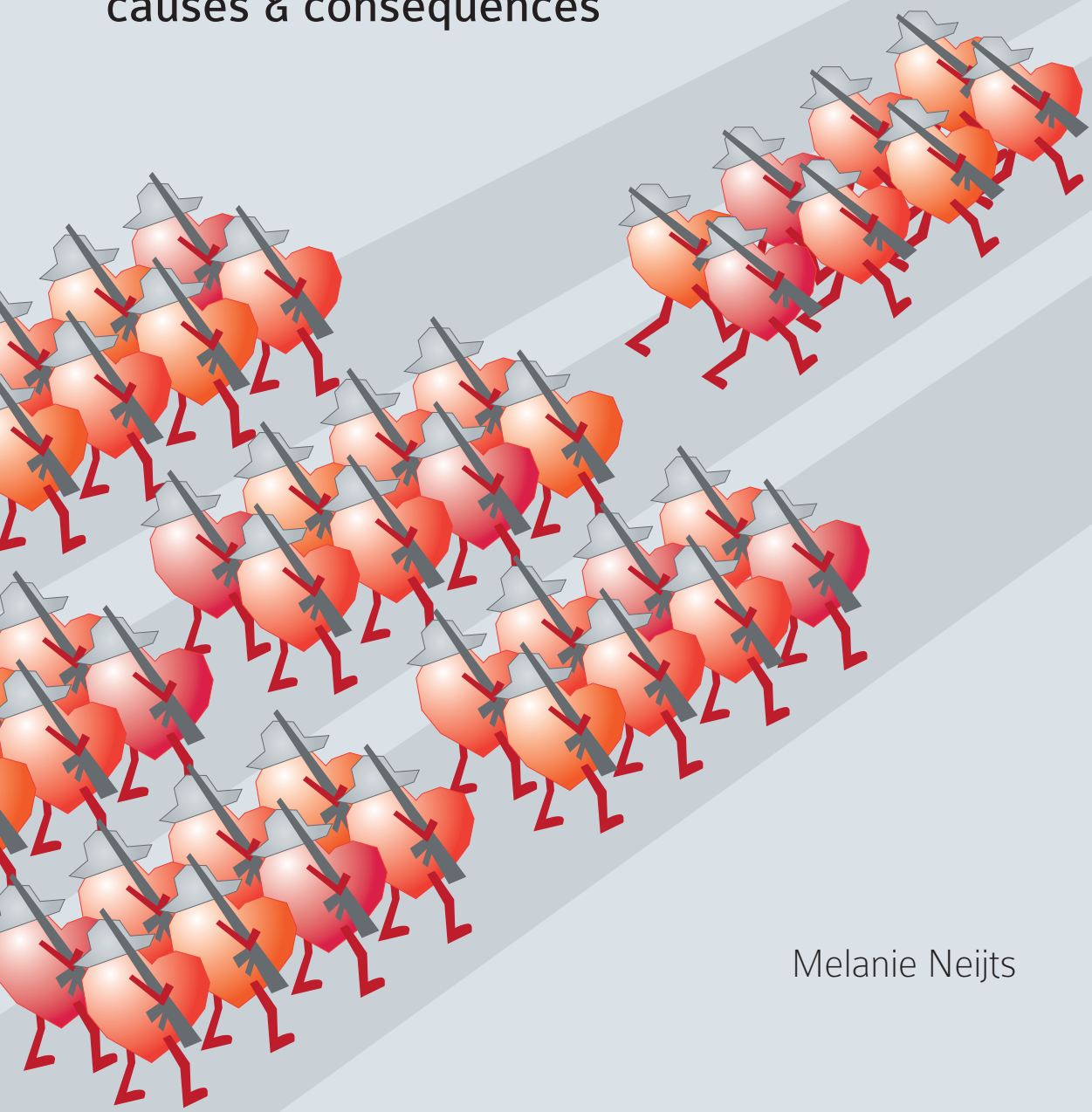
Individual differences in ambulatory autonomic nervous system activity:

causes & consequences



Melanie Neijts

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Individual differences in ambulatory autonomic nervous system activity: causes and consequences

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door
Melanie Neijts
geboren te Eindhoven

promotor:
Prof. dr. J.C.N. de Geus

copromotor:
Dr. A.H.M. Willemsen

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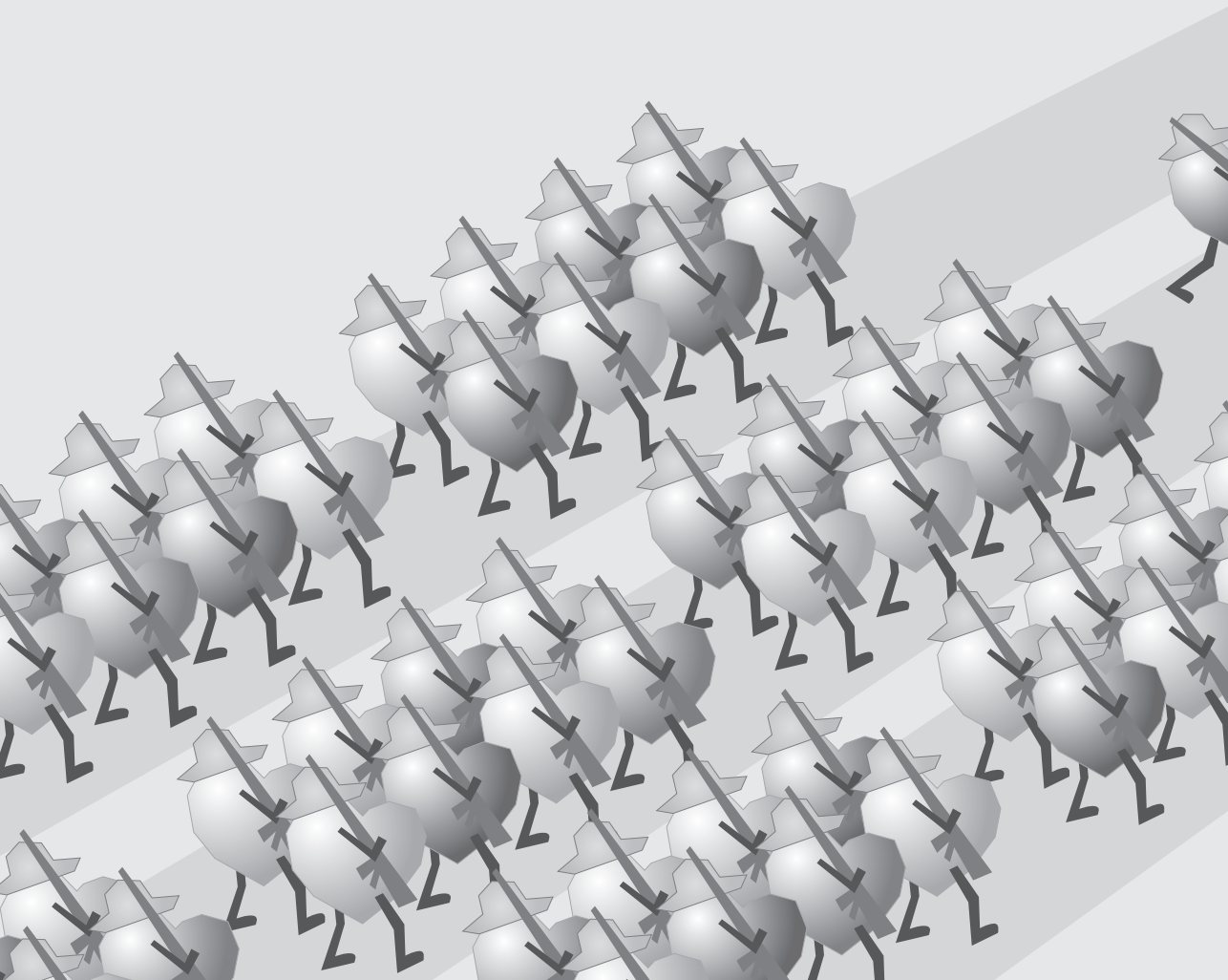
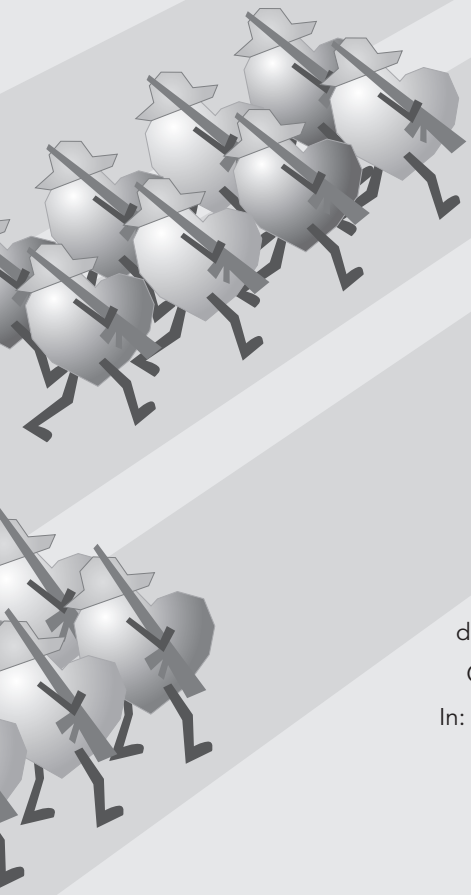


Chapter 1

Introduction



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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., 1997; Dekker et al., 2000); 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 and the North American Society of Pacing and Electrophysiology, 1996; Tracey, 2009; von Kanel R., 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, Kuper, & Hemingway, 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, Cacioppo, & Quigley, 1994; Berntson, Norman, Hawkley, & Cacioppo, 2008; Cacioppo et al., 1994; 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, Kupper, Boomsma, & Snieder, 2007; Houtveen, Rietveld, & de Geus, 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 contribution of genetic factors to the individual differences in ANS activity at rest and during stress. We first present a short overview of the ANS and of the measurement strategies used to study it, with a focus on (ambulatory) noninvasive measures that can be used in population-based samples that are sufficiently large to allow genetic analyses. Next, we review the twin studies on the heritability of these ANS measures and present a list of candidate genes that have already been found to influence ANS activity.

The autonomic nervous system

The ANS can be divided in two branches; the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). The SNS 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 to coordinate 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 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 vagus nerve (CN X) carries fibers 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. 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 α_1-adrenergic (e.g., in arterioles) or β_1- and β_2-adrenergic receptors (e.g., on the heart). Stimulation of the α_1-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 β_2-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. However, 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, Cacioppo, & Quigley, 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 subjects showed increased SNS activity with *increased* PNS activity (Bosch, de Geus, Veerman, Hoogstraten, & Nieuw Amerongen, 2003). Most important, 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; Lambert, Straznicky, Lambert, Dixon, & Schlaich, 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 pro-inflammatory 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. Parasympathetic nervous system activity can be assessed invasively by parasympathetic microneurography, microdialysis, or pharmacological blockade (Berntson et al., 1994; Cacioppo et al., 1994; Cerati & Schwartz, 1991; Jewett, 1964; Kunze, 1972; Martinmaki, Rusko, Kooistra, Kettunen, & Saalasti, 2006; Shimizu et al., 2009), but also less invasively by measuring baroreflex sensitivity (BRS), or noninvasively by measuring heart rate variability (HRV), particularly HRV in the respiratory frequency range or RSA (Akselrod et al., 1981; Cerutti, Bianchi, & Mainardi, 2001; DiRienzo, Parati, Radaelli, & Castiglioni, 2009; Katona & Jih, 1975; La Rovere, Pinna, & Raczak, 2008; Sztajzel, 2004; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). There are different ways to quantify HRV and several commonly used time- and frequency domain indices of HRV are summarized in Table 1.1.

As is the case for PNS activity, SNS activity can also be measured invasively by means of sympathetic microneurography, regional norepinephrine spillover, or pharmacological blockade (Berntson et al., 1994; Cacioppo et al., 1994; Eisenhofer, 2005; Esler et al., 1988; Esler & Kaye, 2000; Grassi & Esler, 1999; Hagbarth & Vallbo, 1968; Julius, Pascual, & London, 1971; Wallin, 1984; Wallin, 2004). Other commonly used measurements of the SNS are plasma or urinary catecholamines, salivary α-amylase activity, or the LF/HF ratio (Esler et al., 1990; Hjemdahl, 1990; Goldstein, Eisenhofer, & Kopin, 2003; Nater & Rohleder, 2009; Pagani & Malliani, 2000). However, together with electrodermal activity (Boucsein, 1992; Dawson, Schell, & Filion, 2000; Fowles, 1986) measurement of cardiac contractility is currently the preferred noninvasive method to measure SNS activity. Contractility is reflected in a larger ejection fraction which can be measured, e.g. by echocardiography, as the fraction of the end-diastolic volume ejected as stroke volume. 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 pre-ejection period (PEP) (Sherwood et al., 1990). The different ways to quantify SNS activity non-invasively are summarized in Table 1.2.

Table 1.1. Several commonly used noninvasive measures of heart rate variability (HRV).

Time domain measures	
SDNN	Standard deviation of all valid interbeat intervals
SDANN	Standard deviation of the average interbeat intervals across all 5-minute segments of the entire recording
RMSSD	Root mean square of differences between valid, successive interbeat intervals
pvRSA	Respiratoy Sinus Arrhythmia derived by peak-valley estimation. In the time domain, 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
BRS	Baroreflex Sensitivity derived by the sequence method in combined continuous blood pressure and ECG recordings (can also be derived in the frequency domain as the cross time-series coherence)
Frequency domain measures	
HF power	Power in the respiratory frequency range of 0.15-0.40 Hz
LF power	Power in the low frequency range (0.04-0.15 Hz)
VLF power	Power at very low frequencies (0.003-0.04 Hz)
ULF power	Power in the ultra low frequency band (< 0.003 Hz)
Total power (TP)	ULF + VLF + LF + HF

Table 1.2. Several commonly used noninvasive measures of sympathetic nervous system activity.

Plasma NE/E	Measurements of concentrations of norepinephrine (NE) or epinephrine (E) in venous (or arterial) blood using liquid chromatography
Urinary NE/E	Measurement of (24-h) urinary excretion of NE or E (relative to creatinine)
LF/HF	The LF/HF ratio 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)
PEP	The Pre-Ejection Period is the time interval between the onset of ventricular depolarization (the QRST complex, or Qonset) and the opening of the semilunar valves (sharp upstroke in the dZ/dt, or the B point in the impedance cardiogram)
EF	Ejection Fraction is the fraction of the end-diastolic volume that is ejected with each heart beat.
SCL	The Skin Conductance Level representing slow tonic shifts in electrodermal activity
nsSCR	Skin Conductance Responses (SCRs) are more rapid transient events. nsSCR is the count or frequency of these nonspecific responses (also called electrodermal lability)
SVR	Systemic Vascular Resistance is the resistance to blood flow that must be overcome to push blood into the peripheral circulation

Genetics of autonomic nervous system activity

To determine the genetic contribution to the individual differences in ANS measures, we can resort to family studies. The essence of all family studies is that they relate the degree of genetic resemblance to the degree of trait resemblance. From basic biometrical principles, we can compute the estimated genetic covariance between two individuals based on the degree of their genetic relatedness (Falconer & Mackay, 1996; Lynch & Walsh, 1997). If two individuals are genetically unrelated, their expected genetic covariance is zero. It is important to note that this does not mean that they cannot show trait resemblance, but that such resemblance must be due to factors other than genetic ones (i.e., environmental factors). Things change when the covariance is computed between individuals who *do* have a genetic relationship. Relatives, for instance, parent and offspring, will more often have identical variants at a DNA locus than will unrelated individuals. If genetic factors contribute to the trait, the covariance between family members will vary systematically with the degree of genetic relatedness.

A general formula for the genetic part of the covariance between a trait measured in two relatives is:

$$\text{Covariance}(P_1,P_2) = \mathbf{u} * V_A + \mathbf{r} * V_D$$

where P_1 is a trait measured in relative 1, P_2 the same trait measured in relative 2, V_A the additive genetic variance caused by all loci contributing to the trait, V_D the dominant genetic variance caused by all loci contributing to the trait, **u** the coefficient of relationship representing the correlation between the relatives for loci acting additive, and **r** the coefficient of dominance.

A locus acts additively if both variants at a locus contribute equally to trait variance, whereas in dominance, one variant exerts a (much) stronger effect than the other variant. For parent–offspring pairs, u= ½ and r =0, for full siblings u = ½ and r = ¼, for uncles (aunts) with the nephews (nieces) u = ¼ and r = 0, for grandparent and grandchild u = ¼ and r = 0, and for single first cousins u = $^1/_8$ and r = 0. When the covariance between family members in a trait is measured across many different family relationships (e.g., different values for **u** and **r**), this yields a set of equations that can be algebraically solved to estimate V_A and V_D. This is the essence of the use of pedigrees (parent–offspring, grandparent–grandchild, uncle–niece, siblings, etc.) studies to estimate the broad heritability of a trait, which is simply the genetic variance (V_A and V_D) in the trait divided by the total variance (V_P) in the trait.

Studying family resemblance is a powerful tool to detect genetic contribution to a trait but, as already noted by Sir Francis Galton (Galton, 1869), a major shortcoming of this type of family studies is that the degree of genetic relatedness can be confounded with the degree of shared family environment. Full siblings, for instance, do not only share more genes than nieces and nephews, they are also typically reared in the same household. The shared family environment includes potentially important factors like parental socioeconomic status (SES), neighborhood, school, sports club, family diet, and parental attitudes and general rearing style. The solution to separating the genetic and shared environmental effects on the trait resemblance of family members has been found in what Galton called "a unique experiment of nature": monozygotic (MZ) and dizygotic (DZ) twin pregnancies.

Monozygotic twinning occurs when, for reasons that are still incompletely understood, a fertilized egg divides before it nestles in the uterus. Monozygotic twins are usually said to inherit identical genetic material. This is not entirely correct. Genetic imprinting patterns can be found to differ in MZ twins as is illustrated, for instance, in an MZ twin pair discordant for Beckwith-Wiedemann syndrome due to differential imprinting (Martin, Boomsma, & Machin, 1997). A number of other occurrences can make the genetic identity of MZ twins less than 100 percent. Since these occurrences are all rare, the assumption that MZs have 100 percent genetic identity is quite defensible, particularly since deviation from perfect identity will lead to an *under*estimation of genetic effects (i.e., the assumption is a conservative one).

If more than one egg is released from the ovaries during a menstrual cycle, and each egg is fertilized by a separate sperm, the result is a nonidentical twin also known as a DZ or fraternal twin. Dizygotic twinning rates have risen in the last decades in most countries because of artificial reproduction techniques and the higher age at which mothers get their first child, which may be paired to higher levels of follicle stimulating hormone. Genetically, DZ twins do not differ from singleton brother–brother, sister–sister, or brother–sister pairs; that is, they share on average 50 percent of their genetic material (Hoekstra et al., 2004). Opposite-sex (DOS) twins are always DZ twins.

In a twin study, four possible factors and their interactions and correlations are assumed to contribute to the total variance in a trait: unique environmental factors (E), shared environmental factors (C), additive genetic factors (A), and dominant genetic factors (D). Shared environmental factors and additive and dominant genetic factors can cause twin resemblance, whereas the extent to which twins do not resemble each other

is ascribed to the unique (or nonshared) environmental factors. These include all unique experiences such as differential jobs or lifestyle, accidents, or other life events, and, in childhood, differential treatment by the parents and nonshared peers. Twin researchers typically use structural equation modeling (SEM) to estimate the relative contribution of the A, D, C, and E factors to the individual differences in the trait. In SEM, the relationships between several latent unobserved variables (e.g., genetic and environmental factors) and observed variables (e.g., ANS measures) are summarized by a series of equations. Additional equations can specify the correlation between the latent genetic and environmental factors based on the known genetic relationship. For instance, the latent additive and dominant genetic factors influencing an ANS measure are correlated unity in MZ twins, but only 0.5 and 0.25, respectively, in DZ twins.

It is possible to derive the expected variance-covariance matrix implied by the total set of equations through the use of covariance algebra. Using maximum likelihood estimation, the fit of the expected covariance/variance matrix to the actual observed covariance/variance matrix is iteratively tested in a sample of hundreds or thousands of twins over a range of possible values for the path coefficients. From the best fitting model, the estimates for the path coefficients (e.g., a, c, d, and e) are used to estimate the relative contribution of the latent factors A, C, D, and E to the total variance in, for instance, an ANS measure. Heritability of the ANS measure, defined as the relative proportion of the total variance explained by genetic factors, is obtained as the ratio of $a^2 + d^2/(a^2 + d^2 + e^2 + c^2)$. The heritability can also be expressed as a percentage by multiplying this ratio by 100.

Using the classic twin design, we can only estimate three components of variance at the same time (either A, C, and E, or A, D, and E). One solution is to add parents or offspring of twins to create an extended twin-family design (Keller et al., 2009). If such family data are not available, one needs to make the assumption that either C or D is absent. This can be inferred in twin studies by first inspecting the MZ and DZ correlations to see whether dominance or shared environmental effects are actually likely to play a role. The presence of dominance can be recognized because it yields DZ correlations that are much lower than half the MZ correlation. In contrast, the presence of shared environmental effects yields DZ correlations that are much higher than half the MZ correlations. If the MZ correlation is about twice as high as the DZ correlation an AE model will fit best.

Twin studies of Indicators of Autonomic Nervous System Activity

Tables 1.3 and 1.4 summarize the results from twin studies on the heritability of various measures of ANS activity. Only studies with at least 50 twin pairs were included. Table 1.3 focuses on studies that have estimated the heritability of cardiac vagal activity using the different measures of RSA. The table excludes a study on HF power in 5-month-old infants, in which HF power was only weakly influenced by genetic factors (11 percent), and unique environmental factors explained most of the HF variance, particularly in boys (Dubreuil et al., 2003). Because myelinization of vagal fibers is still incomplete up until age 2 (Loeliger, Tolcos, Leditschke, Campbell, & Rees, 2000), it is uncertain whether the relation between vagal activity and RSA in such young children is comparable to that in adolescents and adults. For completeness, Table 1.3 also includes the heritability estimates for the BRS and total heart rate variability, either measured as the standard deviation of all IBIs (SDNN) or the TP of the IBI spectrum, and for the VLF and ULF power. These HRV measures also reflect PNS effects on the heart but are less 'purely' PNS than RSA. Table 1.4 focuses on studies that have estimated the heritability of measures of SNS activity that, to date, only include muscle sympathetic nerve activity (MSNA), plasma catecholamines, SCL/nsSCRs, and PEP. The table excludes a study on nine MZ twin pairs that used the golden standard MSNA approach (Wallin, Kunimoto, & Sellgren, 1993). This study reported that the MZ twin resemblance was as good as the average test–retest reliability of the method.

Heritability of Measures of Cardiac Vagal Activity

In laboratory twin studies that record RSA in quiet resting conditions, a significant but modest genetic contribution has been systematically reported (Busjahn et al., 1998; de Geus et al., 2007; Kupper et al., 2004; Kupper et al., 2005; Riese et al., 2006; Riese et al., 2007; Su et al., 2010; Tank et al., 2001; Tuvblad et al., 2010; Uusitalo et al., 2007; Wang, Thayer, Treiber, & Snieder, 2005; Wang et al., 2009; Zhang et al., 2007). Heritability estimates at rest for pvRSA range from 25 to 55 percent, for RMSSD from 36 to 71 percent, and for HF from 37 to 63 percent. Heritability estimates for BRS ranged from 22 to 55 percent. Osztovits et al. (2011) were the only exception to this as heritability of RSA in their study was not significant. Heritability estimates for European Americans and African Americans were very similar (Wang et al., 2005), and none of the studies reported a sex difference in heritability or evidence for different genes being expressed in males and females.

The difference in heritability estimates across studies and measures probably does not reflect a truly different genetic architecture. Instead, it may simply reflect the large age range across the studies (mean age 9 to mean age 55) and the different duration of the resting condition and the posture of the subject (sitting, supine). One study that used multiple measures in the same set of subjects found a very high correlation among SDNN, RMSSD, and HF, indicating that these three RSA measures may reflect the same underlying genetic factor (Wang et al., 2005).

Strikingly, heritability of ambulatory values of RSA tends to be higher than the values at rest. Three studies that measured pvRSA, HF, or BRS at rest and during a series of mental stressors reported increased genetic variance in these measures under stress (de Geus et al., 2007; Riese et al., 2006; Wang et al., 2009). This suggests that genetic influences on cardiac vagal activity become more pronounced when the subject is challenged by mentally and emotionally "engaging" conditions.

Table 1.3. Studies reporting heritability of cardiac parasympathetic nervous system activity.

Reference	Number of subjects	Age	Sex	Protocol	Measure	Heritability (%)	Best fitting model
Boomsma, et al., 1990	MZ = 140 DZ = 180	16.7	m/f	Laboratory (8-minute rest - quiet sitting)	pvRSA	25	AE
Snieder, et al., 1997	MZ = 182 DZ = 234	44.2	m/f	Laboratory (8-minute rest - quiet sitting)	pvRSA	31	AE
Busjahn et al., 1998	MZ = 190 DZ = 92	33		Ambulatory 30-minute recording	SDNN	60	ADE
					RMSSD	65	ADE
					HF	39	AE
Tank et al., 2001	MZ = 176 DZ =122	33	m/f	Laboratory (10-minute rest – semi-supine)	BRS	43	AE
Kupper et al., 2004	MZ = 218 DZ = 301 Sibs = 253	31	m/f	Ambulatory 24-hour recording, analysis restricted to sitting activities and sleep	SDNN SDNN SDNN SDNN	35 (morning) 36 (afternoon) 47 (evening) 43 (night)	AE AE AE AE
					RMSSD RMSSD RMSSD RMSSD	41 (morning) 48 (afternoon) 48 (evening) 40 (night)	AE AE AE AE

Reference	Number of subjects	Age	Sex	Protocol	Measure	Heritability (%)	Best fitting model
Kupper et al., 2005	MZ = 222 DZ = 305 Sibs = 253	31	m/f	Ambulatory 24-hour recording, analysis restricted to sitting activities and sleep	pvRSA pvRSA pvRSA pvRSA	40 (morning) 49 (afternoon) 55 (evening) 54 (night)	AE AE AE AE
Wang et al., 2005	MZ = 104 DZ = 102	15.2	m/f	Laboratory (10-minute rest – supine)	SDNN	66	AE
					RMSSD	71	AE
					HF	63	AE
					LF	45	AE
de Geus et al., 2007	Re-analysis of Boomsma 1990 dataset	16.7	m/f	Laboratory (8-minute rest - quiet sitting)	pvRSA	31	AE
				Laboratory (mental stress)	pvRSA	54	AE
				Laboratory (reactivity)	pVRSA	ns	
	Re-analysis of Snieder 1997 dataset	44.2	m/f	Laboratory (3-minute rest - quiet sitting)	pvRSA	32	AE
						44	AE
				Laboratory (mental stress)	pvRSA		
				Laboratory (reactivity)	pvRSA	ns	
Riese et al., 2006	MZ =148 DZ = 102	23.2	f	Laboratory (5-minute rest - quiet sitting)	BRS	22	AE
				Laboratory (mental stress)	BRS	42	AE
Riese et al., 2007	MZ = 115 DZ = 91	22.8	f	Laboratory (rest + stress in a single latent factor)	BRS	53	AE
					TP	51	AE
Zhang et al., 2007	MZ = 336 DZ = 106	15-84	m/f	Laboratory (5-minute rest – sitting)	BRS	55	AE
					TP	23	AE
Uusitalo et al., 2007	MZ = 208 (104 chronic diseased) DZ = 296 (173 chronic diseased)	51.5	m	Laboratory (5-minute rest – supine)	RMSSD	36	ADE
					LF	28	AE
					HF	37	ADE

Reference	Number of subjects	Age	Sex	Protocol	Measure	Heritability (%)	Best fitting model
Wang et al., 2009	MZ = 282 DZ = 372 Singleton twins = 81	17.8	m/f	Laboratory (15-minute rest – supine)	RMSSD	48	AE
				Laboratory (mental stress)	RMSSD	58	AE
				Laboratory (reactivity)	RMSSD	18	AE
				Laboratory (15 minute rest – supine)	HF	50	AE
				Laboratory (mental stress)	HF	58	AE
				Laboratory (reactivity)	HF	49	AE
Su et al., 2010	198 MZ = 121 DZ = 77	55.0	m	Ambulatory 24-hour recording, physical activity restricted to light walking	TP	63	AE
					ULF	59	AE
					VLF	57	AE
					LF	43	AE
					HF	56	AE
Tuvblad et al., 2010	MZ = 482 DZ = 361	9.6	m/f	Laboratory (3-minute rest – quiet sitting)	pvRSA	39	AE
Osztovits et al., 2011	MZ = 126 DZ = 76	44.3	m/f	Laboratory (15-minute rest – supine)	RMSSD	ns	CE
					pNN50	ns	CE
					LF	ns	CE
					HF	ns	CE
					BRS	ns	CE
Hightower et al., 2013	MZ = 350 DZ = 198	38.6	m/f	Laboratory (5-minute rest – sitting)	BRS	25/37 [a]	-

MZ, monozygotic; DZ, dizygotic, Sibs, singleton siblings of the twins added in an extended twin design.
[a]Different estimate for upward/downward deflection

Heritability of Measures of Sympathetic Nervous System Activity

Table 1.4 summarizes the twin studies that have estimated the heritability of (putative) measures of SNS activity. The studies on plasma and urinary norepinephrine levels reported heritability estimates of 42 to 70 percent for plasma and 46 to 76 percent for urinary levels. For epinephrine, heritability estimates of 64 to 74 percent were found for plasma and 65 to 78 percent for urinary levels (Jedrusik et al., 2004; Rao et al., 2007; Williams, Puddey, Beilin, & Vandongen, 1993; Hightower et al., 2013).

For electrodermal lability, heritability estimates between 30 and 43 percent have been found (Crider et al., 2004; Lykken, Iacono, Haroian, McGue, & Bouchard, Jr., 1988), and, for skin conductance level, heritability was 26 percent in males and 34 percent in females (Tuvblad et al., 2010). An echocardiographic study computing the sibling correlations in the ejection fraction estimated the familial variance in left ventricular contractility at 40 percent (Fox et al., 2010). These familial factors appear to be of a genetic nature as shown by a more recent twin study estimating the heritability of contractility at 40 percent (Hightower et al., 2013). In our own studies in the Netherlands Twin Registry, we used the PEP to index left ventricular contractility (de Geus et al., 2007; Kupper, Willemsen, Boomsma, & de Geus, 2006). We found substantial heritability of the PEP in laboratory and ambulatory settings, which varied from 48 percent in nighttime recordings to 74 percent during mental stress. In an adolescent sample, PEP reactivity to a mental stressor was also heritable (54 percent). This converges with the finding of significant genetic contribution to the stress reactivity of heart rate and blood pressure, the variables that strongly depend on cardiac SNS reactivity. Mental stress-induced increases in heart rate and systolic and diastolic blood pressure ranged between 26 percent and 43 percent (Wu, Snieder, & de Geus, 2010), respectively.

In the middle-aged groups, but not in the adolescents, the DOS correlation for PEP was lower than the correlation in same-sex DZ pairs (de Geus et al., 2007; Kupper et al., 2006). This indicates that different genes play a role in individual differences in cardiac sympathetic activity in adult men and women. The most likely explanation for this sex difference is an interaction between adrenoceptor signaling and the male and female sex hormones. Several studies have shown the presence of such interaction. Testosterone regulates gene expression of the major calcium regulatory proteins in isolated ventricular myocytes (Golden, Marsh, & Jiang, 2004; Golden, Marsh, Jiang, & Moulden, 2005). A role for female sex hormones is supported by several studies showing that estrogen inhibits β_1-adrenergic receptor activation on the heart (Thawornkaiwong, Preawnim, & Wattanapermpool, 2003).

Table 1.4. Studies reporting heritability of measures of sympathetic nervous system activity.

Authors	Number of subjects	Age	Sex	Protocol	Measure	Heritability (%)	Best fitting model
Williams et al., 1993	MZ + DZ = 196	17–65	m/f	Venipuncture in supine rest	Plasma [NE]	57	AE
					Plasma [E]	74/64[a]	AE
Crider et al., 2004	MZ = 325 DZ = 291	47.8	m	4 + 3 minutes rest – quiet sitting	nsSCR	43	AE
Jedrusik et al., 2004	MZ = 78 DZ = 74	34.5	m/f	Venipuncture in rest	Plasma [NE]	42	AE
					Urinary [NE]	76	AE
					Plasma [E]	69	AE
					Urinary [E]	65	AE
O' Connor group (data re-published in multiple papers; here (Rao et al., 2007) was used	MZ = 238 DZ = 106	15–84	m/f	Venipuncture in rest	Plasma [NE]	70	AE
					Urinary [NE]	46	AE
					Plasma [E]	67	AE
					Urinary [E]	68	AE
Wang et al., 2005	MZ = 104 DZ = 102	15.2	m/f	Laboratory (10-minute rest – supine)	LF /HF	32	AE
Kupper et al., 2006	MZ = 218 DZ = 301 Sibs = 253	31	m/f	24 hour recording, analysis restricted to sitting activities & sleep	PEP	62 (morning)	AE AE
					PEP	62 (afternoon)	AE AE
					PEP	55 (evening)	
					PEP	48 (sleep)	
de Geus et al., 2007	MZ = 140 DZ = 180	16.7	m/f	Laboratory (8 minute rest - quiet sitting)	PEP	70	AE
				Laboratory (mental stress)	PEP	74	AE
				Laboratory (reactivity)	PEP	54	AE

Authors	Number of subjects	Age	Sex	Protocol	Measure	Heritability (%)	Best fitting model
de Geus et al., 2007	MZ = 182 DZ = 234	44.2	m/f	Laboratory (3 minute rest - quiet sitting)	PEP	64	AE
				Laboratory (mental stress)	PEP	56	AE
				Laboratory (reactivity)	PEP	ns	
Uusitalo et al., 2007	MZ = 208 (104 chronic diseased) DZ = 296 (173 chronic diseased)	51.5	m	Laboratory (5-minute rest – supine)	LF/HF	28	AE
Tuvblad et al., 2010	MZ = 512 DZ = 484	9.6	m/f	Laboratory (3 minute rest – quiet sitting)	SCL	26/34[a]	AE
					nsSCR	30	AE
Bosker et al., (2012)	MZ = 94 DZ = 74 Singleton twins = 24	18	m/f	Overnight urine	Urinary [NE]	68	AE
					Urinary [E]	74	
Hightower et al., 2013	MZ = 350 DZ = 198	38.6	m/f	Venipuncture in rest	Plasma [NE]	65	-
					Urinary [NE]	49	-
					Plasma [E]	67	-
					Urinary [E]	78	-
				Laboratory (5-minute rest – sitting)	LV contractility (dP/dT max)	40	-
					Stroke volume	73	-
					Stroke volume index	58	-
					Systemic vascular compliance	53	-
					Systemic vascular resistance (SVR)	57	-

Authors	Number of subjects	Age	Sex	Protocol	Measure	Heritability (%)	Best fitting model
Wu, Treiber, & Snieder, 2013	MZ = 540 DZ = 522	14.7	m/f	Laboratory (15-min rest – supine)	Stroke volume (SV)	51/58[a]	AE
					SVR Index	49/44[a]	AE
				Laboratory (15-min mental stress)	SV	53/58[a]	AE
					SVR Index	55/39[a]	AE
				Laboratory (reactivity)	SV	ns/6[a]	
					SVR Index	8/10[a]	AE
Noh et al., 2015	MZ = 596 DZ = 124 Sibs = 567 Parents = 354	42.5 (parents = 65.7)	m/f	Laboratory echocardiography	Left Ventricular Ejection Fraction	27	-

NE, norepinephrine; E, epinephrine; MZ, monozygotic; DZ, dizygotic; Sibs, singleton siblings of the twins added in an extended twin design.
[a]Different estimate in males/females

Which Genetic Variants Cause the Heritability of SNS and PNS Activity?

Although twin studies show that individual differences in basal ANS activity and its responses to stress are significantly influenced by genetic factors, these factors are modeled as latent factors, and the actual gene networks that harbor the DNA variants underlying the heritability of the ANS are left unspecified. Identification of these genes and their functional variants is an important next step because they could help elucidate the biological pathways through which ANS activity contributes to CVD risk.

One of the main gene finding strategies is a candidate gene association study. Such a study tests whether a particular variant in a candidate gene and a trait co-occur above chance level, given the frequency of the variant and the distribution of the trait in the population (McCaffery, Snieder, Dong, & de, 2007). The selection of genes is often based on the known biological pathways involved in the trait of interest. For the PNS, genes involved in biosynthesis, transport, and breakdown of ACh seem obvious candidates, as are the genes for the muscarinic receptors. For the SNS, likely candidates are genes controlling catecholamine synthesis and metabolism, neuronal norepinephrine reuptake, and adrenergic receptor function. The variants to be typed within the candidate gene – for example, single nucleotide polymorphisms (SNPs), repeat polymorphisms, or insertion/deletion polymorphisms – are prioritized by their location within coding,

promoter, or splice regions, or, if known, their functional effects on the gene product or on gene expression.

Table 1.5 presents a sample of the genetic variants that have been found, at least once, to be significantly associated with an ANS measure in human subjects (Adam et al., 2014; Baccarelli et al., 2008; Beetz et al. (2009); Boccardi et al., 2010; Busjahn et al., 1998; Chang, Chang, Chen, Fang, & Huang, 2014; Chang, Fang, Chang, Chen, & Huang, 2014; Huntgeburth et al., 2011; Ellis, Beevers, Hixon, & McGeary, 2011; Kurnik et al., 2007; Masuo et al., 2005; Matsunaga et al., 2005; Matsunaga et al., 2007; Matsunaga et al., 2009; Matsunaga et al., 2010; Milan et al., 2005; Nagai, Sakane, Tsuzaki, & Moritani, 2011; Neumann, Lawrence, Jennings, Ferrell, & Manuck, 2005; Neumann et al., 2006; Neumann et al., 2009; Neumeister et al., 2005; Newton-Cheh et al., 2007; Nishikino et al., 2006; Park et al., 2006; Probst-Hensch et al., 2008; Rao et al., 2008; Seppala et al., 2014; Shihara et al., 1999; Shihara et al., 2001; Stolarz et al., 2004; Su et al., 2009a; Suzuki et al., 2003; Thayer et al., 2003; Tingley et al., 2007; Viola, James, Archer, & Dijk, 2008; Yang et al., 2010; Yasuda et al., 2004; Ylitalo et al., 2000; Zhang et al., 2010). Such a list is, by its nature, unfinished, because ongoing progress in the understanding of the biology of the ANS will increase the list, whereas failure to replicate typically does not lead to removal of the gene as a "candidate" (because it still meets the criterion "associated at least once"). If the more rigorous demands were made that (1) an association needs to be detected in a sample that was a priori sufficiently powered and (2) the association needs to be replicated exactly (same SNP, same variant, same direction of effect, same ANS variable) in independent samples (Sullivan, 2007), then none of the candidates on the list would survive. This is probably too rigid, and at least two genes are very likely to be truly involved in ANS activity based on experimental confirmation in animal studies; namely, the genes encoding the G-protein-regulated phosducin and the dual-specific A kinase-anchoring protein 2 (Beetz et al., 2009; Tingley et al., 2007, respectively).

Mice with a targeted deletion of Pdc displayed elevated catecholamine turnover, and their postganglionic sympathetic neurons showed prolonged action potential firing after stimulation with Ach and increased firing frequencies during membrane depolarization (Beetz et al., 2009). Interestingly, Pdc –/– mice displayed exaggerated increases in blood pressure in response to stress, and in two different human populations several SNPs in the *PDC* gene were associated with stress-induced blood pressure reactivity. These findings suggest that phosducin is an important modulator of sympathetic activity. For the PNS, subjects with a variant in the dual-specific A kinase-anchoring protein 2 (*AKAP10*) gene

were found to have lower SDNN in a small patient sample (Tingley et al., 2007). In a much larger sample of healthy middle-aged adults, the same variant was again found to be associated with SDNN and also with RMSSD. The importance of this gene for cardiac vagal activity was further confirmed by experimental disruption of the gene in mouse embryonic stem cells that were differentiated into cardiac cells (Tingley et al., 2007). Mutation of mouse *AKAP10* increased the sensitivity of cultured cardiac cells to cholinergic signals, and this result was reproduced in living mice.

In a very recent study by Riese et al. (2014) data of seven cohorts were used in a large candidate gene study including 443 genotyped and imputed common genetic variants in eight key genes (CHAT, SLCI8A3, SLC5A7, CHRNB4, CHRNA3, CHRNA, and ACHE) of the acetylcholinergic pathway. It was tested whether these variants were associated with the RMSSD. Results showed that none of the variants were significantly associated with RMSSD after correction for multiple testing.

A fair summary of the current state of the art is that the yield of the candidate gene approach has been modest. This may reflect a general disadvantage of an approach that capitalizes entirely on existing biological disease models. Many of the hundreds or thousands of genes relevant to the disease may not have been properly annotated, or they may reside in pathways that have not been linked to the disease before. Increasingly, gene finding attempts in large-scale samples have used the more agnostic strategy of a genome-wide association study (GWAS) that makes no a priori assumptions on the biological pathways involved (Manolio et al., 2009). Genome-wide association studies exploit the availability of the human genome sequence and the rapid development across the past decades of affordable, high-accuracy, high-throughput technologies to genotype from SNPs scattered across the entire genome. Although a single subject will deviate in around 4 million SNPs from the major variant in the base population, a subset of 500,000 to 1 million well-chosen tagging SNPs can capture the whole genome of that subject. Single-nucleotide polymorphisms that lie in proximity on the same chromosome are often transmitted together across generations as a block of SNP genotypes (also called a *haplotype block*). Recombination among the SNPs in the haplotype block is infrequent, and combining the tagSNPs with the detailed inventory of the haplotype structure of the world's main populations in the HapMap/1000 genomes projects allows the imputation of the unmeasured SNPs with high precision. The final set of (imputed) SNPs can be used to test genetic association to ANS parameters on a genome-wide scale.

At the time of writing, two GWA studies had been published that focused specifically on

the ANS (Fox et al., 2013; Newton-Cheh et al., 2007). In the Framingham Heart Study, 548 subjects were typed on the Affymetrix 100K SNP chip (Newton-Cheh et al., 2007). No genome-wide association test results for SDNN/TP nor for the LF/HF ratio met the stern *p*-value criterion of $5 \times 10^{e-8}$ commonly applied in GWAS to deal with the huge multiple testing burden when half a million SNPs are tested. Taken the current experience of the GWA field, this study was a priori underpowered to detect a significant effect of a single SNP, which will typically contribute no more than 0.1–0.01 percent to the genetic variance in the trait. To detect significant SNP contribution in GWAS, analyses on tens to hundreds of thousands of subjects are needed for which whole-genome SNP data and ANS traits are available. No single research group can mount such numbers, and large GWA consortia have been formed worldwide that exchange their association results in an across-study meta-analysis. These meta-analyses have been hugely successful (http://www.genome.gov/gwastudies/) for many traits, including cardiovascular risk factors (Demirkan et al., 2011; Dehghan et al., 2011; Eijgelsheim et al., 2010; Dupuis et al., 2010; Wain et al., 2011).

For this reason, Fox et al. (2013) joined data of nine cohorts on several variables related to cardiac structure and systolic function, including ejection fraction, as measured by fractional shortening on two-dimensional echocardiography. A genome-wide significant hit on chromosome 13 for rs9530176 in *KLF5* was found. This locus contains two genes that are associated with left ventricular systolic dysfunction and ejection fraction, Chromogranin B (*CHGB*) and forkhead box A2 (*FOXA2*), respectively. *CHGB* is involved in catecholamine secretion and may contribute to beta-adrenergic effects on contractility. *FOXA2* controls multiple genes implicated in metabolism-secretion coupling of glucose-induced insulin release and hyperinsulinemia may ultimately affect ejection fraction because of an increase in mitogen-activated protein kinase activity that influences myocyte hypertrophy (Fox et al., 2013).

Table 1.5. Studies reporting association of candidate genes with measures of autonomic nervous system activity.

Reference	ANS measure	Genetic variant	Finding	Remarks
Busjahn et al., 1998	HF RMSSD SDNN	Angiotensin-converting enzyme (*ACE*)	The DD variant of an insertion deletion polymorphism was associated with increased RSA at rest.	Association opposite to that found in African Americans by Thayer et al.
Ylitalo et al., 2000	BRS	Aldosterone synthase gene (*CYP11B2*)	BRS became incrementally lower with more C-alleles in the promoter C-344T SNP.	Findings were stronger in women than in men.
Shihara et al., 2001	VLF	Uncoupling protein 1 (*UCP1*) β3 adrenergic receptor (*β3AR*)	GG homozygotes of the *UCP1* promoter A-3826G SNP had lower VLF at supine rest.	The inhibitory effect of *UCP1* on VLF was observed only with occurrence of the Trp64Arg variant of the *β3AR* gene.
Suzuki et al., 2003	LF VLF	α-adrenergic receptor type 2B (*ADRA2B*)	Short/Short homozygotes of a three-amino acid deletion polymorphism had significantly greater LF and VLF than Long/Long homozygotes.	
Thayer et al., 2003	HF	Angiotensin-converting enzyme (*ACE*)	The II variant of an insertion deletion polymorphism was associated with increased RSA at rest.	Association opposite to that found in European Americans by Busjahn et al.
Stolarz et al., 2004	LF/HF LF HF	Aldosterone synthase (*CYP11B2*)	Supine LF and LF/HF increased whereas HF decreased with the number of T-alleles at the *CYP11B2* C-344T promoter SNP.	Association only seen in subjects with sodium excretion > 190 mmol.
Stolarz et al., 2004	LF/HF LF HF	Type-1-angiotensin receptor (*AT1R*)	Orthostatic changes in LF, HF, and LF/HF were blunted in C-allele carriers of the *AT1R* A1166C SNP.	Association only seen in subjects with sodium excretion > 190 mmol.
Yasuda et al., 2004	HF	α-Subunit Gs-protein (*GNAS1*)	T-allele carriers at the *GNAS1* T393C SNP had lower supine HF, but not standing HF.	
Zhang et al., 2004	Plasma [E] Urinary [NE]	Tyrosine Hydroxylase (*TH*)	Increased number of the (TCAT)10i allele of a repeat polymorphism was associated with higher levels of plasma E and urinary NE.	

