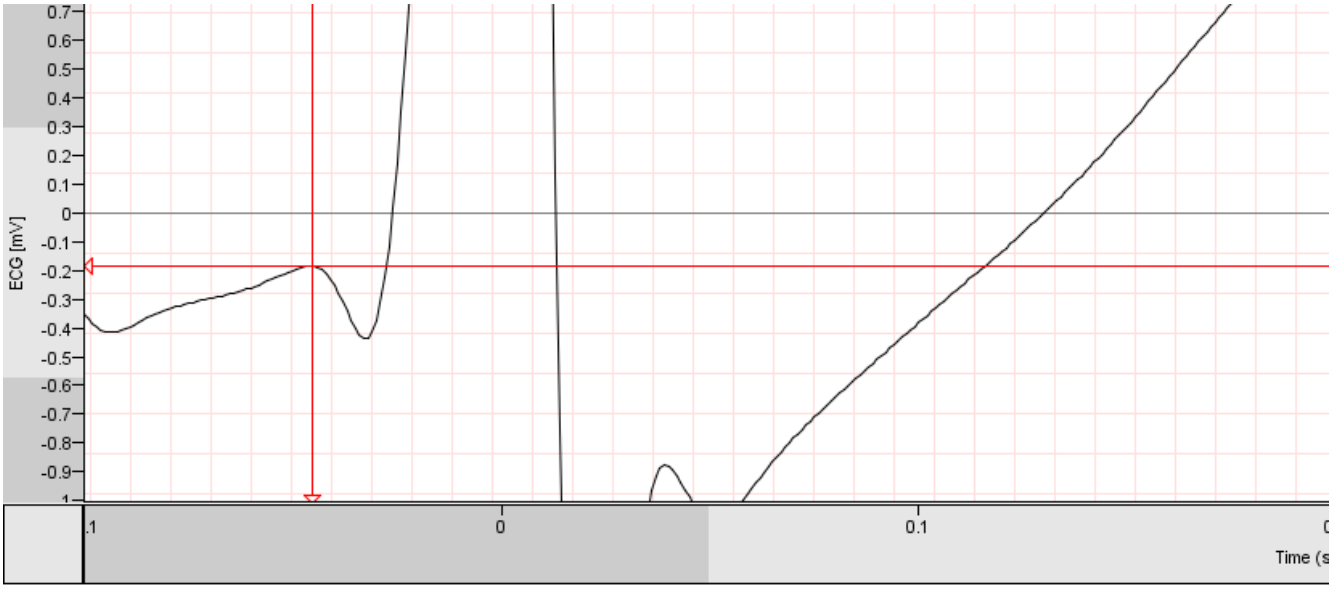
Constant QR vs Q-point scoring.

The ensemble averaged ECG allows for easy Q-point scoring. The Q-point might look obscured but will appear more clearly when zooming in on the Y-axis.

Normal:



Zoomed in on Y-axis:



Calculated Variables

Calculated Parameters

PEP:	121.55 [msec]
HR Average:	84.48 [bpm]
R - dZ/dt minimum:	119.00 [msec]
LVET:	225.29 [msec]
Z0 Average:	10.96 [ohm]

A number of additional variables are computed based on values from the marker positions. The values of these variables reflect the mean across the label. For some variables the values are displayed in the ICG scoring screen:

***PEP**:* Preejection Period (Q-B interval) in [msec].

***HR Average**:* Average heart rate in beats per minute [bpm].

***R - dZ/dt minimum**:* ECG R-peak to ICG C-point in [msec].

***LVET**:* Left Ventricular ejection time (B-X interval) [msec].

***Z0 Average**:* Average thorax impedance in [ohm] during current label.

For other variables values are only given in the *Label Information* tab and in the results files saved from this tab:

***Stroke volume**:* The amount of blood ejected per beat in [cm^3]. Calculated with the following formula

$$\mathrm{SV} = \frac{-rho_{blood} L^2 \left(\frac{dZ}{dt}\right) min * LVET}{{Z_0}^2}$$

***Heather index**:* An alternative contractility measure in [ohm/sec^2]. Calculated with the following formula (2):

$$\mathrm{HI} = \frac{\left(\frac{dZ}{dt}\right) min}{ISTI}$$

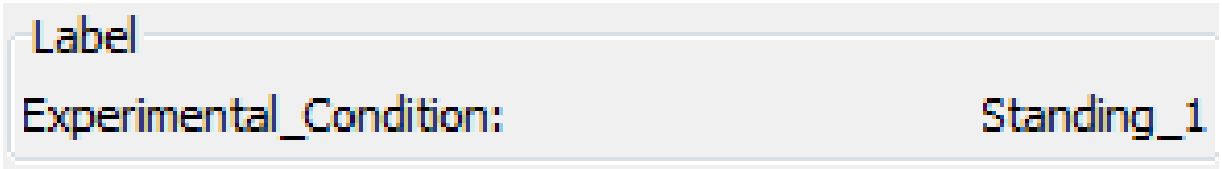
, where ISTI is the time between the R-peak and (dZ/dt)min.

***Minute volume**:* The total amount of blood circulated through the body per minute, calculated as:

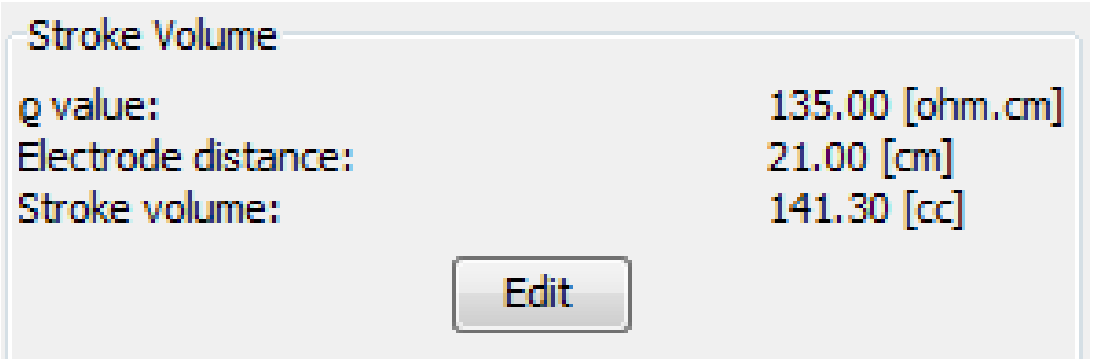
Stroke Volume * Heart Rate * 0,001 in [l/min]

Label

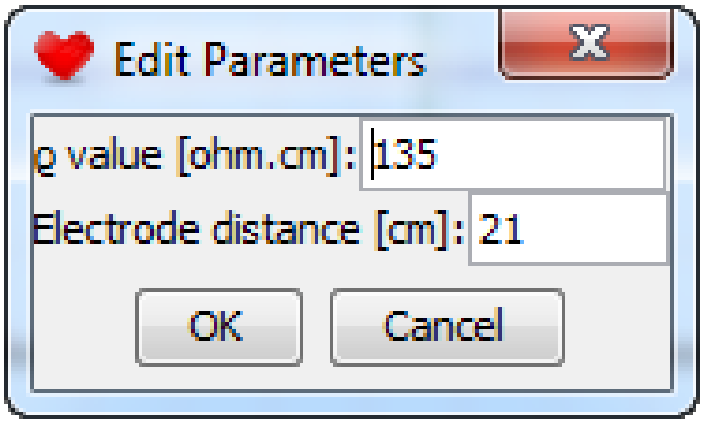
This simply shows the labeled period across which the current ICG complex was ensemble averaged.



2.5.3 Setting Stroke Volume parameters



There are two parameters that influence the calculation of the stroke volume: the distance between the measuring (yellow) ICG electrodes (Le, we advise to provide this information when filling in the subject ID before you start a recording), and the specific blood resistance (ρ). These variables can be changed by clicking the 'Set parameters…' command in the left panel. If hematocrit was not obtained, the standard value of 135 Ohm.cm can be used.



2.5.4 ICG Scoring principles

Below we give 6 scoring principles that can be used during visual inspection and manual correction of the dZ/dt signal. These principles are shown in order of importance:

1 - morphology

The B-point or upstroke should be at a first or second order zero-crossing in the dZ/dt signal. It should be close to the dZ/dt=0 line, and be the starting point of the longest uphill slope before the dZ/dtmin point. However, rather than appearing as a clear incisura, the B-point may sometimes take the form of a subtle inflexion and may vary considerably from beat to beat. It is therefore very important to inspect the dZ/dt signal closely in order to identify it. Occasionally, there is simply no clearly identifiable point that can be chosen to fit the above description of the B-point. In that case the point of the dZ/dt=0 crossing may be appropriate (see also Sherwood et al., 1990).

The dZ/dtmin is normally visible as a clear peak in the window between the B- and the X-point. In some cases the dZ/dt signal shows a double peak, a bit like rabbit ears. If one of the peaks is clearly (40%) higher then this peak is chosen. If the peaks are of comparable magnitude, choose the first peak.

The X-point or incisura is always a local minimum after the dZ/dtmin. Often it is the lowest point in the entire signal, but not necessarily. In the ideal situation it can be seen as a sharp trough in the ICG signal. This is the most clearly identifiable choice for the X-point. It may be that two or more troughs lie in close proximity without one being clearly the lowest point in all complexes. The latter part of the ICG waveform then looks like a "W". In this case choose the second trough (mostly, this trough is usually followed by the longest uphill slope after the dZ/dtmin point).

2 - consistency

Whatever point you choose, choose that point consistently. If a "less-than-ideal" upstroke is present in all complexes, but an "ideal" upstroke is present in some, choose the less-than-ideal one in all complexes, even those featuring a more "ideal" upstroke. Before starting to score the ICG, try browsing through the entire ICG signal first. You can then decide which points can be most consistently identified, and this holds for both for the B-point and the X-point.

3 - in dubio abstine

You may have quite a lot of one-minute ensemble averages. Sometimes 2 out of the 5 ensembles are ugly, possibly because of arm movement artefacts. Don't try to make the best of these 2 if you feel pretty confident about the other 3 ensembles. The 3 good ones will give a good estimate of the ICG parameters during that particular period. Simply reject the other two. In general: when in doubt, reject the complex altogether.

4 - physiological plausibility

If you have doubts on whether the dZ/dt signal is correct, or should be rejected, you might use the following physiological guidelines as an indication of where the B- and X-point should be in an ideal situation. This is hazardous for at least two reasons: first it stains the independency of the rating which should be based on morphology only ; second large individual differences in physiology exist and the general rules may not always apply.

HR: 40-60 → PEP: 100-140 → LVET 300-450

HR: 60-80 → PEP: 90-130 → LVET 250-400

HR: 80-100 → PEP: 80-120 → LVET 200-350

HR: 100-120 → PEP: 70-100 → LVET 200-300

HR: 120+ → PEP: < 80 → LVET 150-250

Again, if your signal shows B- and X-points outside of these ranges, this does not at all mean that your dZ/dt signals should be discarded. The above table is just a general rule of thumb.

NB: These guidelines are based on adult recordings.

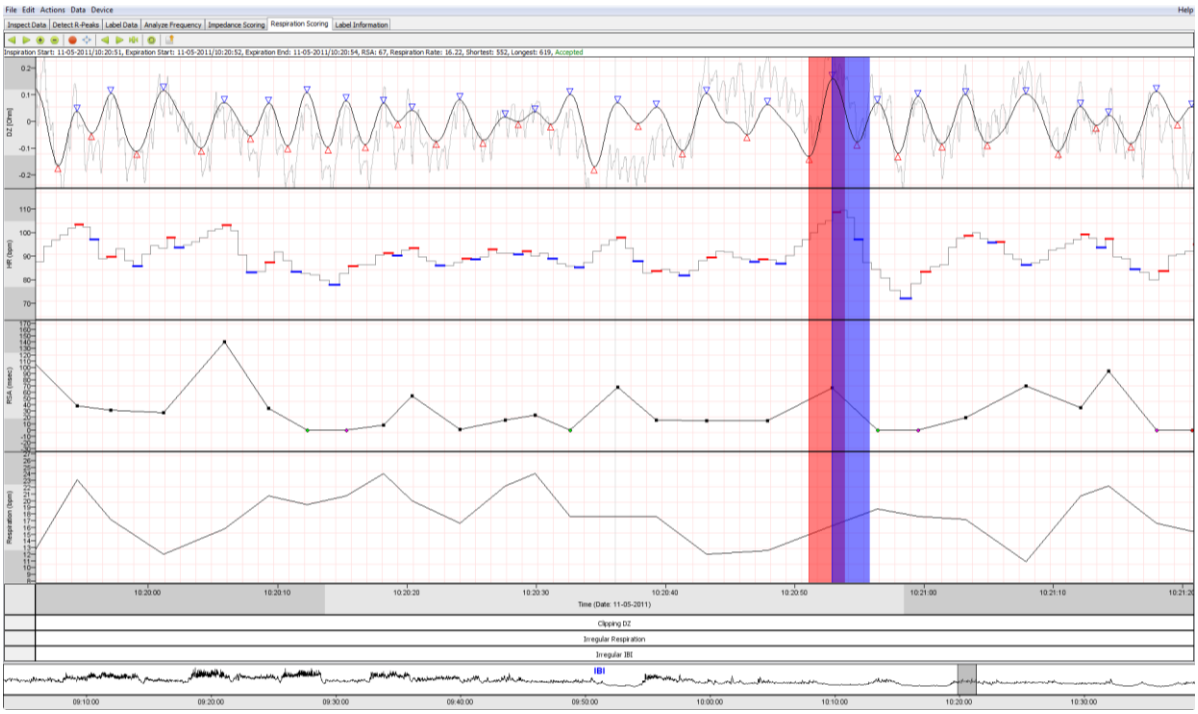
5 - Multiple Rater comparison

Reliability increases if two (or more) raters score the same data set independently. After interrater reliability is established, the various raters should ideally compare their deviant scoring to converge on a single solution, in view of the consistency principle. Mostly one will have picked a different B-point then the other(s). Averaging the B-point location is meaningless. Consensus has to be reached on the correct B-point location to satisfy the consistency criteria.

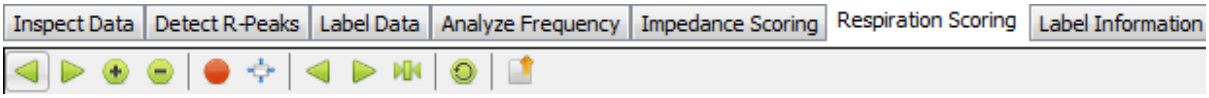
6 - Keep score of the quality of your rating

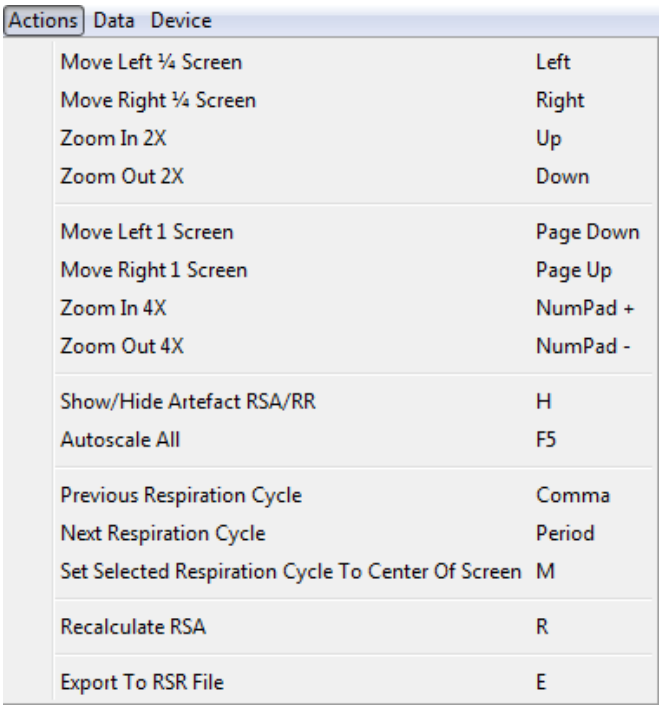
Sometimes, scoring is difficult and doubtful, at other times you feel pretty sure. After scoring you might want to generate three parameters for "scoring-quality". Make separate judgments for B-point scoring, X-point scoring and general signal quality on a scale from 0 (yuk!) to 10 (excellent!). Later on, during statistical analysis, request to see the mean of all parameters as a function of your quality rating.

2.6 Respiration / RSA scoring



All actions for the *Respiration Scoring* tab are presented in the form of buttons:



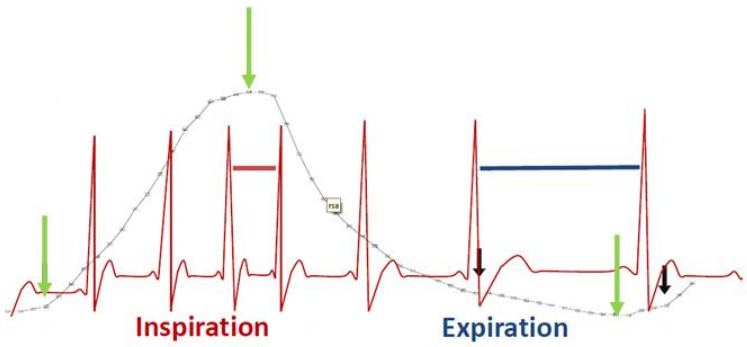


The function of these buttons should be self-explanatory.

2.6.1 RSA explained

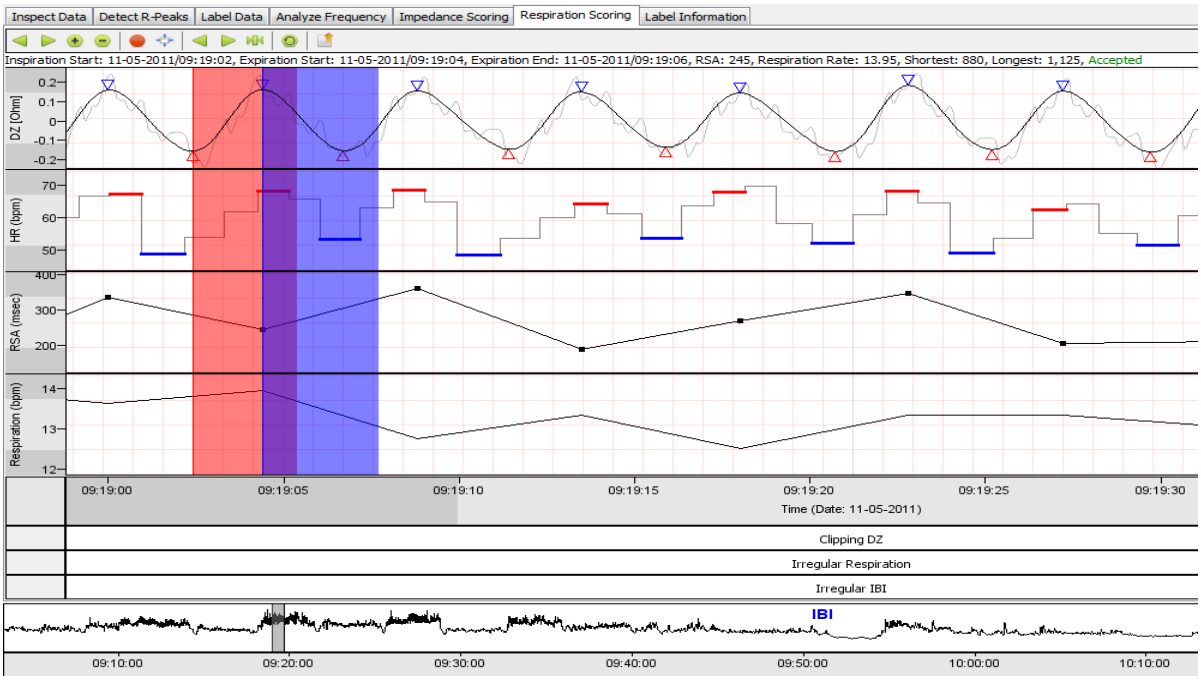
Respiratory Sinus Arrhythmia (RSA) scoring by the DAMS program is based on the peak-valley method (Grossman, van Beek, & Wientjes, 1990 ; de Geus et al., 1995) that uses the IBI time series extracted from the ECG together with the respiration signal obtained from filtered (0.1 – 0.4 Hz) dZ signal to obtain heart period variability that is associated with respiration. This heart period variability is referred to as RSA. The DAMS program contains an automatic scoring algorithm for detecting the beginning and end of inspiratory and expiratory phases in each respiratory cycle. Inspiratory and expiratory phases include the inspiratory and expiratory pauses which are not detected separately.

For each respiratory cycle the total cycle time between begin of inspiration and end of expiration is extrapolated to a per-minute respiration rate (RR). In addition, RSA is computed per respiratory cycle from two IBIs: The shortest IBI during an interval starting at the begin of inspiration and ending 1000 msec (default) after the end of inspiration and the longest IBI during an interval starting at the begining of expiration and ending 1000 msec (default) delay after the end of expiration. RSA is calculated by the subtraction of the shortest IBI from the longest IBI, provided that the shortest IBI (highest HR) is part of an accelerating series within the inspiratory interval and the longest IBI (lowest HR) of a decelerating series within the expiratory interval. This is illustrated in the figure below.



If either the decelerating longest or accelerating shortest IBI is missing for a breath cycle, or a negative RSA value is obtained on subtraction, we set RSA in these breaths as missing. Under the *Label Information* tab two different mean RSA variables are calculated: the mean RSA across all breaths in the label with a valid RSA only, and the "RSA-zero" in which the RSA value is set to be zero for breaths with an invalid RSA. The DAMS labels these variables in the results files as RSA and RSA0 respectively.

2.6.2 Visual inspection and manual correction



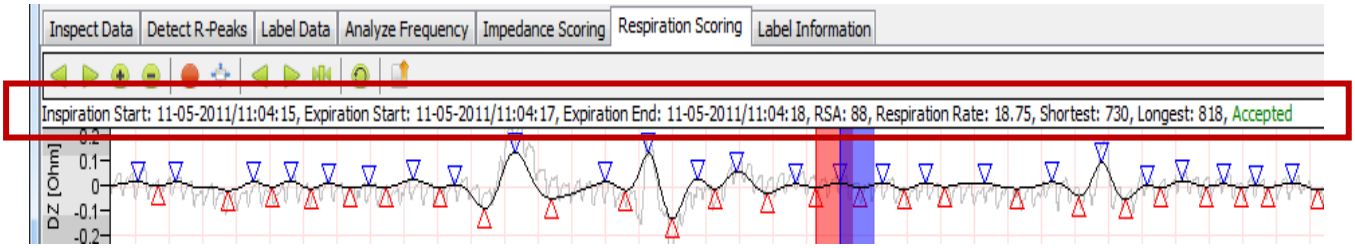
The windows of the *Respiration Scoring* tab shows 2 physiological and 2 derived signals for the time period indicated at the X-axis of the graph:

The raw impedance signal (dZ in Ohm, grey) with the **filtered impedance signal** representing respiratory thorax movement plotted on top of it. For each respiratory cycle, red triangles indicate the start of the inspiration and blue triangles mark the beginning of expiration. The currently selected respiratory cycle is indicated by the combination of a red, purple and blue box. The purple box reflects the overlap of the inspiration phase which is extended by a dZ-HR shift (1000 msec) with the expiration phase. You can activate/de-activate the raw impedance signal in the settings screen. This is accessed from the main menu by selecting *Edit* → *Settings* → *Respiration Scoring*.

CardioTachogram: A beat-per-beat estimate for the Heart Rate (in beats/min, grey with red and blue marks). On this graph the highest heart rate (shortest IBI) during inspiration, provided that it is part of an accelerating IBI series, is indicated by a fat red mark. The lowest heart rate (longest IBI) during expiration, provided it was part of a decelerating IBI series, is indicated by a fat blue mark

The time series of RSA values across the consecutive breaths (in msec).

Respiration Rate (extrapolated from the respiratory cycle time) across the consecutive breaths (in breaths per minute).



When selecting a single breath you will see the following information per breath on top of the upper window: Inspiration start, expiration start, expiration end, RSA (in msec), respiration rate (in breath/pm), shortest IBI (highest heart rate) during inspiration on decelerating slope (msec), Longest IBI (lowest heart rate) during expiration on a accelerating slope (msec) and weather the breath is accepted or rejected. Rejection codes signal one of the following reasons why RSA was not accepted:

RSA : -1 undetectable 'shortest IBI'

RSA : -2 undetectable 'longest IBI'

RSA : -3 both 'longest IBI' and 'shortest IBI' were undetectable

RSA : -4 'longest IBI' is shorter than the 'shortest IBI'

RSA : -5 'Irregular IBI detected'

RSA : -6 'Irregular respiration rate'

RSA : -7 'Clipping dZ'

Fortunately, automatic scoring of the respiration signal works quite well in most subjects. Mostly, it will suffice to just load the *.amsdata* file into DAMS and browse through the signal after having set the time axis at a low temporal resolution (e.g. ten minutes per screen). While browsing through the respiration signal from the beginning to the end of the file check the following:

Did the program mark more than 10% of the recording as artifact in either the "clipping dZ", "Irregular respiration" or "Irregular IBI" bars (pink, blue and yellow markers respectively) at the bottom of the screen? If so the parameters of the scoring algorithm may need to be changed (see below).

Do all inspirations and expirations appear to be appropriately scored in the upper respiration signal (indicated by blue and red triangles)? If erroneous breaths are scored did the program mark them as artefacts in the bar at the bottom of the screen labeled "Irregular respiration"? If this is not the case you can manually delete a fragment of the signal by clicking and dragging the mouse in this window. NOTE: Pay special attention to the breath cycles measured during the night. Some subjects show strong abdominal breathing which seriously affects detection of the respiration signal by thorax impedance. This can often be repaired by 'rescoring the cycles' (under the main menu item *Edit* → *Settings* → *Respiration Scoring*) and changing the 'Relative Threshold' parameter of the scoring algorithm (see below).

Check whether the program has rejected all deviant IBIs (spikes) without removing IBIs that reflect large but true heart rate variability. The difference between a spike and a truly high heart rate variability is rapidly gleaned from the shape of the tachocardiogram. If there is a staircase pattern rather than a sudden single-beat change, the subject may have a generally high heart rate variability which can be verified in the RSA window (e.g. RSA > 200 msec). If there is a sudden single-beat drop or jump then there is a spike. Spikes often represent extrasystolic beats or very delayed beats (which often occur jointly). Note that these beats do not represent an error in judgment of the R-wave

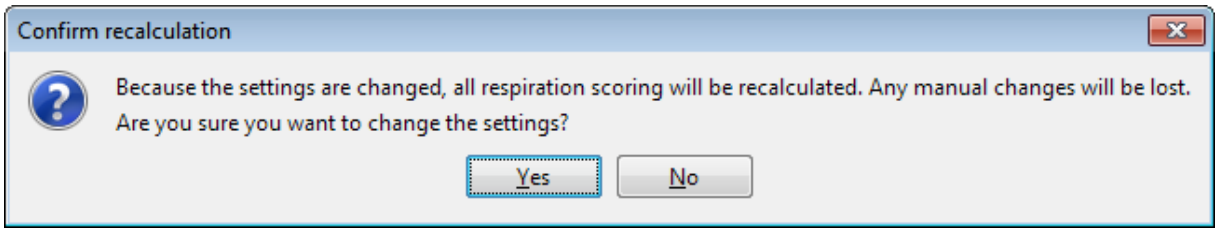
detection algorithm (which should have been dealt with earlier during R-peak detection and correction). They do result in IBIs that are twice the length or half the length of most of the other IBIs. This can inflate the RSA value for the breaths in which they occur very strongly, and it is advised to remove these. Hence, make sure all spikes are marked as artefacts in the bar at the bottom of the screen labeled "Irregular IBI"? If this is not the case you can manually delete a fragment of the signal by clicking and dragging the mouse in this window.

Finally, check whether the per breath estimate of the respiration rate (in the Respiration window) takes on expected values between 7-14 at night, 12-22 across most daily activities except moderate to high physical activity where respiration rate can increase to 30 breath per minute.