Reference	ANS measure	Genetic variant	Finding	Remarks
Matsunaga et al., 2005	LF/HF	G-Protein β_3 Subunit (*GNB3*)	CC homozygotes for the *GNB3* C825T SNP had lower LF/HF and higher HF/TP ratio's than CT/TT genotypes while standing.	
Milan et al., 2005	BRS	Bradykinin β_2 receptor gene (*B2R*)	The BRS increased with the number of T-alleles at the *B2R* C258T SNP.	B2R genotype was a strong independent predictor of BRS, accounting for 12% of its variation.
Neumann et al., 2005, 2006.	HF LF LF/HF	Acetylcholine transporter (*CHT1*, *SLC5A7*)	G-allele homozygotes for a *CHT1* non-coding 3′ UTR SNP (rs333229) had lower HF power and higher LF power and higher LF/HF ratio.	Same SNP is also associated to depressed mood and brain activity in regions of the "central autonomic network."
Neumeister et al., 2005	Total body NE spillover	α-Adrenergic receptor type 2C (*ADRA2C*)	At rest, homozygotes for the Del322-325 polymorphism had higher total body NE spillover than heterozygotes or noncarriers.	The same In-frame deletion of *ADRA2C* increases the risk of congestive heart failure.
Kurnik et al., 2006	plasma [NE]	α-Adrenergic receptor type 2A (*ADRA2A*)	Two uncommon variants (G>C at -1903 and C>G at -1607, identified in 3 black subjects) were associated with higher plasma NE levels.	
Nishikino et al.,2006	LF	Type-1-angiotensin receptor (*AT1R*)	Higher LF in C-allele carriers of the *AT1R* A1166C SNP.	
Park et al., 2006	HF	Hemochromatosis gene (*HFE*)	C282Y(rs1800562) and H63D (rs1799945) major alleles were associated with lower HF.	Association was conditional on high level of particulate air pollution.
Matsunaga et al., 2007	HF LF/HF LF	β-Adrenergic receptor type 1 (*ADRB1*) β-Adrenergic receptor type 2 (*ADRB2*)	At supine rest, *ADRB1* Arg16 homozygotes had lower LF/HF and higher HF than the Gly16 allele carriers. *ADRB2* Glu27 allele carriers had higher LF than Gln27 homozygotes.	
Newton-Cheh et al., 2007	SDNN	α-Adrenergic receptor type 1A (*ADRA1A*)	6 SNPs in the gene reached significance at classical $p < .05$	As the SNPs were part of a genome-wide association scan, the significance level may have been too liberal.

Reference	ANS measure	Genetic variant	Finding	Remarks
	SDNN/TP	α-Adrenergic receptor type 1B (*ADRA1B*)	1 SNP(rs2195926) in the gene reached significance at classical $p < .05$	As the SNP was part of a genome-wide association scan, the significance level may have been too liberal.
	TP	α-Adrenergic receptor type 2B (*ADRA2B*)	1 SNP(rs9325124) in the gene reached significance at classical $p < .05$	As the SNP was part of a genome-wide association scan, the significance level may have been too liberal.
Tingley et al., 2007	SDNN	Alpha-kinase anchoring protein 10 (*AKAP10*)	Homozygotes for the G-allele (Val) at the *AKAP10* I646V SNP (rs203462) have lower SDNN.	In mice *AKAP10* was shown to modulate the sensitivity of cardiac cells to cholinergic stimulation.
Baccarelli et al., 2008	SDNN	Methylene-tetrahydrofolate reductase (*MTHFR*)	T-allele carriers of the *MTHFR* C677T SNP (rs1801133) had lower SDNN than CC homozygotes.	
Rao et al., 2008	plasma [NE]	Tyrosine hydroxylase (*TH*)	TH promoter haplotype #2 (TGGG) increased NE excretion during stress.	
Beetz et al., 2009	Plasma [NE]	G-protein regulated phosducin (*PDC*)	Compared with Pdc +/+ mice, Pdc -/- mice showed elevated catecholamine turnover, their post-ganglionic neurons showed prolonged action potential firing after stimulation with Ach and increased firing frequencies during membrane depolarisation	Pdc -/- mice also showed greater increases in BP in response to stress compared with Pdc +/+ mice. And in two different human populations, several SNPs in the PDC gene were associated with stress-induced BP reactivity
Matsunaga et al., 2009	HF LF/HF	Uncoupling proteins 2 & 3 (*UCP2*, *UCP3*)	At supine rest the II variant of the UCP2 45-bp insertion/deletion polymorphism was associated higher LF/HF ratio. Carriers of the T-allele of the *UCP3* S55 C/T SNP had lower LF/HF ratio and higher HF than CC homozygotes.	The II variant of the UCP2 insertion/deletion polymorphism was also associated with higher blood pressure.

Reference	ANS measure	Genetic variant	Finding	Remarks
Neumann et al., 2009	SDNN RMSSD	Alpha-kinase anchoring protein 10 (*AKAP10*)	G-allele (Val) carriers at the *AKAP10* I646V SNP (rs203462) have lower SDNN and RMSSD.	SDNN exactly replicates Tingley et al., 2007
Rana et al., 2009	plasma [E]	Nicotinic acetylcholine receptor (*CHRNA*)	*CHRNA3* K95K (G) allele (rs3743075) was associated with higher E levels.	
Su et al., 2009a	ULF	C-reactive protein (*CRP*)	Subjects homozygous for the T-allele at *CRP* SNP rs1205 had higher ULF than CC homozygotes.	The SNP was significantly associated with both CRP and ULF and explained 11% of their genetic covariance.
Viola et al., 2009	SDNN LFnu	Circadian clock gene PERIOD3 (*PER3*)	Homozygotes for the longer allele (5/5) in a variable number tandem repeat polymorphism in the coding region of *PER3* had higher LFnu and lower SDNN compared to 4/4 homozygotes, in particular during baseline sleep.	The *PER3* VNTR polymorphism also affected slow wave sleep duration.
Boccardi et al., 2010		Transcription factor 7-like 2 (*TCF7L2*)	TT homozygotes of a *TCF7L2* G/T SNP (rs12233572) had higher LF/HF ratio during glucose ingestion compared to heterozygotes and CC homozygotes.	No effects of the *TCF7L2* SNP were found on baseline LF/HF.
Ellis et al., 2010	HF	Serotonin transporter (5-HTT) repeat polymorphism	The short variant of the 5-HTTLPR was associated with lower HF at rest.	
Matsunaga et al., 2010	HF LF VLF TP	Estrogen receptor α gene (*ESR1*)	Haplotype analysis based on the PvuII and XbaI polymorphisms showed that *ESR1* haplotype 2 (PvuII C allele and XbaI A allele) had lower TP, VLF, LF, and HF at supine rest.	Carriers of the ESR1 haplotype 2 also had a higher systolic and mean arterial blood pressure.
Probst-Hensch et al., 2010	TP LF	Glutathione S-transferase (*GST*)	Participants missing both copies of the *GSTT1* gene had lower TP and LF.	
Yang et al., 2010	HF RMSSD LF/HF	Brain-derived neurotrophic factor (*BDNF*)	Met/Met homozygotes at the *BDNF* Val66Met polymorphism had lower HF, RMSSD and higher LF/HF ratio compared to the Val/Val homozygotes.	

Reference	ANS measure	Genetic variant	Finding	Remarks
Hunt-geburth et al., 2011	Fractional shortening (FS) Rate-corrected velocity of circum-ferential fiber shortening (Vcfc)	Arg389Gly β1-adrenoreceptor gene polymorphism	Homozygous Arg389 carriers showed significant higher FS at baseline. In response to dobutamine treatment both FS and Vcfc were higher in the homozygous Arg389 carriers. Pre-treatment with metropolol decreased the levels of the Arg/Arg carriers to the levels of the Gly-carriers.	In Arg/Gly or Gly/Gly participants, modest effects of dobutamine-induced increase in FS only at low not at higher concentrations of dobutamine was found after pre-treatment with metropolol
Nagai et al., 2011	VLF LF HF TP	Uncoupling protein 1 (*UCP1*)-3826 A/G	G/G carriers showed lower VLF	
Adam et al., 2014	SDNN TP ULF VLF LF HF LF/HF	Functional IL-6-174 G/C polymorphism	Compared with IL6-174 GG genotypes, participants with one or no G-risk allele showed higher SDNN, TP, and lower LF/HF.	An inverse association between traffic-related particulate matter exposure and the IL6-174 G/C on change in HRV was found with higher exposure being related to lower SDNN and LF for -174 GG genotypes.
Chang et al., 2014	RMSSD TP VLF LF HF LF/HF	*BDNF*	Males bearing the Val/Val genotype had higher LF and LF/HF ratio, compared to Met/Met homozygotes at the BDNFVal66Met polymorphism.	Females showed the opposite association, but nonsignificant
Chang et al., 2014	HF RMSSD	Functional nerve growth factor (*NGF*) polymorphism	Males with any T-allele showed lower HF and RMSSD compared to men with the C/C genotype.	Females showed the opposite association, but nonsignificant
Seppälä et al., 2014	LF HF LF/HF	Alanine-glyoxylate aminotransferase 2 (*AGXT2*)	Both rs37369 and rs16899974 were associated with LF/HF and rs16899974 was additionally associated with the HF component alone.	This finding was part of a GWAS in which symmetric dimethylarginine (SDMA) levels were significantly associated with both *AGXT2* variants.

NE, norepinephrine; E, epinephrine

Conclusion

Although the molecular genetics of ANS activity remains to be elucidated in full, twin studies demonstrate significant genetic contributions to the main indices of the SNS and PNS. For the main indicator of cardiac vagal activity, RSA, heritability estimates range between 25 and 71 percent and for a second indicator, BRS, heritability estimates range between 22 and 53 percent. For the SNS, diverse indicators of activity to different organs converge on an even more substantial contribution of genetic factors. Heritability of plasma and urinary norepinephrine varied between 42 and 76 percent, heritability of epinephrine levels between 64 and 74 percent, heritability of nsSCR/SCL between 26 and 43 percent, and heritability of PEP between 48 and 74 percent.

This thesis

A weakness of the research conducted so far is that the majority of studies on the genetics of ANS activity have focused on laboratory recordings. Laboratory studies generally involve the measurement of ANS activity 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 have been instrumental in establishing the existence of stable individual differences in basal ANS activity and its responses to stress. However, these individual differences in ANS (re)activity may not transfer well to actual real-life situations because the association between laboratory and ambulatory measurements has been shown to be moderate at best (Gerin, Rosofsky, Pieper, & Pickering, 1994; Johnston, Tuomisto, & Patching, 2008; Kamarck, Debski, & Manuck, 2000; Kamarck & Lovallo, 2003; van Doornen, Knol, Willemsen, & de Geus, 1994). It is possible that the psychological and physiological processes induced by laboratory conditions are only a poor reflection of the actual processes in everyday real-life situations. Perhaps as a consequence, the predictive value of cardiovascular reactivity 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 to perform 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 amply demonstrated (Mallion, Baguet, Siche, Tremel, & De, 1999; Palatini & Julius, 2004; Verdecchia, 2000; Verdecchia, Schillaci, Reboldi, Franklin, & Porcellati, 2001).

For the research project described in this thesis, an already existing dataset of 816 adult participants with 24-h ANS activity measurements collected between August 1998 and June 2003 (ANS Study 1, [Kupper et al., 2006; Kupper et al., 2005]) was further expanded with a new data collection round in 592 adult participants between November 2010 and June 2012 (ANS Study 2). This led to a unique dataset large enough to allow extensive genetic analyses. Data from these two studies was used to investigate the role of genetic and environmental factors in individual differences in ambulatory levels and reactivity (calculated as the absolute difference between ambulatory rest and stress levels) of the two main indicators of ANS activity, PEP and RSA. Between these two ANS studies, the NTR undertook a large biobank study, assessing biodata from blood samples in over 9000 twins and their family members between January 2004 and July 2008 (Willemsen et al., 2010). Data of all three studies was combined to investigate the prospective bi-directional association between ambulatory PEP/RSA level and reactivity and pro-inflammatory and metabolic risk factors. In chapter 2, an overview of the data and variables that were used for the current research project is provided. The main focus is on the data collection procedures for ANS Study 2, as this data was collected within the current PhD project.

In the research described in chapter 3, data of the two large 24-h ambulatory ANS studies was combined to explore heritability of the three most commonly used HRV measures in the largest ambulatory dataset to date. Because previous research has shown that ceiling effects may strongly impact HRV at low heart rates - a result that was recently confirmed by our group (van Lien et al., 2011), a secondary goal of this research was to test whether these effects confounded heritability estimates, at different levels of physical activity.

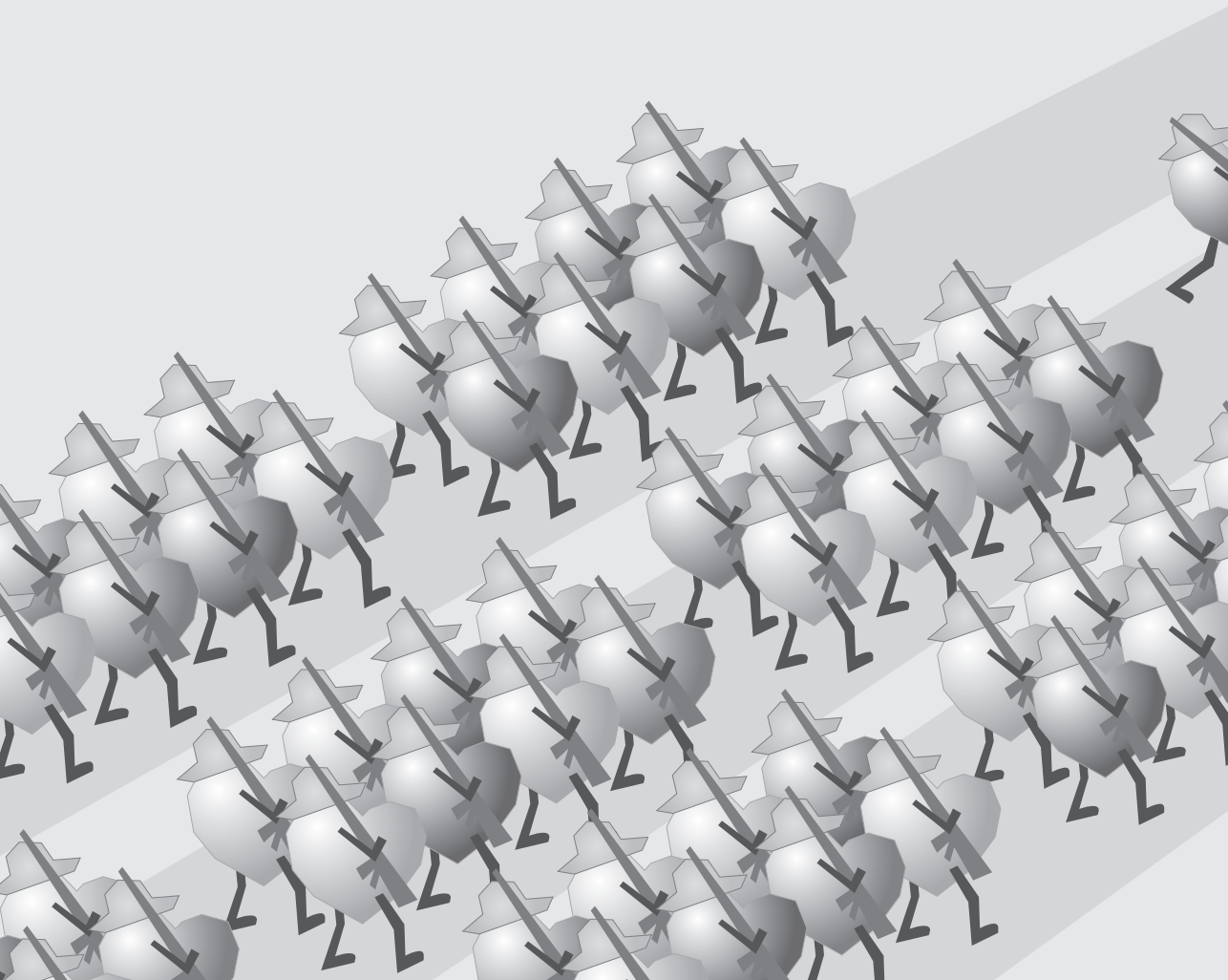
Chapter 4 focuses on the quantification of ambulatory ANS reactivity to real-life situations. ANS activity during real life stress is typically measured during a concrete event or stressor taking place in the daily life of the subject. In this chapter, we systematically

explored the possibilities of capturing real life stress in unstructured settings without preset (stressful) events. Several resting baseline and periods of stress were quantified and the temporal stability and heritability of these measures was inventoried. We further tested whether we could replicate the increase in genetic variance when going from rest to stress, as previously observed in a laboratory setting (de Geus et al., 2007).

In chapter 5, several clinically relevant cardiac repolarization and depolarization parameters were extracted from the continuous ECG registrations that were assessed in the second 24-hour ambulatory ANS study. The reliability of this approach was inventoried by researching the heritability of these parameters and by comparing the heritability estimates to those of the same parameters obtained from a typical 10 sec resting ECG as commonly used in clinical practice. Among the repolarization parameters studied was the T-wave amplitude (TWA). Because of its alleged link to sympathetic influences on cardiac repolarization, the TWA was recently coined as a valuable addition to the PEP in characterizing cardiac sympathetic activity (van Lien, Neijts, Willemsen, & de Geus, 2015). As heart rate was a major co-determinant of TWA, we specifically tested whether the same or different genetic factors operated on repolarization at different heart rates. Furthermore, it was tested whether the same or different genetic factors were at the basis of the different repolarization parameters.

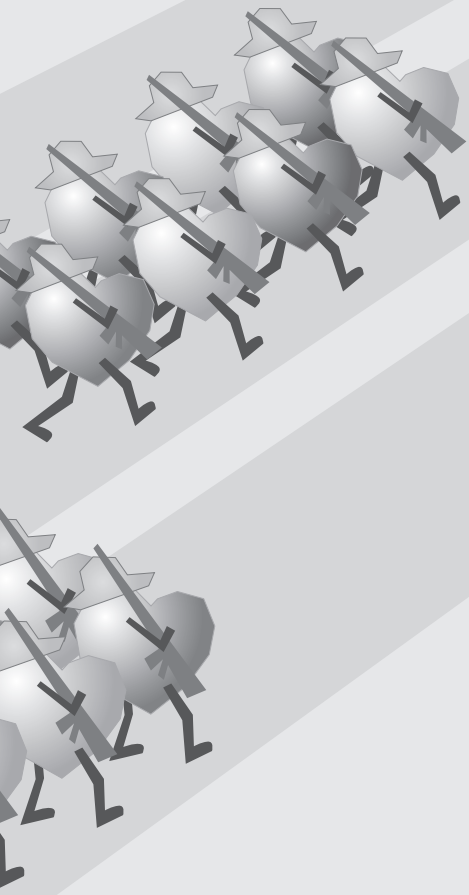
In chapter 6, focus temporarily shifted from ANS functioning to inflammation processes, more specifically to pro-inflammatory activity which forms yet another important correlate of cardiovascular disease risk. The heritability of the plasma levels of two important cytokines and two acute phase reactants was assessed in an extended twin design which included not only twins and their siblings, but also the mothers and fathers of the twins. Various sources of noise were taken into account (batch effects, oral contraception-induced or cyclic variation in estrogen, and seasonal variation) after which the heritability of these parameters was established. These data were then used in chapter 7 to research the prospective association between ambulatory ANS level and reactivity, and pro-inflammatory and metabolic risk in two independent tests. In the first test ANS functioning was linked to inflammatory and metabolic risk factors measured 4.9 years later. In the second test the reverse association was researched linking inflammatory and metabolic risk to ANS functioning measured 5.4 years later.

Chapter 8 summarizes the main results of this thesis and provides new hypotheses for future cardiovascular research regarding the causes and consequences of ANS regulation in daily life.



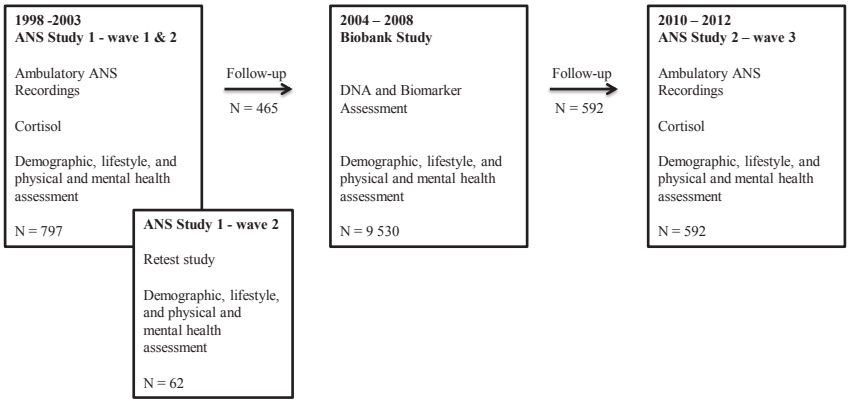
Chapter 2

Research design and data collection



All data used in this project was collected in participants who were registered with the (adult) Netherlands Twin Register (NTR). Since 1991, ongoing large-scale longitudinal data is being collected by means of surveys on health, lifestyle and personality and through experimental projects in which data on biological, psychological and/or behavioral processes are actively collected. For an overview of the data that is collected in adults by the NTR, see Willemsen et al. (2013). For this project we used data from a large biobank study conducted by the NTR and two large 24-hour ambulatory ANS studies. The first ambulatory ANS study took place between August 1998 and June 2003 (ANS Study 1) and biobank data was collected between January 2004 and July 2008. As part of this PhD project, additional ambulatory 24-hour ANS data was collected between November 2010 and June 2012 (ANS Study 2). Figure 2.1 gives an overview of these three studies. A total of 465 participants who took part in the first ambulatory ANS study, also took part in the biobank study and all participants participating in the second ambulatory ANS study, were selected based on participation in the biobank study.

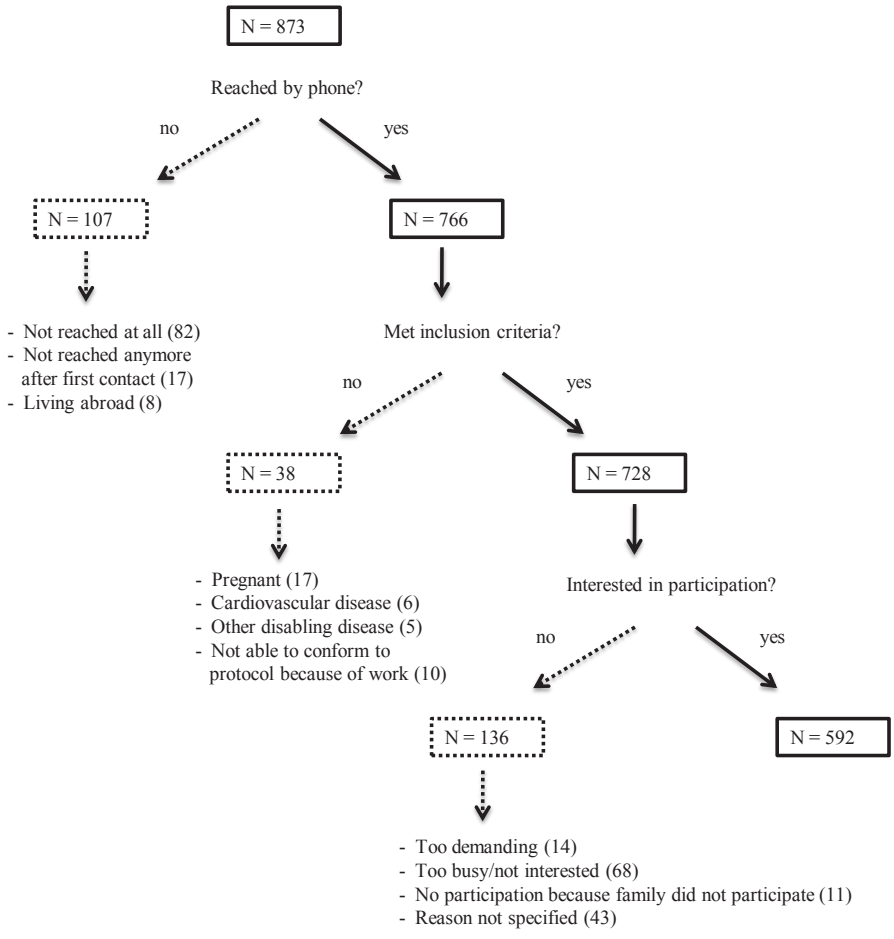
Figure 2.1. Overview of the data(structure) used for this thesis.



The study protocol and the data collection for the two ambulatory studies was essentially the same. The main differences between the first ANS data collection round and the second one, is that software and hardware improvements have been made over time. The ANS assessments in Study 2 were performed with an improved version of the VU Ambulatory Monitoring System (VU-AMS) and, compared with Study 1, the protocol of Study 2 was complemented by several (subscales of) questionnaires concerning physical health and

psychological wellbeing. We also made some changes in the protocol concerning the cortisol data collection. For more detailed information on the previous ANS study, you are referred to the dissertations of Mireille van den Berg who was involved in the first wave of data collection of Study 1 (van den Berg, 2002), Nina Kupper who was involved in the data collection of wave 2 of Study 1 (Kupper, 2005), and Annebet Goedhart who was mainly involved in the retest part of this study (Goedhart, 2008). Below, a detailed description of the research set up and protocol for the current 24-hour ambulatory ANS study is provided, followed by a brief overview of the biobank study.

Figure 2.2. Overview of the subject selection for the current ambulatory ANS recording study.



Ambulatory ANS Study 2

Participants

Healthy twins aged between 21 and 50, and non-twin siblings within an age range of three years of the twins were considered eligible. Additional requirements were that they had also participated in the biobank study and that they had not participated in the first ambulatory ANS study. Furthermore, only participants with DNA data for at least two multiples within the family were selected for participation. The resulting sample was then pre-screened for known cardiovascular disease or other disease related states that could compromise the ambulatory recordings (i.e. multiple sclerosis). This screening was based on their health status as assessed during the biobank study. A total of 873 participants that met the inclusion criteria were contacted for participation in the current ANS Study. Ultimately, 592 (81.3%) of the 728 participants who were reached by phone and who met the inclusion criteria as verified by telephone interview participated in the current study (see figure 2.2 for an overview).

Testing procedure

All participants that were eligible for participation received a letter by mail informing them about the study and providing them with background information. The invitation letter and brochure are in Appendix I. About one to two weeks after the invitations were sent, participants received a phone call and were asked if they were interested in participating in the current study. During the phone call their health status was shortly screened. A priori reasons for exclusion were pregnancy (until half a year after delivering the baby), heart transplantation, presence of a pacemaker, known ischemic heart disease or congestive heart failure or other physical complaints that could compromise the ambulatory assessment (i.e. severe illness, broken leg). When the inclusion criteria were met, an appointment was made on a representative weekday, preferably a working day. The appointment was then confirmed in a letter (Appendix II) which was accompanied by the informed consent form (Appendix III), three Salivettes, instructions for saliva collection (Appendix IV) and a survey. Included in the survey were a questionnaire on sleep quality (Pittsburgh Sleep Quality Index, Buysse, Reynolds III, Monk, Berman, & Kupfer, 1989), life events, and neuroticism and extraversion were assessed by means of the Amsterdamse Biografische Vragenlijst which is a personality questionnaire similar in content to the Eysenck Personality Questionnaire (Eysenck & Eysenck, 1975). Participants were asked

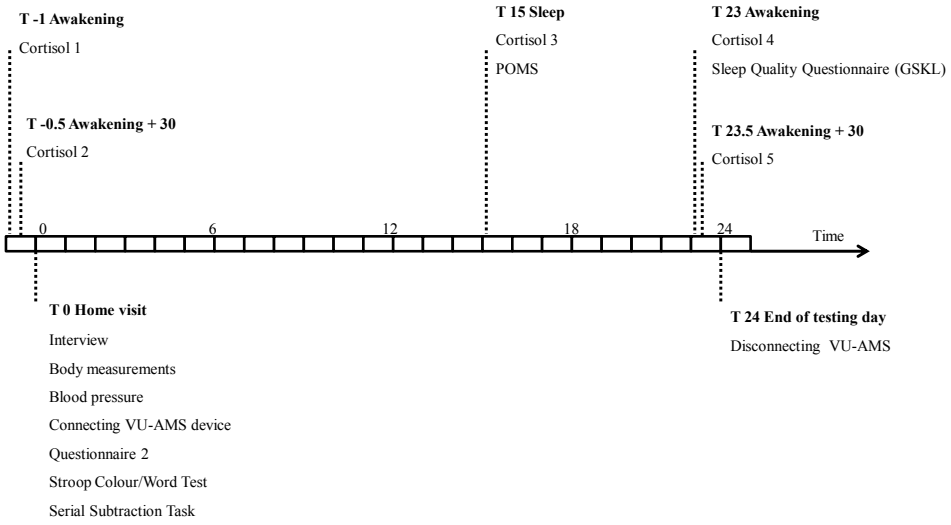
to fill out the survey the day(s) prior to the ambulatory recording. They were asked to refrain from excessive physical exertion the day prior to the ambulatory recording and on the recording day itself. On the first morning of the testing day, participants collected two fasting saliva samples; the first one was collected immediately after awakening. The second was collected 30 minutes later. They were then visited by the researcher and the research assistant in the morning, between 6 am and 12 pm at home, or when preferred at work. Figure 2.3 shows an overview of the testing day.

During the visit, the procedure was explained, the informed consent was signed and the Salivettes that were used prior to the visit were collected. The researcher then conducted an interview on socioeconomic status, lifestyle and health status, including an inventory of medication use, current health complaints and adherence to the protocol. Meanwhile participants were asked about their height, weight was measured with a calibrated scale, and waist and hip circumference were assessed using measuring tape, and consecutively the VU-AMS was attached by the research assistant (see Appendix V for the interview/protocol of the visit). After this, participants were asked to sit down in a secluded part of the house/work area, they were attached with a blood pressure monitor and asked to fill out a second survey about their perceived quality of life (Cantril, 1965), life satisfaction (Satisfaction with Life Scale, Diener, Emmons, Larsen, & Griffin, 1985), anxiety by means of the Young Adult Self Report (Verhulst, Van der Ende, & Koot, 1997) and the State Trait Anxiety Inventory (Van der Ploeg, Defares, & Spielberger, 1979), and a questionnaire assessing self-esteem (Rosenberg Self-Esteem Scale, Rosenberg, 1965). Consecutively, two 2-min cognitive performance (mental stress) tasks were completed (the Stroop Colour/Word Test and the Serial Substraction Task). Blood pressure was assessed twice during the resting period while the participant filled out the survey, and twice during the stress period, once during each task. The total protocol took about 45 minutes. Finally, participants received a diary (Appendix VI), that also included the shortened Dutch version of the Profile of Mood States (POMS) that contains 32 items (Wald & Mellenberg, 1990), and a questionnaire on their perceived sleep quality concerning the night of the ambulatory recording (GSKL, Groningse Slaap Kwaliteit Lijst Meijman, de Vries-Griever, de Vries, & Kampman, 1988). They were additionally provided with three more Salivettes, and instructions for the rest of the day, the following night and the next morning.

During waking hours, participants were asked to chronologically report about their activities, social situation, location and posture every 60-min in the diary to relate to the

continuously recorded tri-axial accelerometer and ECG signals of the VU-AMS device. At 11.30 pm, or earlier if participants wanted to go to bed earlier, participants were asked to collect one saliva sample after a 30-min period of fasting. At the end of the day, before going to bed, they were asked to fill out the POMS. The next morning, participants collected two fasting saliva samples again, the first one immediately after awakening and the second one half an hour later. They additionally filled out the GSKL. After having worn the ambulatory recording device for 24 hours, participants detached the VU-AMS themselves. The VU-AMS, the diary and the saliva samples were then sent back by mail to the VU University Amsterdam.

Figure 2.3. Overview of the testing day.



The Salivettes were stored immediately at -20˚C upon arrival at the VU. They were sent to the laboratory in Dresden for cortisol determination in two waves, once after the first year of data collection (fall 2011) and once after the second year, at the end of the data collection (fall 2012).

The ANS data was visually inspected immediately after delivery of the device by mail. The ECG signal was checked and scored, and generally within two weeks, the participants received a report on their blood pressure and their heart rate during the 24-hour ambulatory recording (see Appendix VII for an example). The respiration, RSA, and

impedance signals were scored at the end of the data collection. Methods on how the ECG and ICG signals were derived with the VU-AMS and details on how this data was scored is outlined in the chapters specifically focusing on these measures. Table 2.1 gives an overview of the variables we extracted from the 24-h ECG and ICG for this research project.

Table 2.1. The ANS variables extracted from the ECG and ICG that were used in this research project.

Measures	From	Comment
Heart rate, Heart Rate Variability (SDNN, RMSSD, HF-IBI power, LF IBI-power, VLF-IBI power, peak-valley RSA), Respiration Rate	Ambulatory ECG & respiration signal; 24-hour recording	HF, RSA, and RMSSD assess relatively pure cardiac vagal control.
Pre-ejection period (PEP)	Ambulatory ECG & ICG signal; 24-hour recording	PEP assesses relatively pure cardiac sympathetic control.
T-Wave Amplitude (TWA)	Ambulatory ECG signal	TWA is associated more strongly with PEP than with RSA

Data loss

Of the 592 participants who wore the VU-AMS device, VU-AMS data of 16 participants were discarded for the following reasons: Data of one participant were missing because we never received back the device. ECG and ICG data of four participants were lost due to equipment failure, data of eight participants were discarded because their ECG showed many arrhythmias or preventricular contractions, and data of three participants were excluded because we did not receive back the diary. The resulting sample comprised 297 families with 576 family members, of which 3 participants were triplets and 518 participants were twins: 282 MZ twins (128 complete pairs) and 236 DZ twins (98 complete pairs). Furthermore, 55 non-twin siblings, 21 brothers and 34 sisters, of twins participated (see Table 2.2).

Biobank study

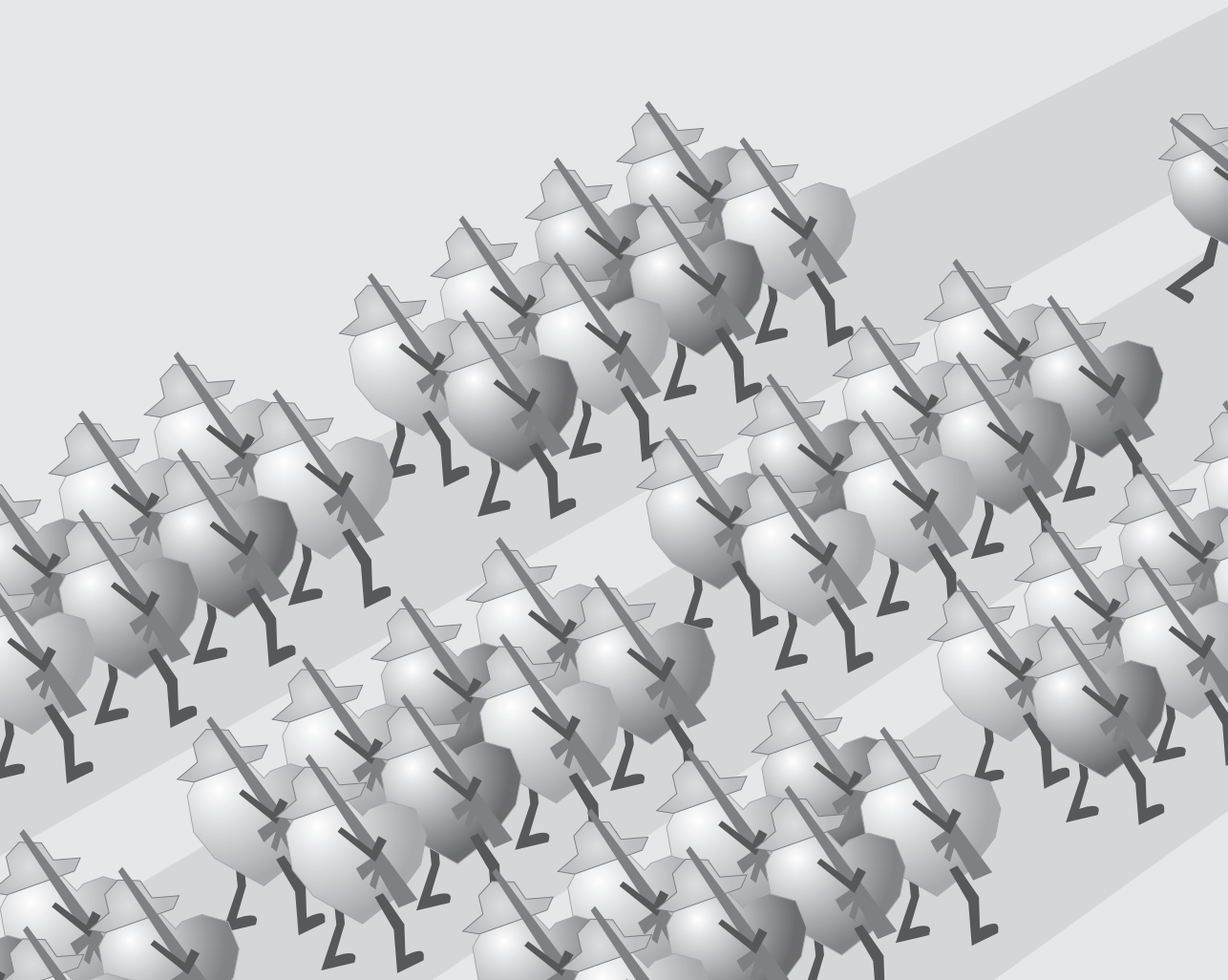
For the biobank study, a total of 9 530 adult twins and their family members were visited between 7 am and 10 am at home or, when preferred, at work, to collect fasting blood and urine samples. They were instructed to abstain from physical exertion and, if possible, not to take medication at the day of the home visit, and to refrain from smoking one hour before the home visit. Fertile women were visited on the 2nd-4th day of their menstrual cycle or, if they took oral contraceptives, in their pill-free week. During the home visit, a brief interview was conducted on health status, including an inventory of medication use, illness (last time occurrence, duration and type of illness), and adherence to the protocol. Also, height, weight and waist circumference were assessed. Blood tubes were collected in the following order; 2 × 9 ml EDTA, 2 × 9 ml heparin, 1 × 4.5 ml CTAD, 1 × 2 ml EDTA, 1 x 4.5 ml serum. From these samples, the levels of several biomarkers were determined, including inflammatory and metabolic parameters. Table 2.3 provides an overview of the biomarkers that were used in this project. For more details about this study, you are referred to the paper of Willemsen et al. (2010), which gives an overview of the data that was collected during this study.

Table 2.2. Family composition ANS Study 2.

		Number of additional non-twin siblings				
		0	**1**	**2**	**3**	**Total**
MZ	Twin pair	112	13	3	-	128
	Single twin	23	3	-	-	26
DZ	Twin pair	84	10	2	2	98
	Single twin	33	5	2	-	40
Triplet		1	-	-	-	1
No twin		-	4	-	-	4
Total number of families		253	35	7	2	297

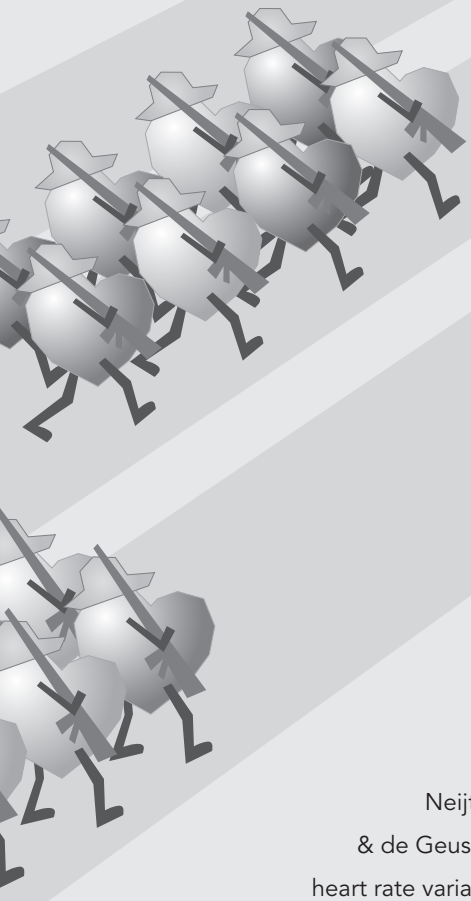
Table 2.3. Variables that were collected in the biobank study and used in this thesis.

Measures	*From*
Pro-inflammatory markers	
Tumor Necrosis Factor (TNF)-alpha, Interleukin-6 (IL-6)	EDTA plasma, using an UltraSensitive ELISA (R&D systems, Minneapolis, USA, Quantikine HS HSTA00C)
C-Reactive protein (CRP)	CRP was measured in heparin plasma, using Immulite 1000 CRP assay (Diagnosic Product Corporation, USA)
Fibrinogen	Fibrinogen levels were measured in citrate plasma, on a STA Compact Analyzer (Diagnostica Stago, France), using STA Fibrinogen (Diagnostica Stago, France)
Metabolic markers	
Fasting glucose	Glucose was measured using the Vitros 250 Glucose assay (Johnson & Johnson, Rochester, USA)
Total Cholesterol (TC), High Density Lipoprotein Cholesterol (HDL-C), Triglycerides (Trig)	TC, HDL-C, and triglycerides were measured in heparin plasma, using Vitros 250 direct HDL cholesterol and Vitros 250 Triglycerides assays (Johnson & Johnson, Rochester, USA)
Low Density Lipoprotein Cholesterol (LDL-C)	LDL-C was calculated using the Friedewald Equation (Friedewald, 1972)



Chapter 3

Heritability of cardiac vagal control in 24-hour heart rate variability recordings: Influence of ceiling effects at low heart rates



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Abstract

This study estimated the heritability of 24-h heart rate variability (HRV) measures, while considering ceiling effects on HRV at low heart rates during the night. HRV was indexed by the standard deviation of all valid interbeat intervals (SDNN), the root mean square of differences between valid, successive interbeat intervals (RMSSD), and peak-valley respiratory sinus arrhythmia (pvRSA). Sleep and waking levels of cardiac vagal control were assessed in 1,003 twins and 285 of their non-twin siblings. Comparable heritability estimates were found for SDNN (46%-53%), RMSSD (49%-54%), and pvRSA (48%-57%) during the day and night. A nighttime ceiling effect was revealed in 10.7% of participants by a quadratic relationship between mean pvRSA and the interbeat interval. Excluding these participants did not change the heritability estimates. The genetic factors influencing ambulatory pvRSA, RMSSD, and SDNN largely overlap. These results suggest that gene finding studies may pool the different cardiac vagal indices and that exclusion of participants with low heart rates is not required.

Introduction

Reduced heart rate variability (HRV) is a predictor for all-cause mortality and adverse cardiovascular events, including atrial fibrillation, myocardial infarction, congestive heart failure, and coronary artery disease in pre-morbid populations and samples of cardiac patients (Bigger, Jr., Fleiss, Rolnitzky, & Steinman, 1993; Bigger, Jr. et al., 1992; Bigger, Jr., Hoover, Steinman, Rolnitzky, & Fleiss, 1990; Buccelletti et al., 2009; Dekker et al., 1997; Dekker et al., 2000; Kleiger, Miller, Bigger, Jr., & Moss, 1987; Singer et al., 1988; Tsuji et al., 1996; Vikman et al., 2003). A proposed mechanism that can explain the risk conveyed by low HRV is that it reflects a decrease in cardiac vagal activity which increases the chance of arrhythmic events (La Rovere et al., 2001; Schwartz, Billman, & Stone, 1984; Schwartz et al., 1988).