2.6.3 Adjustment in respiration and RSA scoring

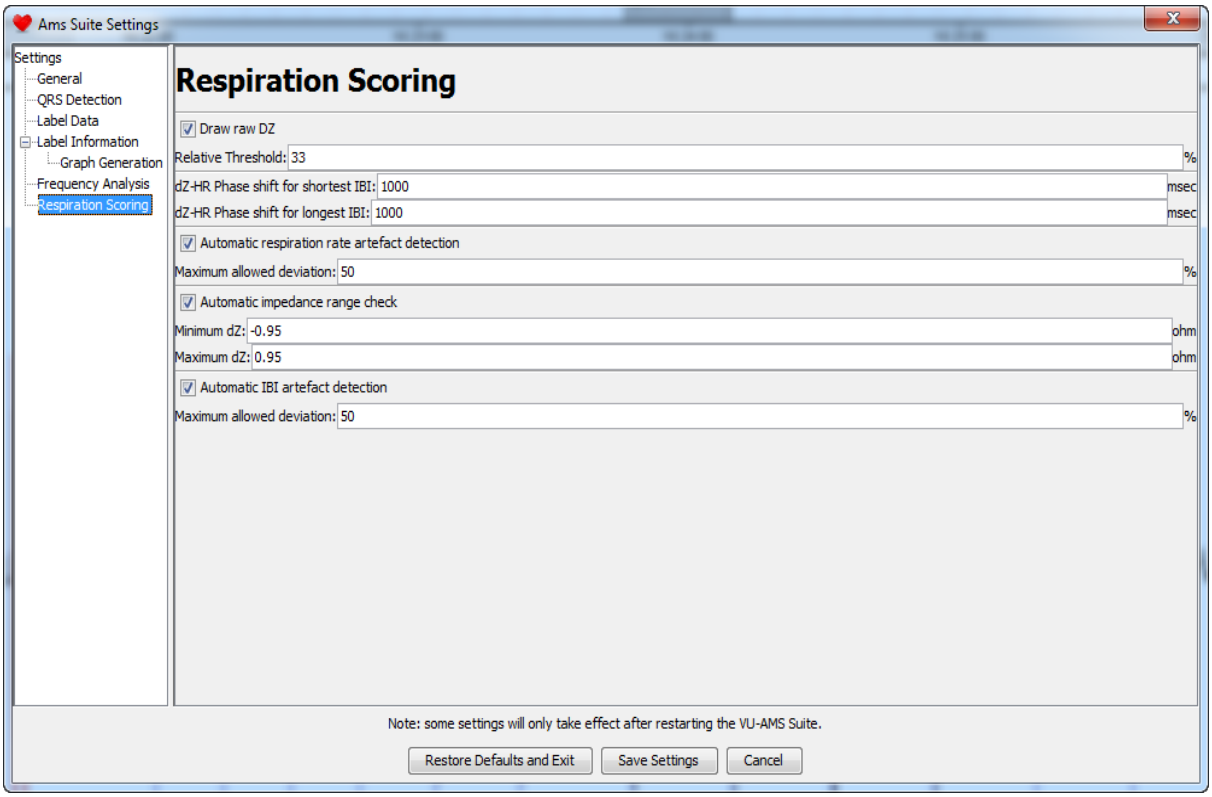
The DAMS program filters the raw thorax impedance change (dZ) signal to obtain the respiration signal and then detects the beginning and the end of inspiration and expiration in the entire registration using both amplitude and frequency modulation. The RSA scoring algorithm first uses three artefact detection algorithms: it will check for dZ clipping, irregular respiration rates (based on a maximal percentage for deviation of the duration of consecutive breaths) and irregular IBIs (based on a maximal percentage for deviation of the duration of consecutive beats). Parameters governing these artifact detections can be modified in the main menu by selecting *Edit* → *Settings* → *Respiration scoring.*

NOTE CAREFULLY: After changing the settings, the respiration signal and RSA are recalculated on the original signal. This means that manually rejected fragments will be restored, so first make sure you have the chosen the optimal settings before manually rejecting fragments of the recording. It will warn you before recalculation.



Relative threshold [0…1] : 0.33 (default)

Purpose: The purpose of this parameter is to alter the sensitivity for the detection of breaths. A breath is defined by two zero crossings (a peak and a valley) in the first derivative of the filtered impedance signal that are separated by a minimum amplitude. The *Respiration Scoring* tab calculates a running average over the 20 seconds preceding the current selected breath cycle of the difference in amplitude at peaks and valleys in the dZ signal. The relative threshold defines the percentage of this average that is used as the minimum amplitude for the 'tidal volume' in a respiratory cycle.



Adjustment: A higher threshold decreases the sensitivity (less of the consecutive peak-valley pairs in the filtered impedance will be counted as true breaths – use when too many small wobbles are counted as breaths), and a lower threshold value increases the sensitivity for amplitude differences (more of the consecutive peak/valley pairs will be counted as true breaths – use when the amplitude of the actual breaths becomes low, for instance in nighttime belly breathing).

dZ-HR Phase shift (in msec) :1000 (default)

Purpose: This defines the delay added to the inspiratory and expiratory phases in which the *Respiration Scoring* tab is allowed to search in the IBI series for a shortest IBI in inspiration or longest IBI in expiration, respectively. Increasing the dZ-HR Phase shift can increase the number of valid RSA values in subjects with low respiration rates whereas at high respiration rates, the default 1000 msec interval may lead to erroneously used IBIs from the next respiratory cycle.

Adjustment: Increasing the phase-shift increases the time-delay.

Automatic respiration rate artefact detection : is "on" when ticked

Purpose: The purpose of this option is to automatically reject breaths with an unusually small or unusually high respiration rate as compared to the running average of the 20 preceding breaths. For participants with irregular breathing, the maximum allowed deviation might need to be increased in order to prevent false rejects.

Adjustment: The 'maximum allowed deviation' (default 50%) enables the scorer to specify how much the respiration rate of a breath needs to deviate from the running average in order to be excluded by the automatic scoring program. Increasing the percentage means allowing for larger deviations from the running average.

Clipping dZ : is 'on' when ticked.

Purpose: The purpose of this option is to automatically reject clipping (i.e. where the raw respiration signal turns into a flat line at dZ = 1 ohm or dZ = –1 ohm).

Adjustment: Although the default values generally seem to work well, some recordings may require an automatic impedance range check that is a fraction more or a fraction less strict. It can be a waste to reject breaths that are not deviant from the normal respiration rate nor show any other deviant features, just because the signal clips for a short moment. Therefore, the option of 'minimal duration' (default 2000 msec) is added so clipping is only rejected when it occurs for the time specified by the scorer or longer.

Irregular IBI check : is 'on' when ticked.

Purpose: The purpose of this option is to automatically reject spikes in the IBI time series, that represent extrasystolic beats or very prolonged beats. Note that these beats do not represent an error in R-peak placement by the DAMS program (which should have been dealt with earlier during manual inspection and correction). They do result in IBIs that are twice the length or half the length of most of the other IBIs. This inflates can inflate the RSA value for the breaths in which they occur very strongly. When the irregular IBI check is 'on' these beats are removed from the set of IBIs that are considered when selecting the shortest IBI and longest IBI to compute the RSA.

Adjustment: The allowed magnitude of the difference between consecutive IBIs can be adjusted by changing the 'maximum allowed deviation' (default 50%).

2.6.4. Exporting raw breath to breath data

You have the option to export breath to breath results to a tab delimited text output file using the button "Export To RSR File". These *.rsr* files give the following information on each respiratory cycle on a single line:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1105016	16	11-05-2011	09:04:13	1800	1800	754	755	16.67	1	768.5	-34.16	114.64	148.81	A	10
1105016	17	11-05-2011	09:04:16	1700	1200	748	-1	20.69	-2	751.67	-99.87	-1.6	98.27	A	10
1105016	18	11-05-2011	09:04:19	2000	3200	716	812	11.54	96	742.17	-35.45	149.61	185.06	A	10
1105016	19	11-05-2011	09:04:24	1900	1200	695	-1	19.35	-2	737.33	-137.62	56.78	194.4	A	10
1105016	20	11-05-2011	09:04:27	2600	1900	683	748	13.33	65	717.2	19.24	74.63	55.39	A	10

column 1 : Subject ID

column 2 : Respiratory cycle number

column 3 : Date (dd-mm-yy)

column 4 : Start of respiratory cycle (hh:mm:ss)

column 5 : Inspiration time [msec]

column 6 : Expiration time [msec]

column 7 : Shortest accelerating IBI in inspiration [msec]

column 8 : Longest decelerating IBI in expiration [msec]

column 9: RR [breath per min]

column 10: RSA [msec]

column 11: Mean IBI across the cycle [msec]

column 12: Amplitude dZ at start inspiration [milliOhm/sec]

column 13: Amplitude dZ at start expiration [milliOhm/sec]

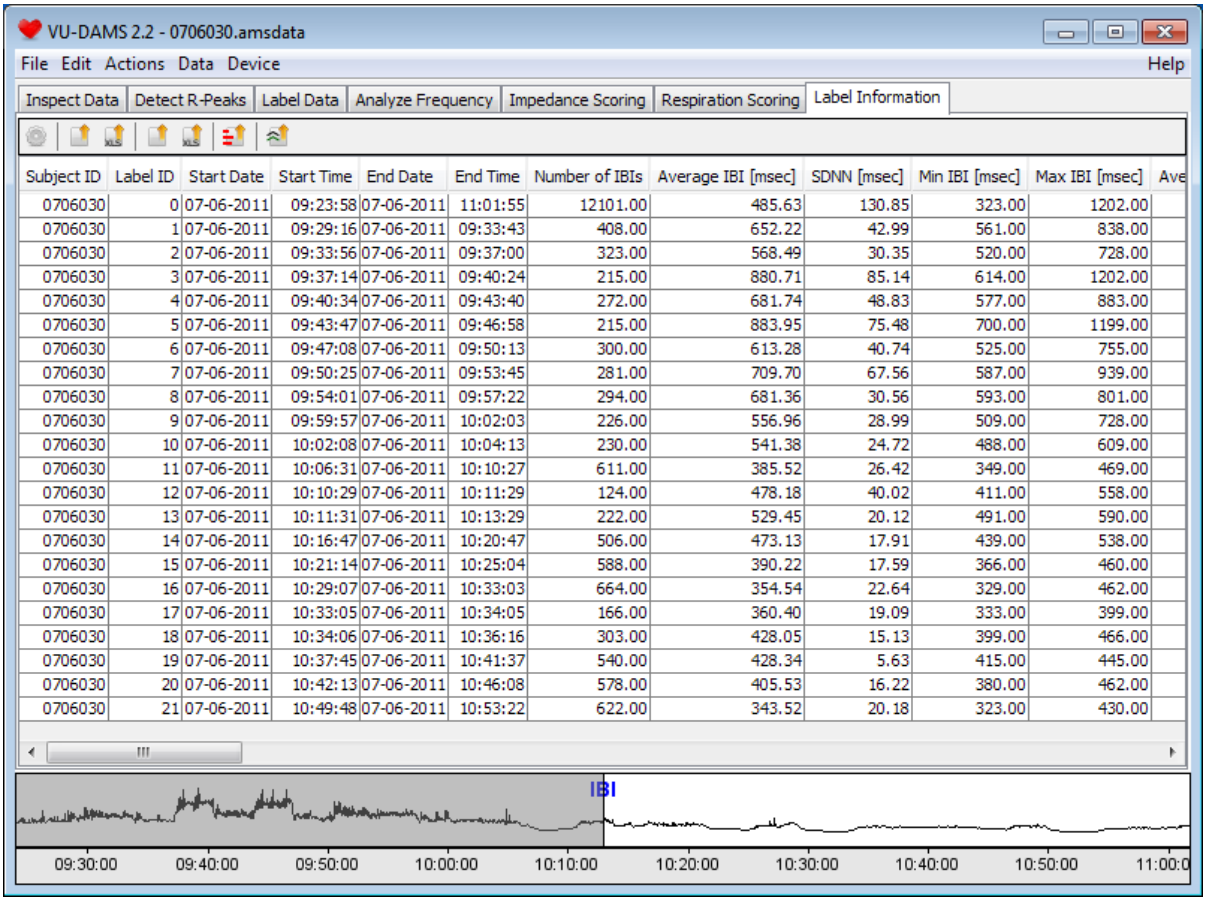
column 14: Tidal volume [milliOhm/sec] -- calibration is needed to translate this to ml

column 15: Rejected (R) as artefact or accepted (A)

column 16+: Labels (-9999 = not available)

You can import this text file in e.g. SPSS for more fine grained analyses that use breath-to-breath information rather than the averaged values per label that are typically produced under the *Label information* tab. Please note that amplitude is in milliOhms/sec and needs calibration before volumes have physiological meaning. For respiratory time intervals careful outlier detection is needed before you do further statistical analysis on these breath-to-breath data. In view of the huge number of breath cycles to be quality controlled in 24-hour ambulatory monitoring, some automation of these checks is desirable, for instance by scripting in MATLAB, R or even SPSS.

2.7 Label Information/ Exporting results

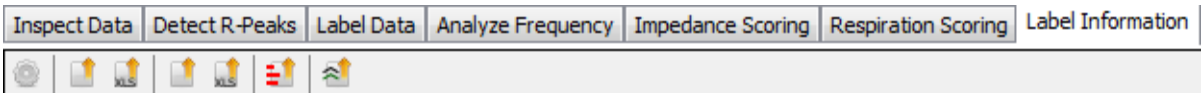


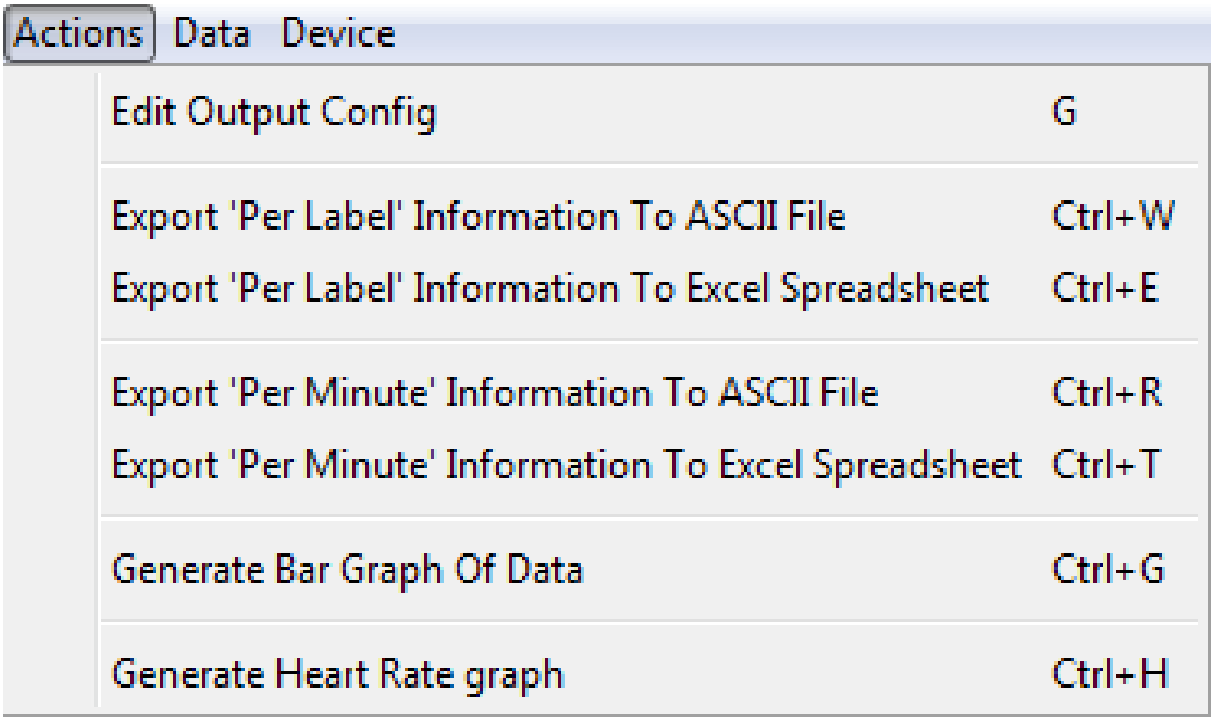
Subject ID	Label ID	Start Date	Start Time	End Date	End Time	Number of IBIs	Average IBI [msec]	SDNN [msec]	Min IBI [msec]	Max IBI [msec]	Ave
0706030	0	07-06-2011	09:23:58	07-06-2011	11:01:55	12101.00	485.63	130.85	323.00	1202.00	
0706030	1	07-06-2011	09:29:16	07-06-2011	09:33:43	408.00	652.22	42.99	561.00	838.00	
0706030	2	07-06-2011	09:33:56	07-06-2011	09:37:00	323.00	568.49	30.35	520.00	728.00	
0706030	3	07-06-2011	09:37:14	07-06-2011	09:40:24	215.00	880.71	85.14	614.00	1202.00	
0706030	4	07-06-2011	09:40:34	07-06-2011	09:43:40	272.00	681.74	48.83	577.00	883.00	
0706030	5	07-06-2011	09:43:47	07-06-2011	09:46:58	215.00	883.95	75.48	700.00	1199.00	
0706030	6	07-06-2011	09:47:08	07-06-2011	09:50:13	300.00	613.28	40.74	525.00	755.00	
0706030	7	07-06-2011	09:50:25	07-06-2011	09:53:45	281.00	709.70	67.56	587.00	939.00	
0706030	8	07-06-2011	09:54:01	07-06-2011	09:57:22	294.00	681.36	30.56	593.00	801.00	
0706030	9	07-06-2011	09:59:57	07-06-2011	10:02:03	226.00	556.96	28.99	509.00	728.00	
0706030	10	07-06-2011	10:02:08	07-06-2011	10:04:13	230.00	541.38	24.72	488.00	609.00	
0706030	11	07-06-2011	10:06:31	07-06-2011	10:10:27	611.00	385.52	26.42	349.00	469.00	
0706030	12	07-06-2011	10:10:29	07-06-2011	10:11:29	124.00	478.18	40.02	411.00	558.00	
0706030	13	07-06-2011	10:11:31	07-06-2011	10:13:29	222.00	529.45	20.12	491.00	590.00	
0706030	14	07-06-2011	10:16:47	07-06-2011	10:20:47	506.00	473.13	17.91	439.00	538.00	
0706030	15	07-06-2011	10:21:14	07-06-2011	10:25:04	588.00	390.22	17.59	366.00	460.00	
0706030	16	07-06-2011	10:29:07	07-06-2011	10:33:03	664.00	354.54	22.64	329.00	462.00	
0706030	17	07-06-2011	10:33:05	07-06-2011	10:34:05	166.00	360.40	19.09	333.00	399.00	
0706030	18	07-06-2011	10:34:06	07-06-2011	10:36:16	303.00	428.05	15.13	399.00	466.00	
0706030	19	07-06-2011	10:37:45	07-06-2011	10:41:37	540.00	428.34	5.63	415.00	445.00	
0706030	20	07-06-2011	10:42:13	07-06-2011	10:46:08	578.00	405.53	16.22	380.00	462.00	
0706030	21	07-06-2011	10:49:48	07-06-2011	10:53:22	622.00	343.52	20.18	323.00	430.00	

After clicking on the *Label Information* tab wait until all cells are finished calculating. The first column in the *Label Information* tab is the subject name, which is the ID that we gave when programming the device for recording. Each row represents a single labeled experimental/ambulatory condition and

each consecutive labeled condition is rank-ordered by the *Label ID* field. The rest of the columns contain values for a large number of physiological variables. ICG-based variables only have a value when the scoring under the *Impedance Scoring* tab has been done (e.g. PEP) and the LF and HF power values from spectral analyses on the IBI time series are only present for labels with a minimum length of 4 minutes.

All actions for the Label Information tab are presented in the form of buttons:

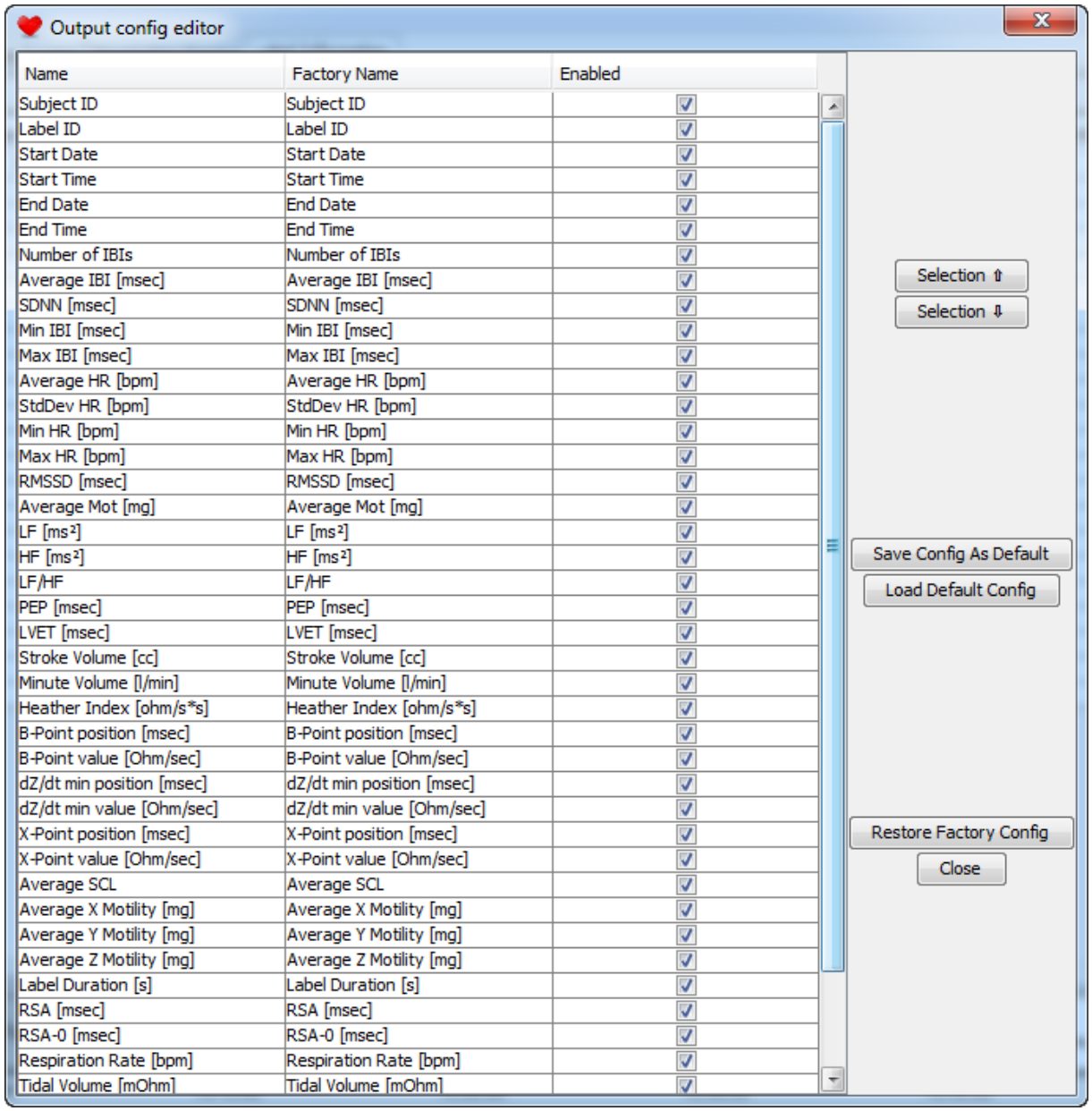




2.7.1 Output configuration editor

Click in the main menu → *Actions* → *Edit Output Config* to adjust variable names, order or omit names.

A menu with all variables will appear:



To change variable names click in the left column and type in the new name. The second column will show the factory configured name of that particular variable. To restore the output configuration to the factory configuration, click on *Restore Factory Config*.

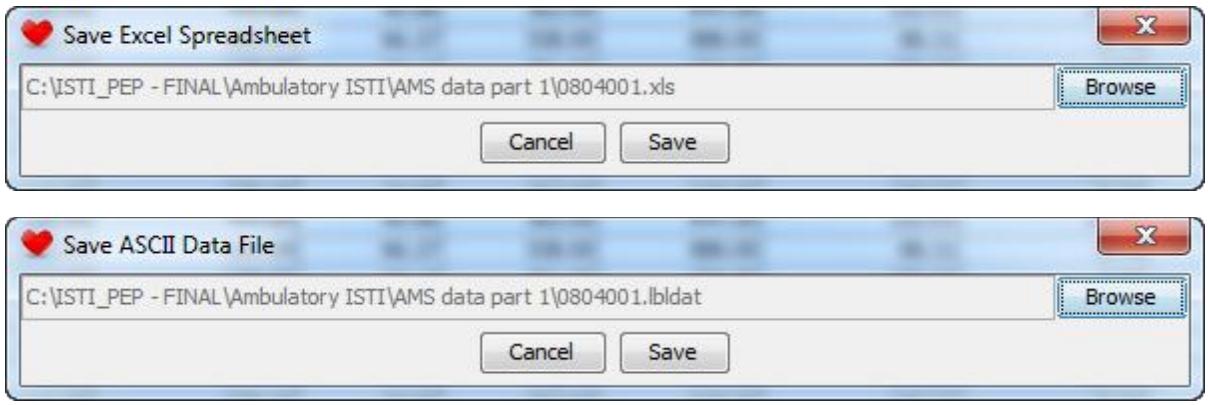
To omit variables simply uncheck the box behind the variable.

To change the order of variables in the output, use *Selection up* and *Selection down* buttons. To restore the output order to the factory configuration, click on *Restore Factory Config*.

After changing the output configuration, you can *Save Config As Default*. This output configuration will then be applied to all .amsdata files that are opened / processed with this DAMS version by this user on this particular computer. You can switch between factory configuration and default configuration by using *Load Default Config* and *Restore Factory Config.*

2.7.2 Export per label

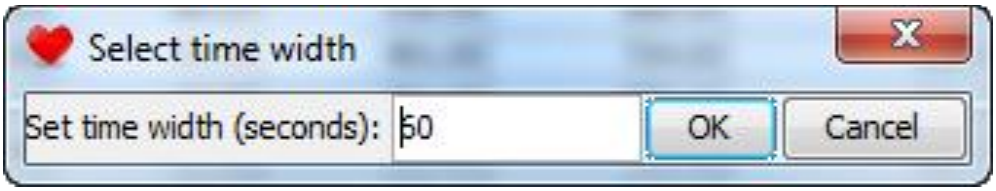
You can either export your data to an excel file (with the extension .xls) or a text file (with the extension .lbldat). Click on the export button in the menu and you will be prompted to enter the location to the output file.



***Be aware that there is a label_ID with the number **0** by default. This label is the average of the entire recording, and not the average of all the labels. If you do not want this in your output you can permanently make sure the program does not show the **label_ID** with the number **0** in the Label information tab. To do so select in the main Edit → Settings → Label information. Then deselect: Include label 0 for entire data recording and save the new settings.*

2.7.3 Export for fixed time based labels

By default the exported data will reflect the averaged values across the labels that were generated during *Labeling of your data*, using experimental condition or diary information to define labeled time periods. However there is also the option to export across fixed periods of time. Click on either the *Export 'Per Minute' Information to ASCII File* or the *Export 'Per Minute' Information to Excel Spreadsheet* button. You will be prompted to enter the duration of the fixed time periods. The output will give averages across consecutive periods of this length in the chronological order of recording. The start and stop times of the fixed time period 'labels' will help you link the generated data to the real time of the experiment.

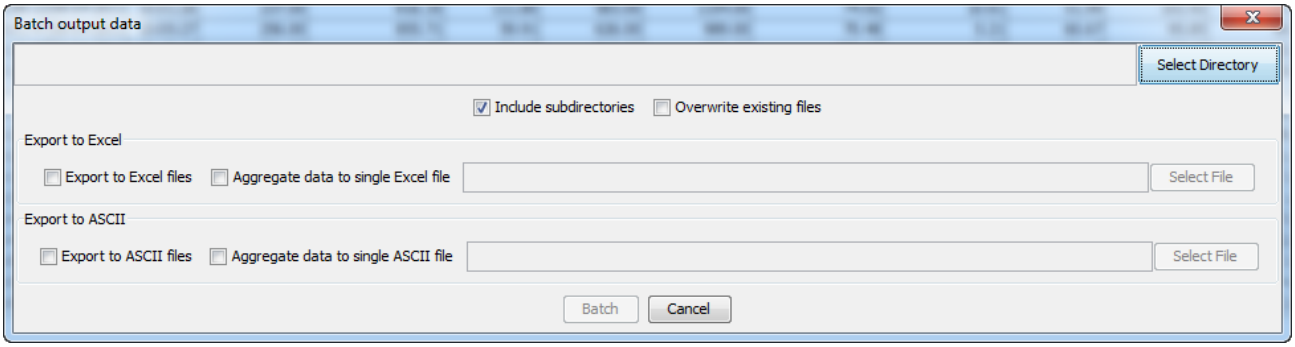


**Note 1: The PEP etc. will be set to missing (-9999 by default), as the ensemble averaged impedance complexes always need manual scoring before values for ICG-derived variables are generated. You need to use Add Time-Based Labels during labeling if you want to produce e.g. one-minute average ensemble PEPs.*

**Note 2: Spectral analysis will only output values for LF and HF labels if the fixed periods are chosen longer than 4 minutes.*

2.7.4 Batch export

You can export text files in batch mode. This function will generate output files per subject or a merged output file of many subjects from single .amsdata files as long as they are placed in the same directory (or a subdirectory in that directory). So place all files that need to be exported for a certain project in one directory and click on *Batch export data* under *File* in the menu bar.



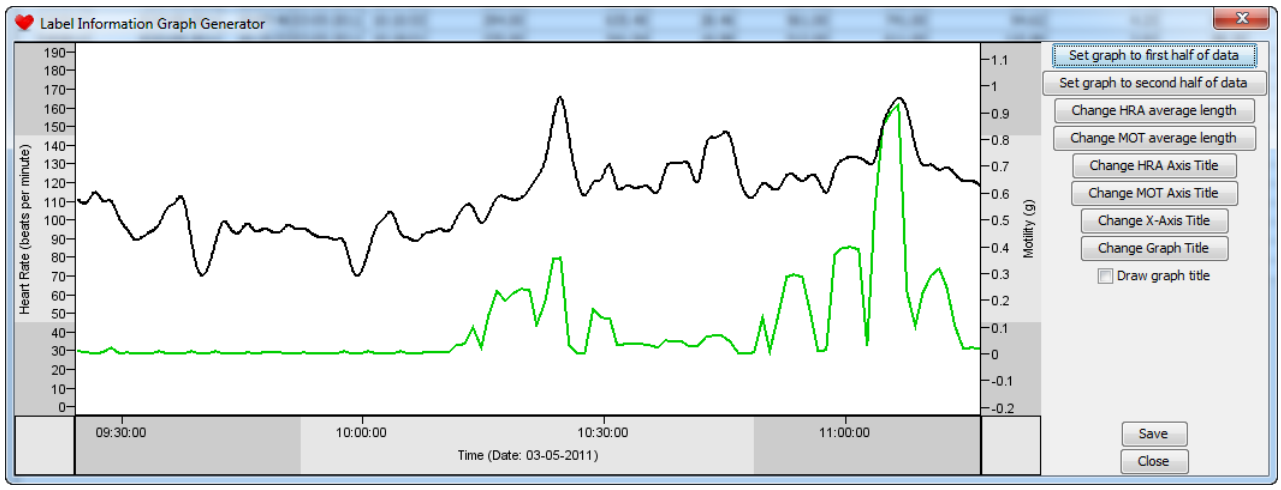
Simply select to export to XLS or to ASCII in single mode or merged mode. Then select the output directory and name the output file.

2.8 Generate reports for the participant

There is an option to generate feedback reports for participants in the forms of heart rate graphs and bar graphs.