A useful noninvasive measure of vagal activity is the HRV in the respiratory frequency range (0.15-0.4 Hz), also called respiratory sinus arrhythmia (RSA). RSA is generated when tonic firing of motor neurons in the nucleus ambiguus is modulated by phasic inhibition and excitation coupled to the respiratory cycle (Berntson, Cacioppo, & Quigley, 1993). This modulation is caused by connections between the nuclei that control the respiratory generator in the pre-Bötzinger and Bötzinger complexes and the vagal motor neurons, which lie in close proximity in the brainstem (Rekling & Feldman, 1998) and is further influenced by input from baro-, mechano-, and chemoreceptors. Respiration-autonomic nervous system (ANS) coupling yields an oscillatory pattern in the release of acetylcholine in the sinoatrial (SA) node, such that acetylcholine levels increase during expiration and decrease during inspiration. The effect of this respiratory 'gating' (Eckberg, 2003) is that heart rate increases during inspiration and decreases during expiration. The effect of the respiratory-related changes in vagal gating on RSA shows relatively little sensitivity to sympathetic blockade but is affected in a dose-response way by muscarinic blockers in humans (Martinmaki et al., 2006) and vagal cooling in animals (Katona & Jih, 1975). This has led to the use of RSA as a proxy for individual differences in cardiac vagal activity (Berntson et al., 1997; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996), although it must be acknowledged that differences in the sensitivity of the muscarinic receptor signaling pathway and differences in respiratory behavior can influence RSA independently of true differences in cardiac vagal activity (Grossman & Kollai, 1993; Grossman, Wilhelm, & Spoerle, 2004; Ritz & Dahme, 2006). RSA, therefore, is more appropriately considered a measure of cardiac vagal control, rather than of vagal activity.

RSA can be measured directly from the combined ECG and respiration signal as peak valley RSA (pvRSA), but two HRV measures derived only from the interbeat interval (IBI) time series are also often used to index vagal control over the heart, namely the standard deviation of all valid IBIs (SDNN) and the root mean square of differences between valid, successive IBIs (RMSSD). Age is a major source of interindividual differences in cardiac vagal control, with younger participants having higher RSA levels than older participants across the entire adolescent/adult age range (de Geus et al., 2007; De Meersman & Stein, 2007; Quilliot, Fluckiger, Zannad, Drouin, & Ziegler, 2001; Vallejo, Marquez, Borja-Aburto, Cardenas, & Hermosillo, 2005). Sex, BMI and lifestyle variables like smoking and regular exercise have also been shown to affect RSA, although these effects are generally modest (de Geus et al., 2007; De Meersman & Stein, 2007; McNarry & Lewis, 2012; Sacknoff, Gleim, Stachenfeld, & Coplan, 1994; Umetani, Singer, McCraty, & Atkinson, 1998; Valentini & Parati, 2009; van Lien et al., 2011).

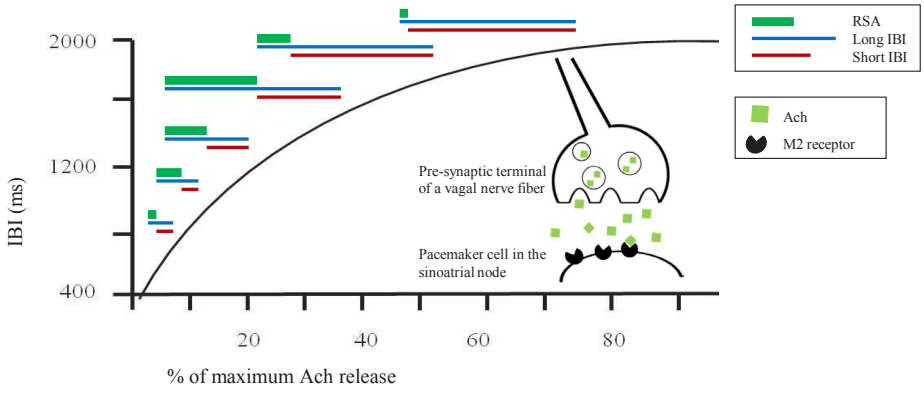
In contrast, a substantial portion of the interindividual variance in RSA appears to be due to genetic variation. Twin studies that record RSA in quiet resting conditions in the laboratory systematically reported a significant genetic contribution to RSA (Boomsma et al., 1990; de Geus et al., 2007; Riese et al., 2006; Riese et al., 2007; Snieder et al., 1997; Tank et al., 2001; Tuvblad et al., 2010; Uusitalo et al., 2007; Wang et al., 2009; Wang et al., 2005; Zhang et al., 2007). Heritability estimates at rest range from 25% to 71%. Ambulatory studies report heritabilities ranging from 35% to 65% (Busjahn et al., 1998; Kupper et al., 2004; Kupper et al., 2005) and from 35% to 55% when confined to sleeping or sitting conditions only (Kupper et al., 2004; Kupper et al., 2005). Estimates were very similar for European and African Americans (Wang et al., 2005), across the three different measures used (Goedhart, van der Sluis, Houtveen, Willemsen, & de Geus, 2007; Kupper et al., 2004), and none of these twin studies reported a sex difference in heritability or evidence for different genes being expressed in males and females.

Interestingly, three independent studies that measured cardiac vagal control at rest during a series of mental stressors all reported increased genetic variance in these measures under stress (Riese et al., 2006; de Geus et al., 2007; Wang et al., 2009). Compared to the heritability of resting baseline levels, the genetic contribution to the variance in measures of vagal control increased on average up to 10-20% when participants were exposed to various stress tasks. These findings have been taken to suggest that genetic influences on cardiac vagal control become more pronounced when the participant is challenged by mentally and emotionally 'engaging' conditions, i.e. they seem to provide evidence

of Gene x Stress interaction (de Geus et al., 2007). This would lead to the prediction that RSA heritability estimates might vary across an ambulatory recording day, for instance by being higher during daytime than at night. In addition, physical stressors may also give rise to higher genetic variance, as vagal withdrawal may not be as strong in each individual. Therefore, across the daytime, heritability of RSA measures may be lower during sitting conditions than during more physically active conditions.

Previous ambulatory twin studies have only indirectly addressed these questions and, importantly, failed to take into account the key observation that RSA can be paradoxically lowered at very low heart rates due to ceiling effects (Malik & Camm, 1993; van Lien et al., 2011). Normally, respiratory gating will result in a larger difference between the shortest IBI in inspiration and the longest IBI in expiration if tonic levels of vagal control over the heart are larger (Berntson et al., 1993; Eckberg, 2003). However, when cardiac vagal control is very high, a ceiling effect may prevent the lengthening of the IBI during expiration more than during inspiration (Malik & Camm, 1993). The biological basis of this ceiling effect is that high cardiac vagal control causes a large occupancy of the available muscarinic receptors on the 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 control (illustrated in Figure 3.1). As expiration is characterized by higher vagal control than inspiration the beats during expiration suffer more strongly from the ceiling effect than beats during inspiration.

Figure 3.1. Graphical representation of the occurrence of a ceiling effect, showing that there is little room left for RSA at very long inter beat intervals (IBIs).



This ceiling effect is expected to cause a quadratic relationship between IBI and RSA at low heart rates. This quadratic shape of the IBI-RSA relationship has indeed been found in laboratory studies inducing variance in vagal control by phenylephrine and nitroprusside infusion (Goldberger, Challapalli, Tung, Parker, & Kadish, 2001). More recently, the occurrence of the quadratic shape was shown under naturalistic settings in 24-hour recordings (van Lien et al., 2011). In a subset of 13 out of 52 participants, with half of them selected as being engaged in regular vigorous exercise, a ceiling effect on RSA was found during the nighttime. Based on these findings it was hypothesized that cardiac vagal control in conditions of low heart rate levels will be underestimated by RSA in participants with a quadratic shape of the IBI-RSA relationship compared to participants with a linear shape of the IBI-RSA relationship (van Lien et al., 2011). Here, we further hypothesize that the variation in the shape of the IBI-RSA curve may lead to an underestimation of the heritability of nighttime cardiac vagal control when it is based on RSA measures.

In this study we aim to test whether heritability of RSA at night differs from that of daytime RSA during sitting and physically active conditions. For this, we use the largest sample with 24-h ambulatory cardiac recordings to date. We assessed the shape of the IBI-RSA relationship in 24-h ambulatory recordings of 486 monozygotic (MZ) twins, 517 dizygotic (DZ) twins, and 285 of their singleton siblings. Based on the scatter plots of the mean of RSA and IBI in 10-min bins we made a qualitative distinction between linear IBI-RSA shapes showing no evidence of ceiling/saturation effects and quadratic IBI-RSA shapes suggesting reduction of RSA at long IBIs through ceiling/saturation effects. First, heritability of three often used RSA measures were computed during sleep and daytime by comparing MZ and DZ/sibling resemblance using the established twin methodology (Neale & Cardon, 1992). Next, heritability estimates of these RSA measures were re-estimated after removing the participants with evidence of ceiling effects. Furthermore, heritability of pvRSA was estimated after replacing the nighttime pvRSA values by (a) the maximal observed pvRSA at the peak of their quadratic IBI-RSA curve, and (b) by the pvRSA value at the longest nighttime IBI obtained from extrapolating from the linear part of their IBI-RSA curve. Although these virtual maxima may still underestimate the true level of cardiac vagal control, they preserve all the participants for the analysis and may better capture the total genetic variance. The rationale behind this approach is akin to the correction of blood pressure in medicated participants by adding the average effect of the antihypertensive medication to the observed blood pressure. This was shown to preserve genetic variance compared to removing participants with medication (Cui, Hopper, & Harrap, 2003).

Our foremost goal was to establish the heritability of RSA measures across the 24-h period while accounting for ceiling effects on RSA at low heart rate levels during the night. We expected the heritability estimates for the nighttime RSA measures to be significantly moderated by the IBI-RSA shape, such that heritability of cardiac vagal control is underestimated unless the ceiling effect is taken into account. As a secondary goal we tested the degree of overlap in the genetic factors influencing pvRSA, RMSSD and SDNN, and their potential sensitivity to the ceiling effects. As these measures are all used to capture individual differences in cardiac vagal control, the expectation is that the genetic factors influencing them are highly correlated.

Method

Participants

Participants were all registered in the Netherlands Twin Register and took part in a large cardiac ambulatory monitoring project in which 24-h recordings were collected in three data collection waves. A priori reasons for exclusion were pregnancy, heart transplantation, presence of a pacemaker and known ischemic heart disease, congestive heart failure, or diabetic neuropathy. We excluded data of 8 participants showing much arrhythmias. Valid ambulatory HRV recordings were available for 1,373 participants, of which 797 participated in the first two waves between 1998 and 2003. Sixty-seven of these participants took part in both waves, as part of a study on temporal stability of the ambulatory recordings (Goedhart, Kupper, Willemsen, Boomsma, & de Geus, 2006). In Wave 3, running between 2010 to 2012, the sample was further expanded with 576 new participants.

Data for 71 participants (75 recordings) were excluded due to the use of cardiovascular medication (beta blockers, ATC C07, cardiac therapy, ATC C01), or antidepressants (ATC N06A) at the time of the ambulatory assessment. Of the remaining 1,302 participants, 63 had duplicate ambulatory recordings from which we selected a single recording only. When the difference in the duration between the duplicate recordings was greater than or equal to 200 min, the shorter recording was excluded (12 recordings). Next, preference was given to the recordings of the data collection wave in which both twins participated, by excluding the recordings for the wave when only one of the twins participated (25 recordings excluded). For the remaining duplicate recordings, the recording of the wave in which most family members participated was retained (26 recordings excluded).

To simplify genetic modeling, we excluded the third member of triplets and included a maximum of two singleton brothers and two singleton sisters per family. Therefore, eleven participants from larger families were additionally excluded: the third member of a triplet (N = 1), nine siblings when more than two brothers and two sisters participated, and one twin who belonged to a second twin pair in the family. For the latter, we selected the siblings who were closest in age to the twins. We additionally excluded three participants who took part in the first two waves while all other family members participated in the third wave.

The final sample comprised 1,288 participants belonging to 624 families, with 486 MZ twins (210 complete pairs), 517 DZ twins (205 complete pairs), and 285 non-twin siblings. Mean age was 33.5 years (SD = 9.2 years), and 61.6% of the sample was female. Zygosity of the twins was determined by DNA typing for 97.9% of the same-sex twin pairs. For the remaining 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 is 96.1% (Willemsen et al., 2013). The Medical Ethics Committee of the VU University Medical Center approved of the study protocol and all participants gave written consent before entering the study.

Ambulatory Measurements of Heart Rate and Heart Rate Variability

For the first two data collection waves, the VU University Ambulatory Monitoring System (VU-AMS) version 4.6 was used (VU University, Amsterdam, The Netherlands, www.vu-ams.nl). This version of the VU-AMS continuously recorded the ECG and changes in thoracic impedance (dZ) from a six-electrode configuration (de Geus & van Doornen, 1996; de Geus, Willemsen, Klaver, & van Doornen, 1995; Riese et al., 2003). The device automatically detects each R wave in the ECG signal, at which it reads out and resets a millisecond counter to obtain the heart period time series. The thoracic impedance (Z), assessed against a constant current of 50 KHz, 350 microamperes, was amplified and led to a precision rectifier. The rectified signal was filtered at 72 Hz (low pass) to give basal impedance Z. Filtering Z at 0.1 Hz (high pass) supplied the dZ signal, which was band pass filtered with 0.1 and 0.4 Hz cutoffs, after tapering with (sin(x))2, to yield the respiration signal.

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. Artifact processing was performed on the IBI data offline. When the IBI deviated

more than 3 SD from the moving mean of a particular period it was automatically coded as an artifact and the IBI was either rejected during visual inspection or new IBIs were created by summing too short IBIs or too long IBIs were split in two IBIs of equal length.

For the third wave, the 5fs version of the VU-AMS was used, which improved on the 4.6 version in that it stores the entire ECG for offline analysis rather than online R-wave peak-detection (van Dijk et al., 2013). The ECG signal was imported into the VU-DAMS software (version 3.2, VU University Amsterdam, www.vu-ams.nl). After automated detection of bad ECG signal fragments (artifacts), R-wave peak detection was done using a modified version of the algorithm by Christov (Christov, 2004). From the R-wave peaks, the IBI time series was again constructed and visually displayed for interactive correction of missed or incorrect R-wave peaks. In addition to the ECG, the 5fs version also stores the entire dZ at 1000 Hz to obtain the respiration signal. The dZ signal is filtered using a second order band pass filter that passes all frequencies in the range of 0.1 to 0.4 Hz. An Exponential Smoothing Average technique is then applied on the filtered DZ signal, which acts as an additional low pass filter. The output of this filter is a weighted combination of previous smoothed value and the newest measured data, or in formula:

$$S_t = \alpha * S_{t-1} + (1 - \alpha) * x_t$$

where S_t is the smoothed average, α is the tunable smoothing factor (which is in the range of 0 to 1), x_t is the observation at time t, and S_{t-1} is the previous smoothed value.

Computation of the RSA measures was done in the same way for all three waves. Combining the IBI time interval series with the respiration signal extracted from the thorax impedance signal (dZ), the 'peak–valley' RSA method was used to asses pvRSA (de Geus et al., 1995; Grossman, van Beek, & Wientjes, 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 according to the procedures detailed elsewhere (de Geus et al., 1995; Houtveen, Groot, & de Geus, 2005; van Lien et al., 2011). Breathing cycles that showed irregularities like gasps, breath holding and coughing, were considered invalid and were removed from further processing. If no shortest or longest IBI could be detected in inspiration and expiration respectively, the breath was either set to missing or to zero when computing the average per condition for pvRSA. Similar results were found for pvRSA computed either way and we employed only one (breaths set to missing) in further statistical analyses. The two other measures of RSA were derived from the IBI time series by taking the standard deviation of all valid IBIs (SDNN) and the root mean square of differences between valid,

successive IBIs (RMSSD):

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

Procedure

Participants were visited at home, before starting their normal daily activities. During a short interview, information on health status and current medication use was obtained. The VU-AMS was attached and its operation explained. Participants were instructed to wear the device the entire day and night up until awakening the next morning. Instructions were supplied that explained how to respond to potential alarm beeps (e.g., on loose electrode contacts), and telephone assistance was available during waking hours. Participants were requested to keep a diary and to write down a chronological account of activity, posture, location, and social situation over the time period. For Wave 1 and 2 this was done every 30 min, for Wave 3 every 60 min. Participants were instructed to refrain from vigorous exercise during the ambulatory recording day.

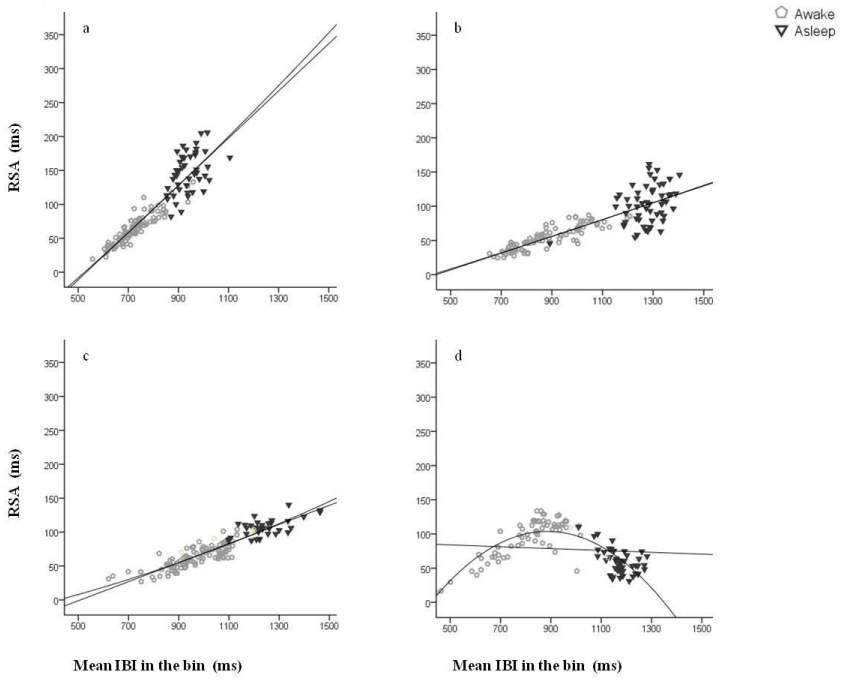
Data Reduction

Using the activity diary entries in combination with a visual display of the output of an inbuilt 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 min and maximum duration was 1 h. If periods with similar activity and posture lasted more than 1 h (e.g., during sleep), they were divided into multiple periods of maximally 1 h. All periods were classified into three main ambulatory conditions: (1) lying asleep, (2) sitting during the day, or (3) mild physical activity (e.g. 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.

To determine the shape of the relationship between IBI and pvRSA we divided the entire 24-h recording into bins no longer than 10 min, thereby making a distinction between waking and sleeping periods. For the majority of the bins (83%), condition within the bin was uniform. The other bins did not fall within a single condition because it was not always determined for the entire bin, or the bin covered more than one condition.

The mean IBI and pvRSA were determined per bin and the correlation across these IBI and pvRSA means was depicted in a separate scatter plot for each of the participants in the study. Four examples of the IBI-RSA relationship are shown in Figure 3.2 (full set of scatter plots available upon request from the corresponding author). Significance of the regression weights (β1 and β2) in the linear and quadratic terms was tested by the SPSS CURVEFIT procedure. First automated classification of the shape was used. 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 human raters (MN and GW) independently verified this algorithmic classification of the scatter plots by visual inspection; a third rater (EG) resolved remaining discrepancies. For all participants, the intercept and standardized beta (daytime_slope) of the IBI-RSA curve was assessed in the waking part of the data, where it was found to be non-quadratic in all participants.

Figure 3.2. Representative IBI-RSA curves of three participants without ceiling effects (panel a,b,c) and one participant with ceiling effects (panel d). The lines represent the best fitting linear and quadratic function for each participant.



For IBI, respiration rate and the three HRV measures (SDNN, RMSSD, pvRSA) a mean value was computed across the sleep, sitting and physically active periods in the recordings. In addition two separate measures were computed to index maximal cardiac vagal control during the night. First, the median value of the six 10-min bins with the highest pvRSA value during the night (pvRSAmax) was obtained. For participants without ceiling effects these bins occurred mostly around the end of the night. For participants with a ceiling effect, the highest pvRSA values were obtained in an earlier phase of the night, corresponding to the moment of occurrence of the peak in the quadratic IBI-RSA curve. Second, a virtual pvRSAmax (pvRSAmax_virtual) was calculated on the basis of the intercept and slope of the daytime IBI-RSA association during sitting activities, by extrapolating the pvRSA to the value that would have been obtained at the nighttime 10-min bin with the longest IBI value (IBImax in seconds) by the following formula:

pvRSAmax_virtual = intercept + (daytime_slope * IBImax)

The rationale behind this virtual value is that by extrapolating the observed daytime IBI-RSA relationship to nighttime RSA, an RSA value may be obtained that may prove a valid alternative to excluding data of participants showing ceiling effects.

Statistical Analyses

SDNN and IBImax showed a continuous normal distribution. A LogN (LN) transformation (for pvRSAmax, pvRSAmax_virtual, pvRSA, and RMSSD) or a squared transformation (for daytime_slope) was applied to obtain a normal distribution.

Group and Condition effects

We used a mixed model ANOVA (IBM SPSS 20.0) and included age, sex, and respiration rate (the latter for pvRSA and RMSSD only) as covariates and family as a random factor. Respiration rate was only included as a covariate in the analyses for pvRSA and RMSSD, because SDNN does not specifically capture HRV related to cadiorespiratory coupling. We tested the fixed effects of group (ceiling vs no ceiling), ambulatory condition (sleep, sitting, active) and the Group x Condition interaction. Significant interaction was followed by post-hoc tests of the ceiling effect within each of the ambulatory conditions. Mixed model ANOVA was also used to test the effects of group on the two alternative measures of maximal pvRSA at nighttime, pvRSAmax and pvRSAmax_virtual. Effects were considered significant when $p < .01$.

Genetic Analyses

In a twin study, the observed variance can be decomposed into four possible sources of variance: variance due to additive genetic effects (A), non-additive genetic effects (D), common environment (C) shared by family members, and nonshared or unique environment (E) (Boomsma & Gabrielli, Jr., 1985). However, in a design that includes identical twins, fraternal twins, and sibling pairs, estimates of C and D are confounded and the observed variances and covariances only provide sufficient information to model either an ACE model or an ADE model, but not both. Based on the pattern of twin and sibling correlations we chose to model A, C, and E. For identical twins, fraternal twins, and sibling pairs alike, common environmental factors are correlated 1. Genetic factors are correlated 0.5 in siblings and DZ twins and 1.0 in MZ twins. By definition, nonshared, or unique, environmental factors are uncorrelated in family members.

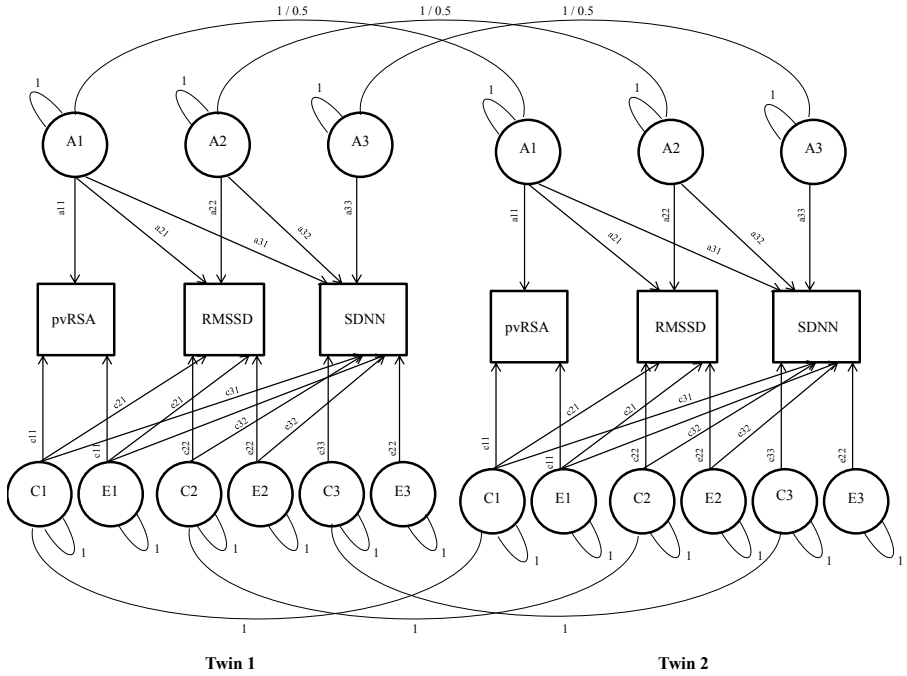
To answer the question to what extent A, C, and E factors contribute to the variance in the RSA measures, biometrical genetic models were fitted to the observed data using the structural equation modeling program Mx (Neale, Boker, Xie, & Maes, 2006). First, fully saturated models were fitted for each variable separately. In these fully parameterized models, means, and variances were estimated freely for both sexes. Then, we tested for sex differences in means and variances by constraining these to be equal for males and females and tested whether these more constrained models led to a significant worse fit to the data. Next, we tested for heterogeneity of correlations of males versus females and of fraternal twins versus singletons. The resulting most parsimonious saturated model indicated to what extent we could limit the specification of the variance components models.

As the individual differences in the ambulatory RSA measures were expected to be sensitive to three main confounding variables: differences in age, sex, and respiration rate (de Geus et al., 2007; De Meersman & Stein, 2007; Eckberg, 2003; Quilliot et al., 2001; Umetani et al., 1998; Vallejo et al., 2005), the above models specifically regressed the effects of sex and age on RSA. Respiration rate was additionally included as a covariate for pvRSA and RMSSD.

Furthermore, we tested for effects of the version of the ambulatory recording device by comparing the means and variances of the three waves. In keeping with the highly similar strategies used to obtain the RSA measures, no device version effect was found so that all three waves were pooled during all genetic modeling. To examine whether the genetic architecture of the RSA measures changed from nighttime to daytime and, within

the daytime, from sitting only to more physical active activities, full trivariate genetic ACE models in Cholesky decomposition were fitted to the mean values for the three RSA measures separately for the nighttime sleep, daytime sitting and daytime physically active periods. Because the MZ twin correlations were always at least twice as high as the DZ and non-twin sibling correlations, it is more likely that familial resemblance derives from genetic factors rather than from shared environmental influences. The ACE model was therefore tested against the nested AE model only. The resulting most parsimonious model was used to further test the source of the observed covariance in the different HRV measures. Figure 3.3 depicts a schematic representation of the full trivariate genetic model that was fitted to the data.

Figure 3.3. Example of a path model decomposing trait variance into additive genetic (A), and shared (C) and unique environmental (E) factors in one twin pair. Unique A, C and E factors load on all three HRV measures. Additionally, A, C and E factors can be shared between the phenotypes. This is depicted by the 'a', 'c' and 'e' paths running from the former A, C and E factors of a HRV phenotype to the next (a21, a31, a32, c21, c31, c32, and e21, e31, and e32). With these paths, the genetic and environmental covariance can be studied. Finally, MZ twins correlate 1.0 regarding the A factor scores because they are assumed to share all of their genetic material, whereas DZ or sibling pairs correlate 0.5.



The significance of A, C, and E factors was tested by comparing the fit of the more parsimonious nested models to the fit of the full model using the likelihood-ratio (χ^2) test in which the difference in minus twice the logarithm of the likelihood (-2LL) was calculated. When the χ^2 test was significant ($p < .01$), the more parsimonious model was considered to fit significantly worse to the data than the fuller model it was tested against. In addition, Akaike's Information Criterion (AIC = χ^2 - 2df) (Akaike, 1987) was calculated for each model, which offers a quick approach to judging the fit of nested models. Those with lower values fit better than models with higher values. For more background information on the heritability estimation procedures, see (de Geus, 2010).

To test whether the heritability estimates were significantly affected by participants whose data showed ceiling effects, the trivariate genetic analyses were repeated excluding those participants. As an alternative to correct for a potential underestimation of the heritability of nighttime vagal control two additional genetic analyses were performed on pvRSAmax and pvRSAmax_virtual and it was tested whether the heritability estimates for these alternative measures were higher compared to the estimates obtained for uncorrected nighttime pvRSA.

Results

In 52 participants no valid nighttime recording of the RSA measures was obtained for at least five 10-min bins. Analyses of the scatter plots of mean RSA and IBI across the 24-h period in the remaining 1,236 participants, showed a significant quadratic relationship in 132 participants (10.7%). From Table 3.1 it can be seen that the nighttime pvRSA and RMSSD may be underestimated in these participants. Mixed ANOVA analysis with correction for family relatedness showed a significant Group x Condition interaction for pvRSA, $F(2,3061) = 36.20$, $R^2 = 0.64$, $p < .01$; and RMSSD, $F(2,3063) = 17.30$, $R^2 = 0.30$, $p < .01$; but not for SDNN, $F(2,3056) = 3.08$, $R^2 = 0.04$, $p = .046$. Whereas participants with a ceiling effect due to a quadratic IBI-RSA curve have a significantly longer IBI throughout the entire recording (hinting at higher cardiac vagal control) compared to the participants without a ceiling effect, their pvRSA and RMSSD are only higher during the two daytime conditions but not at night. Nighttime SDNN appears less affected by the ceiling effect, although the group differences in HRV values during the night were also less pronounced compared to the daytime conditions. Previously, a similar pattern was observed by our

group (van Lien et al., 2011).

Using the maximal observed pvRSA at night still suggests that the participants with a ceiling effect have comparable cardiac vagal control ($F_{group} = 0.29$, $R^2 = 0.10$, $p = .588$) in spite of their longer nighttime IBI. Only when the linear relationship between RSA and IBI was extrapolated to the maximal IBI (pvRSAmax_virtual) was a higher value found in the ceiling group ($F_{group} = 10.47$, $R^2 = 0.84$, $p < .01$). Daytime_slope for the participants with a ceiling effect was steeper compared to the daytime_slope of the participants without a ceiling effect ($F_{group} = 12.19$, $R^2 = 0.73$, $p < .01$).

Table 3.1. Means and (standard deviation) for IBI, pvRSA, RMSSD, SDNN, daytime_slope, pvRSAmax and pvRSAmax_virtual by ceiling status (yes/no) and ambulatory condition.

	No ceiling effects	**Ceiling effects**	**Group difference**
Variable	(1100 < N < 1104)	(N=132)	
IBI (ms)			
Sleep	975.34 (125.42)	1053.08 (165.02)	77.74*
Sitting	804.93 (102.13)	844.62 (115.90)	39.69*
Active	712.60 (87.53)	741.08 (100.49)	28.48*
pvRSA (ms)			
Sleep	56.10 (24.43)	54.05 (26.32)	-2.05
Sitting	44.49 (17.22)	58.18 (21.98)	13.69*
Active	35.24 (12.63)	44.96 (14.72)	9.72*
RMSSD (ms)			
Sleep	53.08 (25.58)	60.31 (31.88)	7.23
Sitting	36.03 (16.61)	51.24 (24.83)	15.21*
Active	29.54 (12.85)	40.24 (18.65)	10.70*
SDNN (ms)			
Sleep	91.49 (26.90)	104.21 (31.37)	12.72*
Sitting	68.92 (19.80)	86.82 (25.01)	17.90*
Active	81.73 (21.34)	98.26 (24.89)	16.53*
daytime_slope	108.50 (64.17)	110.84 (65.86)	2.34*
pvRSAmax (ms)	86.03 (34.83)	89.21 (33.42)	3.18
pvRSAmax_virtual (ms)	73.31 (32.45)	89.62 (39.92)	16.31*

All variables were corrected for family relatedness, age and sex. pvRSA, RMSSD, pvRSAmax, and pvRSAmax_virtual values were additionally corrected for respiration rate.
**= significant main effect of ceiling (within ambulatory condition) (p < .01)
Note. Abbreviations: IBI = inter beat interval; pvRSA = peak valley respiratory sinus arrhythmia; RMSSD = root mean square of differences between valid successive IBIs; SDNN = standard deviation of all valid IBIs.

Table 3.2. Twin and sibling correlations as estimated from the full saturated model.

	MZ twins		**DZ/sibs male**		**DZ/sibs female**		**Opp. sex sibs**	
	MZM	MZF	DZM	twin/ sib-sib	DZF	twin/ sib-sib	DOS	twin/sib-OS sib
SDNN / sleep[1]	0.70	0.56	0.17	0.09	0.17	0.21	0.45	0.19
SDNN / sitting[1]	0.64	0.55	-0.07	0.24	0.49	0.30	0.27	0.38
SDNN / active[1]	0.65	0.56	0.01	0.31	0.46	0.36	0.35	0.32
RMSSD / sleep [2]	0.74	0.60	0.15	0.31	0.17	0.27	0.43	0.29
RMSSD / sitting[2]	0.58	0.61	0.01	0.34	0.40	0.32	0.26	0.31
RMSSD / active[2]	0.57	0.50	0.10	0.40	0.27	0.31	0.28	0.30
pvRSA / sleep [2]	0.69	0.56	0.22	0.26	0.31	0.34	0.34	0.29
pvRSA / sitting[2]	0.60	0.65	0.07	0.35	0.44	0.33	0.28	0.23
pvRSA / active[2]	0.68	0.63	0.07	0.46	0.37	0.40	0.35	0.19
pvRSAmax[2]	0.64	0.64	0.25	0.24	0.30	0.35	0.25	0.28
pvRSAmax_ virtual[2]	0.63	0.72	0.19	0.32	0.44	0.29	0.33	0.34
IBImax[1]	0.53	0.55	0.17	0.27	0.38	0.27	0.31	0.27
daytime_slope[1]	0.46	0.48	-0.06	0.21	0.26	0.25	0.20	0.24

[1] = corrected for age, sex
[2] = corrected for age, sex, respiration rate
Note. Abbreviations: SDNN = standard deviation of all valid inter beat intervals (IBIs); RMSSD = root mean square of differences between valid successive IBIs; pvRSA = peak valley respiratory sinus arrhythmia; pvRSAmax = the median value of the six 10-minute bins with the highest pvRSA value during the night; IBImax = the nighttime 10-minute bin with the longest IBI value;daytime_slope = the slope of the IBI-RSA curve in the waking part of the data; pvRSAmax_virtual = a non-observed experimental variable that extrapolates the pvRSA from the intercept and slope of the daytime IBI-RSA association during sitting activities to the value that would have been obtained at the longest IBI value (IBImax); MZM = monozygotic male, MZF = monozygotic female; DZM = dizygotic male, DZF = dizygotic female, DOS = dizygotic opposite sex, sib = non-twin sibling.

Twin-Sibling Resemblance and Heritability

Table 3.2 shows the twin and sibling correlations for all measures. From the pattern of the correlations it is clear that there is a substantial genetic contribution to all measures. The overarching pattern seen is that DZ and sibling correlations are about half of the MZ correlations, with the exception of the male DZ correlations which are low and suggestive of nonadditivity. Formal testing, however, showed that the DZ twin and non-twin sibling

correlations were homogeneous (all *p's* > .01) and could be equalized in further model fitting. Correlations of MZ males and MZ females also did not differ significantly, nor did the same-sex DZ and opposite-sex DZ twin and non-twin sibling correlations (all *p's* > .01) so no quantitative or qualitative sex differences were modeled.

Table 3.3.a shows the best trivariate models for pvRSA, RMSSD and SDNN separately for the sleep, sitting, and physically active conditions. An AE model provided the best fit. Heritability of pvRSA was lower at night than during the day, but as can be seen from the confidence intervals, the heritability estimates were not significantly different during sleep, sitting and active periods. For RMSSD and SDNN, heritability was also very comparable across all periods.

Genetic and Unique Environmental Correlations

Table 3.4.a shows significant phenotypic correlations between the various HRV measures in all three periods. Generally, the phenotypic, the genetic, and the unique environmental correlations between pvRSA and RMSSD and RMSSD and SDNN were higher compared to the phenotypic, genetic, and unique environmental correlations between pvRSA and SDNN for the entire recording time. A single genetic and unique environmental factor influenced all three RSA measures, but RMSSD and SDNN were also influenced by independent genetic and unique environmental factors that did not affect pvRSA. The observed correlation between the three possible dyads of the RSA measures was for 51% to 56% attributable to shared genetic factors, and for 44% to 49% attributable to shared unique environmental factors.

Correcting for Ceiling Effects on Nighttime RSA

To test the impact of the ceiling effects on the RSA measures on the heritability of cardiac vagal control, we repeated the above trivariate analysis after excluding participants with the ceiling effects. Results are depicted in Table 3.3.b. No significant changes in heritability estimates were noticeable after excluding participants with ceiling effects.

Likewise, excluding the participants with ceiling effects did not significantly change the phenotypic, the genetic, and the unique environmental associations between the RSA measures, nor was a meaningful change observed in the genetic and unique environmental contribution to their covariance (see Table 3.4.b).

Table 3.3. Proportion of variance and confidence intervals (CI) due to A and due to E.

Table 3.3.a. Full sample (1232 < N < 1288).

Condition	Model	df	Model	AIC	-2LL	vs	$\Delta\chi^2$	Δ df	p	phenotype	A	E
Sleep	1	3669	ACE	3632.224	10970.224							
	2	3675	AE	3620.358	10970.358	1	0.135	6	1	pvRSA	0.48 (0.37-0.57)	0.52 (0.43-0.63)
										RMSSD	0.53 (0.43-0.61)	0.47 (0.39-0.57)
										SDNN	0.46 (0.36-0.56)	054 (0.44-0.65)
Sitting	1	3831	ACE	2488.129	10150.129							
	2	3837	AE	2477.869	10151.869	1	1.740	6	.942	pvRSA	0.53 (0.44-0.62)	0.47 (0.38-0.56)
										RMSSD	0.54 (0.45-0.62)	0.46 (0.38-0.55)
										SDNN	0.48 (0.39-0.57)	0.52 (0.43-0.61)
Active	1	3834	ACE	2830.663	10498.663							
	2	3840	AE	2823.792	10503.792	1	5.129	6	.527	pvRSA	0.57 (0.48-0.65)	0.43 (0.35-0.52)
										RMSSD	0.49 (0.40-0.57)	0.51 (0.43-0.60)
										SDNN	0.53 (0.45-0.60)	0.47 (0.40-0.55)

Note. Abbreviations: df = degrees of freedom; Model = specification of the model that is tested; AIC = Akaike's Information Criterion; -2LL = minus twice the logarithm of the likelihood; vs = the model against which this submodel is tested; $\Delta\chi^2$ = model fit statistic: difference in -2LL of two nested models; Δdf = difference in the number of parameters between the two models; p = p-value; phenotype = specification of the HRV parameter; A and E = proportions of variance explained by additive and unique environmental effects for the most parsimonious AE model; pvRSA = peak valley respiratory sinus arrhythmia; RMSSD = root mean square of differences between valid successive inter beat intervals (IBIs); SDNN = standard deviation of all valid IBIs.

Table 3.3.b. Excluding the participants with a ceiling effect (1100 < N > 1104).

Condition	Model	df	Model	AIC	-2LL	vs	$\Delta\chi^2$	Δ df	p	phenotype	A	E
Sleep	1	3273	ACE	3144.299	9690.299							
	2	3279	AE	3132.299	9690.299	1	0	6	1	pvRSA	0.53 (0.42-0.63)	0.47 (0.37-0.58)
										RMSSD	0.53 (0.42-0.63)	0.47 (0.38-0.58)
										SDNN	0.47 (0.35-0.57)	053 (0.43-0.65)
Sitting	1	3282	ACE	2017.696	8581.696							
	2	3288	AE	2006.158	8582.158	1	0.462	6	.998	pvRSA	0.57 (0.46-0.66)	0.43 (0.34-0.54)
										RMSSD	0.52 (0.41-0.61)	0.48 (0.39-0.59)
										SDNN	0.46 (0.35-0.56)	0.54 (0.44-0.65)
Active	1	3282	ACE	2327.274	8891.274							
	2	3288	AE	2327.386	8903.386	1	12.112	6	.060	pvRSA	0.57 (0.47-0.66)	0.43 (0.34-0.53)
										RMSSD	0.46 (0.35-0.55)	0.54 (0.45-0.65)
										SDNN	0.54 (0.44-0.62)	0.46 (0.38-0.56)

Note. Abbreviations: df = degrees of freedom; Model = specification of the model that is tested; AIC = Akaike's Information Criterion; -2LL = minus twice the logarithm of the likelihood; vs = the model against which this submodel is tested; $\Delta\chi^2$ = model fit statistic: difference in -2LL of two nested models; Δdf = difference in the number of parameters between the two models; p = p-value; phenotype = specification of the HRV parameter; A and E = proportions of variance explained by additive and unique environmental effects for the most parsimonious AE model; pvRSA = peak valley respiratory sinus arrhythmia; RMSSD = root mean square of differences between valid successive inter beat intervals (IBIs); SDNN = standard deviation of all valid IBIs.

Table 3.4. Phenotypic and genetic correlation between dyads of RSA measures and the % of the covariance explained by overlapping genetic factors.

Table 3.4.a. Full sample (1232 < N < 1288).

	Phenotypic correlation	Genetic correlation	Contribution of A to the phenotypic covariance	Unique environmental correlation	Contribution of E to the phenotypic covariance
Sleep					
pvRSA - RMSSD	0.82 (0.80-0.84)	0.85 (0.80-0.90)	52% (41-62%)	0.79 (0.74-0.83)	48% (38-59%)
RMSSD - SDNN	0.84 (0.82-0.85)	0.87 (0.82-0.91)	51% (40-61%)	0.81 (0.76-0.85)	49% (39-60%)
pvRSA - SDNN	0.57 (0.53-0.61)	0.63 (0.50-0.74)	52% (37-65%)	0.51 (0.42-0.60)	48% (35-63%)

Sitting					
pvRSA - RMSSD	0.89 (0.88-0.91)	0.94 (0.91-0.96)	56% (47-65%)	0.85 (0.81-0.87)	44% (35-53%)
RMSSD - SDNN	0.86 (0.84-0.87)	0.89 (0.84-0.92)	53% (43-62%)	0.83 (0.79-0.86)	47% (38-57%)
pvRSA - SDNN	0.70 (0.67-0.73)	0.74 (0.65-0.81)	54% (42-64%)	0.66 (0.59-0.72)	46% (36-58%)
Active					
pvRSA - RMSSD	0.86 (0.84-0.87)	0.91 (0.87-0.94)	56% (46-64%)	0.80 (0.76-0.84)	44% (36-54%)
RMSSD - SDNN	0.81 (0.79-0.83)	0.84 (0.79-0.88)	53% (43-61%)	0.78 (0.73-0.82)	47% (39-57%)
pvRSA - SDNN	0.68 (0.65-0.71)	0.66 (0.58-0.73)	53% (42-63%)	0.71 (0.65-0.77)	47% (37-58%)

Note. Abbreviations: pvRSA = peak valley respiratory sinus arrhythmia; RMSSD = root mean square of differences between valid successive inter beat intervals (IBIs); SDNN = standard deviation of all valid IBIs.

Table 3.4.b. Participants with ceiling effects excluded (1100 < N < 1104).

	Phenotypic correlation	**Genetic correlation**	**Contribution of A to the phenotypic covariance**	**Unique environmental correlation**	**Contribution of E to the phenotypic covariance**
Sleep					
pvRSA-RMSSD	0.82 (0.80-0.84)	0.85 (0.78-0.89)	55% (43-65%)	0.79 (0.73-0.83)	45% (35-57%)
RMSSD-SDNN	0.83 (0.81-0.85)	0.87 (0.81-0.92)	52% (40-63%)	0.80 (0.74-0.84)	48% (37-60%)
pvRSA-SDNN	0.57 (0.53-0.61)	0.63 (0.50-0.74)	55% (39-69%)	0.52 (0.41-0.61)	45% (31-61%)
Sitting					
pvRSA-RMSSD	0.90 (0.89-0.91)	0.94 (0.91-0.96)	57% (45-66%)	0.85 (0.81-0.89)	43% (34-55%)
RMSSD-SDNN	0.85 (0.83-0.86)	0.88 (0.83-0.92)	51% (39-61%)	0.81 (0.77-0.85)	49% (39-61%)
pvRSA-SDNN	0.70 (0.66-0.73)	0.75 (0.65-0.82)	55% (41-66%)	0.65 (0.57-0.73)	45% (34-59%)
Active					
pvRSA-RMSSD	0.86 (0.84-0.87)	0.92 (0.88-0.95)	55% (43-65%)	0.80 (0.75-0.84)	45% (35-57%)
RMSSD-SDNN	0.80 (0.78-0.82)	0.82 (0.76-0.87)	51% (39-61%)	0.79 (0.73-0.83)	49% (39-61%)
pvRSA-SDNN	0.68 (0.64-0.71)	0.63 (0.54-0.71)	52% (39-63%)	0.73 (0.66-0.79)	48% (37-61%)

Note. Abbreviations: pvRSA = peak valley respiratory sinus arrhythmia; RMSSD = root mean square of differences between valid successive inter beat intervals (IBIs); SDNN = standard deviation of all valid IBIs.

Table 3.5. Model fit statistics and variance decomposition (CI) for the univariate pvRSA measure, correcting for ceiling effects.

Table 3.5.a. Maximal pvRSAmax obtained during the night.

Phenotype	Model	df	Model	AIC	-2LL	vs	$\Delta\chi^2$	Δ df	p	A	E
pvRSAmax	1	1230	ACE	-1641.008	818.992						
	2	**1231**	**AE**	-1643.008	818.992	**1**	**0**	**1**	**1**	**0.53 (0.42-0.62)**	**0.47 (0.38-0.58)**
	3	1232	E	-1570.323	893.677	1	74.685	2	.000		

Note: Abbreviations: pvRSAmax = the median value of the six 10-minute bins with the highest pvRSA value during the night; df = degrees of freedom; Model = specification of the model that is tested; AIC = Akaike's Information Criterion; -2LL = minus twice the logarithm of the likelihood; vs = the model against which this submodel is tested; $\Delta\chi^2$ = model fit statistic: difference in -2LL of two nested models; Δdf = difference in the number of parameters between the two models; p = p-value; phenotype = specification of the HRV parameter; A and E = proportions of variance explained by additive and unique environmental effects for the most parsimonious AE model.

Table 3.5.b. Virtual pvRSAmax that is extrapolated from the daytime IBI-RSA intercept and slope to the maximal IBI obtained during the night.

Phenotype	Model	df	Model	AIC	-2LL	vs	$\Delta\chi^2$	Δ df	p	A	E
pvRSAmax_virtual	1	1230	ACE	-1376.033	1083.967						
	2	1231	AE	-1378.033	1083.967	1	0	1	1	0.55 (0.45-0.64)	0.46 (0.36-0.55)
	3	1232	E	-1296.892	1167.108	1	83.141	2	.000		

Note: Abbreviations: pvRSAmax_virtual = a non-observed experimental variable that extrapolates the pvRSA from the intercept and slope of the daytime IBI-RSA association during sitting activities to the value that would have been obtained at the nighttime 10-minute bin with the longest IBI value (IBImax) ; df = degrees of freedom; Model = specification of the model that is tested; AIC = Akaike's Information Criterion; -2LL = minus twice the logarithm of the likelihood; vs = the model against which this submodel is tested; $\Delta\chi^2$ = model fit statistic: difference in -2LL of two nested models; Δdf = difference in the number of parameters between the two models; p = p-value; phenotype = specification of the HRV parameter; A and E = proportions of variance explained by additive and unique environmental effects for the most parsimonious AE model.

Table 3.5.c. Maximal IBI obtained during the night.

Phenotype	Model	df	Model	AIC	-2LL	vs	$\Delta\chi^2$	Δ df	p	A	E
IBImax	1	1282	ACE	-3918.594	-1354.594						
	2	1283	AE	-3920.412	-1354.412	1	0.182	1	.670	0.52 (0.43-0.60)	0.48 (0.40-0.57)
	3	1284	E	-3814.549	-1246.549	1	108.45	2	.000		

Note: Abbreviations: IBImax = the nighttime 10-minute bin with the longest IBI value; df = degrees of freedom; Model = specification of the model that is tested; AIC = Akaike's Information Criterion; -2LL = minus twice the logarithm of the likelihood; vs = the model against which this submodel is tested; $\Delta\chi^2$ = model fit statistic: difference in -2LL of two nested models; Δdf = difference in the number of parameters between the two models; p = p-value; phenotype = specification of the HRV parameter; A and E = proportions of variance explained by additive and unique environmental effects for the most parsimonious AE model.

Table 3.5.d. Daytime RSA-IBI slope.

Phenotype	Model	df	Model	AIC	-2LL	vs	$\Delta\chi^2$	Δ df	p	A	E
daytime_slope	1	1282	ACE	-1113.303	1450.697						
	2	1283	AE	-1115.303	1450.697	1	0	1	1	0.35 (0.25-0.44)	0.65 (0.56-0.75)
	3	1284	E	-1072.076	1495.924	1	45.227	2	.000		

Note. Abbreviations: daytime_slope = the slope of the IBI-RSA curve in the waking part of the data; df = degrees of freedom; Model = specification of the model that is tested; AIC = Akaike's Information Criterion; -2LL = minus twice the logarithm of the likelihood; vs = the model against which this submodel is tested; $\Delta\chi^2$ = model fit statistic: difference in -2LL of two nested models; Δdf = difference in the number of parameters between the two models; p = p-value; phenotype = specification of the HRV parameter; A and E = proportions of variance explained by additive and unique environmental effects for the most parsimonious AE model.

Alternative Measures of Nighttime Cardiac Vagal Control

As an alternative measure of nighttime cardiac vagal control, that might be less sensitive to the impact of ceiling effects, we used the median value of the six 10-min bins with the highest RSA value in all participants (Table 3.5.a). This led to heritability estimates that were higher (5%), but not significantly different from those for the pvRSA during the entire sleep period (Table 3.3.a).

A second alternative replaced the nighttime pvRSA values by the estimated pvRSAmax (pvRSAmax_virtual) value based on the daytime linear association of RSA and IBI and extrapolating to the bin with the longest IBI at night (Table 3.5.b). The heritability estimate now increased 7% compared to the uncorrected nighttime pvRSA measure, but this increase was again not significant as confidence intervals still overlapped. For completeness, Table 3.5.c and 3.5.d also present the heritability of the daytime_slope and (nighttime) IBImax.

Discussion

Using a twin family design this paper shows that genetic factors explain around half of the individual differences in ambulatory cardiac vagal control as measured by pvRSA, RMSSD or SDNN. Our expectation that the heritability estimates for the nighttime RSA measures would be significantly affected by the IBI-RSA shape, such that heritability of cardiac vagal control is underestimated at low heart rates at nighttime, was not supported by the data. In spite of significant changes in mean RSA values across the 24-h period, there was no evidence for differences in heritability of cardiac vagal control at night compared to daytime conditions and, during daytime conditions, heritability was very comparable between sitting-only conditions and conditions in which participants were physically active. The genetic factors influencing ambulatory pvRSA, RMSSD, and SDNN largely overlapped, providing support for the idea that pvRSA, RMSSD and SDNN capture the same biological phenomenon. Nonetheless, the overlap in genetic factors influencing pvRSA and RMSSD was higher than the overlap in the genetic factors shared between pvRSA and SDNN.

Earlier twin studies on the heritability of HRV were mainly based on laboratory recordings and the heritability estimates that were found varied substantially between conditions and across the different HRV measures that were used. Laboratory studies reported heritability estimates of pvRSA in resting conditions between 25% to 39%

(Boomsma et al., 1990; de Geus et al., 2007; Snieder et al., 1997; Tuvblad et al., 2010). Estimates for resting RMSSD values in the laboratory ranged from 36% to 71% (Uusitalo et al., 2007; Wang et al., 2005; Wang et al., 2009). Only one laboratory study researched resting SDNN and estimated heritability to be 66% (Wang et al., 2005). These resting laboratory levels can be best approximated by the sitting-only condition in our sample. Heritability of sitting-only pvRSA was estimated at 53%, RMSSD at 54% and SDNN at 48%.

During (mental) stress, laboratory studies reported increases in heritability of pvRSA and RMSSD of 8% to 23% when compared to heritability estimates of resting levels (de Geus et al., 2007; Wang et al., 2009), suggesting that more genetic variance is expressed under conditions in which participants were aroused. This Gene x Stress interaction that was observed in controlled laboratory situations was not recaptured by a nighttime – daytime effect. Instead the genetic variance was rather stable across the 24-h period. This finding was supported by previous analyses within a subset of the present sample, in which we did not observe significant differences in heritability estimates across different times of day (Kupper et al., 2004; Kupper et al., 2005). This raises the important but possibly misleading question of which of these two paradigms 'is right'. The ambulatory situation is by definition a less standardized setting compared to the laboratory setting in which physical activity and postures can be carefully controlled. On the other hand, ambulatory 24-h measurements in real life may give a better reflection of the actual day-to-day situation of participants and thereby capture daily life better. Cardiac recordings in an ambulatory setting presumably also contribute more reliably to risk prediction compared to a recording in the laboratory, which is generally shorter and more sensitive to momentary or lab-specific influences (e.g. the white coat effect) (Zanstra & Johnston, 2011). Although not tested here, it would be interesting to elaborate on this topic and explore different operationalizations of real life reactivity in future research.

Previous research has already shown that RSA can be reliably measured under naturalistic conditions with the use of ambulatory monitoring (de Geus et al., 1995; Wilhelm, Roth, & Sackner, 2003). Such recordings yield large individual differences in RSA that appear to reflect stable trait characteristics. For the average 24-h levels of RSA, high test-retest correlations ($.63 < r < .90$) have been 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) and moderate to high long-term temporal stability ($.58 < r < .76$) has been shown over periods of 7 months to 3.4 years

(Goedhart et al., 2007; Pitzalis et al., 1996). Our results corroborate the stability of these individual differences and suggest that they have a genetic basis.

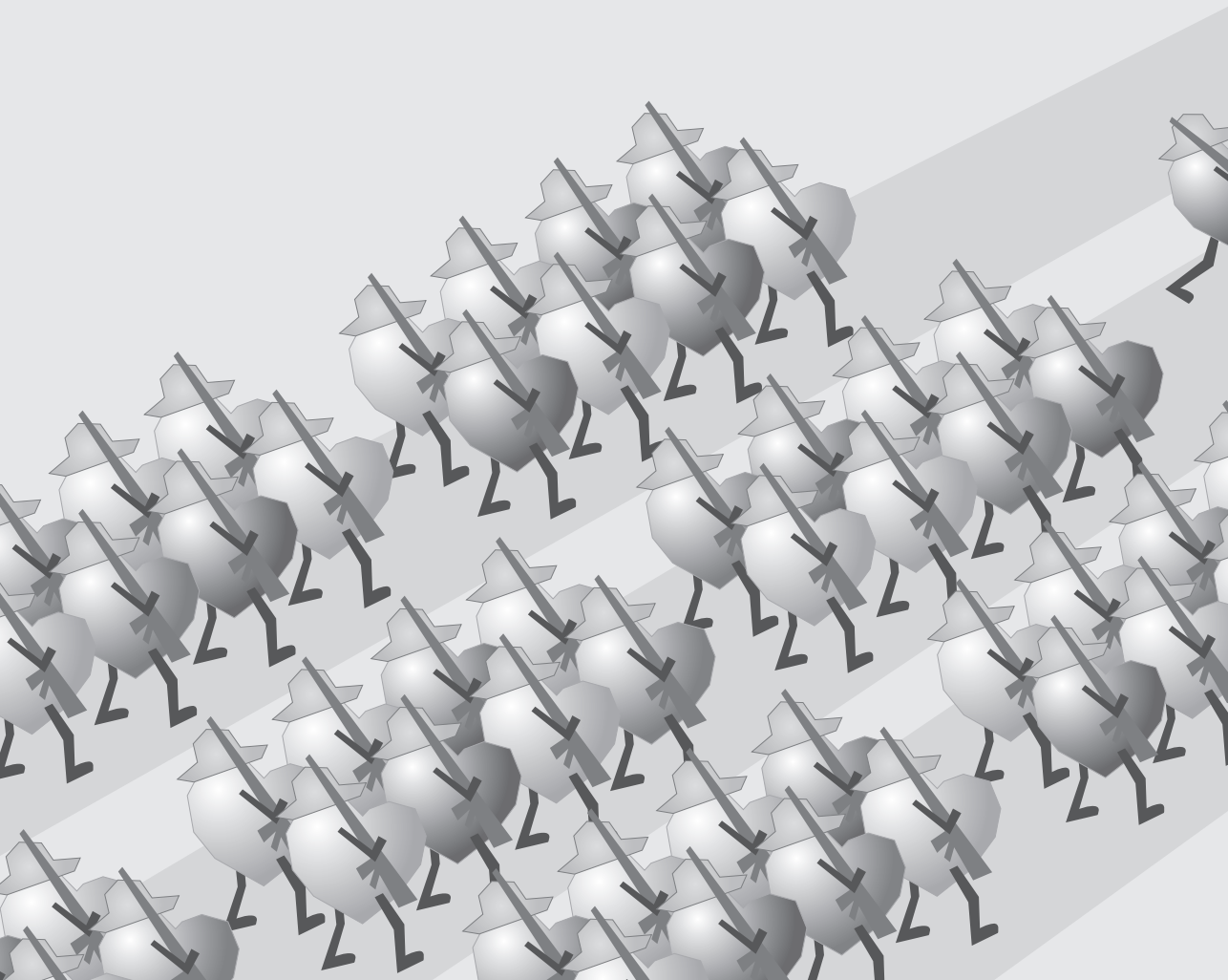
In keeping with the substantial genetic contribution found across different samples and the proven reliability of the assessment of RSA over time, several studies have tried to identify the actual genetic variants underlying RSA heritability. These mainly concerned candidate gene studies, in which a few genes were selected based on their known involvement in processes leading to differences in HRV levels. Candidate gene studies have found the angiotensin converting enzyme (ACE) (Busjahn et al., 1998), alpha-kinase anchoring protein 10 (AKAP 10) (Neumann et al., 2009; Tingley et al., 2007), methylene-tetrahydrofolate reductase (MTHFR) (Baccarelli et al., 2008), the circadian clock gene PERIOD3 (PER3) (Viola et al., 2008), and the brain-derived neurotrophic factor (BDNF) (Yang et al., 2010) to be associated with RMSSD and/or SDNN levels. Candidate gene studies do, however, need to be interpreted with caution before they have been confirmed in independent replication (Sullivan, 2007) and, ideally, functional studies exist that confirm a plausible pathway through which the genetic variant can influence RSA. In that sense, of all candidates found, the evidence of AKAP10 to be involved in HRV levels is strongest as its involvement was confirmed in animal research as well (Tingley et al., 2007). Candidate gene studies are by definition confined to current knowledge, and are selected from the biological mechanisms already expected to be involved in heart rate regulation. In genome wide association studies (GWAS), no a priori assumptions concerning biological mechanisms are made and the entire genome is considered to be 'candidate' (Tabor, Risch, & Myers, 2002; Manolio et al., 2009). To our knowledge, the Framingham Heart Study is the only group that has performed a GWAS on HRV thus far, but none of the associations reached genome-wide significance (Newton-Cheh et al., 2007). The sample studied was however small (N = 548). In a secondary analysis, this group did find significant candidate gene associations between the alpha-adrenergic receptor type 1A (ADDRA1A) and the alpha-adrenergic receptor type 1B (ADRA1B) genes and SDNN at $p < .05$. However, to be able to find genome wide significant hits, analyses on tens of thousands of participants are needed for which whole genome single nucleotide polymorphism and HRV data is available. Because no single research group can mount such numbers, the Genetic Variability in Heart Rate Variability (VgHRV) consortium was set up with the aim to share association results between different research groups and perform across-study meta-analyses on HRV (Nolte et al., 2011). This solves the problem of small sample sizes but may introduce yet another pitfall, namely the lack of unity regarding the HRV measures

that are used across different studies. With this study, we show that the genetic overlap between pvRSA and RMSSD, and RMSSD and SDNN, is high (genetic correlations are estimated at .94 and .89 in resting ambulatory sitting conditions) while the genetic overlap between pvRSA and SDNN, albeit lower, was still .74. From this it follows, that HRV studies that assessed pvRSA and RMSSD, or RMSSD and SDNN, can be safely pooled as the genetic architecture is expected to be highly similar.

A major limitation of this study is that exercise status was not obtained for all participants at the time of testing. Therefore, we cannot rule out that exercise status may explain part of the heritability that is found for HRV since previous work has shown that exercise behavior and RSA are genetically correlated (de Geus, Boomsma, & Snieder, 2003). A second limitation is that we used a crude approach in pooling all 24-hour HRV data in only three ambulatory conditions (sleep, sitting, or physically active) thereby potentially introducing heterogeneity within conditions, considering the wide range of activities that meet these criteria. This may be particularly pertinent for physical activity, where the range of variety is largest. On the other hand, ambulatory activities need to be generalized to some extent to make data of different participants comparable. Finally, as nighttime HRV recordings in twins and siblings, to our knowledge, have only been performed by our group we cannot compare our results to those of independent twin studies.

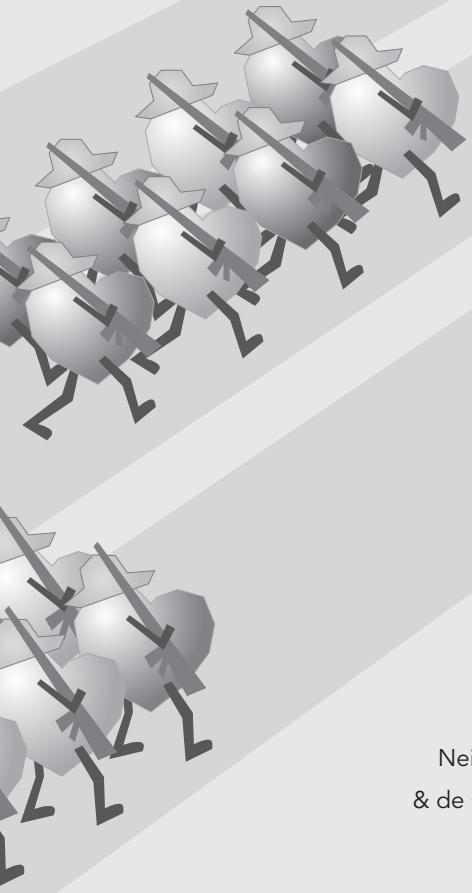
The major strength of this study is that this is the largest 24-h ambulatory cardiac monitoring sample to date in which the heritability of three widely accepted HRV measures is studied, including pvRSA, which is taken to be the most 'pure' HRV measure. Additionally, for the first time, within-participant IBI-RSA associations were inventoried to assess ceiling status and the impact of this potential confounding factor on the heritability estimates has now been thoroughly tested.

We conclude that about half of the variation that is seen in the levels of the three HRV measures that are currently used most in the fields of cardiology and psychophysiology, is genetically determined in the healthy adult population. The heritability estimates were robust against confounding by IBI-RSA ceiling effects that were observed in a subgroup of participants that took part in the study. There is no pressing need to exclude these participants, who may be overrepresented among healthy exercisers, in genetic studies of HRV. The genetic overlap between the three RSA measures studied is large, especially for pvRSA and RMSSD and RMSSD and SDNN, thereby implicating that these measures can be pooled in future GWASs to obtain larger sample sizes and increase power to find the actual genetic variants being responsible for individual differences in cardiac vagal control.



Chapter 4

Heritability and temporal stability of ambulatory autonomic stress reactivity in unstructured 24-h recordings



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Abstract

Measurement of ambulatory autonomic reactivity can help understand the long term health consequences of exposure to psychosocial stress in real-life settings. In this study, unstructured 24-h ambulatory recordings of cardiac parasympathetic and sympathetic control were obtained in 1288 twins and siblings, spanning both work time and leisure time. These data were used to define two ambulatory baseline (sleep, leisure) and four stress conditions (wake, work, work_sitting, work_peak) from which six ambulatory stress reactivity measures were derived. The use of twin families allowed estimation of heritability and testing for the amplification of existing or emergence of new genetic variance during stress compared to baseline conditions. Temporal stability of ambulatory reactivity was assessed in 62 participants and was moderate to high over a three-year period ($0.36 < r < 0.91$). Depending on the definition of ambulatory reactivity employed, significant heritability was found, ranging from 29 to 40% for heart rate, 34 to 47% for cardiac parasympathetic control (indexed as respiratory sinus arrhythmia), and 10 to 19% for cardiac sympathetic control (indexed as the pre-ejection period). Heritability of ambulatory reactivity was largely due to newly emerging genetic variance during stress compared to periods of rest. Interestingly, reactivity to short standardized stressors was poorly correlated to the ambulatory reactivity measures implying poor lab-real-life correspondence. We conclude that ambulatory autonomic reactivity extracted from an unstructured real-life setting shows reliable, stable and heritable individual differences. Real-life situations uncover new and different genetic variation compared to that seen in resting baseline conditions, including sleep.

Introduction

Exaggerated cardiovascular responses to stress have been associated with an increased risk for cardiovascular disease (CVD) (Chida & Steptoe, 2010; Treiber et al., 2003), although the effects will be modulated by genetic sensitivity and the frequency of stress exposure in daily life (Light, 2001; Steptoe & Kivimaki, 2012). To assess the propensity towards exaggerated reactivity, many studies have used standard mental stressors under controlled laboratory conditions. The individual differences in stress reactivity detected by such procedures are substantial, have proven to be reliable and may be associated with long term adverse cardiovascular outcome (Kamarck et al., 1992; Kamarck, Jennings, Stewart, & Eddy, 1993; Matthews et al., 2004). Nonetheless, there is valid concern about the extent to which laboratory tasks actually translate to stress situations in real-life (Johnston et al., 2008; Kamarck et al., 2000; Kamarck, Schwartz, Janicki, Shiffman, & Raynor, 2003; Schwerdtfeger, Schienle, Leutgeb, & Rathner, 2014). The laboratory setting, although important for inventorying the stress response in all its facets, cannot adequately capture prolonged activation and subsequent recovery processes (McEwen, 1998; Steptoe, Cropley, & Joekes, 1999; Schwartz et al., 2003).

Cardiovascular stress research has therefore shifted from using mental stress tasks (e.g. mental arithmetic, reaction time tasks) to also using tasks with a more social evaluative character (e.g. public speaking), see Chida & Hamer (2008) for an overview of the studies conducted in this area). Since the past two decades, cardiovascular stress research has also increasingly moved from the laboratory to the real-life situation of the participants. The rationale behind the ambulatory approach is that the daily life situation can give a more accurate and ecological valid reflection of the psychophysiological state of the participant and might therefore serve as a better indicator or predictor of disease risk (Zanstra & Johnston, 2011). This comes at a price. Compared to the laboratory setting, the ambulatory situation is per definition a much less controlled environment in which confounding factors like physical activity, posture, and time of day come into play (Steptoe, Cropley, & Joekes, 2000). Moreover, there is no standard baseline and there are no standard stressors to which all participants are exposed. Two strategies have been used to deal with this. First, ambulatory recordings have been scheduled during a concrete and relatively comparable stressful event like a school exam (Lucini et al., 2002; van Doornen, 1986; van Doornen et al., 1994) or an oral presentation (Houtman & Bakker, 1991a; Houtman & Bakker, 1991b; Johnston et al., 2008). The second strategy is to use

prolonged ambulatory recordings that span periods at work and in leisure time. Reactivity is then defined by comparing work levels to leisure time levels, under the assumption that the working day will be enriched for mentally and emotionally engaging events compared to leisure time (Riese, van Doornen, Houtman, & de Geus, 2004; Steptoe et al., 1999; Steptoe et al., 2000; Steptoe & Cropley, 2000; Vrijkotte, van Doornen, & de Geus, 2000; Vrijkotte et al., 2004).

A further challenge in ambulatory recording is that it does not allow the same in-depth assessment of physiology as is possible within a laboratory setting. Ambulatory recording has been typically confined to measurements of blood pressure and heart rate, whereas decrease in vagal tone and increased sympathetic nervous system (SNS) activity are the key drivers of stress-induced increases in blood pressure and heart rate (HR). Fortunately, two non-invasive key measures of cardiac sympathetic and vagal control can also be recorded with high fidelity in ambulatory settings: the pre-ejection period (PEP) and respiratory sinus arrhythmia (RSA) (de Geus, van Lien, Neijts, & Willemsen, 2015). When ambulatory HR, PEP and RSA are assessed in a 24-hour recording across a working day, followed by leisure time and sleep recording, there are various ways to define cardiac autonomic reactivity, for instance by defining either sleep or leisure time sitting as the baseline, or using periods of peak reactivity while sitting at work or the entire work period. Until now very little systematic study of the reliability and stability of individual differences in such ambulatory autonomic reactivity has been performed.

In the present study, we recorded 24-h ambulatory HR, RSA and PEP in over 1300 participants. During waking hours, participants filled out detailed activity diaries that were used to divide the entire signal of every participant into fixed periods of distinctive activities and postures. The periods were used to define two different resting (baseline) conditions and four conditions reflecting more mentally and/or emotionally engaging episodes (referred to as 'stress' from this point forward). Mean sleep levels were used as the ultimate resting baseline condition for each participant. As it is not always possible to measure during sleep, we also used an alternative baseline condition by selecting a period in leisure time during sitting activities which were relaxing in nature (internet, watching TV, reading). As a first stress level we used the entire waking period. Since participants were visited on a weekday and more than half of the participants were measured on a working day at the working location, we also extracted the mean working day level including all postures and the mean working day level for sitting activities only as alternative stress levels. We also extracted the most (psychological) arousing activities at work for each

participant by searching for periods with low physical activity but accompanied by high heart rates.

Two to five year retest data were available for 62 participants which allowed estimation of the temporal stability of these ambulatory reactivity measures. In 576 participants we also embedded a standard stress testing protocol assessing autonomic reactivity to two mental stress tasks. This allowed for a comparison of ambulatory reactivity to classical short-term stress reactivity. Measurements were done in monozygotic (MZ) and dizygotic (DZ) twins and their family members to be able to estimate the reliability and heritability of the various ambulatory reactivity measures. Significant heritability indicates that a measure is reliable and taps into stable biological differences. It was further tested whether there is amplification of existing or emergence of new genetic variance during the ambulatory stress conditions compared to ambulatory baselines as has previously been observed for laboratory stressors (de Geus et al., 2007; Riese et al., 2006; Wang et al., 2009). The overarching aim was to identify ambulatory autonomic reactivity measures from unstructured 24-h recordings for use in stress research that have good temporal stability, significant heritability, and the ability to detect stress-specific genetic variance.

Methods

Participants

Participants were all registered in the Netherlands Twin Register (NTR). The NTR has been collecting survey and biological data for over 25 years. In the biennial surveys, data on health, lifestyle, and personality is assessed (Willemsen et al., 2013). A subset of these participants were included in a large cardiac ambulatory monitoring project in which 24-hour recordings were collected in two separate studies (Kupper et al., 2005; Kupper et al., 2006; Neijts et al., 2014). Study 1 was conducted between August 1998 and June 2003 and included two waves of data collection with partial retest data. This sample was further expanded with a new data collection round, Study 2, that took place between November 2010 and June 2012. In the latter study, data was collected in a single wave. For both studies, adult twins and siblings without known CVD or other relevant health complaints were selected and informed about the study by mail. This was followed by a short telephone interview in which the health status of the participants was verified. A priori reasons for exclusion for all studies/waves were pregnancy, heart transplantation, presence of a pacemaker and known ischemic heart disease, congestive heart failure,

or diabetic neuropathy. We excluded data of 8 participants showing many arrhythmias or preventricular contractions. Of the remaining sample (N=1373), data of participants that were on cardiovascular medication, cardiac therapy, or antidepressant medication were excluded (N=71). To simplify genetic modeling, we excluded the third member of triplets (N=1) and used only one pair from families with multiple twins. We further restricted the number of siblings to a maximum of two singleton brothers and two singleton sisters per family, selecting the siblings who were closest in age to the twins and removing data for nine siblings. Three more participants were excluded because the rest of their family members participated in a different wave.

Table 4.1. Sample characteristics for Study 1, the retest study, which was part of Study 1, and Study 2. All twins and siblings that participated in Study 2 also participated in the laboratory protocol as this was embedded within the same 24-h ambulatory measurement.

	Ambulatory Study 1			*Ambulatory Study 2*
	Wave 1 (1998 – 2000)	**Wave 2 (2001 – 2003)**		**Wave 3 (2010 – 2012)**
	New	**New**	**Retest**	**New**
N individuals	367	380	62	541
MZ (N belonging to complete pair)	103 (86)	117 (104)	12 (0)	266 (230)
% MZ females	66.0	62.4	50.0	64.3
DZ (N belonging to complete pair)	144 (110)	120 (147)	29 (4)	180 (226)
% DZ females	75.9	76.3	65.5	72.9
Siblings (% female)	120 (57.5)	116 (61.2)	21 (57.7)	49 (59.2)
Age (SD)	28.5 (9.6)	32.9 (10.7)	33.6 (9.6)	37.2 (5.4)

In the end, recordings of 1288 participants were available with 486 monozygotic (MZ) twins (210 complete pairs), 517 dizygotic (DZ) twins (205 complete pairs), and 285 non-twin siblings. The data of participants belonging to an incomplete twin pair were included because they could still be paired to their non-twin sibling and/or contribute to the estimates of the means and variances. Incomplete pairs occurred because only one of the two participated or because data of the other twin was excluded for reasons mentioned earlier. Mean age was 33.5 years (SD = 9.2 years), and 61.6% of the sample was female. Zygosity of the twins was determined by DNA typing for 97.9% of the same-sex twin pairs. For 2.1% of the 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 is 96.1% (Willemsen et al., 2013). For 62 participants in wave 1 of Study 1, measurements were repeated in a wave 2 of that study after an average period of 3.3 years (range 2.1 to 4.7 years). For more detailed characteristics of the Study 1, Study 2, and the retest sample separately, see Table 4.1. The study protocol was approved by the Medical Ethics Committee of the VU University Medical Center Amsterdam and all participants gave written consent before entering the study.

Procedure

Participants were visited at home, before starting their normal daily activities. During a short interview, information on health status and current medication use was obtained. They were fitted with the VU University Ambulatory Monitoring System (VU-AMS) that records the electrocardiogram (ECG) and impedance cardiogram (ICG) continuously. For participants taking part in wave 1 and wave 2 of Study 1 the VU University Ambulatory Monitoring System (VU-AMS) version 4.6 was used. For Study 2, the 5fs version of the VU-AMS was used. A standard laboratory protocol was embedded within the ambulatory recording protocol of this study. During the home visit two typical laboratory tasks, each lasting two minutes, were executed by the participants in a fixed order. For this we used the computerized Stroop Color-Word conflict task and a Serial Subtraction task, because both cognitive tasks have proven capable of eliciting a psychophysiological stress response (Boutcher & Boutcher, 2006; Renaud & Blondin, 1997; Sung, Izzo, Jr., Dandona, & Wilson, 1999; Tulen, Moleman, van Steenis, & Boomsma, 1989). For the Stroop task, stimuli consisted of one of four color names that were printed in incongruent colors. In total, 99 presentations of 12 combinations of incongruent stimuli were presented in a random order for 2 minutes. As we only included a 2 min Stroop conflict task and none of the non-conflicting control tasks, the test was preceded by a 45 sec practice session. Participants verbally responded to the stimuli. In the Serial Subtraction task, participants were asked to sequentially subtract backward by 7 aloud as quickly as possible for 2 min. Each participant began with the number 1256. When an error was made, the participant was corrected and instructed to continue from that point on. The cognitive tasks were preceded by four minutes of quiet sitting in a secluded part of the house/work area.

Subsequently, participants were instructed to wear the VU-AMS device the entire day and night up until the next day, after having worn the device for 24 hours. Instructions were supplied that explained how to respond to potential alarm beeps (e.g.,

on loose electrode contacts), and telephone assistance was available during waking hours. Participants were requested to keep a paper-and-pencil diary and to write down a chronological account of their activity, posture, location, and social situation over the past time period (free recall). For wave 1 and 2 in Study 1 participants were prompted by an alarm beep to do so every 30 minutes, for Study 2 the diary was filled every 60 minutes. In addition, participants were instructed to write down at which time they had breakfast, lunch, and dinner and they were asked to refrain from vigorous exercise during the ambulatory recording day. The next day, the VU-AMS device was detached and collected by the researcher or returned by mail.

RSA and PEP measurement

RSA is considered a reliable index of parasympathetic control over the heart, whereas PEP is considered a reliable index of sympathetic control over the heart (de Geus et al., 2015). The assessment and quantification of respiration and RSA from VU-AMS recordings has been described previously (Neijts et al., 2014). Briefly, the dZ signal at the respiration frequency (0.1 to 0.4 Hz), combined with the inter-beat interval (IBI) series was used to compute 'peak–valley' RSA (pvRSA). In this method, RSA is scored by detecting the shortest IBI during inspiration and the longest IBI during expiration on a breath-to-breath basis according to procedures detailed elsewhere (de Geus et al., 1995; Houtveen et al., 2005). If no shortest or longest IBI could be detected in inspiration and expiration respectively, pvRSA was set to zero.

For the assessment of the PEP, a measure of cardiac contractility, both the ECG and the ICG are used. The ICG signal was ensemble averaged across the diary-coded activity periods (described in the section on Ambulatory data reduction), time-locking the signal to the R-wave peaks (Riese et al., 2003). The PEP is defined as the time interval between the Q-wave onset of the ECG and the B-point of the dZ/dt signal. The Q-wave reflects the onset of left ventricular activity and the B-point reflects the opening of the aortic valves. In both VU-AMS versions, the R and B points are scored automatically by the software. In the newer 5fs version of the VU-AMS, the entire ECG signal is stored, so the Q-onset time was available as well. All automated scoring was visually checked by the experimenter. For the calculation of PEP in the two waves of Study 1, a fixed Q-R interval of 48 msec was added to the duration of the R-B interval (Willemsen, de Geus, Klaver, van Doornen, & Carroll, 1996). For Study 2, the true Q-onset point was used when present; otherwise the grand average of the Q-R interval was summed to the R-B interval of the individual

participant. If R-onset was additionally missing, we subtracted the grand average Q-onset time from the individual participants' B-point (van Lien, Schutte, Meijer, & de Geus, 2013).

Ambulatory data reduction

Using the activity diary entries in combination with a visual inspection of the output of an inbuilt 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), physical load (no load, light, intermediate and heavy) and location of the participant (e.g. at home, at work, public space). Minimum duration of periods was 5 min 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. For each of the coded periods the mean IBI, RSA and PEP was calculated. The periods belonging to the standard baseline and stress conditions were additionally coded for the participants that took part in Study 2 with one period representing the resting baseline ('standardized_baseline') condition and one period representing the stress condition, averaged over the two tasks ('standardized_stress').

From the ambulatory autonomic nervous system (ANS) recording, two ambulatory baseline and four ambulatory stress conditions were defined. For the first ambulatory baseline condition, the mean IBI, pvRSA and PEP value across all sleeping periods was calculated ('sleep'). A period was classified as a sleeping period based on the reported bedtime in the diary and physical activity was verified by accelerometry. For the second ambulatory baseline, per participant the mean of periods spent sitting while being engaged in recreational activities in the evening, from 6 pm till bedtime, was determined to represent the alternative baseline condition ('leisure'). Periods summed to at least half an hour. For the first ambulatory stress condition, the mean waking level of IBI, pvRSA, and PEP was used, including both sitting and light physical (non-sitting) activities ('wake'). As participants were explicitly instructed not to engage in vigorous exercise during the recording day, these periods did not include high physical activity. Periods of light physical activity were classified as such based on the activity information obtained from the diary and the accompanying accelerometer signal. The additional ambulatory stress conditions were defined only for participants who reported the testing day to be a working day and who actually reported in the diary to have been at the work location during the testing day. For these participants all periods in which the participant was engaged in sitting

activities or light physical activity at work during a working day between 9 am and 6 pm were defined as work. Because there were multiple periods that fit this condition, mean IBI, pvRSA and PEP levels were determined across the entire working period ('work') and across a selection of periods at work when the participants were sitting ('work_sitting'). Finally, an ambulatory stress condition was created from a time frame of at least half an hour with the *highest* HR while the participant was sitting at work between 9 am and 6 pm. Because this timeframe only consisted of few coded periods and we wanted to prevent that relatively high weight would be given to extreme values, the *median* instead of the mean IBI, pvRSA, and PEP values of the periods with the highest HR was selected to represent 'work_peak'.

Table 4.2. Ambulatory conditions for ambulatory reactivity assessment. The reactivity measures that were calculated are listed separately.

Ambulatory condition	Description
Baseline	
sleep	Mean level during sleep
leisure	The mean of periods while the participant is sitting and engaged in recreational activities like internet, reading, watching television, but not eating or drinking. Periods sum to at least half an hour. Only periods in the evening, from 6 pm till bedtime, are considered eligible.
Stress	
wake	The mean waking level – including sitting and light physical activity only
work	The mean of all periods in which the participant was engaged in sitting or light physical activity between 9 am and 6 pm during a working day at the work location
work_sitting	Similar to 'work', except that the mean of all periods in which the participant was engaged in *sitting activities only* between 9 am and 6 pm during a working day at the work location was calculated
work_peak	A time frame of periods with the highest heart rate while the participant is sitting between 9 am and 6 pm during a working day at the work location. Periods sum to at least half an hour. The median of the selected periods is taken to represent work_peak
Reactivity	
Δ wake - sleep	Absolute difference score between wake and sleep level
Δ work - sleep	Absolute difference score between work and sleep level
Δ work_sitting - sleep	Absolute difference score between work_sitting and sleep level
Δ work_sitting - leisure	Absolute difference score between work_sitting and leisure level
Δ work_peak - sleep	Absolute difference score between work_peak and sleep level
Δ work_peak - leisure	Absolute difference score between work_peak and leisure level

Six different ambulatory reactivity measures were calculated by computing absolute diffe-

rence scores between wake minus sleep (Δ wake – sleep), work minus sleep (Δ work – sleep), work_sitting minus sleep (Δ work_sitting – sleep), work_sitting minus leisure (Δ work_sitting – leisure), work_peak minus sleep (Δ work_peak – sleep), and work_peak minus leisure (Δ work_peak – leisure). The standard stress reactivity was computed as the mean of the two mental stress tasks minus the baseline rest condition. For an overview of the ambulatory conditions and ambulatory reactivity measures, see Table 4.2.

Statistical analyses

Sample selection, data preparation, and all non-genetic statistical analyses were performed using IBM SPSS 20.0. The distributions of ambulatory IBI and PEP levels and all ambulatory reactivity measures were normal. For ambulatory pvRSA levels, a natural logtransformation was applied to obtain a normal distribution. Significant differences between ambulatory conditions were tested by a MIXED model ANOVA with age and sex (and with respiration rate for pvRSA only) as covariates and family as a random factor and ambulatory condition as a repeated fixed factor (sleep, leisure, wake, work, work_sitting, and work_peak). A similar ANOVA was used to test the difference between mental stress and baseline conditions during the standard part of the recording. Temporal stability of the ambulatory measures across the two waves of Study 1 was calculated as an intraclass correlation. Associations between ambulatory reactivity scores amongst themselves and with the standard stress reactivity were assessed by Pearson correlations. For all statistical testing, effects were considered significant when $p < .01$.

Genetic analysis

In a twin-study, variance is typically decomposed into latent genetic and environmental components. Genetic variance can be further decomposed into shared additive (A) and non-additive (D) components. Environmental variance can be decomposed in a component that is common in family members (C) or that is unique (E) to the individual. In a study design that only includes twins and siblings, estimates of C and D are confounded, and cannot be estimated simultaneously. In that case, the pattern of the twin correlations is used to guide the experimenters' choice to model either an ACE or an ADE model. An ACE model is chosen when the MZ correlations are less than twice as high as the DZ and sibling correlations. Dominance or non-additive genetic factors may be present when the MZ correlations are more than twice as high as the DZ and sibling correlations, and in that case an ADE model will be chosen (de Geus, 2010). MZ correlations can also be useful to

explore the reliability of the trait. Estimation of the ratio of Var(E) to the total variance (e^2) provides a first impression of the unreliability of the trait. The E factor contains true unique environmental effects on the trait plus measurement error. As MZ twins are correlated perfectly for genetic and for common environmental factors, $1 - e^2$ is equal to the MZ twin correlation (r_{MZ}). Therefore, r_{MZ} is a lower bound for the test-retest reliability coefficient, because at the level of an individual unique environment (E) may also lead to stability.

Genetic analyses were performed using structural equation modeling (SEM) in the software package Mx (Neale et al., 2006). In SEM, models are fitted to the data and a goodness of fit statistic is calculated for each model. Subsequently, the fit of the more parsimonious nested models is compared to the fit of the full model by means of the likelihood ratio test in which the difference in minus twice the logarithm of the likelihood (-2LL) is calculated, this difference has a χ^2 distribution. When the χ^2 test is significant ($p < .01$), the more parsimonious model is considered to fit significantly worse to the data than the fuller model it is tested against. Before the variance was decomposed into genetic and environmental components, saturated models were fitted to the data. In these fully parameterized models, we tested for heterogeneity in male and female variances and family correlations. More specifically, we tested if sex differences were present and if there was evidence for a twin-specific resemblance. The allowed limitations were carried forward in the specification of the genetic models. Overall, we did not find evidence for twin specific resemblance nor for systematic quantitative or qualitative sex differences. We therefore continued estimating all parameters by combining data from males and females. Sex and age (and respiration rate for pvRSA) effects on the mean were regressed out simultaneously with variance decomposition.

Bivariate genetic models were specified to examine the genetic architecture and the change in the genetic influences from baseline ambulatory conditions to ambulatory stress conditions (Supplemental Figure 4.1). More specifically, the heritability of the baseline condition, or the effect of A1, is estimated by the relative contribution of genetic variance to the total variance in the baseline condition and is assessed by the ratio of $a_{11}^2 / (a_{11}^2 + c_{11}^2 + e_{11}^2)$. Because the genetic variance during stress conditions can consist of the genetic variance that is shared with baseline activity and with new genetic variance that emerges during stress, heritability of the ambulatory stress condition is calculated as follows: $a_{21}^2 + a_{22}^2 / (a_{21}^2 + a_{22}^2 + c_{21}^2 + c_{22}^2 + e_{21}^2 + {}_{22}^2)$. The effects of the genetic factors that are expressed during baseline can be amplified ($a_{21} > a_{11}$) or deamplified ($a_{21} < a_{11}$) during stress. The significance of stress-specific genetic effects can be assessed by testing

if the path coefficient a_{22} is significantly different from zero. The part of the heritability that is due to these new genetic factors (A2) can be calculated as: $a_{22}^2 / (a_{21}^2 + a_{22}^2 + c_{21}^2 + c_{22}^2 + e_{21}^2 + {}_{22}^2)$. The heritability of ambulatory reactivity was also calculated by procedures described in more detail elsewhere (de Geus et al., 2007). Briefly, it was calculated as a change score within the bivariate model by adding a latent factor with fixed loadings of +1 and -1 on baseline and stress, respectively. The total variance of the ambulatory reactivity score is equal to the sum of the variance during baseline and stress, minus the covariance between baseline and stress conditions. So the genetic part of the variance of ambulatory reactivity is $a_{11}^2 + a_{21}^2 + a_{22}^2 - 2\ a_{11} \times a_{21}$ which is equal to $(a_{21} - a_{11})^2 + a_{22}^2$. Heritability of the ambulatory reactivity score is calculated by $((a_{21} - a_{11})^2 + a_{22}^2) / ((a_{21} - a_{11})^2 + a_{22}^2 + (c_{21} - c_{11})^2 + c_{22}^2 + (e_{21} - e_{11})^2 + e_{22}^2)$.

Results

Table 4.3 gives the mean levels and standard deviations of the three variables of interest for the six conditions and the means and standard deviations of the different reactivities that were calculated based on these conditions. The table shows that all participants have data during waking time. However, only 56.7% of the participants who reported that the testing day was a working day actually reported to be at the work location during the testing day. 13.9% of participants reported that the testing day was a working day, but they did not spend time in an external working environment (housewives, people working at home).

A significant main effect of ambulatory condition was found for all ANS variables; IBI, ($F(5, 4422) = 3127.954$, $p < .001$), pvRSA ($F(5, 4458) = 335.281$, $p < .001$), and PEP ($F(5, 4337) = 235.841$, $p < .001$). Post hoc testing of the reactivity values showed this to mainly reflect the four daytime versus sleep contrasts for all three ANS variables (see Table 4.3). For IBI and pvRSA, the two contrasts including leisure time as baseline measure were also significant (p's < .001).

Table 4.3. Means and standard deviations (SD) of IBI, pvRSA and PEP in the six ambulatory conditions. Reactivity is listed separately.

	IBI (ms)		pvRSA (ms)		PEP (ms)	
	N	*Mean (SD)*	*N*	*Mean (SD)*	*N*	*Mean (SD)*
Levels						
sleep	1242	983.62 (132.37)	1242	55.80 (24.63)	1225	106.28 (15.72)
leisure	1075	837.67 (115.93)	1075	48.30 (22.31)	1044	99.20 (16.32)
wake	1288	759.58 (95.46)	1288	40.86 (15.10)	1285	96.74 (15.67)
work	730	749.92 (113.52)	730	41.27 (16.65)	725	97.12 (16.79)
work_sitting	686	784.63 (112.70)	686	44.89 (18.25)	677	99.49 (17.78)
work_peak	633	739.26 (105.45)	633	39.83 (16.49)	624	97.93 (21.01)
Reactivities						
Wake-sleep	1242	-223.51 (85.46)	1242	-15.07 (19.00)	1225	-9.42 (12.89)
Work-sleep	701	-240.58 (106.43)	701	-14.89 (22.16)	685	-8.77 (14.81)
Worksit-sleep	657	-205.89 (100.75)	657	-11.48 (21.30)	639	-6.23 (15.17)
Workpeak-sleep	609	-254.71 (104.33)	609	-16.99 (22.83)	591	-8.06 (15.83)
Worksit-leisure	564	-58.70 (84.00)	564	-3.66 (14.77)	544	1.70 (10.03)
Workpeak-leisure	523	-106.08 (87.13)	523	-8.91 (16.75)	506	0.02 (11.11)

There were significant intercorrelations between all six reactivity measures for IBI, pvRSA and PEP (see Supplemental Table 4.1), but the choice of baseline was critical; the four reactivity measures using sleep as a baseline were highly correlated amongst each other (*r*'s > 0.87) and so were the two reactivity measures using leisure time sitting as a baseline (*r*'s > 0.89). Correlation was less strong between to the reactivity measures based on sleep versus those based on leisure time sitting ($0.30 < r < 0.61$ for IBI, $0.28 < r < 0.58$ for pvRSA, and $0.29 < r < 0.60$ for PEP).

Table 4.4 presents the levels attained during the standard stress testing protocol that took place at home preceding the ambulatory monitoring in Study 2. In this setting, task levels also significantly declined compared to rest for IBI ($F(1, 520) = 602.974$, $p < .001$), pvRSA ($F(1, 519) = 33.785$, $p < .001$), and PEP ($F(1, 482) = 98.296$, $p < .001$). The standard reactivity measure was very poorly correlated with each of the six ambulatory reactivity measures (see Supplemental Table 4.2), particularly for PEP.