2.8.1 Heart rate graph

Click on the *Generate Heart Rate graph* button. You will see the following screen.



The graph will; show the heart rate and motility signal of the entire recording. You can change names of the x-axis, y-axis of the HR and the y-axis of the motility as well as the graph title itself (or hide it by unchecking the *`Draw graph title`* box).

You can adjust the degree of smoothing of the signals by *Change HRA average length* or *Change MOT average length.* Increasing the length will give a more smooth signal.

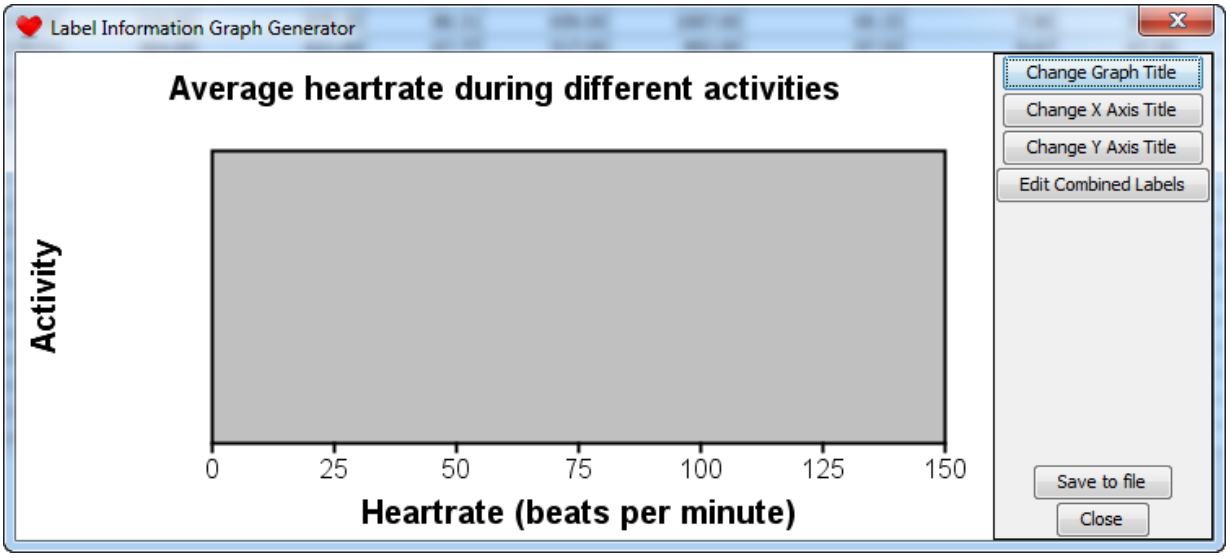
For long recordings (e.g. > 24H) you have the option to display the first or second half of the data by using the buttons of the heart rate graph menu.

When finished, you can save the graph to a .png file by clicking on *Save.*

2.8.2 Generate bar graph

With the heart rate graph it is possible to display the average heart rate across single activities or sets of combined activities. Select or combine various labels into a single bar.

Click on the *Generate Bar Graph Of Data* button. You will see the following screen:

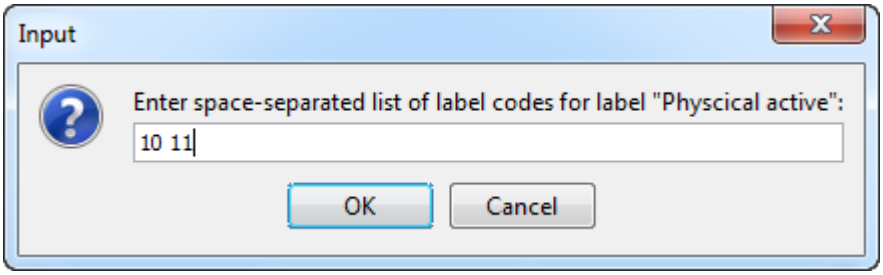


Now click on *Edit Combined Labels*, you will see the following screen:

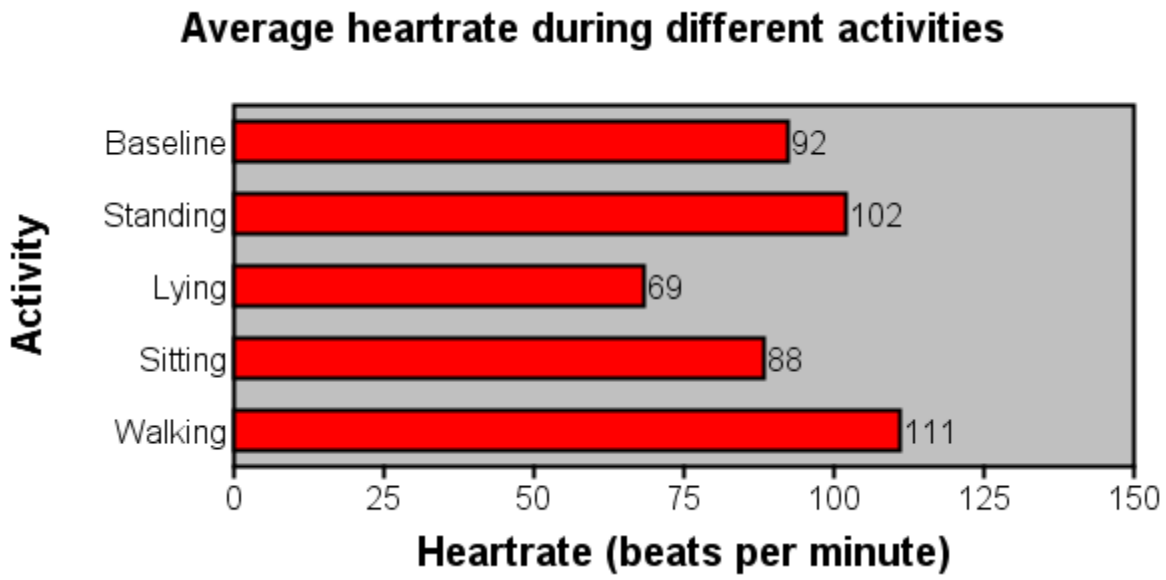
To add bars to the bar graph you need to define what activities should be averaged into a single bar. You can either choose to have a bar represent a single level of a labeling category for example Cycling, or you can combine multiple levels of a category, i.e. cycling and walking into one bar that represents 'physical active' periods.

To do this click on *Add New Label*, and enter the category name you want to give to this specific bar (e.g. physical active).

Click *OK*. Then enter the space separate list of level codes that need to be averaged for this bar. e.g. 10 for cycling and 11 for walking would be entered like this:



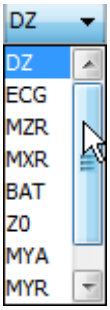
When you entered all desired bars, you can save the bar graph setting as default. You can change the name of the X-axis, Y-axis and Graph by clicking on the *Change … Title* buttons of the bar graph menu. The end result might look like this:



2.9 Export VU-AMS signals

A raw data dump can be obtained from each recorded signal into a text file.

Click on *Data* in the main menu and select to *Export Signal To ASCII*. You can choose to export any signal to an ASCII file and choose the resolution of the output.

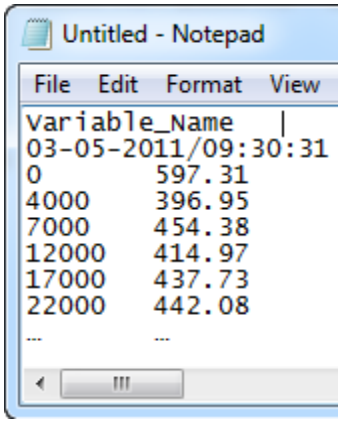


The file has a fixed format, that includes the raw signal but also the cumulative time index and the label codes. Each line represents a single sample from the raw data, with samples spaced in time by the specified resolution. NB: This can generate very large data files in 24-hour recordings!

2.10 Import external signals

Click on *Data* in the menu and select to (re)import a raw signal dump from a text file. This option allows you to use downsampled data or to import an entirely different signal (as long as the format complies with the DAMS raw data dump format).

Click on *Data* in the menu and select *Load external signal*. In the pop-up screen select the ASCII file containing the external signal. This ASCII file should be structured as follows:



Variable_Name → Name of the external signal

03-05-2011/09:30:31 → Start date and start time of the recording

0 597.31 → The first column is the time in msec (starting at zero) and the second

4000 396.95 column is the corresponding value of the external signal

7000 454.38

12000 414.97

17000 437.73

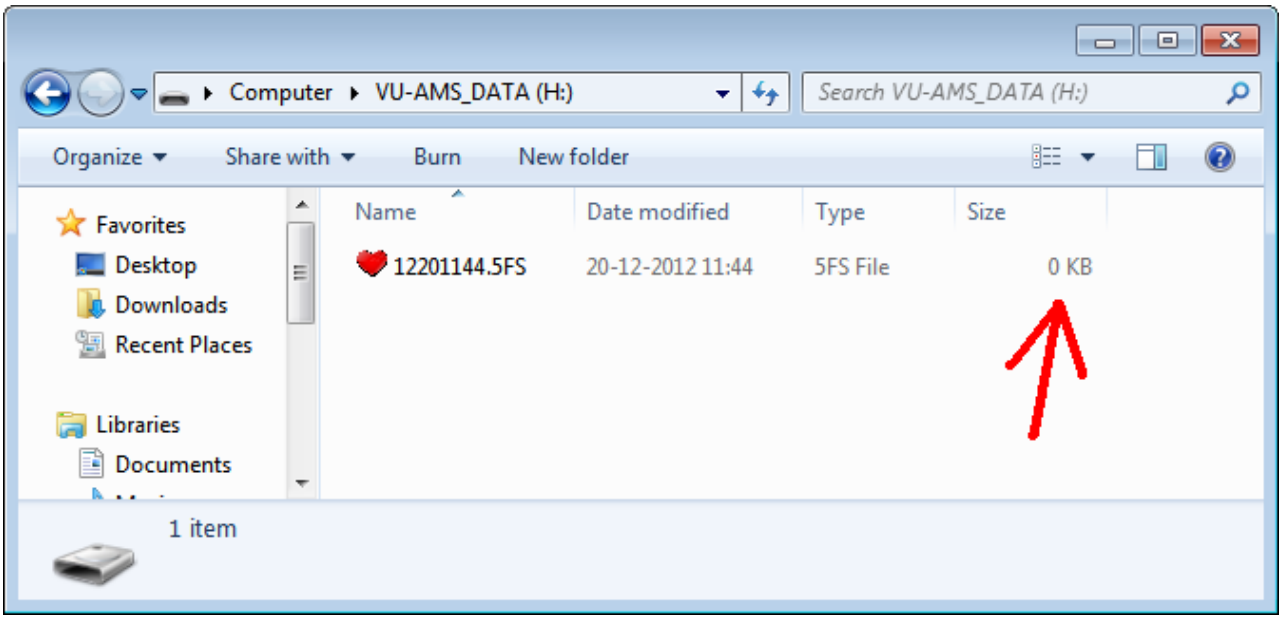
22000 442.08

… …

The external signal will appear above the whole IBI recording panel. The mean value of the external signal over each condition will appear in the Label Information tab under 'External Signal Average'. Click on *Data* in the menu and select *Clear external signal* to remove the external signal.

3. Trouble shooting

3.1 VU-AMS file has zero bytes.



Cause: the VU-AMS has made a recording and probably all data are there but an end-of-file summary has not been placed and the FAT table isn't updated.

Solution: This can be repaired by closely following the instructions on www.vu-ams.nl > Support> Tutorials > Troubleshooting > Video manual: How to repair a 0KB file recorded with the VU-AMS5fs.

3.2 Deviant flashing

When on standby, the VU-AMS device flashes once every 10 seconds, when recording the VU-AMS device flashes once every three seconds. Faster flashing signals problems:

The green light is flashing very rapidly.

Cause: The Compact Flash card is not (properly) installed.
Solution: Install the Compact Flash card in the proper way.

The green light is flashing rapidly.

Cause: The battery lid is not (properly) fastened.
Solution: Fasten the battery lid in the proper way.

3.3 Warning beeps

When the VU-AMS detects that something is amiss it can generate various warning beeps:

You hear a double beep (the 'alert beep'), which is repeated after increasingly shorter intervals (from 30 to 10 seconds).

Cause: The battery voltage is becoming low.
Solution: Replace the batteries with fresh ones.

You hear a triple beep (the 'warning beep').

Cause: An electrode comes off, a lead wire gets detached, or the lead wire connector is pulled out by accident.
Solution: No worries. Just attach the electrode again (use a spare one if necessary), reattach the lead wire, or plug the connector back into the socket.

3.3 Frequently asked questions.

http:/forum.vu-ams.nl

APPENDIX II

Protocol text for the VU-AMS instruction video published by JoVE :

René van Lien, Aimée E. van Dijk, Manon van Eijsden, Reinoud J. B. J. Gemke, Tanja G. M. Vrijkotte, and Eco J.C. de Geus

Measuring Cardiac Autonomic Nervous System (ANS) Activity in Children. Journal of Visualized. Experiments. (74), e50073, doi:10.3791/50073 (2013).

jove Journal of Visualized Experiments www.jove.com

Video Article

Measuring Cardiac Autonomic Nervous System (ANS) Activity in Children

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URL: http://www.jove.com/video/50073
DOI: doi:10.3791/50073

Keywords: Medicine, Issue 74, Neurobiology, Neuroscience, Anatomy, Physiology, Pediatrics, Cardiology, Heart, Central Nervous System, stress (psychological effects, human), effects of stress (psychological, human), sympathetic nervous system, parasympathetic nervous system, autonomic nervous system, ANS, childhood, ambulatory monitoring system, electrocardiogram, ECG, clinical techniques

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Abstract

The autonomic nervous system (ANS) controls mainly automatic bodily functions that are engaged in homeostasis, like heart rate, digestion, respiratory rate, salivation, perspiration and renal function. The ANS has two main branches: the sympathetic nervous system, preparing the human body for action in times of danger and stress, and the parasympathetic nervous system, which regulates the resting state of the body.

ANS activity can be measured invasively, for instance by radiotracer techniques or microelectrode recording from superficial nerves, or it can be measured non-invasively by using changes in an organ's response as a proxy for changes in ANS activity, for instance of the sweat glands or the heart. Invasive measurements have the highest validity but are very poorly feasible in large scale samples where non-invasive measures are the preferred approach. Autonomic effects on the heart can be reliably quantified by the recording of the electrocardiogram (ECG) in combination with the impedance cardiogram (ICG), which reflects the changes in thorax impedance in response to respiration and the ejection of blood from the ventricle into the aorta. From the respiration and ECG signals, respiratory sinus arrhythmia can be extracted as a measure of cardiac parasympathetic control. From the ECG and the left ventricular ejection signals, the preejection period can be extracted as a measure of cardiac sympathetic control. ECG and ICG recording is mostly done in laboratory settings. However, having the subjects report to a laboratory greatly reduces ecological validity, is not always doable in large scale epidemiological studies, and can be intimidating for young children. An ambulatory device for ECG and ICG simultaneously resolves these three problems.