Table 4.5 depicts the temporal stability for the ambulatory levels and reactivity measures. Higher temporal stability is more consistently found for the ambulatory levels ($0.71 < r < 0.90$) compared to the reactivity scores which nonetheless show very good stability over time for all three ANS parameters ($0.36 < r < 0.91$). The fact that the ambulatory levels capture stable individual traits is further reinforced by the MZ

twin correlations that can be used as a proxy of the *minimal* test-retest reliability for the ambulatory levels (Supplemental Figure 4.2).

Table 4.4. Means and standard deviations (SD) of IBI, pvRSA, and PEP levels and reactivity during the standard stress protocol.

	IBI (ms)		**pvRSA (ms)**		**PEP (ms)**	
	N	*Mean (SD)*	*N*	*Mean (SD)*	*N*	*Mean (SD)*
Rest	522	834.62 (120.27)	522	48.36 (22.22)	493	107.12 (20.75)
Tasks	521	774.32 (111.43)	521	44.75 (17.73)	493	103.06 (21.01)
Δ task – rest	521	-59.82 (55.65)	521	-3.51 (13.87)	483	-4.22 (9.30)

Table 4.5. Temporal stability of the ambulatory levels and reactivity measures over an average time period of 3.3 years.

	IBI		**pvRSA**		**PEP**	
	N	*ICC*	*N*	*ICC*	*N*	*ICC*
sleep	53	.80	53	.86	51	.84
leisure	48	.68	48	.82	47	.87
wake	62	.79	62	.82	62	.89
work	19	.77	19	.90	19	.87
work_sitting	19	.71	19	.89	18	.86
work_peak	16	.74	16	.85	15	.85
Δ wake - sleep	53	.71	53	.83	51	.49
Δ work - sleep	17	.91	17	.84	16	.48
Δ work_sitting - sleep	17	.89	17	.79	15	.52
Δ work_sitting - leisure	15	.36	15	.58	13	.76
Δ work_peak - sleep	17	.40	17	.68	15	.73
Δ work_peak - leisure	13	.44	13	.85	12	.65

MZ twin correlations were higher than DZ twin correlations suggesting a role for genetic factors in explaining individual differences in IBI, pvRSA, and PEP levels during rest and stress periods in real-life. This was confirmed by genetic structural equation modeling. For all conditions, IBI and pvRSA in both the ambulatory and the standard setting and for PEP in the standard setting only, ACE models were fitted to the data. For PEP in the ambulatory conditions, ADE models were fitted to the data as the MZ twin correlations in these instances were more than twice as high as the DZ twin correlations. Formal testing showed that C and D factors could be dropped from all models and AE models provided

the most parsimonious fit for all three ANS measures in all conditions in both settings. Heritability estimates for the IBI, pvRSA, and PEP levels in all baseline and stress conditions and the ambulatory and standard reactivity measures are listed in Table 4.6. In general, heritability for the baseline levels was lower than heritability of the stress levels but, as can also be judged from the confidence intervals, this difference was only significant for work_sitting compared to leisure and sleep where it went from 51 to 69% and from 53 to 69%, respectively, for IBI. For PEP, heritability of work_sitting (44%) was also significantly higher than leisure (25%) and there was a clear trend for work_peak for which heritability was estimated at 42%.

For all ambulatory reactivity contrasts, there was a common genetic factor that influenced both baseline and stress levels but, in addition, new genetic factors were found to emerge during stress compared to the baseline conditions, yielding significant heritability of the corresponding ambulatory reactivity measures. For IBI, significant heritability of reactivity was found for five reactivity measures, except for leisure compared to work_peak. For the pvRSA, heritability of four reactivity measures were significant, the exceptions being work_sitting compared to leisure and work_peak compared to leisure. For PEP, heritability of ambulatory reactivity was significant for the wake compared to sleep and both the work_sitting and work_peak compared to leisure contrasts. During stress, genes acting at the resting level were *deamplified* for all ambulatory IBI and pvRSA reactivity measures, with the exception of Δ work_sitting – leisure for IBI and pvRSA, and Δ work_peak – leisure for pvRSA only. Deamplification of genetic variance means that the effect of the genes active during rest accounted for a smaller part of the variance during stress. For the ambulatory PEP reactivity measures, no significant deamplification of genetic factors was found when going from rest to stress and the genetic factors influencing leisure time were even significantly *amplified* by the work_sitting and the work_peak conditions.

For standard stress reactivity, too, a common genetic factor was found to influence both rest and task levels and new genetic factors emerged during task stress for all ANS measures. Although these effects were significant, they were less pronounced in the standard stress environment compared to real-life.

Table 4.6. Heritability of the levels of IBI, pvRSA, and PEP during the baseline and stress levels, the cause of changes in heritability from baseline to stress (amplification vs emergence), and the heritability of the reactivity measures.

	Baseline level h2 (99% CI)	Stress level h2 (99% CI)	Amplification / deamplification of genes acting on baseline level (99% CI)	Specific h2 due to genes emerging during stress (99% CI)	Reactivity h2 (99% CI)
IBI					
wake - sleep	0.52 (0.41-0.62)	0.55 (0.44-0.64)	Deamplification, a_{21}/a_{11} = 0.63 (0.53-0.74)**	0.18 (0.11-0.25)**	0.38 (0.24-0.51)**
work - sleep	0.53 (0.41-0.63)	0.61 (0.46-0.72)	Deamplification, a_{21}/a_{11} = 0.68 (0.53-0.86)**	0.24 (0.10-0.36)**	0.33 (0.15-0.49)**
work_sitting - sleep	0.53 (0.42-0.63)	0.69 (0.56-0.78)	Deamplification, a_{21}/a_{11} = 0.71 (0.57-0.88)**	0.27 (0.15-0.39)**	0.40 (0.21-0.56)**
work_sitting - leisure	0.51 (0.38-0.61)	0.69 (0.57-0.78)	Deamplification, a_{21}/a_{11} = 0.89 (0.72-1.10)	0.18 (0.05-0.30)**	0.29 (0.08-0.49)**
work_peak - sleep	0.53 (0.42-0.63)	0.60 (0.44-0.73)	Deamplification, a_{21}/a_{11} = 0.60 (0.46-0.76)**	0.26 (0.12-0.39)**	0.38 (0.19-0.55)**
work_peak - leisure	0.51 (0.39-0.62)	0.60 (0.43-0.73)	Deamplification, a_{21}/a_{11} = 0.78 (0.61-0.99)**	0.14 (0-0.29)	0.21 (0-0.43)
task - rest	0.58 (0.42-0.69)	0.50 (0.34-0.63)	Deamplification, a_{21}/a_{11} = 0.79 (0.69-0.89)**	0.06 (0.02-0.11)**	0.38 (0.19-0.53)**
pvRSA					
wake - sleep	0.46 (0.32-0.58)	0.55 (0.43-0.66)	Deamplification, a_{21}/a_{11} = 0.69 (0.52-0.91)**	0.27 (0.16-0.38)**	0.34 (0.19-0.49)**
work - sleep	0.47 (0.33-0.59)	0.52 (0.33-0.66)	Deamplification, a_{21}/a_{11} = 0.52 (0.26-0.81) **	0.39 (0.21-0.54)**	0.43 (0.24-0.60)**
work_sitting - sleep	0.47 (0.33-0.59)	0.55 (0.34-0.71)	Deamplification, a_{21}/a_{11} = 0.58 (0.30-0.88) **	0.40 (0.21-0.55)**	0.47 (0.25-0.64)**
work_sitting - leisure	0.43 (0.27-0.57)	0.53 (0.33-0.69)	Deamplification, a_{21}/a_{11} = 0.89 (0.62-1.26)	0.15 (0-0.32)	0.22 (0-0.47)
work_peak - sleep	0.47 (0.34-0.59)	0.34 (0.09-0.56)	Deamplification, a_{21}/a_{11} = 0.39 (0.07-0.72) **	0.28 (0.05-0.47)**	0.40 (0.15-0.60)**
work_peak - leisure	0.44 (0.28-0.58)	0.33 (0.14-0.54)	Deamplification, a_{21}/a_{11} = 0.86 (0.56-1.18)	0 (0-20)	0.01 (0-0.31)
task - rest	0.50 (0.30-0.65)	0.53 (0.34-0.67)	Deamplification, a_{21}/a_{11} = 0.86 (0.69-1.09)	0.11 (0.03-0.19)**	0.31 (0.09-0.49)**
PEP					
wake - sleep	0.38 (0.24-0.50)	0.41 (0.28-0.53)	Deamplification, a_{21}/a_{11} = 0.88 (0.67-1.16)	0.11 (0.02-0.20)**	0.18 (0.03-0.32)**
work - sleep	0.38 (0.25-0.51)	0.45 (0.26-0.61)	Deamplification, a_{21}/a_{11} = 0.92 (0.61-1.31)	0.16 (0-0.30)	0.21 (0-0.40)
work_sitting - sleep	0.38 (0.25-0.51)	0.44 (0.23-0.61)	Deamplification, a_{21}/a_{11} = 0.99 (0.66-1.42)	0.13 (0-0.30)	0.18 (0-0.41)
work_sitting - leisure	0.25 (0.11-0.39)	0.44 (0.26-0.58)	Amplification, a_{21}/a_{11} = 1.44 (1.07-1.99)**	0.02 (0-0.13)	0.19 (0.02-0.44)**
work_peak - sleep	0.38 (0.24-0.50)	0.39 (0.17-0.57)	Deamplification, a_{21}/a_{11} = 0.86 (0.50-1.30)	0.17 (0-0.34)	0.22 (0-0.45)
work_peak – leisure	0.26 (0.12-0.39)	0.42 (0.23-0.58)	Amplification, a_{21}/a_{11} = 1.43 (1.07-1.92)**	0 (0-0.11)	0.10 (0.01-0.33)**
task - rest	0.30 (0.09-0.49)	0.32 (0.11-0.49)	Deamplification, a_{21}/a_{11} = 0.97 (0.74-1.29)	0.04 (0.01-0.07)**	0.20 (0.04-0.35)**

Note. ** = significant effect ($p < .01$).

Discussion

This study describes a large twin study on changes in ambulatory levels of IBI, PEP, and pvRSA when going from sleep and resting in leisure time to more socially and mentally engaging activities during the working day. We found that ambulatory autonomic reactivity is a stable, heritable individual trait, showing moderate to high temporal stability over a three-year follow-up period. Depending on the definition of ambulatory reactivity employed, heritability ranged from 29 to 40% for IBI, 34 to 47% for pvRSA, and 10 to 19% for PEP, although not all reactivity measures showed heritability. Heritability of ambulatory autonomic reactivity was largely due to new genetic variance specifically emerging during stress, while the genetic factors influencing resting baseline levels became less prominent under stress.

These results replicate and extend previous findings in laboratory stress testing. In an earlier laboratory study of our group stress-specific heritability for HR was seen in an adolescent and in a middle-aged sample (de Geus et al., 2007). No stress-specific genetic effects for RSA were found in both age groups, but another study in adolescents did report stress-specific genetic effects on heart rate variability (HRV) (Wang et al., 2009). For PEP, stress-specific genetic effects were found in adolescent but not in middle-aged twins (de Geus et al., 2007). Although we did find stress-specific genetic effects on HR, RSA and PEP in the current study during standard task stress, the influence of stress-specific genetic effects in real-life was generally larger. The partly divergent findings may be due to the nature of the stress tasks that were used. De Geus et al. (de Geus et al., 2007) used short mental stress tasks to induce stress while Wang et al. (2009) used tasks that more closely approached stress in real-life by including virtual reality car driving, a video game challenge, and a social competence interview. Combining this with the evidence from the current ambulatory study, we hypothesize that the use of more ecologically valid stressors tends to increase the contribution of novel genetic factors to individual differences in stress-reactivity.

Previous prospective studies on the health effects of reactivity have been limited to short-term reactivity to standard laboratory stress tasks (Chida & Steptoe, 2010; Treiber et al., 2003). Correlations between short-term stress reactivity calculated from our standard rest and stress tasks with ambulatory reactivity were weak which confirms previous research finding poor generalizability of artificial to more realistic reactivity measures (Johnston et al., 2008; Kamarck et al., 2000; Kamarck et al., 2003; Schwerdtfeger et al.,

2014). This again suggests that the stress induced in the typical laboratory setting may not fully capture the individual differences measured during stress in real-life.

An important question is whether our results allow us to select a clear favorite ambulatory autonomic reactivity measure from unstructured 24-h recordings for use in stress research, using the temporal stability, heritability, and the ability to detect stress-specific genetic variance as prioritization criteria. For IBI, these criteria do not point to an optimal reactivity measure nor do they disqualify any of the six reactivity measures. Temporal stability was good, and of similar magnitude or even better as in previous ambulatory studies (Goedhart et al., 2006; Goedhart et al., 2007; Vrijkotte, Riese, & de Geus, 2001). It was particularly good when compared to the temporal stability of laboratory reactivity for HR (Allen, Sherwood, Obrist, Crowell, & Grange, 1987; Burleson et al., 2003; Cohen et al., 2000; Hamer, Gibson, Vuononvirta, Williams, & Steptoe, 2006; Hassellund, Flaa, Sandvik, Kjeldsen, & Rostrup, 2010; Llabre et al., 1993; Manuck & Garland, 1980; Sherwood et al., 1997; Veit, Brody, & Rau, 1997), PEP (Allen et al., 1987; Burleson et al., 2003; Llabre et al., 1993; Sherwood et al., 1997), or HRV (Burleson et al., 2003; Bertsch, Hagemann, Naumann, Schachinger, & Schulz, 2012; Dragomir, Gentile, Nolan, & D'Antono, 2014). Five IBI reactivity measures were heritable and, due to genetic emergence, more than half of the variation in stress levels of heart rate was due to genetic factors. For PEP and RSA, temporal stability was moderate to good and with few exceptions all reactivity measures were also significantly heritable. For PEP during work_sitting and work_peak no new genetic variance emerged compared to leisure time, instead existing variance got amplified. Using MZ correlations as an indicator of reliability, IBI, RSA and PEP levels also performed well for all six conditions, with no clear 'best' baseline or stress condition. We therefore conclude that all six measures as defined here have comparable properties from a psychometric viewpoint.

To select an optimal ambulatory autonomic reactivity measure additional criteria, more related to content, need to be considered. Three of these are the mean absolute size of reactivity, the extent of the individual differences (variance), and the avoidance of contrasts incorporating changes in posture and physical activity. Statistical power to detect correlation of reactivity with health outcomes scales with its variance and effect size. Standard deviations for the reactivity values were of near comparable magnitude as those for the IBI, pvRSA, and PEP levels, implying there is considerable variation in individual responses to daily life situations. This held true for all reactivity measures incorporating either sleep or leisure time as a baseline, although most variation was seen

in the reactivity measures that included sleep as a baseline. When higher absolute values of reactivity (reflecting larger autonomic engagement) are considered, reactivity measures based on sleep also seem most favorable as they yield the strongest reactivity. However, sleep versus wake contrasts also contain changes in posture and physical activity which are known to influence RSA and PEP through effects other than true changes in cardiac autonomic control (Houtveen et al., 2005; Grossman et al., 2004). Validity of PEP as a readout for cardiac sympathetic control is sensitive to distortion by postural shifts as it is affected by both preload and afterload (Houtveen et al., 2005). The contrasts that avoid large influences of posture change and that are characterized by low physical activity may therefore be optimal, i.e. Δ work_sitting – leisure and Δ work_peak – leisure. However, these were also the contrasts with lowest absolute reactivity, making them less attractive. The magnitude of reactivity computed for these two work conditions with sleep as a baseline (Δ work_sitting – sleep and Δ work_peak – sleep) was much higher. Work_sitting and work_peak were likely characterized by active mental and/or social engagement with the environment, but in the presence of minimal physical activity. As the postural shift from supine to sitting is less severe than the shift from supine to standing (which could occur frequently during wake and non-sitting work periods) Δ work_sitting – sleep and Δ work_peak – sleep may be the preferred measures for studies seeking to link (genetic variants for) individual differences in ambulatory reactivity to health outcomes.

A limitation of this study is the use of objective landmarks in unstructured 24-h recordings to delineate periods as stressful without subjective confirmation of stress by the participant. We reasonably assumed that time spent at work would be more enriched than leisure or sleep by mentally and socially engaging activities and that the presence of a high heart rate within a period of accelerometer-confirmed minimal physical activity was likewise attributable to the effects of such activities. However, the selection of periods of high HR at work is meaningful only when all participants encounter comparable work stressors at the recording day and at least one stressor with sufficient salience to trigger substantial HR reactivity. Therefore, our physiology-driven definition of peak stress may have led us to inadvertently label people who did not encounter a strong stressor at work as low HR reactors. To resolve this circularity, we should have assessed individual differences in the amount of subjective stress exposure at work, which we did not. On the other hand, subjective experience is known to be only very poorly correlated to physiological reactivity (a topic recently covered in a special issue of *Biological Psychology*, see for example Friedman, Stephens, & Thayer, 2014). The critical question is whether autonomic

reactivity as defined here is a good predictor of health outcomes. This of course remains to be tested – here we have shown that there is stable, heritable variation in ambulatory reactivity that can be meaningfully used in future predictive studies.

A further limitation is that the ambulatory baseline conditions that were defined for this study, leisure time and sleep, occurred post-stress in the real-life assessment. This means that recovery processes instead of a 'true baseline' level may have been measured. If recovery processes were indeed involved during leisure time or sleep, this may have contributed to the poor correlation between our standard and ambulatory reactivity measures, as a pre-test baseline was used to compute the standard stress reactivity.

In conclusion, ambulatory autonomic reactivity extracted from an unstructured real-life setting shows reliable, stable and heritable individual differences. Real-life situations uncover new and different genetic variation compared to that seen in resting baseline conditions, including sleep. The contrasts between sitting work levels, including the peak stress period, and sleep baseline seem the most promising ambulatory reactivity measures for research in the field of psychosomatic medicine.

Supplement to Chapter 4

Heritability and temporal stability of ambulatory autonomic stress reactivity in unstructured 24-h recordings

Supplemental Table 4.1. Intercorrelations (N) among the ambulatory reactivity measures.

	Δ wake - sleep	**Δ work - sleep**	**Δ work sitting - sleep**	**Δ work sitting - leisure**	**Δ work_ peak - sleep**
IBI					
Δ wake - sleep					
Δ work - sleep	.91 (701) **				
Δ work_sitting - sleep	.90 (657) **	.95 (657) **			
Δ work_sitting - leisure	.31 (552) **	.54 (552) **	.58 (552) **		
Δ work_peak - sleep	.87 (609) **	.88 (609) **	.94 (609) **	.51 (513) **	
Δ work_peak - leisure	.30 (513) **	.47 (513) **	.52 (513) **	.92 (523) **	.61 (513) **
RSA					
Δ wake - sleep					
Δ work - sleep	.96 (701) **				
Δ work_sitting - sleep	.94 (657) **	.96 (657) **			
Δ work_sitting - leisure	.28 (552) **	.44 (552) **	.47 (552) **		
Δ work_peak - sleep	.92 (609) **	.92 (609) **	.94 (609) **	.47 (513) **	
Δ work_peak - leisure	.33 (513) **	.43 (513) **	.44 (513) **	.89 (523) **	.58 (513) **
PEP					
Δ wake - sleep					
Δ work - sleep	.96 (685) **				
Δ work_sitting - sleep	.93 (639) **	.96 (639) **			
Δ work_sitting - leisure	.31 (529) **	.47 (529) **	.55 (529) **		
Δ work_peak - sleep	.90 (591) **	.92 (591) **	.96 (591) **	.52 (494) **	
Δ work_peak - leisure	.29 (494) **	.41 (494) **	.48 (494) **	.90 (506) **	.60 (494) **

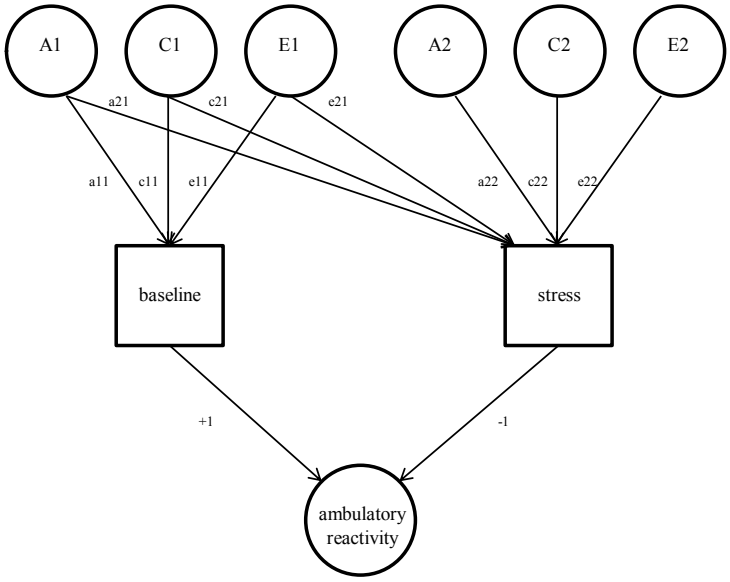
Note. ** = significant correlation ($p < .01$).

Supplemental Table 4.2. 'Lab-real life' correlation (N) between the reactivity to structured stressors and ambulatory reactivity measures.

	Δ wake - sleep	**Δ work - sleep**	**Δ work_ sitting - sleep**	**Δ work_ sitting - leisure**	**Δ work_ peak - sleep**	**Δ work_ peak - leisure**
Δ task – rest IBI	.16 (511) **	.02 (297)	.06 (273)	-.04 (216)	.16 (248) *	.08 (196)
Δ task – rest pvRSA	.14 (511) **	.09 (297)	.05 (273)	.12 (216)	.12 (248)	.20 (196)**
Δ task – rest PEP	-.06 (471)	-.10 (270)	-.12 (245)	-.13 (185)	-.07 (222)	-.06 (168)

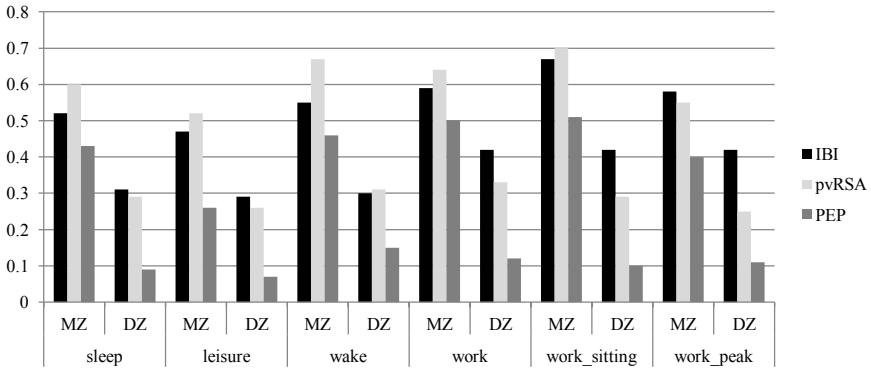
Note. ** = significant correlation ($p < .01$), * = significant correlation ($p < .05$).

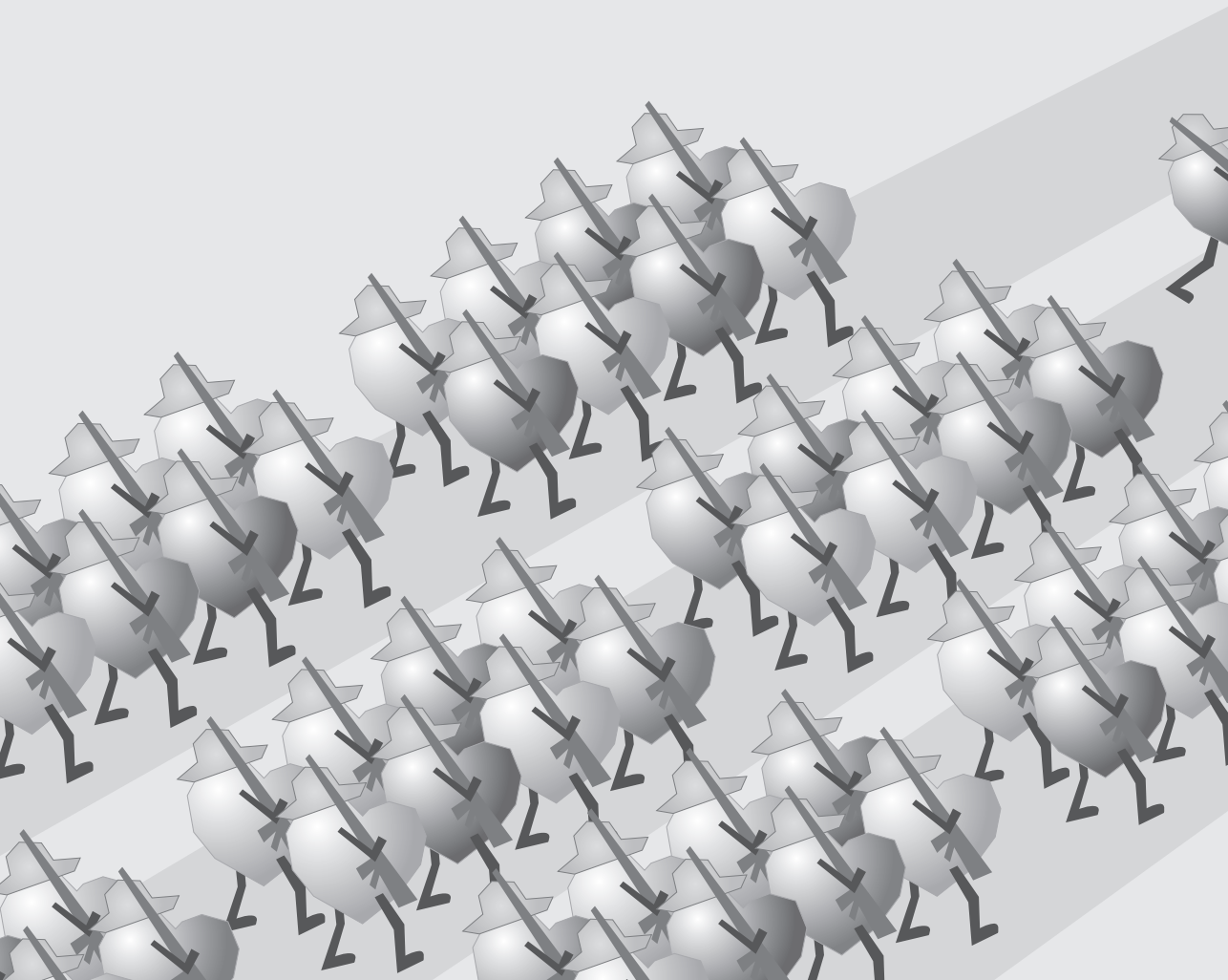
Supplemental Figure 4.1. Example of a bivariate genetic ACE model that was fitted to the ambulatory baseline and stress conditions.



Note. A1 and A2, C1 and C2, and E1 and E2 are the latent genetic, shared environmental, and unique environmental factors, that influence the observed baseline and stress measures. The a11, c11, and e11 paths are unique to the baseline measure, whereas paths a22, c22, and e22 are unique to the stress measure. With the a21, c21, and e21 paths the genetic, shared environmental, and unique environmental factors of the covariance between baseline and stress levels, is described. The model also specifies a 'delta score' ambulatory reactivity which represents the difference between baseline and stress scores.

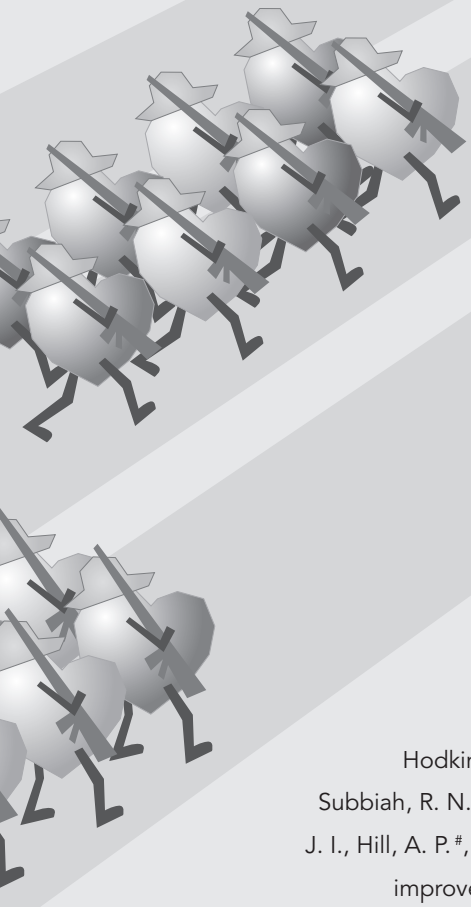
Supplemental Figure 4.2. MZ and DZ correlations of the IBI, pvRSA, and PEP levels as obtained from the saturated Mx models.





Chapter 5

A novel approach to Holter data analysis improves phenotyping and increases the precision of genetic analysis of ECG biomarkers



This chapter is under revision:

Hodkinson, E. C.*, Neijts, M.*, Sadrieh, A.*, Baumert, M., Rajesh, N., Subbiah, R. N., Hayward, C. S., Boomsma, D. I., Willemsen, G., Vandenberg, J. I., Hill, A. P.#, & de Geus, E. J. C #. A novel approach to Holter data analysis improves phenotyping and increases the precision of genetic analysis of ACG biomarkers. *Heart Rhythm*. (*/# Equal first/last authorship).

Abstract

The resting ECG is routinely used to assess cardiac electrical activity, which is a highly dynamic process. We wanted to determine whether analysis of frequency specific data from Holter ECGs allows for a more complete genetic dissection of cardiac electrophysiology. Holter ECGs were recorded from 221 twin pairs and analyzed using a novel multi-parameter beat classifier to extract heart rate specific data. Heart rate dependent estimates of heritability for QRS duration, QT interval, Tpeak–Tend and T-wave amplitude (TWA) were calculated using structural equation modelling. Our data showed that QRS duration is largely determined by environmental factors whereas repolarization is primarily genetically determined. Heritability estimates of both QT interval and TWA were significantly higher when measured from Holter compared to resting ECGs and the heritability estimate of each was heart rate dependent. Analysis of the genetic correlation between repolarization parameters demonstrated that while most of the genes that contribute to covariance of individual ECG parameters at different heart rates overlap, at each specific heart rate there was relatively little overlap between the genes that determine the different repolarization parameters. Here we present the first study of heritability of repolarization parameters measured from Holter ECGs. Our data demonstrate that higher heritability can be estimated from the Holter than the resting ECG and reveals rate dependence in the genetic – environmental determinants of the ECG that has not previously been tractable. Future uses of this analysis include deeper dissection of the ECG of participants with inherited cardiac electrical disease.

Introduction

The electrocardiogram (ECG) represents the summed electrical activity of all the action potentials of individual myocytes in the heart (Einthoven, 1912; Nerbonne & Kass, 2005). The generation of these action potentials reflects the finely tuned and coordinated opening and closing of ion channels that conduct either depolarizing inward currents (sodium and calcium) or repolarizing outward currents (potassium) (Nerbonne & Kass, 2005). Regional variation in ion channel density results in distinct action potential waveforms in different parts of the heart (Gaborit et al., 2007). When combined, they form the P-QRS-T complexes of the electrocardiogram. Specifically, the P wave corresponds to atrial depolarization, the QRS complex reflects ventricular depolarization and the T wave corresponds to ventricular repolarization. As such, the ECG has been an extraordinarily useful non-invasive diagnostic tool for assessing abnormalities of cardiac electrical activity for over a century (Fye, 1994).

Typically, a resting ECG is recorded over ~10 seconds and gives a 'snapshot' of the electrical activity of the heart. The cardiac electrical cycle however, particularly in relation to repolarization, is an incredibly dynamic process modulated by multiple inputs including sympathetic and parasympathetic nervous systems (Coumel, Fayn, Maison-Blanche, & Rubel, 1994), sleep-wake cycles (Jeyaraj et al., 2012), heart rate, and gender (James, Choisy, & Hancox, 2007; Stramba-Badiale, Locati, Martinelli, Courville, & Schwartz, 1997). A wealth of information relating to an individual's cardiac electrical phenotype is therefore missed when recording just a snapshot of the ECG. As an alternative, recording cardiac electrical activity over a 24-hour period using continuous ambulatory ECG (Holter) is a much richer source of information that has the potential to provide a more complete and accurate phenotypic picture (Coumel et al., 1994). Holter ECGs are already a routinely used diagnostic tool in monitoring heart rate variability (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996) and detecting the presence of arrhythmia episodes. They have also been used in a research setting to assess repolarization parameters. However, these previous studies used low temporal resolution recordings (Extramiana et al., 2010; Merri et al., 1992; Neyroud et al., 1998; Sobue et al., 2011; Sugao et al., 2006; Vaglio et al., 2008) and furthermore, the usefulness of ambulatory recording in diseases of repolarization has been questioned (Mauriello, Johnson, & Ackerman, 2011). Two of the reasons for this are the difficulties associated with manipulating and analyzing the large data sets produced over 24 hours

and secondly the problem of accounting for the range of physiological scenarios sampled. One approach to overcome these issues is to use a classification algorithm to "bin" beats that are representative of specific physiological states. Averaged representative beats can then be analyzed quickly and easily. Analyzing ECG parameters in this manner offers two main advantages over the resting ECG. First, one can obtain data that are representative of multiple physiological conditions such as specific heart rates. Second, by averaging multiple beats obtained from specific physiological states, one can obtain a better signal to noise ratio, thus improving the accuracy when measuring the intervals and amplitudes of ECG waveforms.

In this study we have used a novel dual-parameter beat classification algorithm to investigate whether analysis of ambulatory Holter ECG recordings can provide more accurate and detailed information about the genetic basis of cardiac electrical phenotypes relative to that obtained from analysis of the resting ECG. Specifically, we tested: (1) whether heritability of ambulatory recordings, after frequency binning, is larger than heritability of resting ECG, (2) the extent to which genetic influences on the ambulatory depolarization and repolarization parameters are heart rate-dependent, and (3) whether different genes are involved in the manifestation of repolarization in the three different parameters used. Taken together, we test the hypothesis that analysis of frequency binned beats from ambulatory ECG recordings allow for a more complete genetic dissection of individual differences in cardiac electrophysiology than a short resting ECG recording.

Methods

Participants

Holter recordings were carried out on 442 participants – 123 monozygotic (MZ) complete pairs and 98 dizygotic (DZ) complete twin pairs. Participating twins were registered with the Netherlands Twins Registry and all took part in the third wave of a large 24-h ambulatory cardiac monitoring study (Neijts et al., 2014). Zygosity was confirmed by DNA analysis for 97.3% of same-sex twin pairs. For the remaining same-sex pairs, zygosity was based on survey questions regarding physical similarity and the frequency of confusion of the twins by parents, other family members, and strangers (Willemsen et al., 2013). A priori reasons for exclusion from this cohort were heart transplantation, presence of a pacemaker, known ischemic heart disease, congestive heart failure, diabetic neuropathy, and pregnancy. In addition, 79 of the participants were taking medication with the

potential to the ECG (Supplemental Table 5.1) and were excluded from the main analysis. For comparison, twin correlations including participants on cardioactive medications are shown in Supplemental Table 5.2. The study protocol was approved by the medical Ethics Committee of the VU University Medical Center Amsterdam and the Human Research Ethics Committee of the New South Wales Ministry of Health (Australia). All participants gave written consent before entering the study.

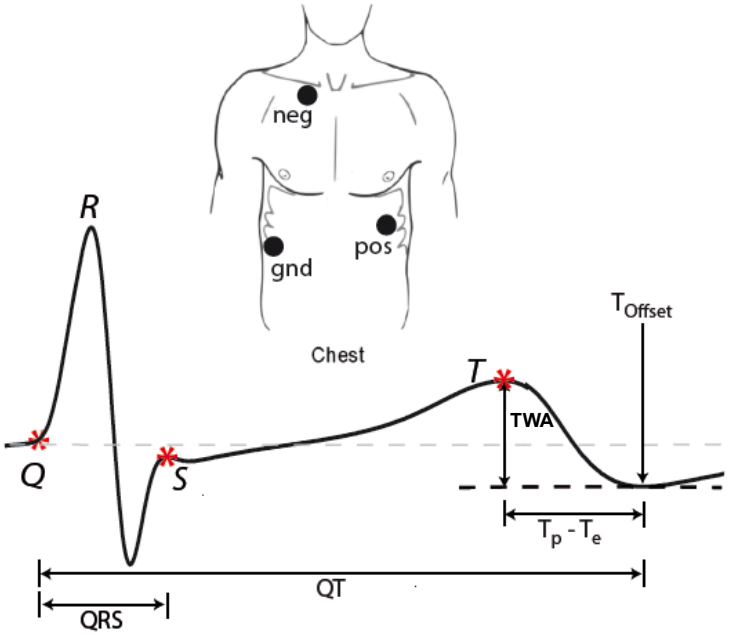
Holter ECG recording procedure

On the recording day, participants were visited either at home or at work and information on health status and current medication use was obtained. Participants were fitted with the 5fs version of the Vrije Universiteit Ambulatory Monitoring System (VU-AMS device, VU University Amsterdam, www.vuams.nl) to acquire both electrocardiogram (ECG) and impedance cardiogram (ICG) data continuously over a 24-h period (de Geus et al., 1995; van Dijk et al., 2013; Willemsen et al., 1996). Participants were asked to refrain from vigorous exercise during the measurement day. For acquisition of ECG data three electrodes were placed in modified CS5 lead positions - right subclavicular region 4 cm to the right of the sternum (negative electrode), under the left breast, 4 cm under the nipple (positive electrode) and the lower right thorax (ground electrode) – to obtain a derived Lead II. A typical averaged ECG obtained using the modified CS5 lead positions is shown in Figure 5.1. The raw ECG signal was imported into the VU-DAMS software (version 3.2, VU University Amsterdam, www.vu-ams.nl) and exported to an ASCII file sampled at 1 kHz for further processing.

Five landmarks were identified on each averaged ECG waveform: (i) Q: first deflection from the isoelectric line after the P-wave, (ii) R: peak of the QRS complex, (iii) S: intersection of S wave upstroke and T-P isoelectric line, (iv) Tpeak: peak of the T-wave, (v) Toffset: the point 95 % of the distance from T-peak to the minimum of the T wave. Since the modified CS5 lead positioning can result in biphasic T-waves (where the end of the T wave overshoots the isoelectric line; Figure 5.1), this estimate of the minimum point is typically taken as the end of the T wave (van Lien et al., 2015).

From these landmarks we measured the QRS duration (time from Q to S), a measure of ventricular depolarization, and QT interval (time from Q to Toffset), Tp-Te (time from Tpeak to Toffset) and TWA (amplitude from Tpeak to Toffset), all measures of ventricular repolarization (Figure 5.1).

Figure 5.1. ECG acquisition. Three electrodes (positive, negative and ground) were placed in modified CS5 lead positions to obtain a derived Lead II. The example averaged waveform shows ECG landmarks and the measured intervals (QRS, QT, Tp-Te) and amplitudes (TWA).



Extraction of averaged ECGs from Holter recording

Recordings were initially processed using a modified version of R-wave detection and waveform boundary recognition software available from the open source PhysioNet resource (Moody, Mark, & Goldberger, 2000; Goldberger et al., 2000). Every beat of the 24 hour recording was classified first by the R-R interval into 0.1 Hz frequency 'bins' and then by R wave amplitude, before each bin was averaged (Figure 5.2). For subsequent analysis of heritability, three frequencies were considered - 1 Hz (Low heart rate, 60 bpm), 1.3 Hz (Medium heart rate, 78 bpm) and 1.6 Hz (High heart rate, 96 bpm).

To permit comparison with previous estimates of heritability for ECG parameters derived from a standard resting ECG, an averaged QRS-T complex was generated for each participant from 10 successive beats with the lowest frequency and lowest standard deviation of R-R interval chosen from the period between 09.50 am and 10.10 am (the period that on average, for the entire participant dataset, had the lowest SD of RR interval) (Supplemental Figure 5.1). This approach ensured that we had the 'best' resting ECG in

terms of both stability and resting heart rate (average heart rate was 67 ± 0.6 bpm or 1.12 ± 0.01 Hz (SEM; n=385)). In addition to the 4 standard ECG measurements, we also calculated corrected QT intervals for the resting ECGs, using Bazett's formula (Bazett, 1920).

Rate corrected QT has consistently been shown to have lower heritability than uncorrected QT (Haarmark, Kyvik, Vedel-Larsen, Budtz-Jorgensen, & Kanters, 2011; Carter et al., 2000), a trend that was confirmed in our first pass twin correlation calculations (see Supplemental Table 5.3). Therefore all further analysis was carried out using uncorrected QT for the resting ECG, as the most stringent comparison to our Holter derived heritability estimates. Both the binned Holter data and the resting ECG data went through a two-step process of quality control. First, data was deemed unusable if there was an insufficient number of beats at the specified frequency or if it was too noisy. This left 323, 365 and 346 participants for low, medium and high heart rate respectively. Usable data was extracted to derive 'resting ECGs' for 427 participants. In stage two, two blinded independent investigators checked the computer-detected points for the Q-wave, the J-point, T-peak and T-offset for every tracing. If the computer-generated data were deemed inaccurate, all measurements for that participant at that particular heart rate were discarded. After quality control, we had data for 322 participants at low heart rate, 357 participants at medium heart rate and 313 participants at high heart rate (this corresponds to 90-99% of traces deemed usable). For the resting ECGs, data from 385 participants were suitable for heritability analysis.

Genetic analysis based on twin data

Genetic models, addressing the etiology of individual differences, were fitted to the data using structural equation modelling (SEM) in the software package Mx (Neale et al., 2006). When data from twins are available, variance in an observed trait, called phenotypic variance, is typically decomposed into variance due to latent additive genetic factors (A), non-additive genetic factors (D), common environment (C) shared by family members, and non-shared or unique environment (E). In the classical twin design, which includes monozygotic (MZ) and dizygotic (DZ) twins, estimates of C and D are confounded as the total phenotypic variance, the MZ covariance, and the DZ covariance only provide sufficient information to estimate three out of four parameters (Boomsma, Busjahn, & Peltonen, 2002). Based on the pattern of twin correlations, we chose to model either an ACE or an ADE model. For all twins (MZ and DZ), common environmental factors are set

to correlate 1.0. Additive genetic factors are set to correlate 0.5 in DZ twins, and 1.0 in MZ twins, and non-additive genetic factors are set to correlate 0.25 in DZ twins, and 1.0 in MZ twins. Nonshared, or unique environmental factors are by definition uncorrelated in family members. These theoretical values among latent factors form the basis of the model to estimate the influence of A, C or D and E on the phenotype.

After establishing the most parsimonious variance components model (ACE or ADE, AE, CE, or E) for each ECG phenotype at each frequency two separate analyses were conducted. First, to test if heritability of the resting ECG differs from the Holter ECG and to examine whether the heritability of the different variables extracted from the Holter ECG was rate dependent, quadrivariate genetic models were fitted to the data for the three rate specific Holter groups (1.0Hz, 1.3Hz and 1.6Hz) and the resting ECG data (Supplemental Figure 5.2). Fit statistics of constrained models that equated the heritability for various combinations of the parameters where compared to those of the full model that estimated a separate heritability for each of the four ECG parameters. Second, to examine whether the three repolarization parameters were influenced by common or different genetic factors, trivariate genetic models were fitted to the rate specific Holter data. (Supplemental Figure 5.3) From these models we computed the genetic correlations between the three parameters and the contribution of the common genetic factor to the phenotypic correlation. The comparison of the fit of restricted models to the full model was performed by means of likelihood-ratio (χ^2) tests in which the difference in -2LL between the two models is calculated. When the likelihood-ratio test is significant, the restricted model is considered to fit significantly worse to the data than the fuller model it is tested against.

All models regressed the effects of age and sex on the phenotype. A priori, we assumed no quantitative or qualitative sex differences in the variance decomposition to be present so only one MZ and one DZ correlation was estimated for each variable.

Results

Dual parameter beat classification of Holter ECGs

To obtain ECG signals representative of specific physiological states, we used a beat binning approach (Figure 5.2A). For every beat in the Holter record, three parameters were measured: 1) The interval from the previous R wave to that of the selected beat (RRn), 2) The interval from the R wave of the selected beat to the subsequent R wave (RRn+1), and 3) The amplitude of the R wave of the selected beat (Ramp).

Based on these criteria, every beat was classified according to RRn and Ramp and a 'heatmap' created showing the frequency distribution of beats as a function of these parameters (Figure 5.2B). From this heatmap, subsets of beats representative of particular heart rates can be extracted. For example, the shaded region in Figure 5.2B corresponds to a heart rate of 1 Hz or 60 bpm. To reduce the number of abnormal and/or ectopic beats included in the analysis, only those beats that had an Ramp within 1 standard deviation (SD) of the mean Ramp at that frequency were included in the average for that frequency bin (Figure 5.2C). Furthermore, beats with an abnormally short or long coupling interval to the subsequent beat (RRn+1), were also excluded from the analysis for similar reasons (Figure 5.2D). A family of beats extracted at 1 Hz according to the criteria described above are shown in Figure 5.2E. This family of beats, all of similar morphology and timing, are then ensemble averaged, based on alignment of the peak of the R wave, to give a beat representative of a particular physiological state that has high signal to noise ratio, and is amenable to further analysis (Figure 5.2F). In order to test the efficacy of our binning approach in extracting beats from the Holter recording that are representative of different heart rates, we examined the rate dependent trends in ECG parameters measured from low, medium and high heart rate beats (Figure 5.3 and Supplemental Table 5.4). All measures of repolarization showed rate dependence. Both QT interval and Tp-Te shortened with faster heart rates, while TWA reduced. In contrast, the QRS duration, a measure of depolarization of the myocardium, was relatively rate independent.

Figure 5.2. Dual parameter beat binning from Holter ECG. A) 10 beats from a typical raw Holter trace. For the beat highlighted in red, three measured parameters are shown: the interval from the peak of the R wave to the previous R peak (RRn), the interval from the R peak to the next R peak (RRn+1) and the R-amplitude (Ramp). B) A typical 'Heatmap' of the distributions of ECG beat characteristics over the 24hr period classified according to RRn and Ramp. The region incorporating beats with a frequency of 1 +/- 0.025 Hz is highlighted in grey. C) Frequency distribution of R peak amplitude (Ramp) within the region defined by 1+/- 0.025Hz in (B). The region incorporating 1 SD of Ramp variation is highlighted in grey. D) Frequency distribution of RRn+1 within the region defined by 1SD variation in (C). The region incorporating 2SD in RRn+1 is highlighted in grey. E) Representative family of 100 beats at 1Hz after the filtering procedure described in B, C and D. F) Averaged beat derived from the data in (E).

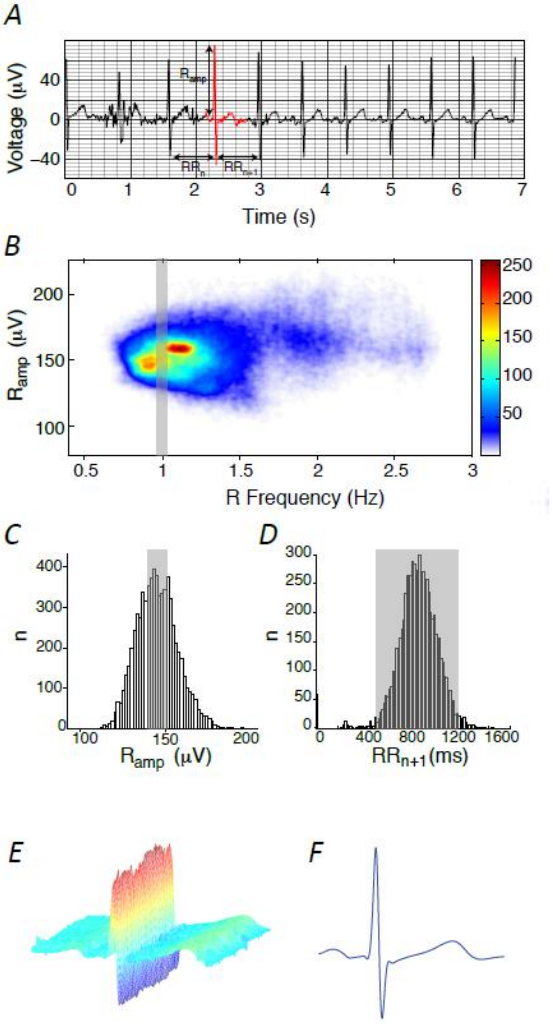
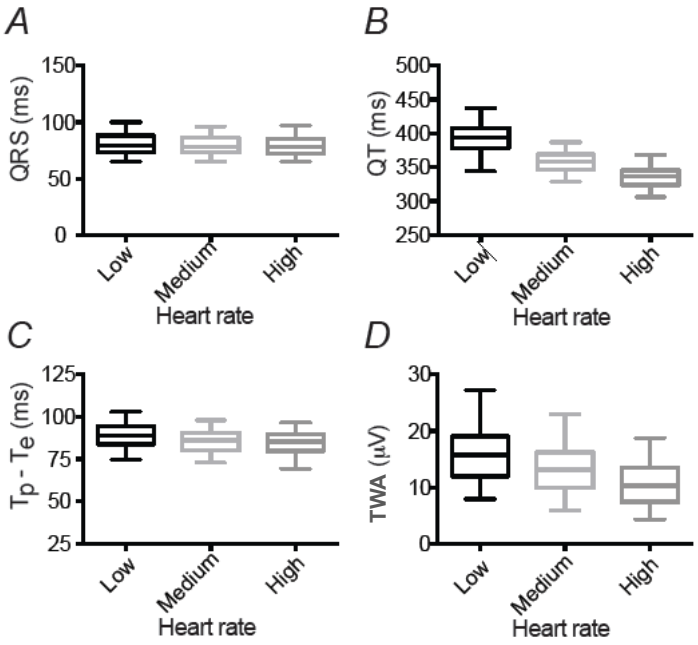


Figure 5.3. Heart rate dependence of ECG parameters. Box plots of the raw frequency-binned ECG measurements showing the rate dependence of QRS duration (A), QT interval (B), Tp–Te (C) and TWA (D). For each box plot, the centre line marks the mean value, the outer box edges the 25th and 75th percentile and the 'whiskers' denote the 5th and 95th percentile.



Heritability of ECG parameters

Scatterplots of the MZ versus DZ pairwise correlations for QT intervals obtained from the low heart rate ECGs and from the resting ECGs are shown in Figure 5.4A & B respectively. From these plots it is clear that there is a greater correlation in the monozygotic (MZ) twins compared to the dizygotic (DZ) twins, consistent with there being a significant genetic contribution to QT interval. The twin correlations for each of the 4 ECG parameters at low, medium and high heart rates as well as from the resting ECG, for participants not taking cardioactive medications are summarized in Table 5.1. MZ pair correlations were higher than the DZ correlation for every parameter at all heart rate categories and in the resting ECG indicating that there is a genetic component for all parameters. As a general trend, the MZ twin correlations were highest for low and medium rate Holter data and lower for high rate Holter and for the resting ECG. Very similar results were also obtained when participants taking cardioactive medication were included (see Supplemental Table 5.2).

To eliminate the potential confounding influence of these medications all subsequent analyses were undertaken solely using participants not taking cardioactive medications.

Table 5.1. Monozygotic and dizygotic twin correlations from the saturated model. 99% CIs are shown in parentheses.

ECG parameter	Rate	MZ correlation	DZ correlation
TpTe	Low	0.62 (0.36-0.77)	0.12 (-0.27-0.46)
	Medium	0.69 (0.49-0.81)	0.07 (-0.40-0.49)
	High	0.55 (0.23-0.73)	-0.39 (-0.71-0.34)
	Resting	0.55 (0.29-0.71)	0.00 (-0.35-0.36)
TWA	Low	0.65 (0.34-0.80)	0.34 (-0.05-0.61)
	Medium	0.66 (0.43-0.79)	0.46 (0.10-0.69)
	High	0.53 (0.22-0.72)	0.42 (0.02-0.67)
	Resting	0.54 (0.26-0.71)	0.06 (-0.25-0.36)
QT	Low	0.71 (0.49-0.82)	0.07 (-0.33-0.45)
	Medium	0.62 (0.38-0.76)	0.15 (-0.20-0.50)
	High	0.50 (0.18-0.69)	0.25 (-0.17-0.57)
	Resting	0.51 (0.23-0.69)	0.14 (-0.16-0.41)
QRS	Low	0.52 (0.19-0.72)	-0.10 (-0.46-0.30)
	Medium	0.52 (0.23-0.71)	-0.10 (-0.43-0.27)
	High	0.42 (0.12-0.62)	0.07 (-0.47-0.55)
	Resting	0.34 (0.06-0.56)	-0.14 (-0.44-0.21)

Having established that there is a genetic component to each of the ECG parameters, we performed formal twin modelling to estimate heritability. Our initial modelling included additive genetic factors (A), non-additive genetic factors (D), and non-shared or unique environment (E). Univariate genetic analyses showed that non-additive genetic effects could be dropped from the ADE models without significant loss of fit. For all parameters, therefore, a model including only additive genetic and unique environmental factors, or an AE model, was retained for further analyses.

A quadrivariate AE model for the ECG parameters for low, medium and high rate Holters and the resting ECG was fitted to the data (see Supplemental Figure 5.2). The heritability estimates obtained from this model are summarized in Table 5.2. On the whole, repolarization parameters (QT, Tp-Te and TWA) showed a larger heritability estimate

(ranging from 56% to 72% at low heart rates) than QRS, the depolarization parameter (41% at low heart rate). Significantly higher heritability estimates could be obtained when measured from the Holter compared to the resting ECG for QT interval ($p < .01$) and TWA ($p < .05$). Likewise, significant rate dependence of the heritability estimate from Holter recordings was observed for QT interval ($p < .01$) and TWA ($p < .05$). A formal statistical analysis of these relationships is presented in Supplemental table 5.5.

Figure 5.4. QT interval correlations in twin pairs. Scatter plots and linear regression of QT interval for monozygotic (MZ) and dizygotic (DZ) twin pairs measured from low rate Holter ECG (A) of resting ECG (B). For each twin pair, Y-axes depict QT interval of the first born twin and X-axes the QT interval of the second born twin.

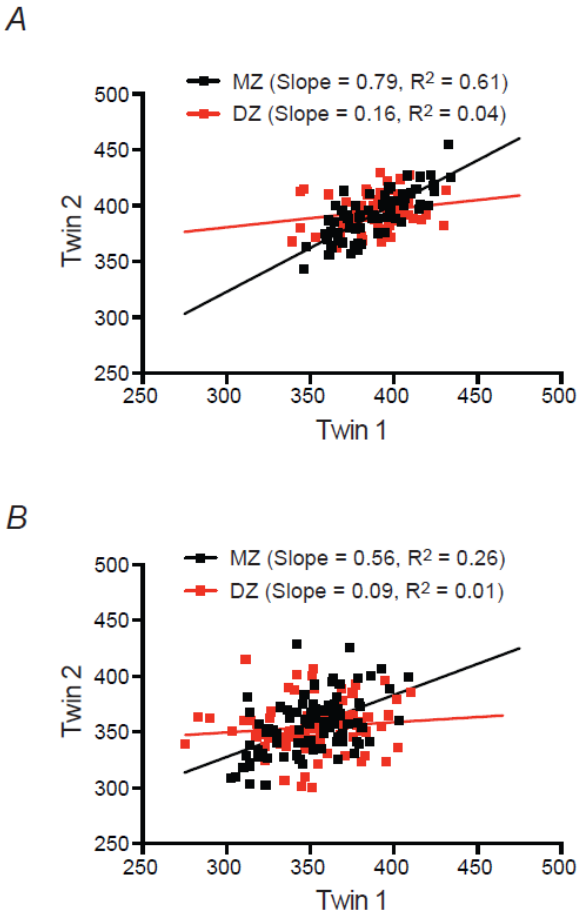


Table 5.2. Heritability of ECG parameters for Low, Medium and High heart rate Holter and resting ECG. Asteriks denote statistical difference compared to low Holter where ** = p < .01 and * = p < .05. Full statistical analysis is presented in Supplemental Table 5.5.

Parameter	Best Model	ECG type	Heritability (99% CI)
TpTe	AE	Low	56% (32-73%)
		Medium	63% (36-79%)
		High	52% (21-73%)
		Resting	56% (30-72%)
TWA	AE	Low	72% (53-83%)
		Medium	68% (49-80%)
		High	58% (36-73%)*
		Resting	55% (31-71%)*
QT	AE	Low	69% (48-82%)
		Medium	58% (33-75%)
		High	34% (14-53%)**
		Resting	40% (18-58%)**
QRS	AE	Low	41% (10-64%)
		Medium	41% (13-63%)
		High	42% (14-62%)
		Resting	32% (7-55%)

Rate dependence of genetic factors influencing Holter ECG parameters

To further investigate the rate dependence observed above, we tested the extent of the correlation among ECG parameters measured at different heart rates and to what extent genetic or environmental factors explained this phenotypic correlation (see Table 5.3). Phenotypic correlations across heart rates ranged from 0.47-0.94 and were highest for TWA and QRS. The genetic contributions to these phenotypic correlations were higher for the repolarization parameters than for QRS. The 3rd column of Table 5.3 lists the genetic correlation for the two ECG parameters being considered in each case. With the exception of the comparisons between Tp-Te at low and high heart rates and QT at low and high heart rates, all the other genetic correlations are very high, up to 1.0 for QRS between medium and high heart rates. This indicates that largely the same genetic factors influence the different ECG parameters irrespective of the heart rate at which they are being measured.

Table 5.3. Phenotypic and genetic correlation between different heart rates for individual ECG parameters. 99% CIs are shown in parentheses.

Parameter / Frequency comparison	Phenotypic Correlation	Genetic Correlation between rates	Contribution of genetic factors to phenotypic co-variance
Tp-Te			
Low-Medium	0.76 (0.67-0.83)	0.88 (0.73-0.97)	68% (39-86%)
Low-high	0.56 (0.41-0.67)	0.56 (0.20-0.80)	54% (14-82%)
Medium - High	0.76 (0.68-0.83)	0.89 (0.72-0.97)	66% (32-87%)
TWA			
Low-Medium	0.93 (0.90-0.95)	0.98 (0.94-1.00)	74% (55-85%)
Low-high	0.82 (0.75-0.87)	0.91 (0.79-1.00)	72% (51-85%)
Medium - High	0.93 (0.90-0.95)	0.97 (0.93-1.00)	66% (45-79%)
QT			
Low-Medium	0.70 (0.61-0.78)	0.77 (0.57-0.92)	69% (42-86%)
Low-high	0.47 (0.32-0.59)	0.52 (0.15-0.78)	54% (14-81%)
Medium - High	0.72 (0.64-0.79)	0.84 (0.62-0.95)	52% (24-72%)
QRS			
Low-Medium	0.94 (0.92-0.96)	0.97 (0.84-1.00)	42% (12-65%)
Low-high	0.86 (0.81-0.90)	0.95 (0.79-1.00)	46% (13-69%)
Medium - High	0.90 (0.87-0.93)	1.00 (0.95-1.00)	46% (15-68%)

Genetic correlations between the repolarization parameters

We next investigated whether the three repolarization parameters (QT, TWA and Tp-Te) were influenced by similar or different genetic factors. This is an important question as it pertains to whether measuring multiple parameters provides significantly more information about the genetic signature of repolarization or whether a single parameter is sufficient. The phenotypic correlations between parameters at any given rate (Table 5.4, second column) are clearly much lower than the correlations for individual parameters at different heart rates (Table 5.3, second column). At low and medium heart rates, phenotypic and genetic correlations between the repolarization parameters were significant but of modest size. However, what phenotypic covariance was observed was almost entirely caused by genetic factors (Table 5.4, last column). For high heart rates, no significant phenotypic and genetic correlations between the repolarization parameters was found. These data therefore show that while there is low phenotypic correlation between the three repolarization parameters, this correlation is almost entirely due to a common genetic factor shared by all three repolarization parameters.

Table 5.4. Phenotypic and genetic correlation between repolarization ECG parameters at each heart rate. 99% CIs are shown in parentheses.

Frequency/parameter comparison	Phenotypic correlation	Genetic correlation between parameters	Contribution of genetic factors to phenotypic covariance
Low			
QT-TpTe	0.38 (0.22 - 0.51)	0.38 (0.05 - 0.63)	65% (9 – 97%)
QT-TWA	-0.28 (-0.43 - -0.12)	-0.42 (-0.70 - -0.12)	100% (39 – 100%)
TpTe-TWA	-0.39 (-0.52 - -0.24)	-0.58 (-0.93 - -0.26)	92% (47 – 100%)
Medium			
QT-TpTe	0.32 (0.16 - 0.45)	ns	ns
QT-TWA	-0.25 (-0.40 - -0.09)	-0.46 (-0.82 - -0.15)	100% (58 – 100%)
TpTe-TWA	-0.18 (-0.33 - -0.02)	-0.36 (-0.75 - -0.06)	100% (58 – 100%)
High			
QT-TpTe	0.31 (0.15 - 0.46)	ns	ns
QT-TWA	ns	ns	-
TpTe-TWA	ns	ns	-

Discussion

In this study we present the first measurement of the heritability of repolarization parameters from Holter ECGs. To achieve this we have developed a novel beat binning algorithm allowing extraction of averaged beats that are representative of specific heart rates and physiological states. Twin modelling based on this approach resulted in similar heritabilities for Tp-Te and QRS, compared to the resting ECG, but higher estimates for TWA and QT interval. Furthermore, our novel analysis allowed us to interrogate the overlap between the genetic factors influencing individual ECG parameters at different heart rates as well as between the genetic factors that determine the different repolarization parameters at each heart rate separately. Our data therefore demonstrates the potential for analysis of Holter ECGs to give a more complete insight into the genetic underpinnings of the cardiac electrical system than the standard resting ECG.

Beat Binning approach

We set out to test the hypothesis that analysis of Holter ECGs can provide more in depth information regarding the cardiac electrical genotype that the standard resting ECG. This hypothesis is based on the assumption that the Holter ECG samples a broad range of physiological states over the 24-hour acquisition period compared to the limited

physiological range of the typical resting ECG based on 10 beats or recordings up to a maximum of 300s, and is therefore potentially a much richer source of information if analyzed effectively. However, extraction of meaningful measurements from the Holter is complicated both by the volume of information that needs to be processed, and the often noisy data, both in terms of physiological variability and signal noise, that result from the varied activity and physiological states of the participant throughout the time-course of the acquisition.

With this in mind we have developed a dual parameter beat binning approach to extract averaged beats that are representative of different physiological states. This approach eliminates the two main problems outlined above. First, by binning beats with similar properties from throughout the Holter recording based on RR interval and R amplitude, we can extract families of beats that are representative of specific heart rates. Furthermore, by eliminating outliers from the analysis we can ensure that we are only considering 'normal' beats, with ectopic and/or abnormal beats excluded from the analysis. Second, by averaging these families of normal beats with common characteristics, we eliminate the large computational overhead of measuring ECG parameters from every individual beats, and obtain low noise, clean waveforms that are amenable to analysis. Not only does this approach allow for more like-for-like analysis between participants, i.e. we can directly compare a 1Hz/60 bpm waveform from each participant, it also allows us to analyze rate dependent trends in phenotype and the genetic contributions to these trends. This is not possible considering the resting ECG alone.

By way of validation, analysis of rate dependent trends in the characteristics of the idealized waveforms were consistent with previously published data. Specifically, QT interval and Tp-Te shortened, and TWA decreased with faster rates (Couderc et al., 2007; Lehmann & Yang, 2001). Conversely, the QRS interval was rate-independent in our data, also consistent with previous observations (Simoons & Hugenholtz, 1975). This consistency of rate dependent trends in ECG parameters in comparison to the previously published literature supports our dual-parameter beat binning approach, and presents us with the opportunity to analyze the heritability and genetic underpinnings of these rate dependent trends.

Heritability of ECG parameters

To assess heritability of ECG characteristics, and to dissect the genetic-environmental interplay in defining features of the ECG, we used a classical twin study. Under the best

fitting models, the parameters were explained by a combination of additive genetic factors and unique environmental influences (which include measurement error). Broad sense heritability (h2) of parameters related to depolarization (QRS duration) was lower than for repolarization-related parameters (QT interval, Tp-Te and TWA), with a maximum heritability of 42 % for QRS duration, compared to between 63 % and 72% for repolarization parameters. Previous studies have not been able to demonstrate a significant contribution of additive genetic factors to QRS duration, possibly related to sample size (Havlik, Garrison, Fabsitz, & Feinleib, 1980; Mathers, Osborne, & Degeorge, 1961; Mutikainen et al., 2009; Russell, Law, Sholinsky, & Fabsitz, 1998). Our study is therefore the first to present a formal measure of the role of additive genetics in determination of this parameter.

The corollary of modest heritability of QRS is a greater contribution of environmental factors. It is well established that ventricular depolarization depends on an intact conduction system and can be affected by many extrinsic factors. In this study, we corrected for gender, age and medications, and excluded participants with overt cardiac pathology (see Methods). However, many other acquired factors known to affect QRS duration (Surawicz et al., 2009) such as body habitus, valvular dysfunction, overall LV function, cardiac afterload and other organ pathology such as pulmonary disease, all have the potential to increase the environmental contribution to the phenotype.

For parameters related to repolarization, heritability measured from our rate specific Holter data was at least comparable, or higher, than our measures based on resting ECGs. In particular, significantly higher heritability of QT (69 % for low rate Holter compared to 40% for the resting ECG, $p < .01$) and TWA (72 % from low rate Holter compared to 55 % for the resting ECG, $p < .05$) were measured from the rate specific Holter analysis compared to the resting ECG. It should be noted that in spite of our good sample size power to detect heritability differences was modest. For example, computation showed that differences of 19% or higher were required for our analysis to show significance of $p < .01$. However, confidence in the added value of Holter data is increased because the same trend (of increased heritability when measured from the Holter ECG) was evident when our estimates were compared to previously published data based on resting ECGs. For example, the most comprehensive twin study of repolarization parameters in resting ECGs to date reported heritability of 67 % for QT interval, 46% for Tp-Te and between 34 % and 47% for TWA, after correction for confounding factors such as age and sex (Haarmark et al., 2011), in comparison to our measures of 69%, 63 % and 72 % respectively.

More broadly, other studies have reported the contribution of additive genetic influences to QT interval of zero (Mutikainen et al., 2009), 25 % (Carter et al., 2000), 36 % (Russell et al., 1998) and 60 % (Dalageorgou et al., 2008).

Likewise for TWA, previous publications report values for the contribution of additive genetic factors between zero and 72% (Haarmark et al., 2011), though these values are heavily dependent on the lead selected from the resting ECG. Of most direct comparison to our data, Mutikainen reported a h2 of 61% when measured from lead II in a cohort of older women (Mutikainen et al., 2009). These data show that by considering data extracted from Holter ECGs, higher heritability can be measured for most ECG parameters and that rate specific ECG waveforms extracted from Holter recordings give a more precise measure of the effect of the underlying genotype, that is confounded by rate effects in measurements from the resting ECG.

Rate dependence of genetic factors influencing the ECG

A major advantage in analyzing the genetic determinates of ECG parameters from Holter records is that by deriving beats that are representative of specific physiological states, we are able to measure the rate dependence of genetic factors influencing ECG parameters – something that is not tractable using the resting ECG. As a result of this, no previous studies have looked at the rate dependence of the genetic influences on the T wave. This is an important consideration in understanding the rate dependent changes that occur in T wave morphology in both physiological (Bazett, 1920; Fisch, 1997) and pathophysiological states (Corrado et al., 2010; Hanna & Glancy, 2011; Moss et al., 1995; Sadrieh et al., 2013; Sadrieh et al., 2014). Our data showed that for QT interval and TWA, heritability was dependent on the heart rate (heritability measured at low versus high heart rates were significantly different for these parameters). Possible explanations for these observations included non-genetically determined adrenergic responses, such as to exposure to chronic stressors or the conditioning effect of exercise, which varies between individuals (Corrado et al., 2010). In contrast, no significant effect of heart rate could be determined for QRS and Tp–Te.

Additional analysis showed that whilst the genetic contribution to phenotypic covariance between rates varied for different parameters (but was higher for all repolarization parameters compared to QRS duration), the overall genetic factors that influenced each of the ECG parameters between heart rates largely overlapped. In most cases around 90% of the genes that contribute to phenotypic covariance of

ECG parameters were shared between rates. This supports the concept that the same components of the rhythmonome define individual parameters regardless of the heart rate. These data therefore demonstrate that in general, the genetic factors contributing to defining the ECG are similar at all heart rates, but that they explain the largest part of the total variance at low heart rates, while at higher heart rates, environmental factors play an increasing role in defining ECG characteristics.

Genetic overlap between repolarization parameters

In addition to examining the contribution of genetic factors to covariance of specific ECG parameters at different heart rates, we also examined the genetic overlap between different repolarization parameters at a given heart rate. Cross-parameter comparison clearly show a much lower genetic correlation (between 0.38 for QT and Tp-Te and 0.58 for Tp-Te and TWA) compared to what we observed for within-parameter comparison at different heart rates. Genetic correlation further decreased at medium and high heart rates. This data therefore suggests that while the phenotypic covariance between parameters is mostly genetically determined, there is relatively little overlap between the genes that influence these parameters. This is consistent with our previous studies examining the molecular basis of T wave morphology that showed that individual ECG parameters had very different sensitivities to variability in cardiac ion channel genes (Sadrieh et al., 2013; Sadrieh et al., 2014). This is an important observation as it establishes that in order to fully reflect an individual's genotype, multiple parameters describing the ECG waveform must be measured. Measuring one individual parameter, the QT interval for example, only represents a fraction of the participants whole cardiac ion channel gene complement.

Limitations

The biphasic T wave produced by the electrode configuration in this study limited the analysis of T wave parameters. Specifically, the QT interval measured using the intersection of a tangent to the downslope of the T wave with the isoelectric line, the most widely used method in clinical practice, could not be accurately determined. As previously published (Goldberger et al., 2000) and described here (see Methods), Toffset was used as the end of the T wave in this study. A further limitation is that we did not model the heritability of the rate corrected QT interval for the resting ECG data. However, this was a deliberate choice, since rate corrected QT has consistently been shown to have lower heritability than uncorrected QT (Haarmark et al., 2011) as was confirmed by our

first pass twin correlation calculations for QT and QTc. Heart rate itself is known to be a heritable phenotype so a ratio separating the overlapping genes between QT and heart rate heritability is problematic (Dalageorgou et al., 2008). Finally, specific limitations apply to all twin research (Boomsma et al., 2002). For the purposes of heritability calculations, monozygotic twins are assumed to be genetically identical and neither epigenetic effects nor somatic mutations are accounted for.

Conclusion

In conclusion, our novel beat binning approach to analysis of the Holter ECG has allowed the first rate specific estimate of the heritability of ECG parameters from the Holter ECG. Our data demonstrates that higher measures of heritability can be estimated from the Holter than the resting ECG, suggesting that this approach allows a more precise measure of the effect of the underlying genotype that is confounded by rate effects in the resting ECG. Furthermore, we show rate dependence in the genetic-environmental determinants of the ECG that has not previously been tractable. Future potential uses of this type of analysis include deeper dissection of the ECG of participants with inherited cardiac electrical disease.

Supplement to Chapter 5

A novel approach to Holter data analysis improves phenotyping and increases the precision of genetic analysis of ECG biomarkers

Supplemental Table 5.1. Participants taking medications with potential to alter ECG intervals. Anatomical Therapeutic Classification (ATC) Codes are shown.

Medication (Anatomical therapeutic chemical classification code)	**Number of participants**
Anti Hypertensives (ATC C02)	1
Diuretics (ATC C03)	8
Beta Blockers (ATC C07)	7
Ca Channel Blockers (ATC C08)	3
Renin-Angiotensin system agents (Renin-Angiotensin C09)	10
Lipid modifiers (Renin-Angiotensin C10)	7
Anti Psoriatics (ATC D05)	2
Hormone replacement therapy (ATC G03)	3
Urologicals (ATC G04)	2
Thyroid therapy (ATC H03)	6
Endocrine therapy (ATC L02)	1
Immunosuppressants (ATC L04)	3
Immunostimulants (ATC L03)	1
Anti Epileptics (ATC N03)	5
Anti Parkinson drugs (ATC N04)	1
Psycholeptics (ATC N05)	15
Psychoanaleptics (ATC N06)	18
Obstructive airway disease agents (ATC R03)	17

Supplemental Table 5.2. Monozygotic and duzygotic twin correlations from the saturated model including participants taking cardioactive medication. 99% CIs are shown in parentheses.

ECG parameter	Rate	MZ correlation	DZ correlation
TpTe	Low	0.58 (0.35-0.72)	0.01 (-0.32-0.33)
	Medium	0.67 (0.51-0.78)	0.11 (-0.27-0.44)
	High	0.63 (0.44-0.75)	-0.18 (-0.57-0.36)
	Resting	0.56 (0.36-0.70)	0.08 (-0.25-0.37)
TWA	Low	0.58 (0.34-0.74)	0.38 (0.08-0.59)
	Medium	0.56 (0.33-0.71)	0.43 (0.15-0.62)
	High	0.54 (0.29-0.71)	0.39 (0.11-0.61)
	Resting	0.45 (0.19-0.63)	0.19 (-0.06-0.41)
QT	Low	0.69 (0.52-0.80)	0.07 (-0.30-0.41)
	Medium	0.57 (0.37-0.71)	0.24 (-0.07-0.50)
	High	0.47 (0.20-0.65)	0.25 (-0.09-0.52)
	Resting	0.52 (0.29-0.68)	0.09 (-0.17-0.34)
QRS	Low	0.46 (0.20-0.64)	0.10 (-0.24-0.41)
	Medium	0.49 (0.25-0.66)	0.08 (-0.25-0.39)
	High	0.39 (0.14-0.59)	0.25 (-0.24-0.58)
	Resting	0.30 (0.07-0.50)	0.00 (-0.29-0.29)

Supplemental Table 5.3. Monozygotic and dizygotic twin correlations for Bazett corrected QT and uncorrected QT. Numbers of twin pairs are shown in parentheses.

	MZ	DZ
QT	0.52 (95)	0.11 (76)
QTc	0.34 (95)	0.10 (76)

Supplemental Table 5.4. Rate dependent analysis of ECG parameters. Mean data +/-SD are shown.

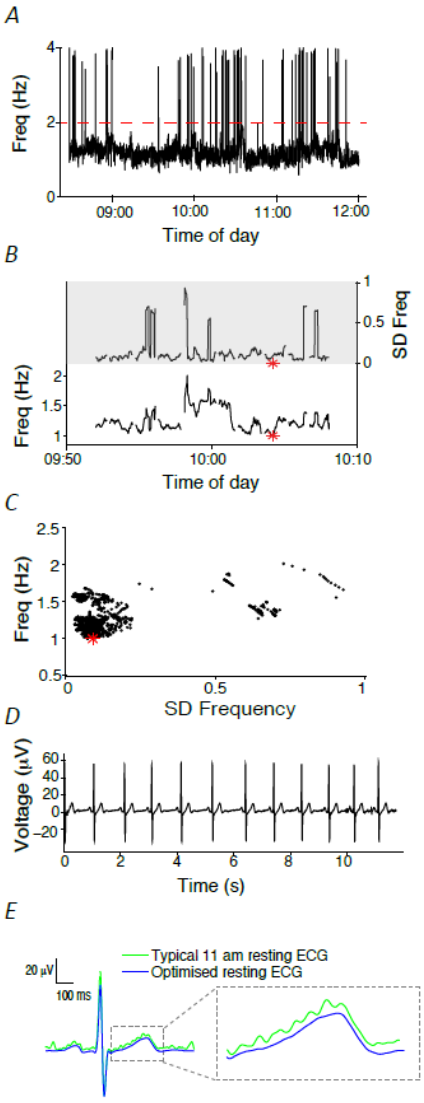
Heart rate	TpTe (ms)	TWA (mV)	QT (ms)	QRS (ms)
Low (60 bpm)	88.9 +/- 8.1	1.6 +/- 0.6	392.9 +/- 20.4	80.4 +/- 10.6
Medium (78 bpm)	85.2 +/- 8.2	1.4 +/- 0.5	357.9 +/- 17.3	79.2 +/- 10.0
High (96 bpm)	84.2 +/- 8.6	1.1 +/- 0.5	335.9 +/- 18.4	79.3 +/- 10.3
Resting	79.0 +/- 10.5	1.5 +/- 0.7	354.1 +/- 25.2	78.8 +/- 9.3

Supplemental Table 5.5. Statistical analysis of the quadrivariate model.

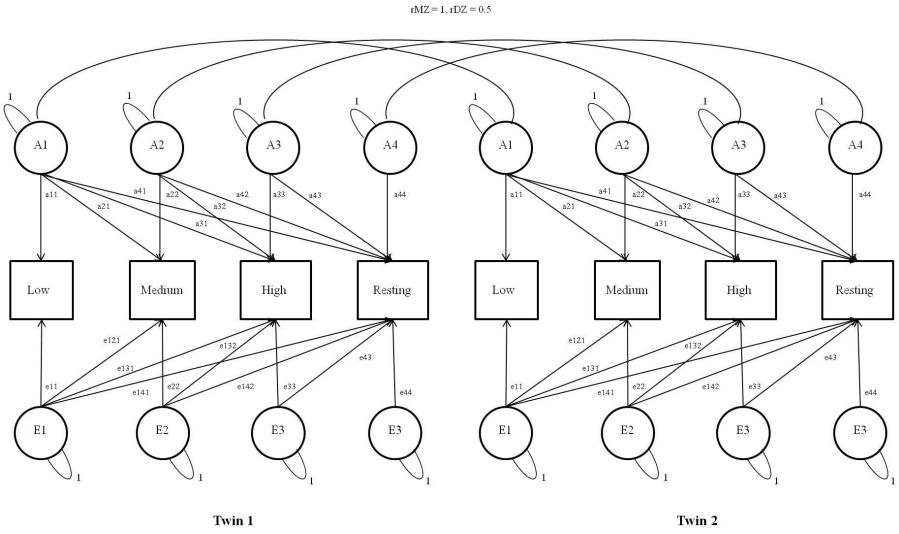
Parameter	Model	df	Model	AIC	-2LL	vs	ΔX2	Δ df	p	Frequency	A	E
Tp_Te	1	1104	AE	90.320	2298.320					Low	0.56 (0.32-0.73)	0.44 (0.27-0.68)
										Medium	0.63 (0.36-0.79)	0.37 (0.21-0.64)
										High	0.52 (0.21-0.73)	0.48 (0.27-0.79)
										Midday	0.56 (0.30-0.72)	0.44 (0.28-0.70)
	2	1107	low=medium=high=midday	86.672	2300.672	1	2.352	3	.503			
	3	1105	low=midday	88.320	2298.320	1	0	1	.995			
	4	1105	medium=midday	89.000	2299.000	1	0.681	1	.409			
	5	1105	high=midday	88.415	2298.415	1	0.096	1	.757			
	6	1106	low=medium=high	88.648	2300.648	1	2.329	2	.312			
	7	1105	low=high	88.419	2298.419	1	0.1000	1	.752			
	8	1105	medium=high	90.073	2300.073	1	1.754	1	.185			
QT	1	1104	AE	2038.801	4246.801					Low	0.69 (0.48-0.82)	0.31 (0.18-0.52)
										Medium	0.58 (0.33-0.75)	0.42 (0.25-0.67)
										High	0.34 (0.14-0.53)	0.66 (0.47-0.86)
										Midday	0.40 (0.18-0.58)	0.60 (0.42-0.82)
	2	1107	low=medium=high=midday	2051.523	4265.523	1	18.722	3	.000			
	3	1105	low=midday	2047.349	4257.349	1	10.548	1	.001			
	4	1105	medium=midday	2041.619	4251.619	1	4.819	1	.028			
	5	1105	high=midday	2037.257	4247.257	1	0.456	1	.499			
	6	1106	low=medium=high	2050.834	4262.834	1	16.033	2	.000			
	7	1105	low=high	2049.598	4259.598	1	12.797	1	.000			
	8	1105	medium=high	2045.416	4255.416	1	8.616	1	.003			

TWA	1	1104	AE	-1753.587	454.413				Low	0.72 (0.53-0.83)	0.28 (0.17-0.47)
									Medium	0.68 (0.49-0.80)	0.32 (0.20-0.51)
									High	0.58 (0.36-0.73)	0.42 (0.27-0.64)
									Midday	0.55 (0.31-0.71)	0.45 (0.29-0.69)
	2	1107	low=medium=high=midday	-1751.869	462.131	1	7.717	3	.052		
	3	1105	low=midday	-1750.011	459.989	1	5.575	1	.018		
	4	1105	medium=midday	-1751.779	458.221	1	3.807	1	.051		
	5	1105	high=midday	-1755.411	454.589	1	0.176	1	.675		
	6	1106	low=medium=high	-1752.387	459.613	1	5.200	2	.074		
	7	1105	low=high	-1751.453	458.547	1	4.134	1	.042		
	8	1105	medium=high	-1750.799	459.201	1	4.788	1	.029		
QRS	1	1104	AE	-599.646	1608.354				Low	0.41 (0.10-0.64)	0.59 (0.36-0.90)
									Medium	0.41 (0.13-0.63)	0.59 (0.37-0.87)
									High	0.42 (0.14-0.62)	0.58 (0.38-0.86)
									Midday	0.32 (0.07-0.55)	0.68 (0.45-0.93)
	2	1107	low=medium=high=midday	-603.382	1610.618	1	2.263	3	.520		
	3	1105	low=midday	-600.656	1609.344	1	0.990	1	.320		
	4	1105	medium=midday	-599.579	1610.421	1	2.067	1	.150		
	5	1105	high=midday	-599.832	1610.168	1	1.813	1	.178		
	6	1106	low=medium=high	-603.624	1608.376	1	.022	2	.989		
	7	1105	low=high	-601.624	1608.376	1	.022	1	.883		
	8	1105	medium=high	-601.641	1608.359	1	.004	1	.947		

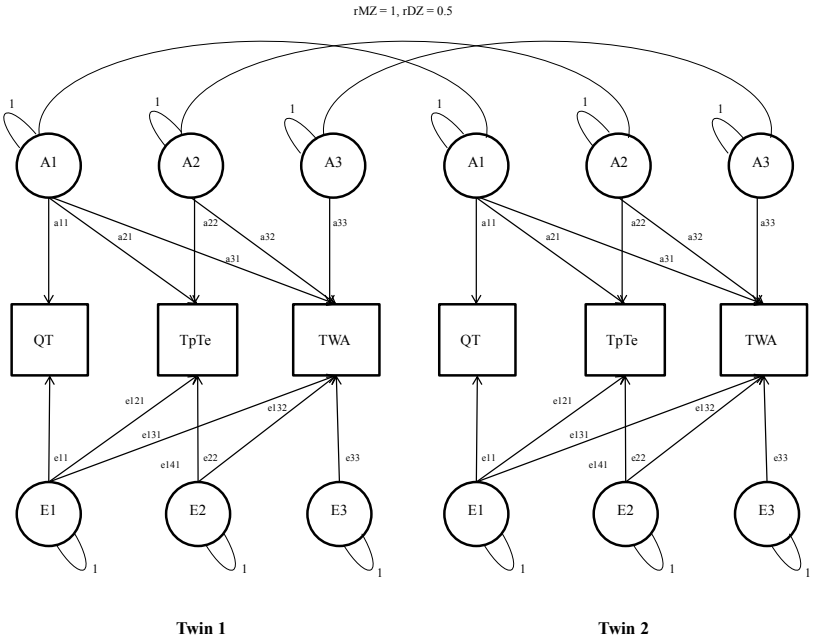
Supplemental Figure 5.1. Extraction of resting ECG. A) R peak frequency in the period between 8 am and 12 pm for a typical participant. Beats with a frequency above 2 Hz (120 bpm, red dashed line) were considered non-physiological and excluded from further analysis. Within the entire patent dataset, the region between 9.50 am and 10.10 am showed the lowest SD of RR interval on average. This region was therefore selected for extraction of a typical resting ECG from each participant. B-C) The 10 s with the lowest frequency and SD (Red aterisk) between 9.50 am and 10.10 am was selected as the typical resting ECG for each participant. D) 10 s of Holter ECG activity selected according to the criteria identified in B-C. E) An average beat from the 10 s of data presented in D (optimized resting ECG, Blue), compared to an averaged beat taken random from 10 s of data at 11 am.



Supplemental Figure 5.2. Quadrivariate model.



Supplemental Figure 5.3. Trivariate model.



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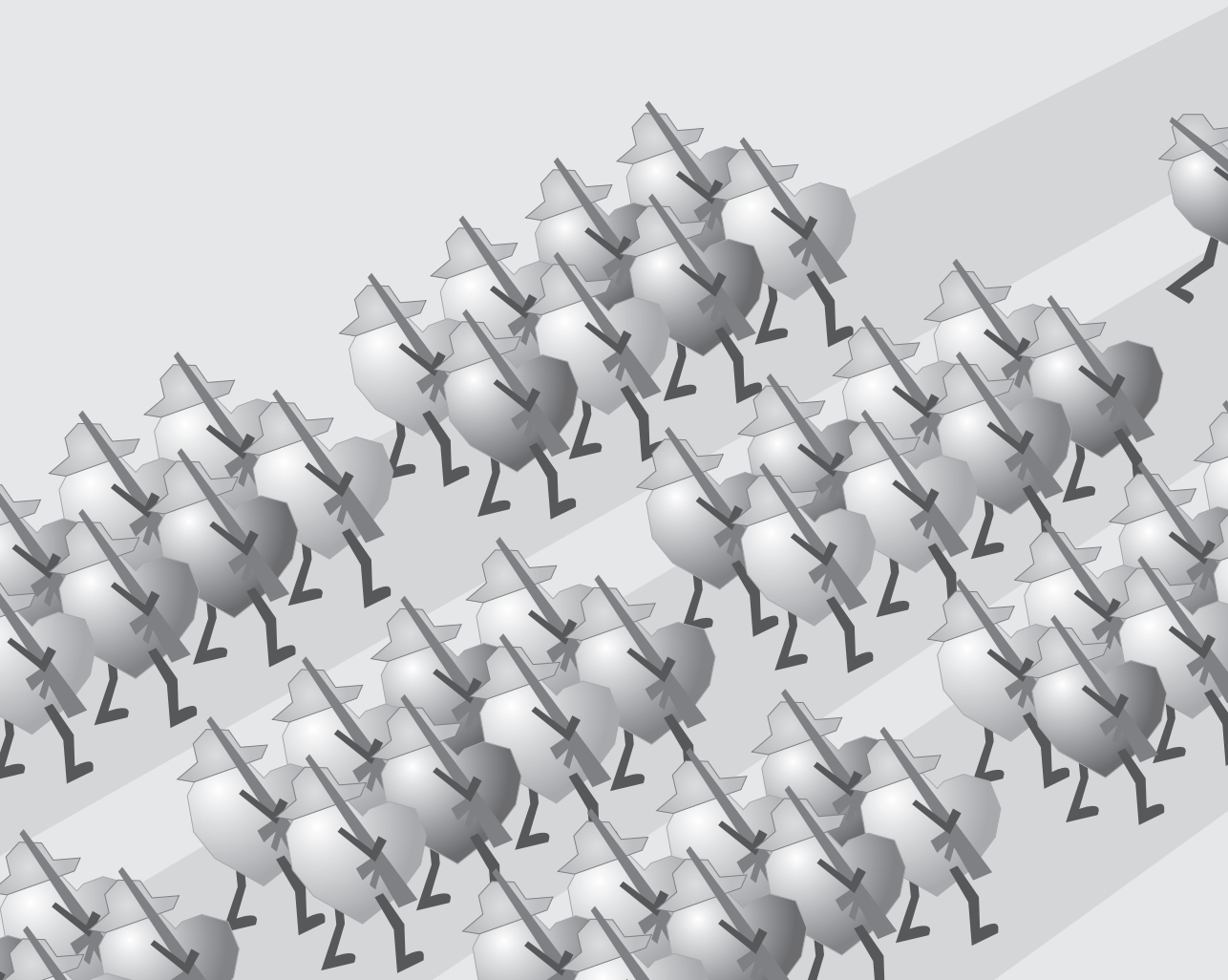
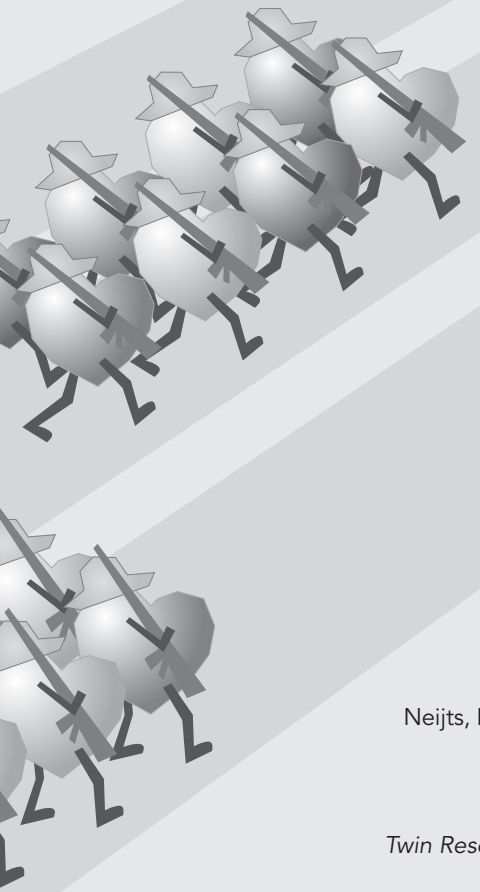


Chapter 6

Genetic architecture of the pro-inflammatory state in an extended twin-family design



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Abstract

In this study we examined the genetic architecture of variation in the pro-inflammatory state, using an extended twin-family design. Within the Netherlands Twin Register (NTR) Biobank, fasting Tumor Necrosis Factor-α (TNF-α), Interleukin-6 (IL-6), C-Reactive Protein (CRP) and fibrinogen levels were available for 3,534 twins, 1,568 of their non-twin siblings and 2,227 parents from 3,095 families. Heritability analyses took into account the effects of current and recent illness, anti-inflammatory medication, female sex hormone status, age, sex, BMI, smoking status, month of data collection, and batch processing. Moderate broad-sense heritability was found for all inflammatory parameters (39%, 21%, 45% and 46% for TNF-α, IL-6, CRP and fibrinogen, respectively). For all parameters, the remaining variance was explained by unique environmental influences and not by environment shared by family members. There was no resemblance between spouses for any of inflammatory parameters, except for fibrinogen. Also, there was no evidence for twin-specific effects. A considerable part of the genetic variation was explained by non-additive genetic effects for TNF-α, CRP and fibrinogen. For IL-6, all genetic variance was additive. This study may have implications for future genome-wide association (GWA) studies by setting a clear numerical target for genome-wide screens that aim to find the genetic variants regulating the levels of these pro-inflammatory markers.