Here, we present a study design for a minimally invasive and rapid assessment of cardiac autonomic control in children, using a validated ambulatory device [1-5], the VU University Ambulatory Monitoring System (VU-AMS, Amsterdam, the Netherlands, www.vu-ams.nl).

Video Link

The video component of this article can be found at http://www.jove.com/video/50073/

Protocol

1. Preparation: Starting Up

1. You need:
   - a VU-AMS5fs ambulatory recording device (including an infrared interface cable that either connects to the RS232 serial port of a PC or to a USB port).
   - 7 electrodes (we used ConMed 1690-003).
   - 2 charged AA-batteries.
   - an empty CompactFlash memory card (the VU-AMS5fs has been extensively tested with the 1GB 80x CF card from Transcend (TS1GCF80), but other CF cards should work too).
   - a laptop or PC with flash card reader and the Data Analysis Management Software (DAMS) suite installed.
   - a stopwatch.
   - music player with children's stories and headphones and a small self-inflatable air mattress are optional.

2. Check the time and date settings on the laptop/PC, since these will be recorded as metadata on your files. Put the empty memory card and full batteries in the VU-AMS device (successful placement is signalled by a triple beep). When the device is on standby, the green light will flash twice every ten seconds. This indicates it is ready, but not recording. Now connect the device to the laptop using the provided cable and start up the DAMS program. Initiate communication with the device (select the tab 'device' and choose the appropriate connection mode, infrared cable or bluetooth).
3. Have the participant take off his/her upper body wear. In the places where the electrodes will be placed,clean the skin with alcohol-wipes, and place the seven electrodes on the chest and back (**Figure 1**). Then attach the lead wires following the color scheme, and connect them to the device.
4. Check the battery type and battery voltage indication (this should be about 3.4 V for alkaline and about 2.4 V for rechargeable NiMH batteries). Fill out the identification field. The typical sampling frequencies are as shown in the figure (**Figure 2**).
5. Measure the distance between the two chest electrodes in millimeters, and fill this out in the field 'ICG-V distance'. Then click 'send settings' to send the current settings/ID to the device.
6. Now, the 'Online' option of the program should be used to display the ECG, ΔZ (this is the respiration) and dZ/dt (this is the ICG). The ΔZ signal reflects the base impedance across the thorax, which after appropriate filtering can be used to extract the respiration signal with high fidelity [7]. The dZ/dt signal is the ΔZ differentiated over time and reflects rapid changes in ΔZ linked to the ejection of blood from the ventricle into the aorta.
    1. A clear QRST-complex should be detectable in the ECG. The R-wave should be upward and it should be the peak with the largest (absolute) amplitude in either direction.
    2. The ΔZ should be within -0.5 and +0.5 Ω most of the time and dZ/dt between -1 and +1 Ω/sec.
    3. Z_0 should always stay within an 8 to 20 Ω range. This variation reflects the fact that the thorax impedance signal depends on the distance between the measuring electrodes which is a function of the child's height, and the 'wetness' of the thorax column enclosed by the measuring electrodes, differences in body composition (*e.g.* BMI) can affect the amplitude of the dZ/dt signal (fat mass containing less water than muscle). Individual differences in absolute Z_0 amplitude are also reflected in the ΔZ signal but this does not affect the determination of systolic time intervals, which are amplitude-independent.
    4. The ΔZ signal should reflect deep breathing of the subject clearly (instruct the child to take a slow deep breath and exhale slowly).
    5. In the ICG the typical upward waveform reflecting the cardiac ejection phase should be clearly detectable. Light movement of the subject should not distort the dZ/dt signal. If these criteria are not met, re-clean the skin and re-attach the electrodes until satisfactory signals are obtained.
7. When good signals are attained, start data recording by pressing the 'start' button. You will hear a beep acknowledging the start of the recording and the green light will start flashing once every three seconds. The registration has now started. Close the VU-DAMS program. You can now disconnect the device from the interface cable.

2. The Registration Period

1. Once the registration has started, ask the child to lie down for the first experimental condition. When the child has been in the supine position (without head-up tilt) for two minutes, you shortly (< 2 s) press the small black button on top of the device. Pressing this button marks a special event, and will later on help you identify the start of this condition in your data.
2. After four minutes, press the event button again. This signals the end of the lying down condition. Now have the child sit up and repeat the procedure for this second condition. Press the button, wait four minutes and press the button again. The children are instructed to rest quietly during these conditions.
3. To stop the measurement, press and hold down the button for at least 3 sec. The light will flash every 10 sec to indicate it has stopped and is in 'stand by' mode. Once the device has stopped, you may disconnect the lead wire plug from the connector and the lead wires from the electrodes.
4. Remove the batteries and flash card form the VU-AMS device and place the flash card in the reader unit. Move the acquired files to a designated directory (typically the name of the directory will be identical to the subject identifier used in the identification field).

3. Processing the Data

1. Upon opening the data with VU-DAMS program, the data will be automatically converted from raw data format (extension *.5fs)* to a new format (extension *.amsdata)*. This is the data file that VU-DAMS will be using in the ensuing steps.
2. First extract the Inter Beat Interval time series from the ECG signal. Select the *Detect R-peaks* tab. An automated algorithm will detect all R-peaks in the ECG signal and select (if present) periods with very low ECG quality for removal. In the upper left hand corner the number of Blue (correct), Yellow (medium suspicious) or RED (highly suspicious) is indicated. By pressing '.' (DOT) the cursor is moved to the next suspicious R-peak and the user can delete or add markers for R-waves by hand. It is recommended that at least all highly suspicious beats are visually inspected.
3. The main aim is to obtain a mean value for the heart rate, the preejection period (PEP) and measures of respiratory sinus arrhythmia (RSA, HF, RMSSD) across the experimental conditions used. Therefore proceed by indicating which periods in the raw data correspond to these conditions. This process is called 'data labeling'. Select the *Label Data* tab. Two panels show the heart rate signal and movement signal respectively, as well as the actual time of the recording.
4. Place the mouse cursor in the top bar where it says "click and drag to add labels" at around the start time of your first condition and drag the mouse to the end time of that condition. These times are either obtained from a written record of start and stop times (that you noted down during data collection) or you can use the start and stop markers obtained from pressing the button at the start and end of each condition, which are the vertical lines running across the HR and movement graphs.
5. Each label can be given a (unique) identifier to signal a particular condition. In our case we have only one category for our labels: experimental condition. This category has two values: lying down and sitting up.
6. VU-DAMS needs to be made aware of the experimental design by a so-called label configuration file (label.cfg). This is an ASCII file that can be opened with most text editors and, for example, looks like this:

exp_condition

10 lying down

11 sitting up

7. By placing the label.cfg file in the directory of the .amsdata files, it will be automatically loaded by the VU-DAMS program. Once a label has been made, a pop up screen will appear with the categories/values listed in the label.cfg file. Select 'lying down' for the first label and 'sitting up' for the second label.
8. After labeling, select the 'Impedance scoring' tab to score the PEP in the impedance cardiogram. For each of the conditions an ensemble averaged dZ/dt waveform is displayed, time-locked to the ECG R-peak. An ensemble averaged ECG is presented below the dZ/dt waveform. Place the four vertical cursors in the correct positions: ECG Q-wave onset (start of electrical activity), ICG B-point (start of the ejection phase), ICG dZ/dt-min (maximal ejection speed), and ICG X-point (aortic valve closure - end of ejection phase).
9. Next, select the 'Respiration Scoring' tab to score the peak-valley RSA using the respiratory and ECG signals. Automated breath-to-breath scoring of the respiratory interval and the shortest interbeat interval during inspiration and the longest interbeat interval during expiration can now be inspected. Typically the automated detection algorithm should not classify more than 15% of the breaths as deviant - otherwise inspect the respiration signal and tune the parameters of the detection algorithm as needed.

Guidelines for visual inspection of the ECG, ICG and respiration signals and interactive PEP and RSA scoring can be found on the VU-AMS website, www.vu-ams.nl.

10. Finally, select the *Label Information* tab. A table with the results appears after calculation. Each row represents the average value of a series of physiological parameters (heart rate, PEP, RSA, RR) for each labeled time period. The first column has the subject identifier (Label_ID). The last column indicates the values of all categories used during labeling (here only a single category 'experimental condition' with two values, 'sitting up' and 'lying down'). Spectral powers of the interbeat interval times series are given only for labels with a minimum length of 4 min (otherwise the missing code is displayed). The spreadsheet in this display can be exported to ASCII or EXCEL for further statistical analyses.

Representative Results

In the Amsterdam Born Children and their Development study, a Dutch prospective, longitudinal birth cohort, the measurement protocol was started in 3,097 children [6]. Approval was obtained from the Academic Medical Center Medical Ethical Committee, the VU University Medical Center Medical Ethical Committee and the Registration Committee of Amsterdam. All participating mothers gave written informed consent for themselves and their children.

As the monitors are lightweight and unobtrusive, the children tolerated these measurements very well. We do not have data on the refusal rate, but experience taught us that only a few children resisted the placement of the electrodes and thereby obstructed further assessment. Of the 3,097 registrations, 0.7% were lost due to either equipment failure or misplacement of files. Out of the 3,074 registrations left, 98.7% were of children who completed the entire protocol (n = 3,056). Within each of the labelled time periods (we originally labelled four time periods, but later summarized these to two), we encountered unclear ICG signals, meaning PEP could not be determined. This led to a loss of 1.5% in the first out of four labelled periods, 2.4% in the second, 2.8% in the third and 4.1% in the fourth period. Complete data on PEP in all time periods was available in 2,797 cases (91.5%, thus 8.5% loss due to unclear ICG signals). Complete data on heart rate (HR), pre-ejection period (PEP) and respiratory sinus arrhythmia (RSA), as well as sex and age, was available from 2,761 children; in this final step, 1.3% data loss occurred, due to unknown reasons. Overall, 89.2% of the started registrations led to full subject data. The mean age of the children was 5.7 years (SD 0.5; interquartile range 5.0:6.5), and their BMI was 15.5 kg/m^2 (SD 1.5; interquartile range 13.9:17.2).

The mean values of the major outcome variables HR, PEP, and RSA are given in **Table 1** and graphically depicted in **Figure 3**, separately for boys and girls. HR (both lying down and sitting up) and PEP (only sitting up) were higher in girls than in boys (both postures). RSA was lower in girls than in boys (both postures). The higher values for HR in girls are likely to be caused by the lower vagal (parasympathetic) cardiac control. Their sympathetic cardiac control was not different or even lower than that in boys (sitting up).

In both sexes, HR was higher when sitting up compared to lying down, whereas RSA was lower when sitting up. This reflects the lower vagal control when sitting up. PEP was shorter lying down then sitting up. This effect was also as expected, and it reflects the outcome of opposite processes: lower sympathetic activity (lengthens PEP) while lying down with increased preload (shortens PEP)[7].

	Boys						Girls				
	Lying down			Sitting up			Lying down		Sitting up		
	Mean	SD		Mean	SD		Mean	SD	Mean	SD	
Heart rate (bpm)	83.9	9.5	*	89.1	10	*†	86.9	10.1	92.4	10.4	†
Pre-ejection period (msec)	76.9	11.8		78.5	12.2	*†	77.7	10.3	81	11.7	†
Respiratory sinus arrythmia (msec)	127.0	60.4	*	115.7	55.8	*†	121.7	56.8	108.7	51.9	†

Table 1. Cardiac autonomic nervous system measures in boys and girls, by posture on posture difference. $p < 0.05$ for one sample T-test on sex difference. † $p < 0.05$ for paired samples T-test.

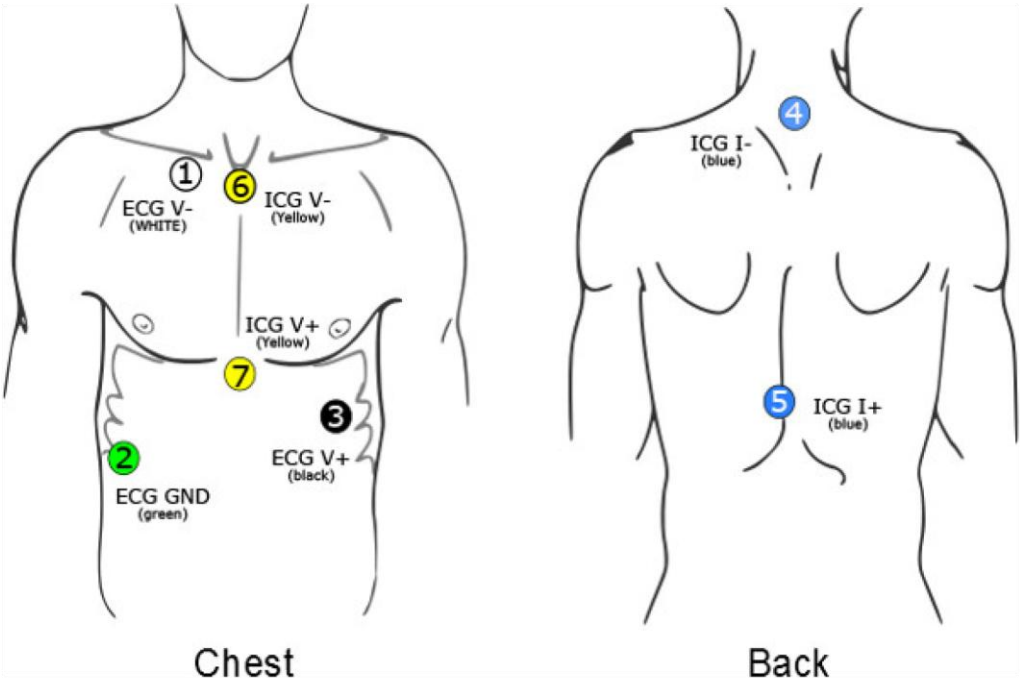

Figure 1. The seven electrodes should be placed on the participant's chest and back. The first ECG electrode (V-) is placed slightly below the right collar bone 4 cm to the right of the sternum. The second ECG electrode (V+) is placed at the apex of the heart over the ninth rib on the left lateral margin of the chest approximately at the level of the processus xiphodius. The third ECG electrode (GND) is a ground electrode and is placed on the right side, between the lower two ribs at the right abdomen. The first ICG measuring electrode (V_1) is placed at the top end of the sternum, between the tips of the collar bones. The second ICG measuring electrode is placed at the xiphoid complex of the sternum, where the ribs meet. The two current electrodes are placed on the back: I- on the spine over the cervical vertebra C4, at least 3 cm (1 in) above the ICG measuring elec-trode V-, and I+ between thoracic vertebrae T8 and T9 on the spine, at least 3 cm (1") below the ICG measuring elec-trode V_2. The ICG electrode placement takes into account that the largest part of the left ventricle driven change in thorax impedance is captured by the column between the suprasternal notch and the processus xiphoideus.