Introduction

Chronic low-grade inflammation plays an important role in numerous diseases including major depression and heart disease, and it has been implicated as one of the major causes for the comorbidity of these diseases (Capuron et al., 2008; Vaccarino et al., 2008; Vaccarino et al., 2007). The inflammatory response is activated by pro-inflammatory cytokines, of which TNF-α and IL-1 are the first to appear (Tracey, 2002). The inflammatory cascade is further promoted by the production of IL-6 that in turn stimulates the acute-phase response which is reflected in the synthesis of fibrinogen and CRP (Gabay, 2006; Gabay & Kushner, 1999; Packard & Libby, 2008). Elevations in TNF-α, IL-6, CRP and fibrinogen have been associated with an increased risk for both cardiac disease (Cesari et al., 2003; Danesh et al., 2008; Danesh et al., 2004; Humphries, Cooper, Talmud, & Miller, 2007; Libby & Theroux, 2005; Packard & Libby, 2008; Woods, Brull, Humphries, & Montgomery, 2000) as well as major depression (O'Brien, Scott, & Dinan, 2004; Penninx et al., 2003).

In spite of the obvious importance of these pro-inflammatory markers in depression and cardiovascular disease, which are both in the top 4 of burden of disease prediction for 2020 (Mathers & Loncar, 2006), very little is known about the etiology of the individual differences in TNF-α, IL-6, CRP and fibrinogen levels. A first important question is to what extent the variance in these biological parameters is innate, caused by environmental factors that are shared by family members, or caused by environmental factors unique to each individual member of a family. This question can be addressed by the classical twin design comparing the resemblance between monozygotic (MZ) and dizygotic (DZ) twins (Boomsma et al., 2002; van Dongen, Slagboom, Draisma, Martin, & Boomsma, 2012). A few twin studies in healthy samples have estimated the heritability of cytokines and acute phase reactants with estimates varying between 21% and 60% for fibrinogen (de Lange, Snieder, Ariens, Spector, & Grant, 2001; de Lange et al., 2006; de Maat et al., 2004; Jermendy et al., 2011; Reed, Tracy, & Fabsitz, 1994; Su et al., 2008), between 20% and 76% for CRP (de Maat et al., 2004; Jermendy et al., 2011; MacGregor, Gallimore, Spector, & Pepys, 2004; Rahman et al., 2009; Su et al., 2008; Su et al., 2009a; Su et al., 2009b; Wessel et al., 2007; Wang et al., 2011; Worns, Victor, Galle, & Hohler, 2006), between 17% and 26% for TNF-α (de Maat et al., 2004; Sas et al., 2012), and between 15% and 61% for IL-6 (de Maat et al., 2004; Grunnet, Poulsen, Klarlund, Mandrup-Poulsen, & Vaag, 2006; Sas et al., 2012; Su et al., 2008; Su et al., 2009a; Su et al., 2009b; Worns et al.,

2006). With a few exceptions heritability estimates of the aforementioned studies have been based on relatively small twin samples. Such studies are fairly accurate in estimating broad-sense heritability but they lack precision and power to estimate the contribution of non-additive genetic effects or shared family environment like the dietary habits or neighborhood factors shared by parents and offspring. As the average sample size of previous studies was around 400 individuals, these studies were not sufficiently powered to detect an effect of shared environmental factors explaining less than 40% of the variance or to discriminate between additive and non-additive genetic factors (Posthuma & Boomsma, 2000). Also, the relatively small sample sizes may explain the large range of heritability estimates based on previous studies.

Here we extend the classical twin design, including only MZ and DZ twin pairs, by including non-twin siblings, and their parents in the largest set of twin- and family data on TNF-α, IL-6, CRP, and fibrinogen described to date. Inclusion of non-twin siblings increases statistical power and offers the possibility to assess twin-specific effects. The inclusion of parents allows taking into account assortative (non-random) mating effects, which can influence heritability estimates. Data from parents also allow for the examination of shared household effects in spouses who share a household, but are not biologically related (e.g. Distel et al., 2010; Rebollo & Boomsma, 2006). The availability of a large sample size allowed for the exclusion of subjects with current and recent illness and for the examination of a number of health-related variables and methodological factors that could affect the reliability of the assessment of plasma levels of inflammatory variables, while retaining adequate power to detect shared environmental factors and to discriminate between additive and non-additive genetic factors.

Methods

Subjects

The data were obtained from the NTR Biobank study that was conducted among twins and their family members registered with the Netherlands Twin Register in the period of 2004-2008 (Willemsen et al., 2010). Subjects were visited between 7 am and 10 am at home or, when preferred, at work, to collect blood and urine samples. Subjects were instructed to fast from the evening before, to abstain from physical exertion and, if possible, not to take medication at the day of the home visit, and to refrain from smoking one hour before the home visit. Fertile women were visited on the 2nd-4th day of their menstrual cycle or, if

they took oral contraceptives, in their pill-free week. During the visit, a brief interview was conducted on health status, including an inventory of medication use, illness (last time occurrence, duration and type of illness), and adherence to the protocol.

The study consisted of 9,405 subjects with data on at least one of the four pro-inflammatory parameters of interest. Values exceeding 15 pg/ml for IL-6 and TNF-α, 15 mg/L for CRP and/or 6 g/L for fibrinogen were set to missing, leading to the exclusion of 11 subjects. Subjects who were on anti-inflammatory medication, medication impacting on the Hypothalamic Pituitary Adrenal (HPA)-axis, or both, were excluded from further analyses (N = 408). We also excluded subjects suffering from a cold, the flu, inflammation, or allergy at the time of blood sampling (N = 1,013). The remaining subjects (N = 7,973) served as the reference group to quantify the effects of the various covariates and to compute residual scores for every immune parameter.

For the twin-family analyses we additionally excluded non-biological parents and siblings (N=35), spouses of twins (N = 409), subjects under 18 years of age (N = 87), the third member of triplets, and additional twins from families with more than one twin pair (N = 4). When zygosity was missing for a twin pair and both twins participated in the study, we randomly selected one of the two to be excluded (N = 10). To simplify the genetic model fitting procedure, we included a maximum of two singleton brothers and two singleton sisters per family and randomly selected two siblings from families with more than two same-sex siblings (N = 99 excluded). The final sample was comprised of 3,095 families with 7,329 family members of which 3,534 subjects were twins, more specifically 590 MZ male (MZM), 320 DZ male (DZM), 1,281 MZ female (MZF), 624 DZ female (DZF) and 719 dizygotic opposite-sex (DOS) twins. The following numbers of complete twin pairs were included: 201 MZM, 96 DZM, 466 MZF, 211 DZF and 217 DOS. Furthermore, 464 non-twin male siblings, 1,104 non-twin female siblings, 1,003 fathers of twins and 1,224 mothers of twins were included. Zygosity of twins was determined by DNA typing for 85.1% 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).

Assessment of TNF-α, IL-6, CRP and fibrinogen

During the home visit, eight blood tubes were collected in the following order; 2 × 9 ml EDTA, 2 × 9 ml heparin, 1 × 4.5 ml CTAD, 1 × 2 ml EDTA, 1 x 4.5 ml serum. To prevent

clotting, all tubes were inverted gently 8–10 times immediately after collection (for detail, see Willemsen et al., 2010).

Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) were measured in EDTA plasma, obtained from one of the 9 ml tubes. During transport this tube was stored in melting ice and upon arrival at the laboratory, it was centrifuged for 20 minutes at 2000x *g* at 4°C. EDTA plasma, buffy coat, and red blood cells were harvested and aliquoted (0.5 ml), snap-frozen in dry ice, and stored at –30°C. Plasma levels of TNF-α and IL-6 were determined using an UltraSensitive ELISA (R&D systems, Minneapolis, USA, Quantikine HS HSTA00C). The inter-assay coefficient of variation (CV) for TNF-α was < 12.8%, for IL-6 the inter-assay CV was < 11.6%.

C-reactive protein (CRP) was obtained from one of the 9 ml heparin tubes. The tube was stored in melting ice during transport. At the laboratory the tube was centrifuged for 15 minutes at 1000x *g* at 4°C, after which heparin plasma was obtained and divided into 8 subsamples of 0.5 ml, snap-frozen and stored at –30°C. The processing took place in a sterile flow cabinet. CRP level in heparin plasma was determined using the Immulite 1000 CRP assay (Diagnostic Product Corporation, USA). The inter-assay CV was < 5.1%.

Fibrinogen. Fibrinogen level was obtained from the 4.5 ml CTAD tube, which was stored in melting ice during transport. Upon arrival at the laboratory, it was centrifuged for 20 minutes at 2000x *g* at 4°C, after which citrated plasma was harvested from the buffy coat and red blood cells, aliquoted (0.5 ml), snap-frozen in dry ice, and stored at –30° C. Fibrinogen levels in CTAD plasma were determined on a STA Compact Analyzer (Diagnostica Stago, France), using STA Fibrinogen (Diagnostica Stago, France). The inter-assay CV was < 6.1%.

Fibrinogen values were normally distributed whereas data on the other variables were skewed. Therefore, we took the natural logarithm of these values.

Assessment of covariates

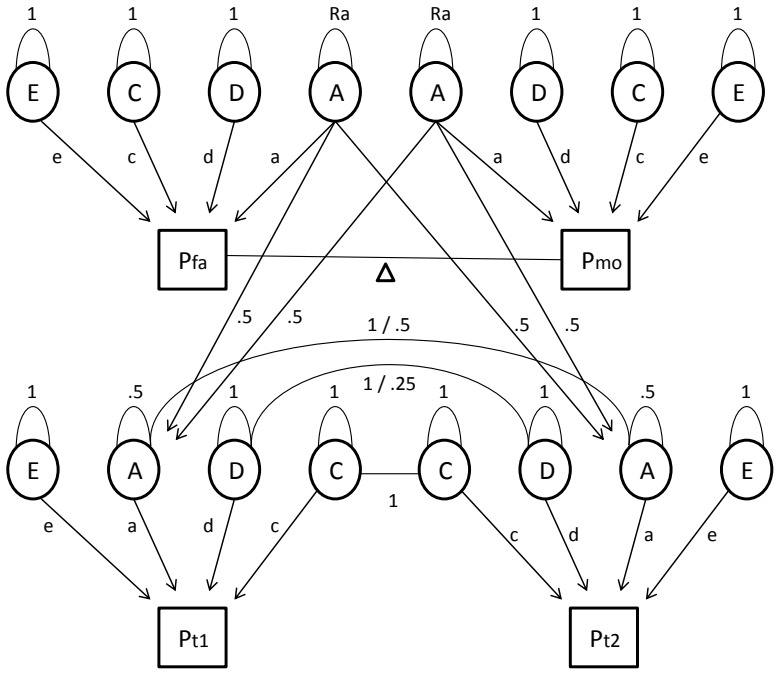
For the heritability analyses, we took into account the effects of age, sex, health-related covariates known to be associated with inflammatory parameters (body mass index (BMI), smoking status), and several methodological covariates that could lead to inflation of family correlations (month of blood sampling and batch effect). During the home visit, height and weight were assessed and BMI was calculated. Subjects were also asked about their past and current smoking behavior and were categorized into one of five groups (never smoked, ex-occasional smoker, ex-regular smoker, current occasional

smoker, current regular smoker). The month of blood sampling was used to correct for the effects of time of year on the four pro-inflammatory markers. For the cytokines, we also took into account differences in values due to the plate on which the samples were processed, by using the plate mean value for the cytokines as a covariate. The levels of the acute phase reactants were determined on a per sample basis, so plate effects for these variables are not applicable. Previous research suggested that when using the ELISA assay of R&D systems, individuals with blood group O may show higher TNF-α and IL-6 levels than other ABO blood groups, which may in part be due to assay-specific cross-reactivity with ABO antigens (Melzer et al., 2008; Naitza et al., 2012). To investigate this potential confounding effect we used a SNP (rs644234) that showed the strongest association with TNF-α and IL-6 in our data, of all SNPs in the ABO gene region plus/minus a 10 Kb border. The rs644234 SNP explained 7% and 4 % of TNF-α and IL-6 values, respectively. Data on this SNP were available for 5,950 healthy subjects with TNF-α data and for 5,947 subjects with IL-6 data. Because the twin-family models yielded similar results with and without taking the effect of the ABO SNP into account, we only report the analyses on the full sample.

Statistical analyses

Data preparation, sample selection and tests for the effects of covariates were conducted using IBM Statistical Package of Social Sciences 20.0. The covariates were included in a multiple regression analysis (forced entry) and the residual scores were saved for the heritability analyses. As there was a significant age-by-sex interaction for CRP, fibrinogen and IL-6, regression coefficients for age were estimated separately for men and women for these variables. Genetic models were fitted to the data using structural equation modeling (SEM) in the software package Mx (Neale et al., 2006). First, a fully parameterized, or saturated, model was fitted and a goodness-of-fit statistic based on minus twice the logarithm of the likelihood (-2LL) was calculated. Next, the fully saturated model was simplified to a more restricted model to test whether constraints were allowed to be put on the data. The comparison of fit of a restricted model to the full model is performed by means of likelihood-ratio (χ^2) tests in which the difference in -2LL between the two models is calculated. When the likelihood-ratio test is significant ($p < .01$), the nested model is considered to fit significantly worse to the data than the fuller model it is tested against.

Figure 6.1. Path diagram of an extended twin family showing four subjects belonging to one family: father (Pfa), mother (Pmo), twin 1 (Pt1) and twin 2 (Pt2), where P is phenotype. In the genetic model (without constraints) a maximum of 10 parameters is estimated. Path loadings a, d, c and e represent the influence of additive and non-additive genetic effects and shared and unique environmental factors on the phenotype. Spousal resemblance is modeled as a function of phenotypic assortment, denoted by the delta path (Δ). When the delta path coefficient is larger than zero, this indicates non-random mating. The residual variance of additive genetic effects in parents (Ra) will then become larger than 1 to account for increased transmission of additive genetic effects from parents to children under conditions of non-random mating. Finally, two means and two age regression coefficients (for males and females separately) are estimated. Fixed parameters include the .5 genetic relatedness of the parents and their children (path running from the A of both parents to the A of their children with a path loading of .5). The residual variance of A in the offspring is constrained at .5. Finally, MZ twins correlate 1 regarding A and D factor scores (because they are assumed to share all of their genetic material), whereas DZ or sibling pairs correlate .5 and .25, respectively.



First, we tested if constraints on the means and variances for men and women were allowed and if different types of family correlations were equal. In the full model, 19 parameters were estimated: separate means, standard deviations, and regression coefficients for age on the phenotype for men and women, and 13 family correlations (for MZM, MZF, DZM, DZF, DOS twin pairs, male siblings, female siblings, opposite-sex siblings and for mother-daughter, mother-son, father-daughter, father-son and one spouse

correlation). Quantitative sex differences, indicating that the heritability of a trait is different in men and women, were assessed by testing whether correlations in male-male and female-female pairs of first-degree relatives (DZ twins and non-twin siblings) were equal. Next, we tested if the same genes regulate cytokine and acute phase reactant levels in men and women (Vink et al., 2012). When correlations for a trait are the same in same-sex and opposite-sex pairs of family members, there is no evidence for qualitative sex differences in the genetic architecture. When the correlations in DZ twin pairs are of similar magnitude as the correlations in sib-sib pairs, there is no evidence for twin-specific resemblance. Generation effects were tested by equating parent-offspring correlations to the correlations between all other first-degree relatives (DZ twins and non-twin siblings). If this constraint is allowed, there is no evidence that gene expression changes with age. Spousal resemblance was assessed by testing if the correlation between the parents of the twins was significantly different from zero. The most parsimonious model with the maximal number of allowable restrictions was carried forward to the genetic structural equation analyses. In these analyses, the family covariance structure is used to estimate the relative contribution of latent additive (A) and non-additive or dominant (D) genetic factors and common (C) and unique (E) environmental factors to the phenotypic variance. Based on the variance estimates from the full genetic model, sequentially constrained submodels were compared to the fit of the full model to arrive at the most parsimonious genetic model describing the total phenotypic variance best (see Figure 6.1 for a schematic representation of the extended twin-family model).

Results

Descriptive statistics for the four immune parameters of interest in the twins, siblings and parents are given in table 6.1. Table 6.2 presents the amount of variance explained by the various technical and biological covariates that were taken into account.

The effect of age on all parameters was significant and positive (p's < .01). Sex differences were present only for CRP and fibrinogen with women having significantly larger mean values than men (*p's* < .01). For TNF-α and CRP values, standard deviations were significantly larger in females than in males. Table 6.3 shows the family correlations for each of the immune parameters estimated for the values only adjusted for age and sex and for the values additionally adjusted for the other covariates.

Table 6.1. Mean values (and standard deviations) and mean age (range) for Tumor Necrosis Factor-α (TNF-α), Interleukin-6 (IL-6), C-Reactive Protein (CRP) and fibrinogen.

Marker	N total	Mean (sd)	Mean age (range)
TNF-α (pg/ml)			
Fathers	987	1.21 (1.25)	61 (33-89)
Mothers	1,215	1.20 (1.10)	60 (26-89)
Male twins/siblings	1,594	1.02 (0.85)	37 (18-82)
Female twins/siblings	3 218	1.07 (1.14)	38 (18-90)
Total	7,014	1.10 (1.10)	45 (18-90)
IL-6 (pg/ml)			
Fathers	984	2.10 (1.77)	61 (33-88)
Mothers	1,213	1.91 (1.50)	60 (26-89)
Male twins/siblings	1,590	1.37 (1.38)	37 (18-82)
Female twins/siblings	3,220	1.41 (1.27)	38 (18-90)
Total	7,007	1.59 (1.44)	45 (18-90)
CRP (mg/L)			
Fathers	975	2.47 (2.58)	61 (33-89)
Mothers	1,171	2.73 (2.75)	60 (26-89)
Male twins/siblings	1,672	1.77 (2.23)	35 (18-82)
Female twins/siblings	3,244	2.59 (2.91)	38 (18-90)
Total	7,062	2.40 (2.71)	44 (18-90)
Fibrinogen (g/L)			
Fathers	983	2.94 (0.70)	61 (33-89)
Mothers	1,188	3.02 (0.68)	60 (26-89)
Male twins/siblings	1,550	2.51 (0.59)	37 (18-82)
Female twins/siblings	3,136	2.69 (0.65)	38 (18-90)
Total	6,857	2.74 (0.68)	45 (18-90)

Table 6.2. Proportion of variance that is explained by the covariate with the number of subjects within brackets .

Covariates	TNF-α	IL-6	CRP	Fibrinogen
Age	.021** (7,566)	.118** (7,559)	.024** (7,684)	.097** (7,397)
Sex	.000 (7,566)	.000 (7,559)	.014** (7,684)	.008** (7,397)
BMI	.013** (7,521)	.098** (7,515)	.139** (7,645)	.100** (7,357)
Smoking	.001* (7,553)	.030** (7,546)	.007** (7,678)	.009** (7,392)
Plate effect	.106** (7,558)	.079** (7,551)	-	-
Month of blood sampling	.006** (7,566)	.007** (7,559)	.002** (7,684)	.009** (7,397)

* = $p < .05$, ** = $p < .01$

For all parameters male and female MZ correlations did not differ significantly and same-sex and opposite- sex DZ twin and non-twin sibling correlations were also similar in all cases (*p's* > .01), so no quantitative and qualitative sex differences were present, nor did we find evidence for twin-specific environmental effects. Parent-offspring correlations were not significantly different from DZ twin and non-twin sibling correlations ($p > .01$), except for the fibrinogen values adjusted for age and sex only ($p = .003$). This effect was not present in the fully adjusted fibrinogen values. These results suggest that genetic regulation of cytokine and acute phase reactant levels does not change significantly across age. Overall, adjustment for BMI, smoking, month and plate effects in addition to age and sex tended to reduce all familial correlations, including the spouse correlations. Only the spouse correlation for the fully adjusted fibrinogen values was significantly different from zero ($r = .16$, $p < .01$), which indicates that the effects of assortative mating or sharing a household without being biologically related are negligible, except for the small resemblance found for fibrinogen.

For the heritability analyses on the fully adjusted values, contributions of A, D, C and E factors to the total phenotypic variance were constrained over sex while taking into account sex differences in phenotypic variance in TNF-α, CRP and IL-6. Assortative mating was only modeled for fibrinogen. Table 6.4 shows the genetic models that were fitted to the data, supplemented with the proportions of the phenotypic variance that can be explained by the different genetic and environmental factors for both the full ADCE model and the model that provided the most parsimonious fit.

The broad-sense heritability was 39%, 21%, 45% and 46% for TNF-α, Il-6, CRP and fibrinogen, respectively. The models that provided the best fit to the data on TNF-α, CRP and fibrinogen included additive and non-additive genetic factors, and unique environmental factors. Non-additive genetic effects explained 22% of the variance of TNF-α, 18% of the variance of CRP, and 16% of the variance of fibrinogen. For CRP and fibrinogen, a small amount of variation was attributed to sibling-shared environmental factors in the full ADCE model, but an ADE model without shared environmental factors did not fit significantly worse. For IL-6, a model with additive genetic factors and unique environmental factors explained the data best, with no role for non-additive genetic factors, nor for shared environmental factors.

Table 6.3. Family correlations (and 95% confidence intervals) as estimated in the saturated model for the cytokines and the acute phase reactants, with the levels of the pro-inflammatory markers only adjusted for age and sex, and adjusted for all covariates.

	TNF-α[1]	TNF-α[2]	IL-6[1]	IL-6[2]	CRP[1]	CRP[2]	Fibr[1]	Fibr[2]
MZ twins								
MZM	.44 (.29-.55)	.44 (.29-.56)	.23 (.09-.35)	.19 (.04-.32)	.42 (.31-.51)	.39 (.27-.49)	.50 (.37-.60)	.46 (.31-.57)
MZF	.38 (.31-.45)	.38 (.31-.45)	.39 (.31-.46)	.35 (.26-.42)	.50 (.43-.56)	.46 (.39-.52)	.48 (.41-.55)	.45 (.37-.52)
DZ/siblings male								
DZM	-.02 (-.19-.15)	-.04 (-.22-.14)	.13 (-.06-.31)	.20 (.01-.36)	.23 (.06-.39)	.15 (-.03-.32)	.39 (.14-.57)	.31 (.00-.52)
MZM/DZM/brother-brother	.14 (.01-.26)	.11 (-.02-.23)	.05 (-.05-.16)	.00 (-.10-.11)	.27 (.16-.38)	.22 (.10-.33)	.41 (.27-.53)	.34 (.18-.47)
DZ/siblings female								
DZF	.17 (.05-.28)	.14 (.02-.25)	.19 (.06-.30)	.13 (.00-.26)	.34 (.22-.44)	.32 (.19-.43)	.30 (.15-.43)	.26 (.09-.40)
MZF/DZF/sister-sister	.19 (.11-.26)	.20 (.12-.27)	.14 (.07-.21)	.10 (.03-.17)	.23 (.16-.30)	.20 (.12-.27)	.25 (.17-.32)	.22 (.14-.29)
Opposite sex siblings								
DOS	.10 (-.03-.24)	.12 (-.01-.25)	.12 (-.01-.25)	.09 (-.04-.22)	.22 (.10-.33)	.23 (.11-.33)	.15 (.01-.28)	.12 (-.04-.27)
DOS/brother-sister	.12 (.04-.20)	.10 (.02-.18)	.09 (.01-.17)	.07 (-.01-.15)	.21 (.14-.28)	.18 (.10-.25)	.28 (.20-.36)	.24 (.15-.32)
Parent-offspring								
Mother-daughter	.14 (.07-.20)	.12 (.05-.18)	.14 (.08-.20)	.06 (.00-.12)	.16 (.09-.22)	.10 (.03-.16)	.22 (.16-.28)	.18 (.12-.24)
Mother-son	.14 (.05-.22)	.09 (.00-.18)	.20 (.13-.27)	.17 (.10-.25)	.14 (.06-.22)	.09 (.00-.17)	.17 (.08-.25)	.15 (.06-.23)
Father-son	.10 (.01-.18)	.13 (.04-.22)	.16 (.07-.24)	.18 (.09-.26)	.22 (.13-.30)	.22 (.13-.30)	.26 (.17-.34)	.26 (.17-.34)
Father-daughter	.08 (.02-.14)	.06 (.00-.12)	.11 (.05-.18)	.03 (-.03-.10)	.20 (.14-.27)	.15 (.08-.21)	.14 (.08-.20)	.14 (.08-.20)
Parents								
Mother-father	.13 (.06-.20)	.09 (.02-.16)	.13 (.05-.20)	.05 (-.03-.13)	.12 (.03-.20)	.05 (-.04-.14)	.18 (.12-.24)	.16 (.10-.22)

[1] = after adjustment for the effects of age and sex.
[2] = after adjustment for the effects of all covariates (age, sex, month, BMI, smoking and for TNF-α and IL-6 plate as well).

Table 6.4. Genetic model fit statistics of the pro-inflammatory markers after adjustment for sex, age, BMI, smoking, plate and month of sampling.

Marker	Model	df	Model	-2LL	vs	$\Delta\chi^2$	Δ df	p	a^2	d^2	c^2	e^2
TNF-α	1	6951	ADCE	19404.352					.17 (.10-.24)	.22 (.12-.31)	.00 (.00-.05)	.61 (.55-.67)
	2	6953	AE	19425.261	1	20.909	2	.000				
	3	**6952**	**ADE**	**19404.352**	**1**	**0.000**	**1**	**1**	**.17 (.10-.24)**	**.22 (.13-.31)**	**-**	**.61 (.55-.67)**
IL-6	1	6944	ADCE	19352.234					.20 (.09-.25)	.00 (.00-.00)	.00 (.00-.06)	.79 (.70-.84)
	2	**6946**	**AE**	**19352.248**	**1**	**0.014**	**2**	**.993**	**.21 (.16-.25)**	**-**	**-**	**.79 (.75-.84)**
CRP	1	7016	ADCE	19296.178					.25 (.18-.33)	.13 (.03-.24)	.05 (.00-.11)	.56 (.51-.62)
	2	7018	AE	19314.325	1	18.148	2	.000				
	3	7017	ACE	19302.277	1	6.100	1	.014				
	4	**7017**	**ADE**	**19298.509**	**1**	**2.331**	**1**	**.127**	**.27 (.20-.34)**	**.18 (.09-.26)**	**-**	**.55 (.50-.61)**
Fibrinogen	1	6807	ADCE	18524.411					.30 (.23-.36)	.11 (.00-.23)	.05 (.00-.12)	.55 (.49-.62)
	2	6809	AE	18538.390	1	13.979	2	.001				
	3	6808	ACE	18527.761	1	3.349	1	.067				
	4	**6808**	**ADE**	**18525.974**	**1**	**1.563**	**1**	**.211**	**.30 (.24-.37)**	**.16 (.09-.24)**	**-**	**.54 (.48-.60)**

Abbreviations: df = degrees of freedom, Model = specification of the model that is tested, -2LL = minus twice the logarithm of the likelihood, vs = the model against which this submodel is tested, $\Delta\chi^2$ = model fit statistic: difference in -2LL of two nested models, Δdf = the difference in the number of parameters between the models, p = p-value (was regarded significant when < .01), a^2, d^2, c^2, e^2 = proportions of variance explained by additive, non-additive, shared and unique environmental effects. The 95% confidence intervals are depicted within brackets. The most parsimonious model is printed in bold.

Discussion

This is the most comprehensive twin-family study of the genetic architecture of the pro-inflammatory state that has been performed thus far. Results replicate the importance of genetic factors in pro-inflammation observed before (de Lange et al., 2001; de Lange et al., 2006; de Maat et al., 2004; Grunnet et al., 2006; Jermendy et al., 2011; MacGregor et al., 2004; Rahman et al., 2009; Reed et al., 1994; Sas et al., 2012; Su et al., 2008; Su et al., 2009a; Su et al., 2009b; Wessel et al., 2007; Worns et al., 2006; Wang et al., 2011) and extend the findings of previous studies by showing that genetic non-additivity is an important factor in explaining individual differences in TNF-α, CRP and fibrinogen levels and by ruling out a large role for environmental factors shared by family members.

There have only been three previous heritability studies employing a sample size of over 1,000 twins for CRP (Wang et al., 2011; Rahman et al., 2009), IL-6 and TNF-α (Sas et al., 2012). For fibrinogen, the study with the largest sample size included 962 subjects (de Lange et al., 2001). None of these studies systematically corrected for recent illness, medication use, menstrual cycle, oral contraceptives use, batch effects, month of sampling, BMI, and smoking status as done in the present study. In spite of the more strict correction for confounders, our heritability estimate for CRP was of comparable magnitude to these previous studies. For CRP, we confirmed the importance of non-additive genetic factors that was found in the largest of the two previous twin studies (Rahman et al., 2009), whereas the smaller of the two (Wang et al., 2011) only detected additive effects, likely reflecting insufficient power. For fibrinogen, our broad-sense heritability estimate was comparable to the estimate reported by de Lange and colleagues (de Lange et al., 2001), but our study additionally indicated that a significant proportion of the heritability was due to non-additive genetic effects.

For TNF-α and IL-6 our results do not completely support the results of the only large (N > 1,000) previous twin study (Sas et al., 2012). For both cytokines, Sas and colleagues (Sas et al., 2012) found substantial family resemblance, but they could not discriminate between genetics and shared environment as the source of that resemblance. The twin correlations reported in a smaller study on IL-6 (Worns et al., 2006) were suggestive of genetic factors and a potential role of shared environment with the MZ correlation being less than twice as high as the DZ correlation. Our study clearly shows that shared genetic make-up rather than shared family environment is the major source of familial resemblance in these parameters. Furthermore, we show a significant effect of non-additive factors

on TNF-α. Four smaller twin studies of TNF-α and IL-6 values in healthy unchallenged subjects are consistent with our findings, as the MZ correlations found in those studies were about twice as high as the DZ correlations, in elderly subjects (de Maat et al., 2004), young adults (Grunnet et al., 2006), and middle-aged twins (Su et al., 2008; Su et al., 2009b). In their sample of young adult subjects, Grunnet and colleagues (Grunnet et al., 2006) even found the MZ correlations for TNF-α to be more than twice as high than the DZ correlations.

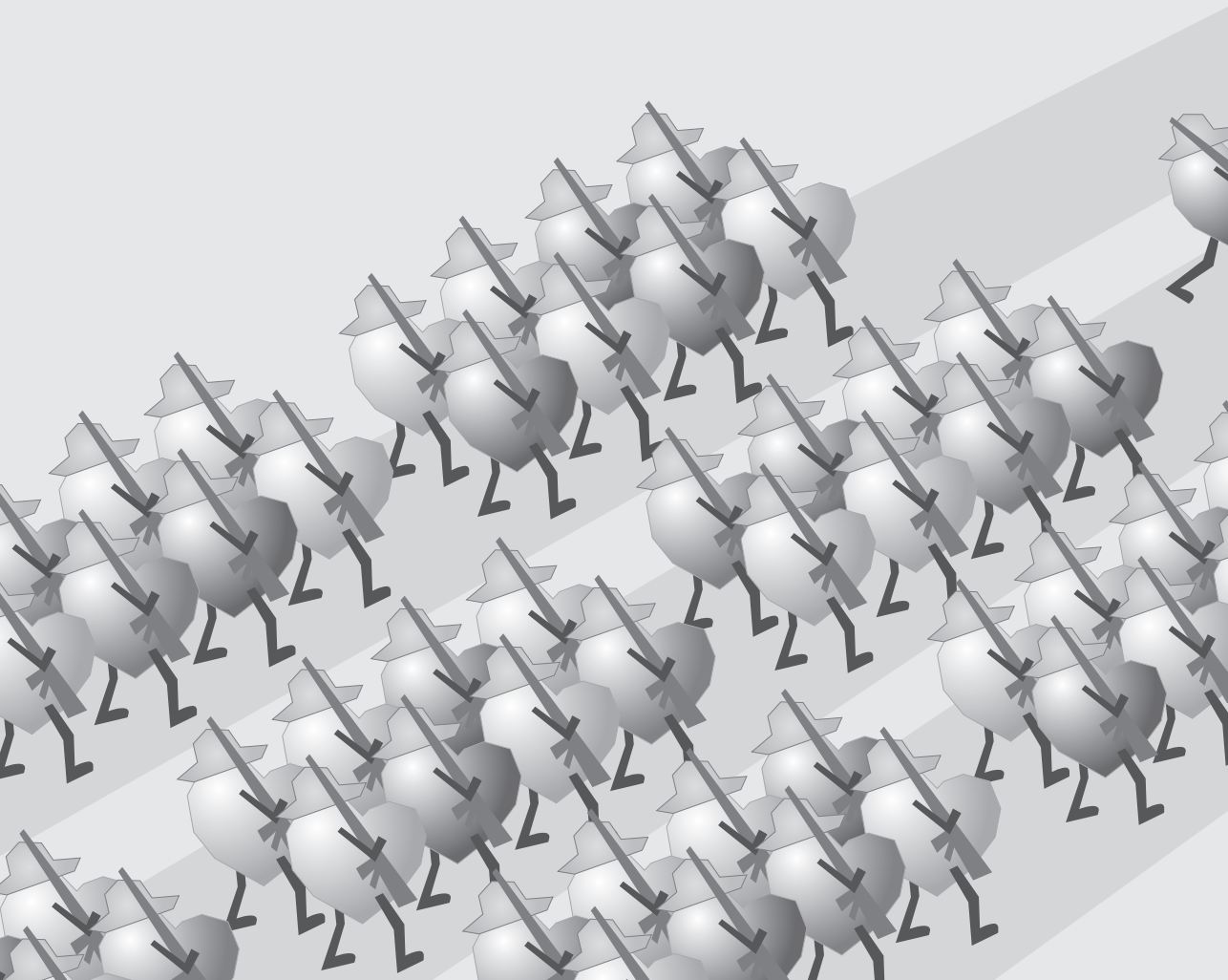
Taken together, our results and those from previous studies suggest that about a third of the variation in these core pro-inflammatory cytokines and acute phase reactants in healthy subjects with values in the non-extreme range is explained by genetic variation. This means that some individuals are more susceptible than others to have higher levels of pro-inflammatory markers and this increased susceptibility is, at least partly, due to genetic differences between individuals. Large scale collaborative attempts to find the actual genes that underlie this genetic variation are under way. In 2011, a meta-analysis of GWA studies of CRP in over 80,000 subjects identified several genes implicated in immune system functioning and inflammation (*CRP, IL6R, NLRP3, IL1F10, IRF1, PPP1R3B, SALL1, PABPC4, ASCL1, RORA* and *BCL7B*) and the metabolic syndrome (*APOC1, HNF1A, LEPR, GCKR, HNF4A* and *PTPN2*) to be associated with circulating CRP levels (Dehghan et al., 2011). In a meta-analysis of six GWA studies on fibrinogen in over 22,000 subjects, significant genome-wide hits were found in the *FGB, IRF1, PCCB* and *NLRP3* genes (Dehghan et al., 2009). For IL-6 and TNF-α no meta-analysis has been published to our knowledge. A single large GWA study on IL-6 (N = 6,145) found significant hits in the IL6R and ABO genes (Naitza et al., 2012). With our study we have accomplished a clear numerical target for these ongoing genome-wide screens that aim to find the actual genetic variants regulating the levels of these pro-inflammatory markers. Heritability studies conducted in large representative samples continue to be valuable, because the heritability of traits can vary between populations and can change across generations. We should also keep in mind that 54 to 79% of the variation found was due to unique environmental factors that are not shared between family members. This estimate may derive from unique environmental factors or measurement error, but it may also result from gene-by-environment interactions that may inflate estimates of unique environmental effects. Unravelling the genetics of these pro-inflammatory parameters may greatly contribute to our understanding of the aetiology of cardiac disease and major depression since chronic low-grade inflammation has repeatedly been shown to be associated with

both (Cesari et al., 2003; Danesh et al., 2004; Danesh et al., 2008; Humphries et al., 2007; Libby & Theroux, 2005; O'Brien et al., 2004; Packard & Libby, 2008; Penninx et al., 2003; Woods et al., 2000).

Because of the large sample size and the extended twin-family design this study had sufficient power to decompose the variance in the levels of an important set of pro-inflammatory markers into additive and non-additive genetic factors, and shared and unique environmental factors. The inclusion of parents and siblings allowed us to detect and correct for assortative mating, quantitative and qualitative sex differences, and effects of age that could potentially affect the heritability estimates. The sample size also allowed us to employ strict exclusion criteria concerning the recent health status and medication use of the subjects without losing power so that we were ensured analyses were run on healthy individuals only. Furthermore, our study design controlled for female sex hormone status.

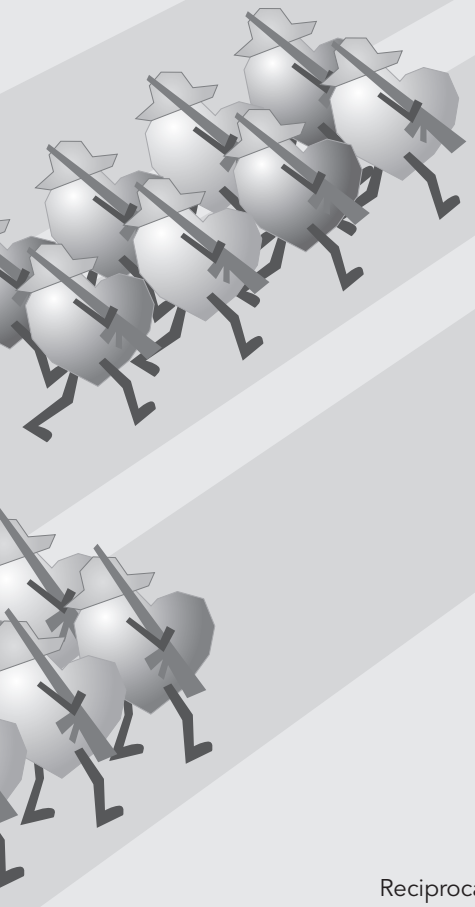
This study also had limitations. First, we selected only a subset of the many immune parameters that co-determine the pro-inflammatory state, including IL-1 and interferon-γ, and we did not take into account the action of the soluble receptors for the cytokines, levels of which may be substantial heritable. Secondly, we used the ELISA assay by R&D systems that may yield higher TNF-α and IL-6 values in individuals of blood group O levels than other ABO blood groups which may in part by due to assay specific cross-reactivity with ABO antigens (Melzer et al., 2008; Naitza et al., 2012). We indeed found a significant association of SNPs in the ABO region to TNF-α and IL-6. Although it explained only a small amount of variance in TNF-α (7%) and IL-6 (4%) compared to the larger effects of plate and BMI, they may cause overestimation of non-additivity or underestimation of shared environment since the shared blood group O will make MZ twins appear more alike than DZ twins or non-twin siblings. Thirdly, we tested whether different genes are expressed at different ages by testing whether parent-offspring correlations and correlations in first-degree relatives (DZ twins and non-twin siblings) were of comparable magnitude. Because there was a partial overlap in age between the parent and the offspring generation, we cannot completely rule out the possibility that the expression of pro-inflammatory genes changes across age.

In conclusion, the familial resemblance in these core pro-inflammatory cytokines and acute phase reactants is explained by genetic variation and not by the shared family environment. For three out of four markers, both additive and non-additive genetic factors contribute to the heritability.



Chapter 7

Reciprocal association between ambulatory autonomic nervous system functioning and pro-inflammatory and metabolic risk profiles



This chapter is submitted:

Neijts, M., Willemsen, G., Boomsma, D., & de Geus, E. J. C. Reciprocal association between ambulatory autonomic nervous system functioning and pro-inflammatory and metabolic risk profiles.

Abstract

Previous cross-sectional research in laboratory settings has shown significant associations between cardiac autonomic functioning and metabolic and inflammatory risk. The nature of this association remains unclear. Prospective associations of cardiac sympathovagal control and inflammatory and metabolic risk were tested in both directions in two independent studies. Study 1 linked autonomic functioning to inflammatory and metabolic risk measured 4.9 years later, Study 2 tested the reverse link with 5.4 years in between. Participants were measured in their natural environment. Study 1 and 2 included 433 and 540 healthy adults registered with the Netherlands Twin Register, respectively. 24-hour autonomic activity was measured and included heart rate (HR), respiratory sinus arrhythmia (RSA, parasympathetic activity), and pre-ejection period (PEP, sympathetic activity). Stress reactivity was computed by contrasting the levels during work with levels during leisure time. Inflammatory and metabolic risk were calculated by summing the Z-scores of several key markers of the pro-inflammatory and the metabolic state. Higher leisure time HR (β_{zIBI} = -0.94, p = .02) and larger PEP reactivity ($\beta_{z\Delta PEP}$ = -0.43, p = .04) were associated with increased inflammatory risk at follow-up. Larger PEP reactivity was also associated with higher metabolic risk at follow-up ($\beta_{z\Delta PEP}$ = -0.80, p = .01). No significant associations in the reverse direction were found. High resting HR together with exaggerated sympathetic reactivity has adverse effects on inflammatory risk. Exaggerated sympathetic reactivity also negatively impacts metabolic risk. No evidence for a reverse association was found.

Introduction

A cluster of metabolic dysregulations, commonly referred to as the metabolic syndrome, is implicated in the development of cardiovascular disease and diabetes (Wilson, D'Agostino, Parise, Sullivan, & Meigs, 2005). Chronic low-grade inflammation has emerged as an additional risk factor (Danesh et al., 2008; Libby & Theroux, 2005; Packard & Libby, 2008) that often accompanies metabolic dysregulation (Grundy et al., 2005; Marques-Vidal et al., 2002). The metabolic and inflammatory risk factors may have a common biological ground: a shift in autonomic nervous system (ANS) activity from parasympathetic towards sympathetic dominance. Previous cross-sectional research showed that such a shift was indeed associated with high waist circumference, and higher levels of several metabolic markers including low density lipoprotein cholesterol (LDL-cholesterol), total cholesterol, triglyceride values, increased systolic blood pressure (SBP), and increased fasting glucose levels (Intzilakis et al., 2013; Licht et al., 2010; Thayer & Fischer, 2013). Licht, de Geus, & Penninx (Licht, de Geus, & Penninx, 2013) tested this association in a longitudinal study in which ANS functioning was used to predict a change in the metabolic components over 2 years. They found that increased cardiac sympathetic control at rest (indexed as the pre-ejection period, or PEP) predicted an increase in the number of metabolic components. In addition, low vagal control at rest (indexed as respiratory sinus arrhythmia, or RSA) predicted a future decrease in HDL cholesterol.

There is solid biological ground to additionally expect that an inflammatory risk profile, in particular the TNF-α/IL-1/IL-6 pathway, is also sensitive to the activity of both branches of the ANS. When the body is inflamed, the acute parasympathetic (PNS) and sympathetic (SNS) nervous system responses act to prevent a further inflammatory cascade by inhibiting the release of pro-inflammatory cytokines and the ensuing production of acute phase reactants, and by stimulating the production of anti-inflammatory cytokines (Elenkov, Wilder, Chrousos, & Vizi, 2000). However, when ANS activity shifts more chronically to SNS dominance, either through decreased PNS activity or through increased sympathetic activity or a combination, chronic low-grade inflammation may be the result (Sternberg, 2006). Cross-sectional HRV studies have indeed shown that reduced cardiac vagal control as assessed during short-term (Haarala et al., 2011; Kon et al., 2006; Sloan et al., 2007; Singh, Hawkley, McDade, Cacioppo, & Masi, 2009; von Kanel, Nelesen, Mills, Ziegler, & Dimsdale, 2008) or prolonged laboratory (Lampert et al., 2008) conditions was associated with higher C-reactive protein (CRP) and interleukin(IL)-6. In addition, 24-h

ambulatory HRV recordings also point to inverse associations of vagal control with CRP, IL-6, and fibrinogen (Araujo et al., 2006; Sajadieh et al., 2004; Stein et al., 2008; Thayer & Fischer, 2009; von Kanel, Thayer, & Fischer, 2009). These associations appear to hold in prospective studies. Lower nighttime HRV at baseline, for instance, predicted higher CRP measured at 3 to 4-year follow-up (Jarczok, Koenig, Mauss, Fischer, & Thayer, 2014; Singh et al., 2009). Two studies found the vagal effect to be independent of urinary epinephrine (Lampert et al., 2008; Thayer & Fischer, 2009). The link between cardiac sympathetic control and chronic low-grade inflammation is not yet clearly established. In humans, cardiac contractility was not associated with CRP (Singh et al., 2009), whereas animal studies pointed to significant positive associations between cardiac contractility and TNF-α levels (Bozkurt et al., 1998; Murray & Freeman, 1996).