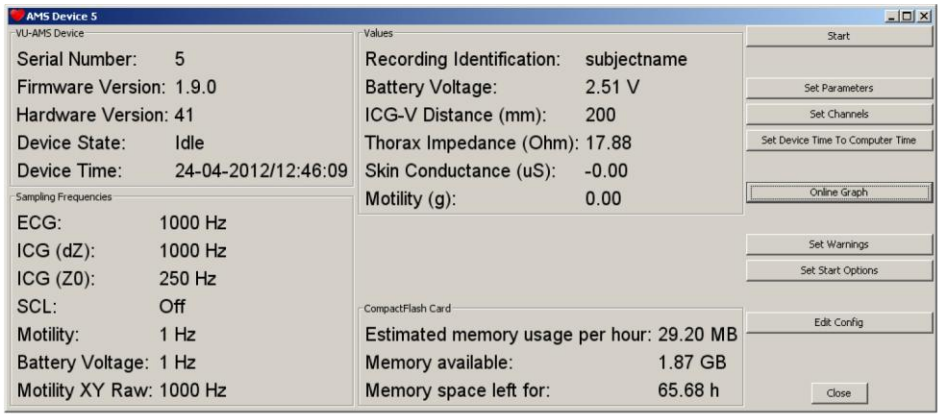

Figure 2. The typical settings used for a recording as displayed by the DAMS software after connecting to the VU-AMS5fs device. Click here to view larger figure.

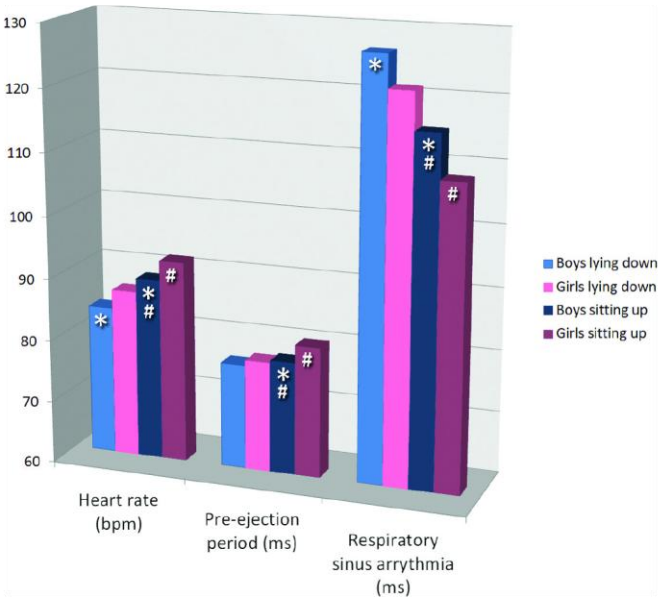

Figure 3. Cardiac autonomic nervous system measures in boys and girls, by posture. * indicates $p < 0.05$ for one sample T-test on sex difference. # indicates $p < 0.05$ for paired samples T-test on posture difference.

Discussion

We used an ambulatory recording device to measure cardiac autonomic control in 3097 children, aged between 5 and 7 years. Seven electrodes sufficed to measure the ECG and ICG from which the heart rate, heart rate variability and the systolic time intervals were extracted. Heart rate variability in the respiratory frequency band (RSA) is a valid indicator of cardiac parasympathetic activity. The systolic time interval, PEP, by reflecting cardiac contractility, is a valid indicator of cardiac sympathetic activity. The mean values obtained for HR, PEP and RSA, the effects of posture changes and the differences between boys and girls were in line with what would be expected from the literature.

As ambulatory monitoring removed the necessity of assessment in a laboratory our recordings could be done in various locations (*e.g.* school, sports center, science museum) without differences in signal recording quality. However, it is of crucial importance to standardize within or between subject comparisons for posture and physical load, as afterload and preload effects can co-determine the PEP without any changes in cardiac sympathetic drive [7]. We conclude that ambulatory recording of the ECG and ICG in large samples of children is highly feasible and propose the current standardized study design as a useful template for future assessments of cardiac autonomic control in children.

Disclosures

We have nothing to disclose.

Acknowledgements

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APPENDIX III

Example of a VU-AMS workshop with assignments

René van Lien and Eco J.C. de Geus

Program overview

Introduction to recording with the VU-AMS and analyzing with DAMS.
Today I am wearing the VU-AMS to provide a fresh recording that we can use to get familiar with the basics while explaining some new features of DAMS 3.0. We will process this file together in a plenary session before we continue with some hands on scoring in the individual sessions. New DAMS features in the plenary session are:

1. VU-AMS 5 lead version
2. Bluetooth connection for online wireless heart rate monitoring
3. Instant Heart Rate during online mode
4. Remote event marker via Bluetooth
5. Automated Scoring for ECG QRST Points
6. Option to remove all HIGH & MEDIUM Suspicious Beats in Detect R-Peaks Tab

Focus on the PEP
After the introduction to DAMS 3.0 we are going to inspect/correct the impedance and respiration scoring of several pre-labeled recordings. The files used in this part of the workshop are from an ambulatory experiment where subjects were guided through common posture and activity changes accompanied by an experimenter. We will come across some new impedance scoring features while doing this.
All participants will score the same recordings and compare them for inter-rater reliability. We will merge data in pairs to see what the consensus between the scoring of two raters was. We will discuss the deviant cases.

24H recordings
We continue with PEP scoring and respiration and RSA scoring on ambulatory data collected in a naturalistic setting. This kind of data usually consists of more and longer complexes and is often prone to noise which makes scoring of the impedance signal quite a challenge!

SCL analyses now available!
For the first time in DAMS we have the opportunity to experience the new SCL analyses consisting of Label and stimulus based export of Skin Conductance Data. We treat this subject separate as it has a different output from ECG and ICG analyses.

Wrap up: some additional features worth mentioning and time for Questions.
At this point we will have seen a lot of data and discussed quite a lot but there are some features worth mentioning.

- Divide a particular label into time labels of chosen interval.
- Batch Export of Raw Signals with & without label information
- Batch Analyze & Export Data (ASCII & Excel) for automatic scoring of ECG & ICG Complexes

Working with DAMS

Step 1: Opening the AMS recording for the first time (.5fs file).

Upon opening the 5fs file, the Inspect Data tab simply gives you an overview of your data. Above the recording time, the Inter Beat Interval (IBI) time series is given as extracted from the ECG. The clock time and IBI signal of the entire recording are presented in all analysis tabs, and functions as orientation point. Vertical lines represent the times at which the event button was pressed.

Assignment 1. Open the example file with the DAMS 3.0 program.

**The example file is named Morning_Recording_Rene.5fs.*

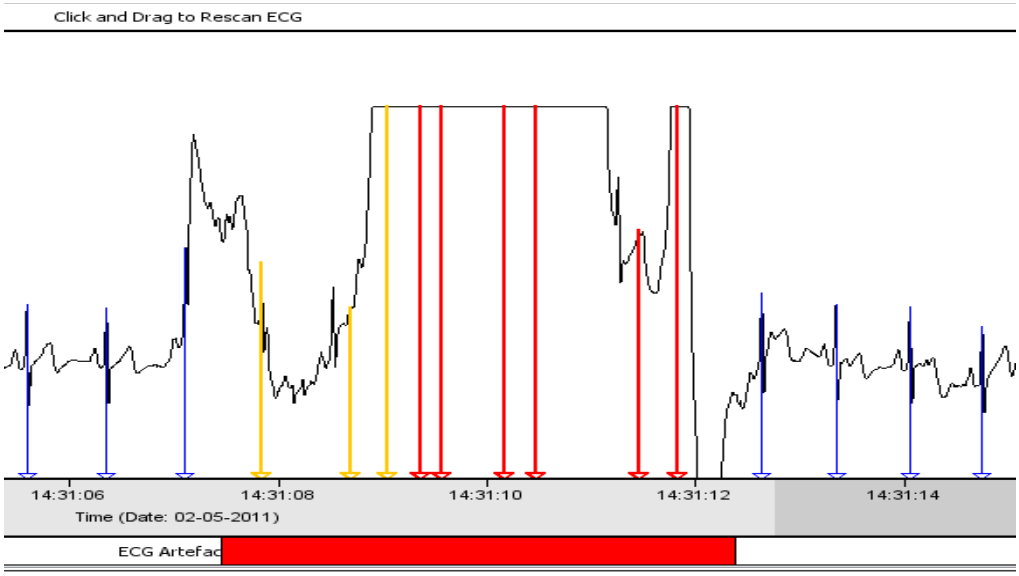
Step 2: R-peak detection

The *Detect R-Peaks* tab will assist you to create an artefact free IBI signal as fast as possible by applying automated artefact and peak detection. The mandatory visual inspection and correction of the resulting IBI signal is made as easy as possible by multiple zoom levels and an automated suspicious beat detector. You can also zoom in or out by using the scroll wheel of the mouse on the part of the data you want to see in more detail. By using the mouse wheel a shorter (scroll forward) or a longer (scroll backward) period of the recorded data can be shown.

The default settings of the R-peak detector work well on most of the ECG recordings but varies with signal quality. Automated artefact labeling reliably detects clipping and signal loss, but detection of noisy ECG is not perfect. Manual deletion of bad ECG signal parts may be additionally needed.

In the main window with the ECG signal the detected R-peaks are marked by vertical lines, mostly blue. A blue line means that the beat was considered to be correct according to the automatic beat detector. Potential mistakes in automated beat detection are termed 'suspicious beats' and are flagged by a red or yellow color. You can easily browse through all suspicious beats from most to least suspicious by pressing *Dot/right pointer* keyboard key for next, and *comma/left pointer* keyboard key for previous suspicious beat. Or you can simply use the menu buttons at the top of the window. When you are at a suspicious beat you can either delete a beat by right clicking on it, e.g. when a beat was placed in an obvious wrong location in between beats. Or you can add a beat by left clicking on the correct location of the R-peak, e.g. when a beat was completely missed. Just remember, suspicious is not always guilty.

To mark an unscorable / bad part of the ECG as an artefact, click with the left mouse button in the artefact bar and drag it from left to right until it covers the entire period that is to be marked as an artefact. All beats in the artefact period will be deleted from further analyses.



Assignment 2: Correct misplaced or missed beats and mark missed artefact periods.

Step 3: Segmenting / labeling the data

Here you divide the continuous data into logical periods for further analysis. We call this labeling. The aim of labeling is to get an average value for each available parameter, such as HR, PEP, and RSA, for each experimental condition or ambulatory activity.

Click on the *Label Data* tab. In the upper window we see the raw IBI time series and/or the smoothed (and hence lightly time lagged) heart rate signal. Because the heart rate signal is dependent on the quality of the IBI time series, make sure the R-peaks have been detected correctly in the *Detect R-Peaks* tab. In the lower window we see the 'motility' signal. This signal is based on the Y axis of the tri-axial accelerometer. The clock time and IBI time series are presented in the two bottom windows.

Before we can actually begin labeling the data, we need to load the blueprint for all possible labels first. This blueprint lists all the experimental conditions or ambulatory activities. This is done in the label configuration file (.cfg file).

Assignment 3: load the label configuration file.

Go to the main menu – *Actions - Edit Label Configuration - Import Label Configuration From File.* Now select the *Morning Recording Rene.cfg* file and click *open*.

Assignment 4: label the activities from the activity diary

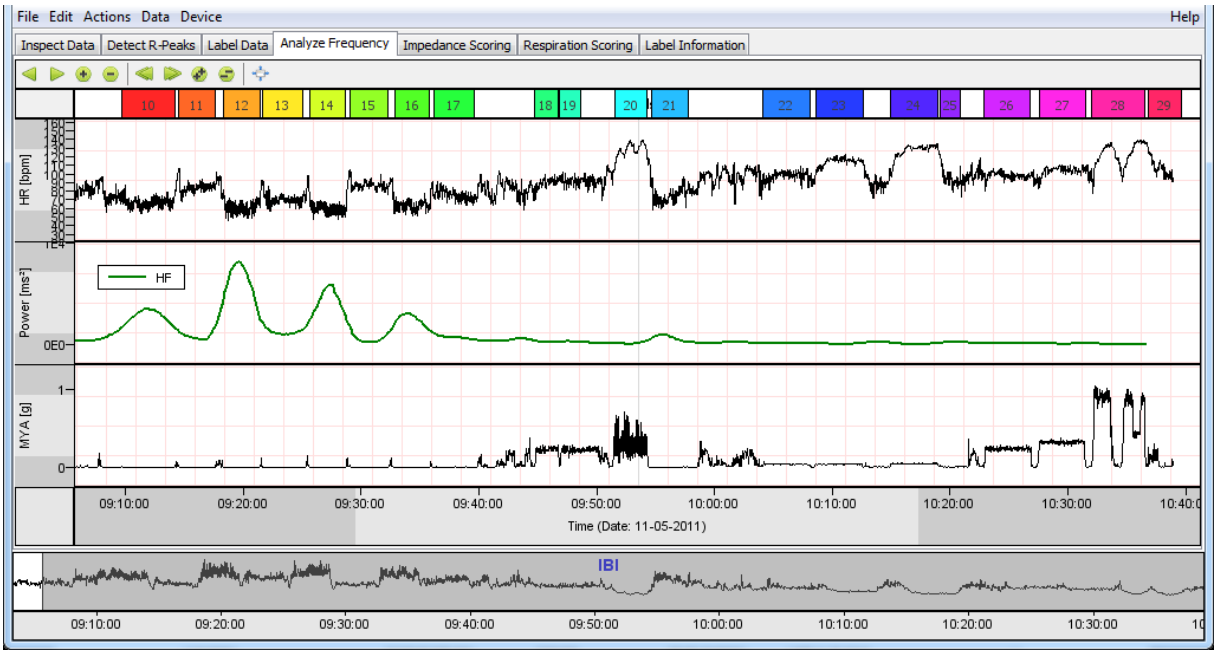
The label configuration is now loaded and now we can start the actual labeling of our data. Use the notes containing the start and stop times of each activity to draw labels in the top bar where it says "Click and Drag to add Labels". When hovering the mouse on the bar you will see an indicator of the time window appear. This will help you place the starting point of your label accurately. Click with the left mouse button in the top bar at your starting point and drag the mouse to the right until the desired end time of the label is reached.

**A dashed vertical line will also appear on both ends of the label extending into the motility signal. This may help you remove the transition periods with movement between two experimental conditions from the final labeled data.*

***During the recording I pressed the event button of the VU-AMS device to mark the beginning and ending of each condition. These markers are displayed as lines here, and help us to more easily detect the start and stop times of each experimental condition. By clicking with right mouse button on the label bar between the two lines, we get the option to place a label between the lines automatically.*

Step 4: Spectral power calculations.

This is the most passive tab in DAMS. Spectral powers are calculated for every label with a label length above 4 minutes and there is no interaction possible. Let's move on!



Step 5: Impedance scoring.

The impedance cardiogram (ICG) is the first derivative of the change in thorax impedance using time as the basis (dZ/dt). This characteristic ICG waveform derives from the change in thorax impedance caused by left ventricular ejection of blood into the descending aorta during the systolic phase of the cardiac cycle. To improve signal quality, the ICG waveform is often obtained by ensemble averaging over beats within in a fixed time period, time locked to the R-wave peak. The typical period for ensemble averaging is one minute. In DAMS we deviate from this practice and instead compute a Large Scale Ensemble Average across the entire label *(see Riese et al., 2003 for the rationale).*

The most important variables extracted from the ICG is the preejection period (PEP). The PEP is an index of contractility which is only influenced by sympathetic but not parasympathetic activity in humans, which makes PEP the measure of choice to monitor changes in cardiac sympathetic activity non-invasively. The PEP is defined as the interval from the onset of left ventricular depolarization, reflected by the Q-wave onset in the ECG, to the opening of the aortic valve (B-point in the ICG).

<u>Assignment 5: Score the ECG and ICG landmarks.</u>

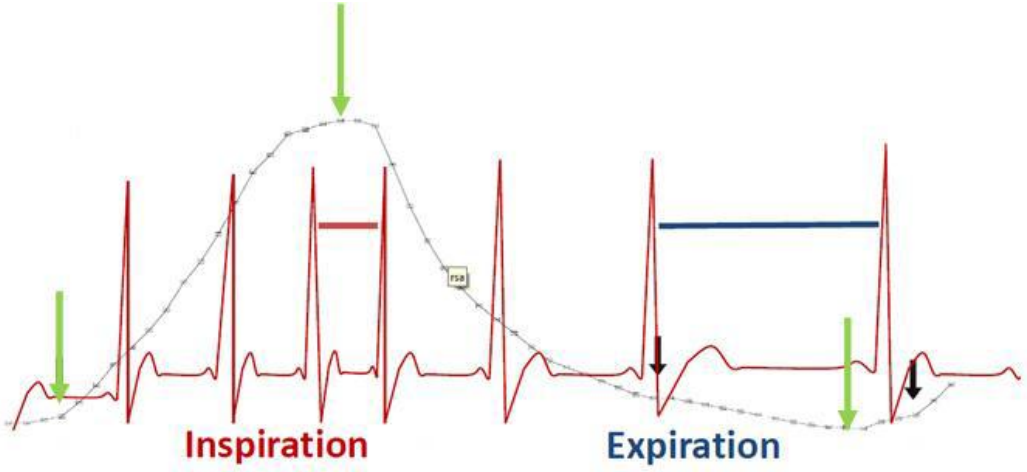
We will score all of these complexes together one by one to get familiar with moving around the impedance tab. The landmarks we will focus on are:

Q-wave onset in the ECG (the start of the electromechanical heart cycle).

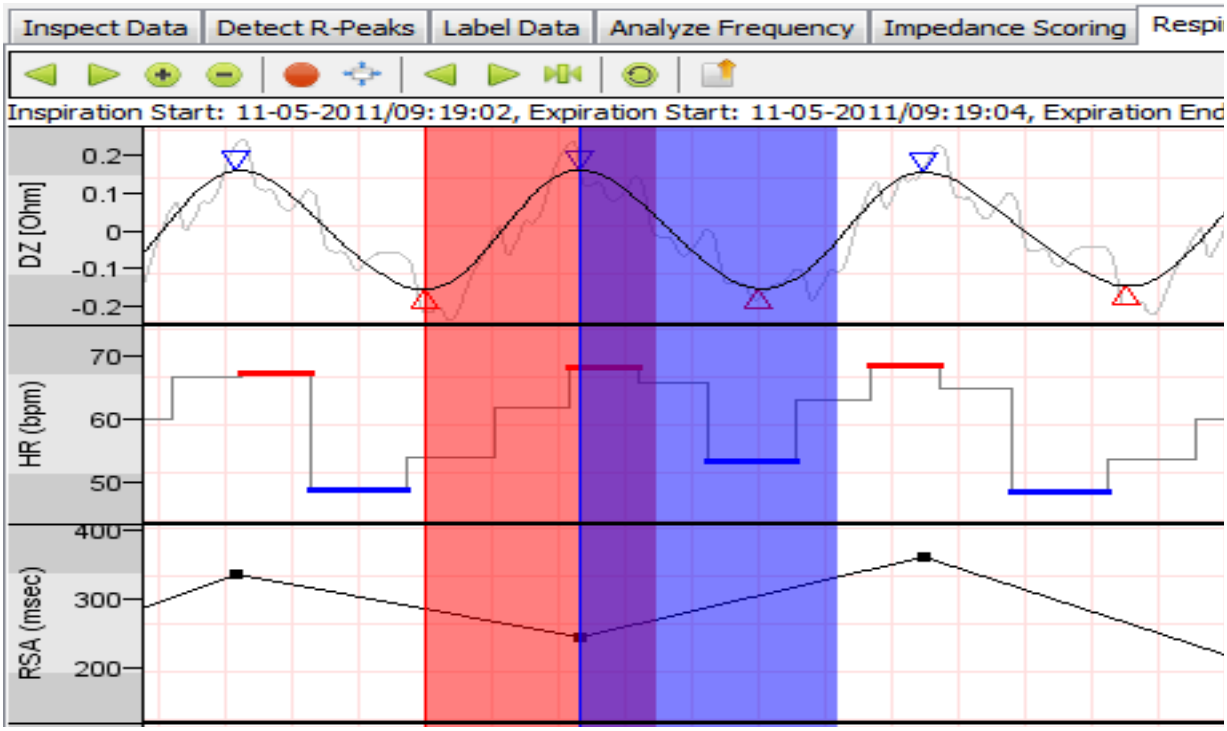
B-point or upstroke(the opening of the aortic valve)

dZ/dt min or C-point (max aortic diameter / velocity of aortic blood flow)
X-point or incisura (closing of the aortic valves = end of LVET

Step 6: Visual inspection of the automated RSA detection.
Respiratory Sinus Arrhythmia (RSA) scoring by the DAMS program is based on the peak-valley method (Grossman, van Beek, & Wientjes, 1990 ; de Geus et al., 1995) that uses the IBI time series extracted from the ECG together with the respiration signal obtained from filtered (0.1 – 0.4 Hz) dZ signal to obtain heart period variability that is associated with respiration. This heart period variability is referred to as RSA.



The DAMS program contains an automatic scoring algorithm for detecting the beginning and end of inspiratory and expiratory phases in each respiratory cycle. For each respiratory cycle the total cycle time between begin of inspiration and end of expiration is extrapolated to a per-minute respiration rate (RR). In addition, RSA is computed per respiratory cycle from two IBIs: The shortest IBI during an interval starting at the begin of inspiration and ending 1000 msec (default) after the end of inspiration and the longest IBI during an interval starting at the begining of expiration and ending 1000 msec (default) delay after the end of expiration. RSA is calculated by the subtraction of the shortest IBI from the longest IBI, provided that the shortest IBI (highest HR) is part of an accelerating series within the inspiratory interval and the longest IBI (lowest HR) of a decelerating series within the expiratory interval. This is illustrated in the figure below.



If either the decelerating longest or accelerating shortest IBI is missing for a breath cycle, or a negative RSA value is obtained on subtraction, we set RSA in these breaths as missing.

Assignment 6: Visually inspect the RSA scoring

Automated scoring of the respiration signal works quite well in most subjects. Mostly, it will suffice to just load the .amsdata file into DAMS and browse through the signal after having set the time axis at a low temporal resolution (e.g. ten minutes per screen). While browsing through the respiration signal from the beginning to the end of the file check the following:

Clipping dZ : Purpose: The purpose of this option is to automatically reject clipping (i.e. where the raw respiration signal turns into a flat line at dZ = 1 ohm or dZ = –1 ohm). The default values usually suffice and detected true clipping very well. Just check if it is true clipping when more than 10% of the recording is marked as a clipping artefact.

Irregular respiration : Do all inspirations and expirations appear to be appropriately scored in the upper respiration signal (indicated by blue and red triangles)? --> If erroneous breaths are scored, did the program mark them as artefacts in the bar at the bottom of the screen labeled “Irregular respiration”? --> Add or remove irregular respiration artefacts where needed.

**Did the program mark more than 10% of the recording as an “Irregular respiration” artefact If so, the parameters of the scoring algorithm may need to be changed under settings in the menu bar.*

Irregular IBI: The purpose of this option is to automatically reject spikes in the IBI time series, that represent extra-systolic beats or very prolonged beats. --> Check whether the program has rejected all deviant IBIs (spikes) without removing IBIs that reflect large but true heart rate variability. Hence make sure all spikes are marked as artefacts in the bar at the bottom of the screen labeled “Irregular IBI".

**The difference between a spike (e.g. caused by premature ventricular contraction (PVC) and a truly high heart rate variability is rapidly gleaned from the shape of the tachocardiogram. If there is a staircase pattern rather than a sudden single-beat change, the subject may have a generally high*

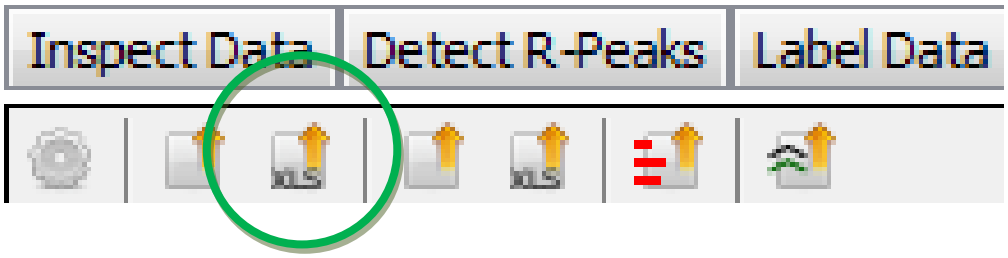
heart rate variability which can be verified in the RSA window (e.g. RSA > 200 msec). If there is a sudden single-beat drop or jump then there is a spike. Spikes often represent extra-systolic beats or very delayed beats (which often occur jointly).

Step 7: Exporting the results

After clicking on the *Label Information* tab wait until all cells are finished calculating. The first column in the *Label Information* tab is the subject name, which is the ID that we gave when programming the device for recording. Each row represents a single labeled experimental/ambulatory condition and each consecutive labeled condition is rank-ordered by the *Label ID* field. The rest of the columns contain values for a large number of physiological variables. ICG-based variables only have a value when the scoring under the *Impedance Scoring* tab has been done (e.g. PEP) and the LF and HF power values from spectral analyses on the IBI time series are only present for labels with a minimum length of 4 minutes.

Assignment 7: Export the results to ASCII

Click on the export button in the menu and you will be prompted to enter the location to the output file.



More practice: PEP and RSA scoring

You will find two folders on the hard drive containing the following:

G:\SAA workshop 2013\EXPERIMENTAL AMS DATA\Yourname

Every folder contains several AMS files and an SPSS script that we will later use for initial read-in and merging of your data.

Assignment 8: Inspect the impedance scoring of each recording

Inspect the impedance scoring of each recording and export the output to a single ASCII file in your directory. Here is a summary of the PEP scoring rules that help you go through the complexes:

Morphology

The B-point or upstroke should be at a first or second order zero-crossing in the dZ/dt signal. It should be close to the dZ/dt=0 line, and be the starting point of the longest uphill slope before the dZ/dtmin point. However, rather than appearing as a clear incisura, the B-point may sometimes take the form of a subtle inflexion and may vary considerably from beat to beat. It is therefore very important to inspect the dZ/dt signal closely in order to identify it.

The dZ/dtmin is normally visible as a clear peak in the window between the B- and the X-point. In some cases the dZ/dt signal shows a double peak, a bit like rabbit ears. If one of the peaks is clearly (40%) higher then this peak is chosen. If the peaks are of comparable magnitude, choose the first peak.

The X-point or incisura is always a local minimum after the dZ/dtmin. Often it is the lowest point in the entire signal, but not necessarily. In the ideal situation it can be seen as a sharp trough in the ICG signal. This is the most clearly identifiable choice for the X-point. It may be that two or more troughs lie in close proximity without one being clearly the lowest point in all complexes. The latter part of the ICG waveform then looks like a "W". In this case choose the second trough (mostly, this trough is usually followed by the longest uphill slope after the dZ/dtmin point).

Consistency
Whatever point you choose, choose that point consistently. If a "less-than-ideal" upstroke is present in all complexes, but an "ideal" upstroke is present in some, choose the less-than-ideal one in all complexes, even those featuring a more "ideal" upstroke. Before starting to score the ICG, try browsing through the entire ICG signal first. You can then decide which points can be most consistently identified, and this holds for both for the B-point and the X-point.

Physiological plausibility
We can use the ECG signal to make decisions a bit easier. e.g. the B point will probably not take place around the Q-onset or T-top. Below are some plausible values for each interval based on recordings in healthy adults.

HR: 40-60 →PEP: 100-140 →LVET 300-450
HR: 60-80 →PEP: 90-130→LVET 250-400
HR: 80-100 →PEP: 80-120 →LVET 200-350
HR: 100-120 →PEP: 70-100 →LVET 200-300
HR: 120+ →PEP: < 80 →LVET 150-250

Again, if your signal shows B- and X-points outside of these ranges, this does not at all mean that your dZ/dt signals should be discarded. The above table is just a general rule of thumb.
** As you will notice some recordings need quite a lot of zooming to see the Q-onset or have no Q-onset no detectable point. We set the Q-onset to missing across all complexes in these latter cases. The PEP will then be calculated from R-B interval + a fixed interval based on the average of a large population of normatives.*

Multiple Rater comparison
Reliability increases if two (or more) raters score the same data set independently. After inter-rater reliability is established, the various raters should ideally compare their deviant scoring to converge on a single solution, in view of the consistency principle. Mostly one will have picked a different B-point then the other(s). Averaging the B-point location is meaningless. Consensus has to be reached on the correct B-point location to satisfy the consistency criteria.

Assignment 9: merge the output ASCII files in SPSS
Run the script with your name in the title to merge all output files.

Assignment 10: inter rater reliability

Run the script mentioned "merging_" in pairs (pre-defined buy your coupled names) and compare the within subject correlations. Where did you differ in choice and is there a consensus to be reached?

List of publications

Articles published

van Lien, R., Goedhart, A., Kupper, N., Boomsma, D., Willemsen, G., and de Geus, E. J.C. (2011). Underestimation of cardiac vagal control in regular exercisers by 24-hour heart rate variability recordings. International Journal of Psychophysiology, 81, 169-176.

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Book Chapter

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List of abbrevations

BMI	Body mass index
BP	Blood pressure
CoAR	measure of ANS co-activation
CVD	Cardiovascular disease
DAMS	Data Analayes and Management Software
DBP	Diastolic blood pressure
DZ	Dizygotic
dz/dt	Change in impedance
ECG	Electrocardiogram
GPS	Global Positioning System
HF	High frequency power
HPA axis	Hypothalamus pituitary adrenal axis
HR	Heart rate
HRV	Heart rate variability
IBI	Inter-beat-interval
ICG	Impedance cardiogram
ISTI	Initial systolic time interval
LF	low frequency power
LF/HF	Ratio of the low and high frequency power
LVET	Left ventricular ejection time
NETAMB	Netherlands twin family ambulatory study
NTR	Netherlands twin register
PEP	Preejection period
RMSSD	Root mean square ofsuccessive differences
RR	Respiration rate
RSA	Respiratory sinus arrhythmia
sAA	Salivary alpha amylase
SBP	Systolic blood pressure
SDNN	Standard deviation of normal-to-normal intervals
TWA	T-wave amplitude
VU-AMS	VU University Ambulatory Monitoring System
Z	Impedance (in Ohm)

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