A first concern about the studies linking autonomic functioning to metabolic or inflammatory risk factors is that they explicitly assume an unidirectional causal effect of the ANS on metabolic and inflammatory risk. The observed association may, however, also derive from reverse effects of metabolic and inflammatory risk on autonomic functioning. Soares-Miranda and colleagues (Soares-Miranda et al., 2012), for instance, found that high levels of SBP, triglyceride, glucose levels and CRP at baseline predicted lower HRV at 2-year follow-up, although these associations disappeared at the 3-year follow-up. The finding of prospective associations in both directions hints at the possibility of reciprocal (bidirectional) causality. Alternatively, parallel patterns of unfavourable ANS functioning and metabolic or inflammatory risk may reflect the effects of underlying factors independently acting on ANS functioning and metabolic/inflammatory risk across time, like socioeconomic position, lifestyle (e.g. physical activity, smoking) or genetic pleiotropy.

A second concern about the extant literature is that many studies were conducted in a laboratory or clinic setting with measures of SNS and PNS activity recorded during periods of quiet rest (Haarala et al., 2011; Kon et al., 2006; Licht et al., 2010; Licht et al., 2013; Sloan et al., 2007; von Kanel et al., 2008). Various studies have indicated that the ecological validity and clinical predictive value of risk factor recording may be increased when they are done in the participant's natural environment by ambulatory monitoring (Bussmann, Ebner-Priemer, & Fahrenberg, 2009; Ebner-Priemer & Trull, 2009a; 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, and better capture the effects of changes in mental and emotional challenge as seen during work-non-work, and wake-sleep transitions.

Here we examined the prospective association between autonomic functioning in daily life and metabolic and inflammatory risk in both directions. We used measures of cardiac vagal and sympathetic control assessed by ambulatory monitoring, which allowed the computation of prolonged resting levels (e.g. sleep, leisure time) as well as stress reactivity to real-life psychological challenge as typically encountered in a work setting. Two independent prospective studies were conducted linking (1) ambulatory parasympathetic and sympathetic cardiac control to inflammatory and metabolic risk measured 4.9 years later, and (2) inflammatory and metabolic risk to ambulatory parasympathetic and sympathetic cardiac control measured 5.4 years later. We expected to find that sympathetic overdrive coupled to vagal withdrawal at rest, and its enhancement during exposure to psychological stress, would be associated with an unfavorable inflammatory and metabolic profiles 4.9 years later. In addition, we expected that the effects would be reciprocal and that subjects with unfavorable inflammatory and metabolic profiles would show sympathetic overdrive coupled to vagal withdrawal at 5.4 year follow-up, and exaggerated autonomic reactivity to psychological stress.

Methods

ANS Study sample 1

A total of 816 participants participated in the first part (Study 1, 1998-2003) of a large cardiac ambulatory monitoring study conducted in families registered with the Netherlands Twin Register (NTR). A subgroup of 68 participants participated twice in this period, so in total 884 ambulatory cardiac recordings were available. A priori reasons for exclusion were heart transplantation, presence of a pacemaker and known ischemic heart disease, congestive heart failure, diabetic neuropathy, or pregnancy. We selected ANS recordings of participants with valid data on both heart rate variability (HRV) and PEP measures (N=21 recordings excluded). Cardiac recordings of participants who were on antidepressant medication (ATC code N06A), beta blocking agents (ATC code C07), or cardiac therapy (ATC code C01) were excluded from further analyses (N=43). Of the remaining recordings, 62 still belonged to participants that participated twice. When the difference in the duration between the two recordings was greater or equal to 200 minutes, the shorter recording was excluded (N=10). When both twins participated, the recording of the twins in which both participated were kept (N=26 excluded). For the remaining duplicate recordings, the recording of the study in which most family members

participated were kept (N=26 recordings excluded). The remaining sample comprised 758 participants. A total of 433 of these participants also participated in a large NTR biobank study (Willemsen et al., 2010) that was conducted between 2004 and 2008, on average 4.9 (SD = 1.7) years later.

ANS Study sample 2

A total of 592 participants who took part in the NTR biobank study also participated in the second part (Study 2, 2010-2012) of the cardiac ambulatory monitoring study. We employed the same a priori reasons for exclusion as for Study 1 and selected ANS recordings of participants with valid data on both heart rate variability (HRV) and PEP measures (N=16 recordings excluded). Cardiac recordings of participants who were on antidepressant medication (ATC code N06A), beta blocking agents (ATC code C07), or cardiac therapy (ATC code C01) were excluded from further analyses (N=32). The remaining sample was comprised of 540 participants having valid IBI, pvRSA, or PEP data during sleep, leisure time, and/or during sitting activities at work. The average time interval between these studies was 5.4 (SD = 1.1) years.

24-hour ambulatory monitoring studies: set up and measures

For both studies, participants were visited at home, before starting their normal daily activities. During a short interview, information on health status and current medication use was obtained. The VU-AMS was attached and its operation explained. Participants were instructed to wear the device the entire day and night up until awakening the next morning. Instructions were supplied that explained how to respond to potential alarm beeps (e.g., on loose electrode contacts), and telephone assistance was available during waking hours. Participants were requested to keep a diary and to write down a chronological account of activity, posture, location, and social situation over the past time period. For Study 1 this was done every 30 minutes, for Study 2 every 60 minutes. Participants were instructed to refrain from vigorous exercise during the ambulatory recording day.

For Study 1, the VU University Ambulatory Monitoring System (VU-AMS) version 4.6 was used (VU University, Amsterdam, The Netherlands, www.vu-ams.nl). This version of the VU-AMS continuously recorded the electrocardiogram (ECG) and changes in thoracic impedance (dZ) from a six-electrode configuration (de Geus et al., 1995; de Geus & van Doornen, 1996; Willemsen et al., 1996). The device automatically detects each R wave in

the ECG signal, at which it reads out and resets a millisecond counter to obtain the heart period time series. The thoracic impedance (Z), assessed against a constant current of 50 KHz, 350 microamperes, was amplified and led to a precision rectifier. The rectified signal was filtered at 72 Hz (low pass) to give basal impedance Z. Filtering Z at 0.1 Hz (high pass) supplied the dZ signal, which was band pass filtered with 0.1 and 0.4 Hz cutoffs, after tapering with $(\sin(x))^2$, to yield the respiration signal.

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. Artifact processing was performed on the IBI data offline. When the IBI deviated more than 3 SD from the moving mean of a particular period it was automatically coded as an artifact and the IBI was either rejected during visual inspection, new IBIs were created by summing too short IBIs or too long IBIs were split in two IBIs of equal length.

For Study 2, the 5fs version of the VU-AMS was used, which improved on the 4.6 version in that it stores the entire ECG for offline analysis rather than online R-wave peak-detection (van Dijk et al., 2013). The ECG signal was imported into the VU-DAMS software (version 3.2, 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, the IBI time series was again constructed and visually displayed for interactive correction of missed or incorrect R-wave peaks. In addition to the ECG, the 5fs version also stores the entire dZ at 1000 Hz to obtain the respiration signal. The dZ signal is filtered using a second order band pass filter that passes all frequencies in the range of 0.1 to 0.4 Hz. An Exponential Smoothing Average technique is then applied on the filtered DZ signal, which acts as an additional low pass filter. The output of this filter is a weighted combination of previous smoothed value and the newest measured data, or in formula:

$$S_t = \alpha * S_{t-1} + (1 - \alpha) * x_t$$

where S_t is the smoothed average, α is the tunable smoothing factor (which is in the range of 0 to 1), x_t is the observation at time t, and S_{t-1} is the previous smoothed value.

Computation of the RSA measures was done in the same way for both studies. Combining the IBI time interval series with the respiration signal extracted from the thorax impedance signal (dZ), the 'peak–valley' RSA method was used to asses pvRSA (de Geus et al., 1995; Grossman & Wientjes, 1986; Grossman et al., 1990). 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 and coughing, were considered invalid and were removed from further processing. If no shortest or longest IBI could be detected in inspiration and expiration respectively, the breath was either set to missing or to zero when computing the average per condition for pvRSA. Similar results were found for pvRSA computed either way and we employed only one (breaths set to missing) in further statistical analyses. As previous research suggests that results may be biased if participants with RSA ceiling effects at low heart rates are included, all analyses were performed twice; once including the participants with RSA ceiling effects at low heart rates and once excluding these participants (Neijts et al., 2014; van Lien et al., 2011).

For the assessment of the PEP, a measure of cardiac contractility, both the ECG and the ICG were used. The ICG signal was large-scale ensemble averaged across the diary-coded activity periods (described below), time-locking the signal to the R-wave peaks (Riese et al., 2003). The PEP is defined as the time interval between the Q-wave onset of the ECG and the B-point of the dZ/dt signal. The Q-wave reflects the onset of left ventricular activity and the B-point reflects the opening of the aortic valves. In both VU-AMS versions, the R and B points were scored automatically by the software. In the newer 5fs version of the VU-AMS, the entire ECG signal was stored, so the Q-onset time was available as well. All automated scoring was visually checked by the experimenter. For the calculation of PEP in Study 1, a fixed Q-R interval of 48 msec was added to the duration of the R-B interval (Willemsen et al., 1996). For Study 2, the true Q-onset point was used when present; otherwise the grand average of the Q-R interval was summed to the R-B interval of the individual participant. If R-onset was additionally missing, we subtracted the grand average Q-onset time from the individual participants' B-point (van Lien et al., 2013).

Ambulatory data reduction and specification of ambulatory conditions

Using the activity diary entries in combination with a visual inspection of the output of the inbuilt accelerometer, 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), physical load (no load, light, intermediate, heavy), and location (e.g. at home, at work, public space). Minimum duration of periods was 5 min 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.

From the ambulatory ANS recording, two resting conditions and one ambulatory stress condition were specified. For the first resting condition, the mean IBI, pvRSA, and PEP value across all sleeping periods was calculated ('sleep'). For the second resting condition the period with the *lowest* heart rate in the evening from 6 pm till bedtime in which the participant was sitting was selected ('leisure'). As ambulatory stress conditions, we used 'work' (the mean of all periods in which the participant was engaged in sitting activities at the work location between 9 am and 6 pm during a reported working day). Reactivity was calculated as the difference score between work and leisure (Δ work - leisure). These ambulatory reactivity measures from unstructured 24-h recordings have been described in more detail in (Neijts et al., submitted).

Biobank study: set up and measures

For the biobank study, the participants were visited in the morning at home, or when preferred at work, to collect blood and urine samples after an overnight fast. They were instructed to abstain from physical exertion and, if possible, not to take medication at the day of the home visit, and to refrain from smoking one hour before the home visit. Fertile women were, when possible, visited on the 2nd- 4th day of their menstrual cycle or, if they took oral contraceptives, in their pill-free week. During the home visit, a brief interview was conducted on health status, including an inventory of medication use, illness (last time occurrence, duration and type of illness), and adherence to the protocol. Also, height, weight and waist circumference were assessed. A total of eight blood tubes were collected. To prevent clotting, all tubes were inverted gently 8–10 times immediately after collection. The blood tubes of the biomarkers that were used for the current study (2 × 9 ml EDTA, 1 × 9 ml heparin, 1 × 4.5 ml CTAD) were transported in melting ice during transport to the laboratory. Both EDTA tubes and the CTAD tube were centrifuged for 20 minutes at 2000x *g* at 4˚C upon arrival at the laboratory. EDTA plasma was harvested and aliquoted (0.5 ml), snapfrozen in dry ice, and stored at -30˚C. Citrated plasma from the CTAD tube was harvested from the buffy coat and red blood cells, aliquoted (0.5 ml), snapfrozen in dry ice, and stored at -30˚C. The heparin tube was centrifuged for 15 minutes at 1000x *g* at 4˚C, heparin plasma was obtained and divided into 8 subsamples of 0.5 ml, snapfrozen and stored at -30˚C (Willemsen et al., 2010). TNF-α and IL-6 were measured in EDTA plasma, using an UltraSensitive ELISA (R&D systems, Minneapolis, USA, Quantikine HS HSTA00C) (Neijts et al., 2013). CRP was measured in heparin plasma, using Immulite 1000 CRP assay (Diagnosic Product Corporation, USA). Fibrinogen levels

were measured in citrate plasma, on a STA Compact Analyzer (Diagnostica Stago, France), using STA Fibrinogen (Diagnostica Stago, France). Glucose was measured using the Vitros 250 Glucose assay (Johnson & Johnson, Rochester, USA). Total cholesterol, HDL-cholesterol, and triglycerides were measured in heparin plasma, using Vitros 250 direct HDL cholesterol and Vitros 250 Triglycerides assays (Johnson & Johnson, Rochester, USA). LDL-cholesterol was calculated using the Friedewald Equation (Friedewald, Levy, & Fredrickson, 1972).

For the current analyses, values of the pro-inflammatory markers were excluded in case of current illness of the participant (allergy, cold, flu, infection), medication use (medication impacting the hypothalamic pituitary adrenal [HPA] axis [ATC codes H01, H02] and anti-inflammatoy medication [ATC codes L01 to L04, M01]). A value > 15 pg/mL for TNF-α, IL-6, and CRP, or > 6 g/L for fibrinogen was considered an outlier and was hence set to be missing. Metabolic variables were only included when participants had been fasting from 00:00h the previous night until the blood sampling took place. Lipid variables were excluded from further analyses when participants were on lipid modifying medication (ATC code C10). Glucose values were set to be missing when antidiabetic drugs were used at the time of the study (ATC code A10). Values deviating more than four standard deviations from the mean were considered outliers and were set to be missing for all metabolic variables (glucose, LDL-cholesterol, HDL-cholesterol, triglycerides, waist circumference, and BMI).

Assessment of covariates

The effects of age and sex were taken into account for all variables. For the inflammatory markers, the month of blood sampling and plate-effects for the cytokines were also taken into account because of their known effects on these variables (Neijts et al., 2013). Age, sex, plate mean and/or month mean for the inflammatory markers were included in multiple regression analyses (forced entry) and the residual scores were saved and used for subsequent risk score computation.

Metabolic and inflammatory risk scores

All residual metabolic and inflammatory variables were transformed to Z-scores. An inflammatory risk score was calculated as: ZTNF-α + ZIL-6 + ZCRP + ZFibrinogen, with higher values representing higher inflammatory risk. A metabolic risk score was calculated as: ZWaist + ZBMI + ZGlucose + ZTriglycerides + ZLDL – ZHDL, with higher waist, BMI,

fasting glucose levels, triglycerides, and LDL-cholesterol and lower HDL-cholesterol representing higher metabolic risk.

Statistical analyses

Data preparation and statistical analyses were conducted using IBM SPSS 21.0. A mixed model ANOVA with age and sex as fixed factors, family as a random factor, and time of day as a repeated measures factor was used to study the effects of time of day and the effects of the covariates. Pearson correlations were calculated to test the associations of the ANS variables with the z-transformed residuals of the variables constituting the metabolic and inflammatory risk scores, and the risk scores themselves.

Since the pattern of bivariate correlations may reflect consistency that may be partly derived from the intercorrelation between the ANS measures and conditions and because there may be chance findings due to multiple testing, we used multiple regression analyses to simultaneously test for an effect of all three measures of ANS functioning during sleep and leisure time and their work-leisure reactivity on metabolic and inflammatory risk scores. These analyses were run after a careful check for potential problems due to multicollinearity, which remained within acceptable boundaries. For Study 1, general linear models were used to predict inflammatory and metabolic risk from the ambulatory IBI, pvRSA, and PEP levels at sleep and leisure and the work-leisure reactivities. Two models were run with either the inflammatory or the metabolic risk score as dependent variable. In these models, the ANS predictors were entered as fixed factors, and family was included as a random factor. ANS predictors included sleep, leisure and the Δ work - leisure reactivity measure. For Study 2, general linear models were used to predict IBI, pvRSA, and PEP levels at sleep and leisure and the work-leisure reactivity from the inflammatory and metabolic risk scores. Nine models were run with the inflammatory or the metabolic risk scores as predictors and IBI, pvRSA, and PEP levels in sleep and leisure and Δ work – leisure reactivity as the nine dependent variables. In these models, the predictors were entered as fixed factors, and family was included as a random factor. All general linear models were rerun without the participants with ceiling effects for RSA during sleep.

Results

Table 7.1 shows the means and standard deviations of the variables for Study 1 and 2. As expected, PEP, RSA and IBI were lower during sleep than during the two daytime recordings for both Study 1 (F (2, 1118) = 1374.596, $p < .001$, for IBI, F (2,1134) = 77.052, $p < .001$, for RSA, and F (2,1093) = 179.600, $p < .001$ for PEP) and Study 2 (F (2, 809) = 789.532, $p < .001$ for IBI, F (2,817) = 64.271, $p < .001$ for RSA, and F (2,799) = 42.935, $p < .001$ for PEP). RSA was lower with increasing age at both time points (F (1,373) = 120.296, $p < .001$ for Study 1 and F (1,268) = 44.307, $p < .001$ for Study 2) but the influence of age on heart rate and PEP was not significant. Sex differences were found for all ANS variables, with females showing higher heart rate (F (1,752) = 71.399, $p < 001$ for Study 1 and F (1,537) = 14.980, $p < 001$ for Study 2), higher RSA (F (1,757) = 20.234, $p < .001$ for Study 1 and F (1,510) = 34.079, $p < .001$ for Study 2), and generally longer PEP (F (1,720) = 1.986, $p = .159$ for Study 1 and F (1,513) = 8.808, $p = .003$ for Study 2) values than males.

Tables 7.2 and 7.3 depict for Study 1 and Study 2, respectively, the correlations between ANS measures and the z-transformed residuals for the variables constituting the metabolic and inflammatory risk scores, and the risk scores themselves. From the pattern of correlations for Study 1 depicted in table 7.2, it becomes clear that higher heart rate during sleep and leisure time, and during sitting activities at work was associated with higher inflammatory risk at follow-up. This effect was most pronounced for IL-6 where heart rate during sleep, leisure time, and work were significantly associated with future IL-6 ($-0.18 < r < -0.20$, $p < .001$). Higher heart rate during sleep and leisure time was also associated with higher future CRP levels ($-0.16 < r < -0.19$, $p < .001$), and higher heart rate during work and sleep was associated with higher future fibrinogen (-0.22 $r < -0.24$, $p < .01$). TNF-α levels were not significantly associated with ANS functioning 4.9 years earlier. Predictors of higher metabolic risk at follow-up were higher heart rate during leisure time ($r = -0.17$, $p = .001$) and lower RSA during leisure time ($r = -0.18$, $p = .001$), and PEP at work ($r = -0.19$, $p = .008$). The main determinants of these associations were lower HDL-cholesterol levels ($0.18 < r < 0.25$, $p < .001$), larger waist circumference ($-0.10 < r < -0.17$, $p < .05$) and, to a lesser extent, higher BMI ($r = -0.14$, $p = .037$ for PEP_{work}), and higher LDL-cholesterol ($r = -0.16$, $p = .001$ for $RSA_{leisure}$) and triglyceride levels ($r = -0.14$, $p = .006$ for $IBI_{leisure}$).

Table 7.1. Means and standard deviations of the ANS and biobank variables included in Study 1 and Study 2.

	Study 1	N	Study 2	N
Age at baseline (mean +/- sd)	32.0 (11.1)	433	31.8 (5.5)	540
Sex (% female)	60.3	433	61.3	540
ANS measures				
IBI (msec)				
Sleep	971.34 (131.61)	421	986.83 (131.42)	533
Leisure	861.97 (116.63)	416	896.77 (129.05)	501
Work	766.07 (115.09)	223	804.30 (106.12)	292
Δ work – leisure	-98.14 (86.56)	214	-97.31 (97.76)	272
pvRSA (msec)				
Sleep	54.95 (25.88)	421	54.29 (23.21)	533
Leisure	52.06 (25.60)	416	49.81 (23.61)	501
Work	44.45 (19.88)	223	43.92 (17.04)	292
Δwork – leisure	-8.78 (17.48)	214	-6.21 (16.82)	272
PEP (msec)				
Sleep	105.08 (15.18)	414	107.84 (16.12)	526
Leisure	97.83 (15.88)	413	102.15 (17.64)	475
Work	98.11 (16.50)	222	101.74 (18.94)	284
Δwork – leisure	0.42 (7.04)	213	1.31 (13.83)	251
Pro-inflammatory markers				
Tumor Necrosis Factor (TNF)-α (pg/ml)	1.14 (1.37)	352	1.06 (1.06)	469
Interleukin(IL)-6 (pg/ml)	1.37 (1.21)	353	1.26 (1.19)	469
C-Reactive Protein (CRP) (mg/L)	2.39 (2.90)	345	2.17 (2.65)	458
Fibrinogen (g/L)	2.58 (0.65)	350	2.46 (0.56)	454
Metabolic markers				
Body Mass Index (BMI)	24.48 (3.92)	427	23.72 (3.57)	537
Waist circumference (cm)	83.82 (11.67)	427	80.77 (10.60)	535
Glucose (mmol/L)	5.30 (0.58)	395	5.20 (0.51)	498
Low density lipoprotein cholesterol (LDL-C) (mmol/L)	3.00 (0.83)	398	2.79 (0.86)	496
High density lipoprotein cholesterol (HDL-C) (mmol/L)	1.39 (0.34)	398	1.42 (0.36)	499
Triglycerides (mmol/L)	1.19 (0.53)	396	1.19 (0.63)	495

Table 7.2. Correlations between ANS (re)activity at baseline and the variables constituting the metabolic and inflammatory risk scores, and the risk scores themselves 4.6 years later.

		ZBMI	ZWaist	ZGlucose	ZLDL-C	ZHDL-C	ZTrig	**Metabolic risk score**	ZTNF-a	ZIL-6	ZCRP	ZFibr	**Inflammatory risk score**
IBI													
Sleep	r	-0.100*	-0.162**	0.003	0.048	0.121*	-0.069	-0.095	-0.063	-0.195**	-0.163**	-0.137**	-0.222**
	p	*.041*	*.001*	*.957*	*.349*	*.017*	*.177*	*.068*	*.245*	*.000*	*.003*	*.012*	*.000*
	N	416	415	383	386	386	384	371	341	342	334	339	324
Leisure	r	-0.071	-0.172**	0.004	-0.088	0.183**	-0.142**	-0.168**	-0.035	-0.175**	-0.189**	-0.081	-0.202**
	p	*.151*	*.000*	*.939*	*.085*	*.000*	*.006*	*.001*	*.517*	*.001*	*.001*	*.141*	*.000*
	N	410	410	378	381	381	379	365	337	338	331	335	321
Work	r	-0.057	-0.167*	0.024	-0.055	0.097	-0.123	-0.122	-0.113	-0.220**	-0.182*	-0.240**	-0.287**
	p	*.398*	*.013*	*.736*	*.431*	*.166*	*.079*	*.086*	*.132*	*.003*	*.014*	*.001*	*.000*
	N	220	220	207	207	207	206	199	179	181	181	178	176
Δ work-leisure	r	0.058	0.074	-0.024	0.053	-0.025	-0.060	0.009	-0.072	0.081	0.068	-0.028	0.046
	p	*.401*	*.285*	*.732*	*.458*	*.730*	*.405*	*.901*	*.553*	*.293*	*.375*	*.717*	*.555*
	N	211	211	198	198	198	197	190	170	172	172	169	167
RSA													
Sleep	r	0.037	0.019	-0.045	-0.101*	0.092	-0.008	-0.040	-0.024	0.022	0.027	0.025	0.032
	p	*.448*	*.694*	*.383*	*.047*	*.071*	*.870*	*.439*	*.661*	*.692*	*.627*	*.651*	*.571*
	N	416	415	383	386	386	384	371	341	342	334	339	324
Leisure	r	-0.070	-0.100*	-0.025	-0.164**	0.228**	-0.071	-0.179**	0.050	0.006	-0.034	0.027	0.010
	p	*.156*	*.044*	*.624*	*.001*	*.000*	*.170*	*.001*	*.360*	*.915*	*.542*	*.623*	*.863*
	N	410	410	378	381	381	379	365	337	338	331	335	321
Work	r	-0.124	-0.127	-0.043	-0.117	0.189**	-0.065	-0.169*	0.062	-0.044	-0.047	-0.083	-0.069
	p	*.067*	*.060*	*.540*	*.092*	*.006*	*.354*	*.017*	*.412*	*.552*	*.527*	*.270*	*.364*
	N	220	220	207	207	207	206	199	179	181	181	178	176

		ZBMI	ZWaist	ZGlucose	ZLDL-C	ZHDL-C	ZTrig	**Metabolic risk score**	ZTNF-a	ZIL-6	ZCRP	ZFibr	**Inflammatory risk score**
Δ work - leisure	r	0.068	0.092	0.043	.077	-0.098	-0.117	0.046	-0.017	-0.065	-0.101	-0.106	-0.116
	p	*.323*	*.184*	*.550*	*.282*	*.170*	*.103*	*.524*	*.826*	*.396*	*.186*	*.171*	*.136*
	N	211	211	198	198	198	197	190	170	172	172	169	167
PEP													
Sleep	r	0.000	-0.043	-0.060	-0.004	0.111*	0.000	-0.072	-0.020	-0.050	0.030	-0.001	-0.037
	p	*.998*	*.382*	*.249*	*.932*	*.031*	*.995*	*.171*	*.712*	*.360*	*.589*	*.989*	*.515*
	N	409	408	376	379	379	377	364	335	336	328	333	318
Leisure	r	-0.049	-0.114*	-0.005	-0.001	0.170**	-0.044	-0.114*	-0.017	-0.008	-0.002	0.048	-0.020
	p	*.324*	*.021*	*.923*	*.978*	*.001*	*.392*	*.030*	*.756*	*.879*	*.976*	*.382*	*.726*
	N	407	407	375	378	378	376	362	334	335	328	332	318
Work	r	-0.141*	-0.174**	0.024	-0.065	0.249**	-0.071	-0.188**	0.057	0.021	-0.003	-0.005	0.012
	p	*.037*	*.010*	*.736*	*.356*	*.000*	*.312*	*.008*	*.448*	*.779*	*.972*	*.990*	*.879*
	N	219	219	206	206	206	205	198	179	181	181	178	176
Δ work-leisure	r	-0.176*	-0.186**	0.079	-0.147*	.082	-0.079	-0.161*	-0.113	-0.067	-0.043	-0.064	-0.090
	p	*.011*	*.007*	*.270*	*.040*	*.251*	*.273*	*.027*	*.141*	*.380*	*.573*	*.412*	*.249*
	N	210	210	197	197	197	196	189	170	172	172	169	167

* Light gray is significant at 0.05
**Dark gray is significant at 0.01

Table 7.3. Correlations between the variables constituting the metabolic and the inflammatory risk scores, and the risk scores themselves, at baseline and ANS (re)activity 5.4 years later.

		IBI				**RSA**				**PEP**			
		sleep	leisure	work	Δ work - leisure	sleep	leisure	work	Δ work - leisure	sleep	leisure	work	Δ work - leisure
ZBMI	r	-0.064	-0.038	-0.095	-0.022	0.008	-0.077	-0.172**	0.022	-0.017	-0.043	-0.183**	-0.063
	p	*.140*	*.398*	*.106*	*.724*	*.848*	*.088*	*.003*	*.712*	*.705*	*.349*	*.002*	*.322*
	N	531	498	291	271	531	498	291	271	524	472	283	250
ZWaist	r	-0.038	-0.026	-0.104	-0.057	.018	-0.034	-0.147*	-0.001	0.008	-0.050	-0.134*	-0.092
	p	*.389*	*.569*	*.078*	*.351*	*.686*	*.453*	*.012*	*.990*	*.860*	*.275*	*.024*	*.151*
	N	528	497	289	269	528	497	289	269	521	471	222	248
ZGlucose	r	-0.009	-0.046	-0.007	0.015	-0.048	-0.018	0.012	-0.058	0.015	-0.017	0.029	-0.081
	p	*.833*	*.321*	*.905*	*.812*	*.288*	*.700*	*.850*	*.363*	*.744*	*.721*	*.636*	*.217*
	N	493	462	270	251	493	462	270	251	487	437	263	231
ZLDL-C	r	-0.037	-0.067	-0.116	-0.039	0.009	-0.058	-0.094	-0.014	-0.120*	-0.096*	-0.102*	-0.074
	p	*.419*	*.150*	*.057*	*.540*	*.837*	*.215*	*.124*	*.822*	*.008*	*.044*	*.101*	*.262*
	N	491	461	269	250	491	461	269	250	485	436	262	230
ZHDL-C	r	0.040	0.034	0.104	0.082	0.019	0.061	0.180**	0.011	-0.067	-0.013	-0.062	-0.059
	p	*.374*	*.468*	*.088*	*.197*	*.668*	*.188*	*.003*	*.860*	*.137*	*.792*	*.313*	*.373*
	N	494	463	271	252	494	463	271	252	488	438	264	232
ZTrig	r	-0.237**	-0.168**	-0.147*	-0.005	-0.020	-0.017	-0.082	-0.057	-0.045	-0.103*	-0.132*	-0.023
	p	*.000*	*.000*	*.016*	*.940*	*.655*	*.709*	*.179*	*.367*	*.325*	*.031*	*.033*	*.723*
	N	490	459	268	249	490	459	268	249	484	435	261	230
Metabolic risk score	r	-0.101*	-0.091	-0.136*	-0.066	-0.008	-0.061	-0.163**	-0.025	-0.011	-0.063	-0.104	-0.053
	p	*.027*	*.056*	*.029*	*.311*	*.869*	*.197*	*.009*	*.704*	*.815*	*.195*	*.100*	*.437*
	N	478	447	259	240	478	447	259	240	472	423	252	221

ZTNF-α	r	0.024	0.024	-0.022	0.024	0.008	-0.022	-0.022	0.024	0.027	-0.039	-0.009	-0.024
	p	*.602*	*.623*	*.719*	*.711*	*.866*	*.652*	*.723*	*.705*	*.560*	*.428*	*.883*	*.727*
	N	463	432	261	242	463	432	261	242	456	408	253	221
ZIL-6	r	-0.153**	-0.117*	-0.134*	0.077	-0.094*	-0.156**	-0.096	0.141*	0.027	-0.018	-0.048	0.036
	p	*.001*	*.015*	*.031*	*.232*	*.044*	*.001*	*.121*	*.029*	*.560*	*.714*	*.444*	*.600*
	N	463	432	260	241	463	432	260	241	456	408	252	220
ZCRP	r	-0.068	-0.043	-0.033	0.043	0.079	0.036	-0.014	-0.034	0.017	0.072	-0.016	-0.081
	p	*.149*	*.380*	*.599*	*.513*	*.093*	*.459*	*.827*	*.598*	*.710*	*.152*	*.798*	*.234*
	N	453	423	257	239	453	423	257	239	456	399	249	218
ZFibrinogen	r	-0.068	-0.035	0.032	0.056	0.084	0.067	0.079	-0.051	0.014	-0.087	-0.065	0.075
	p	*.149*	*.480*	*.610*	*.397*	*.076*	*.174*	*.215*	*.439*	*.776*	*.084*	*.317*	*.277*
	N	453	419	250	232	448	419	250	232	446	395	242	211
Inflamatory risk score	r	-0.084	-0.074	-0.066	0.082	0.036	-0.031	-0.027	0.020	-0.014	-0.024	-0.032	0.022
	p	*.078*	*.132*	*.304*	*.214*	*.452*	*.529*	*.673*	*.763*	*.772*	*.643*	*.620*	*.747*
	N	440	411	247	229	440	411	247	229	433	387	239	208

* Light gray is significant at 0.05
**Dark gray is significant at 0.01

To take the significant intercorrelation between the predictor variables as well as the outcome variables into account, multiple regression with all predictors was used as the main test of the effect of the ANS on metabolic and inflammatory risk scores. These analyses showed that a higher heart rate during leisure time (β_{zIBI} = -0.95, p = .02) and larger PEP reactivity ($\beta_{z\Delta PEP}$ = -0.43, p = .04) were independently associated with higher inflammatory risk after 4.9 (SD = 1.7) years. Larger PEP reactivity also tended to be associated with higher metabolic risk at follow-up ($\beta_{z\Delta PEP}$ = -0.80, p = .01). Figures 7.1 and 7.2 illustrate that the findings did not depend on a set of outliers.

Figure 7.1. Heart rate during leisure time (left panel) and PEP reactivity to work (right panel) predict a higher inflammatory risk score.

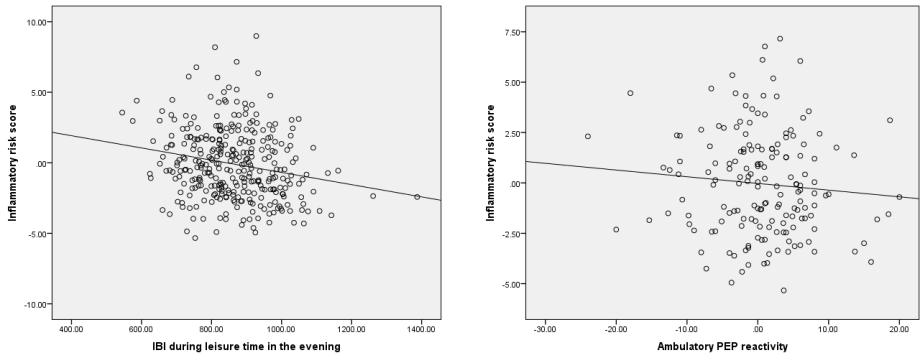
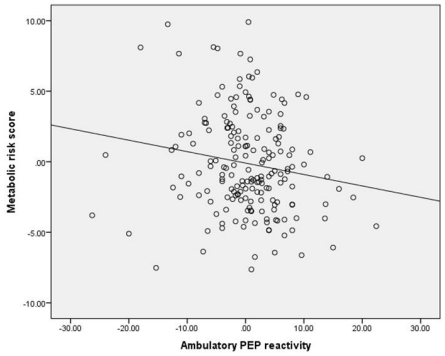


Figure 7.2. PEP reactivity to work predicts a higher metabolic risk.



Correlations for Study 2 are depicted in Table 7.3. In contrast to Table 7.2, few correlations achieved significance. The metabolic risk score predicted future RSA levels at work (r = -0.16, p = .009). The main determinants of this association were higher BMI (r = -0.17, p = .003) and lower HDL-cholesterol levels (r = 0.18, p = .003). Furthermore, higher BMI predicted shorter PEP at work (r = -0.18, p = .002) and higher LDL-cholesterol levels predicted shorter PEP during sleep (r = -0.12, p = .008), and higher triglycerides predicted higher heart rate during sleep and leisure time (-0.17 < r < -0.24, p < .001).

The inflammatory risk score did not predict future ANS functioning, although higher IL-6 levels were associated with higher heart rate during sleep (r = -0.15, p = .001). The metabolic risk score did not predict future ANS functioning. Multiple regression simultaneously using inflammatory and metabolic risk scores as predictors also revealed no significant associations between metabolic or inflammatory risk measured at baseline and ambulatory ANS (re)activity measured 5.4 years later.

Overall, a similar pattern of outcomes was found for Study 1 and 2 when the analyses were repeated excluding the data of participants with a ceiling effect on RSA during sleep (results not shown).

Discussion

This was the first study to jointly investigate whether heart rate, cardiac sympathetic, and cardiac parasympathetic control in a natural environment can predict inflammatory and metabolic risk five years later, as well as the reverse prediction of whether inflammatory and metabolic risk at baseline can predict ambulatory autonomic cardiac control at five year follow-up. We found that higher heart rates during leisure time in the evening together with exaggerated cardiac sympathetic reactivity to work were associated with higher inflammatory risk over time. In addition, exaggerated cardiac sympathetic reactivity to work was associated with higher metabolic risk over time. The reactivity effect was corrected for possible confounding by physical activity by selecting only periods of sitting activities during leisure and work. An unfavorable metabolic or inflammatory risk profile, however, was not associated with later ANS functioning. This pattern of results is thus not compatible with bidirectional causal effects or with an underlying factor model. These would have led to significant associations between inflammatory risk and future heart rate and PEP reactivity, and between metabolic risk and future PEP reactivity. Such

associations were not observed. Instead our results are most compatible with a model that has unidirectional causal effects of resting heart rate and cardiac sympathetic reactivity on inflammatory risk and additional effects of cardiac sympathetic reactivity on metabolic risk. Below we compare these results to previous studies on the association between ANS functioning and inflammatory or metabolic risk.

Inflammatory risk

High mean HR across 24-h (Intzilakis et al., 2013; Sajadieh et al., 2004; Stein et al., 2008), daytime (Kon et al., 2006; Sloan et al., 2007; Whelton et al., 2014) and nighttime recordings (Hartaigh et al., 2014; Intzilakis et al., 2013; Sajadieh et al., 2004) have all been associated with increased inflammation previously. Whereas the group of Sajadieh and colleagues found a significant association of both 24-h and nighttime HR with CRP and TNF-α (Intzilakis et al., 2013; Sajadieh et al., 2004), Hartaigh et al. (Hartaigh et al., 2014) only found nighttime HR, and not 24-h or daytime HR, to be significantly associated with CRP. Our correlational analysis suggests that HR throughout all phases of the day is associated with higher IL-6, CRP and/or fibrinogen, but not TNF-α. When HR, RSA and PEP levels and reactivity were simultaneously used as predictors in multivariate models, only higher heart rate during leisure time in the evening remained a significant predictor of higher future inflammatory risk. HR reactivity to work did not independently contribute to the prediction of future inflammatory risk.

Reduced cardiac vagal control, measured as HRV across different times of day and/or varying physical activities, has repeatedly been associated with an unfavourable inflammatory profile in cross-sectional studies (Araujo et al., 2006; Haarala et al., 2011; Intzilakis et al., 2013; Jarczok et al., 2014; Lampert et al., 2008; Kon et al., 2006; Sajadieh et al., 2004; Sloan et al., 2007; Stein et al., 2008; Thayer & Fischer, 2009; von Kanel et al., 2009; von Kanel et al., 2008; Whelton et al., 2014). To our knowledge, only one longitudinal ambulatory study was performed. This study found that higher nighttime cardiac vagal control was associated with lower CRP assessed 4 years later (Jarczok et al., 2014). A reverse association has also been shown where higher CRP values predicted higher, instead of lower, vagal control in each of the following three years (Singh et al., 2009). Neither of these findings was supported by our study as RSA was not significantly associated with inflammatory risk at follow-up, and inflammatory risk did not predict RSA. Methodological differences between studies may account for the divergent findings. We measured vagal control by the peak-valley method linking ECG and respiratory signals

on a breath-to-breath basis, whereas others used frequency-domain based measures of HRV not always restricted to the respiratory range. Also, some studies recorded vagal control under laboratory conditions as opposed to the more ecologically valid real-life setting used here. The studies that did use ambulatory recording, did not always control as stringently as we did for differences in posture or activities throughout the day (Araujo et al., 2006; Intzilakis et al., 2013; Lampert et al., 2008; Sajadieh et al., 2004; Stein et al., 2008; Thayer & Fischer, 2009; von Kanel et al., 2009).

Research testing the association between cardiac sympathetic control and inflammation is scarce. Previous cross-sectional studies have mainly used the LF/HF ratio as indicator of sympathetic effects despite the controversy surrounding this measure (de Geus et al., 2015). None of these studies found this ratio to be significantly associated with the inflammatory profile (Araujo et al., 2006; Haarala et al., 2011; Stein et al., 2008; von Kanel et al., 2008). The study of Singh et al. (Singh et al., 2009) included the PEP as a measure of cardiac sympathetic control but no significant cross-sectional or longitudinal associations with CRP in either direction was found. In our study we also did not observe a longitudinal association between ambulatory PEP levels and inflammatory risk in either direction, but stronger PEP decreases at work compared to leisure time were associated with higher future inflammatory risk. No such association in the reverse direction was found.

Metabolic risk

In our study, correlational analyses showed that lower daytime HR with lower RSA and shorter PEP were associated with less favourable levels of the metabolic risk factors five years later. These effects were largely driven by reduced HDL-cholesterol levels. This is in line with the finding in a large study performed by Jiang et al. (Jiang et al., 2015) that reported both a cross-sectional (N = 89,860) and a prospective (N = 43,725) positive association between laboratory resting HR and the development of the metabolic syndrome 4 years later. It is also partly in line with the cross-sectional study by Licht and colleagues that found that lower levels of RSA and PEP were independently associated with the presence of the metabolic syndrome (Licht et al., 2010). Surprisingly they found an association for all individual components measured, *except* HDL-cholesterol. When they retested the same participants 2 years later, they again found that increased heart rate and cardiac sympathetic control were predictive of an increase in the number of metabolic components, but now high heart rate and low RSA also predicted a 2-year

decrease in HDL-cholesterol, which is again in keeping with our findings (Licht et al., 2013). In a systematic review of the literature on the association between cardiac vagal activity and the metabolic syndrome, Stuckey and colleagues (Stuckey, Tulppo, Kiviniemi, & Petrella, 2014), reported consistent reduced vagal activity in women with the metabolic syndrome while studies on men were inconsistent. We have however not investigated sex differences as this would greatly reduce the sample size and the power of both studies. Instead, sex was entered as a covariate in both studies.

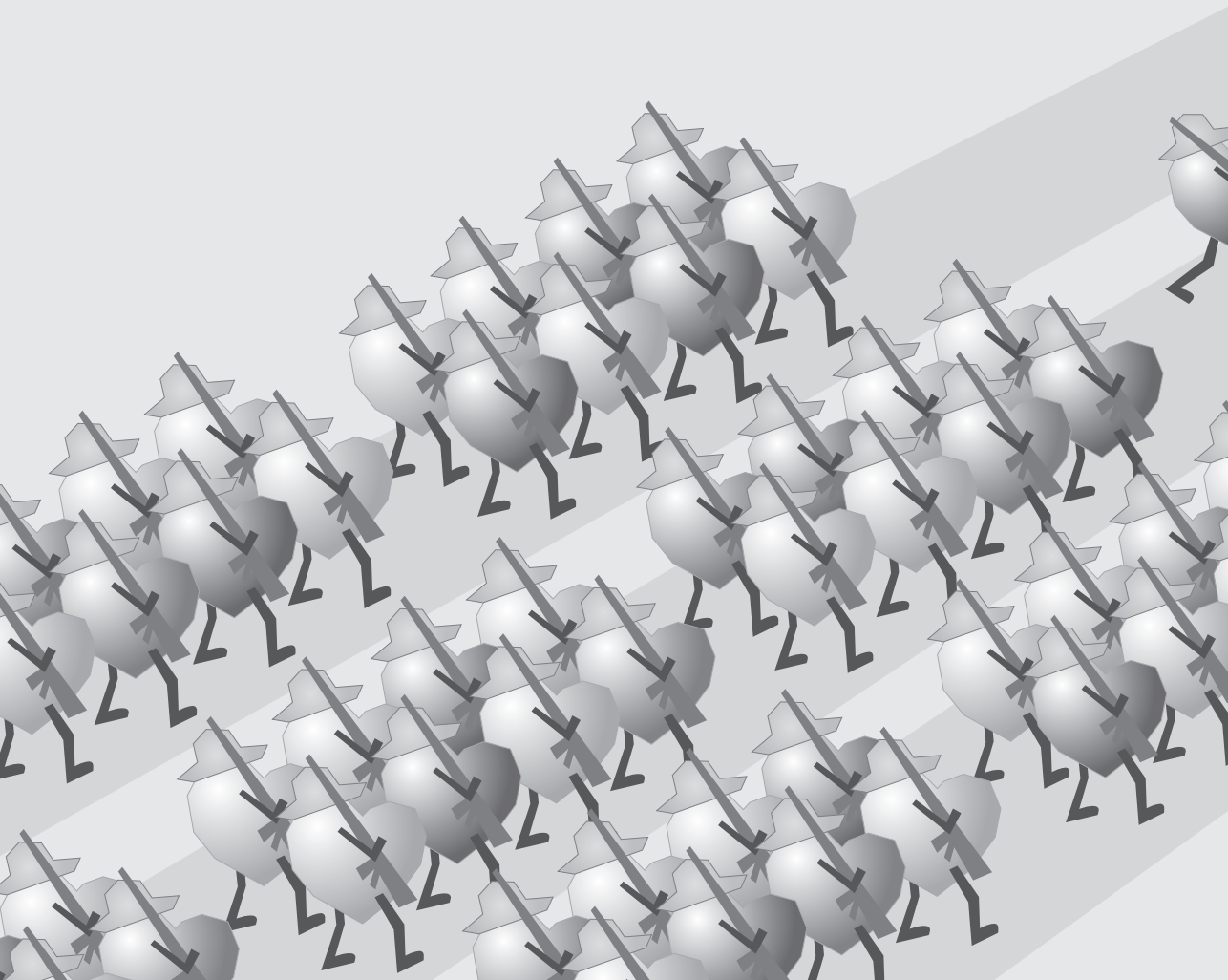
In spite of the congruence with previous studies caution is in order in the interpretation of our study. Using the full metabolic risk score, only a significant prediction of the metabolic risk by ambulatory PEP reactivity was found over a 5 year period. Levels of HR, RSA or PEP did not predict the full metabolic risk score. We also found no evidence for a reverse association between metabolic risk measured at baseline and ANS functioning measured at 5 year follow-up. It is important to note, however, that our metabolic risk factor cannot be equated with the metabolic syndrome as used in some of the above studies, as we did not measure blood pressure.

Gentile and colleagues (Gentile, Dragomir, Solomon, Nigam, & D'Antono, 2014) were the first to include autonomic responses to psychological laboratory stress in their prediction of metabolic burden few years later. They found no effect of HR reactivity to laboratory stressors in the prediction of metabolic burden, but they did find a positive association between vagal reactivity and metabolic burden at 3 year follow-up, though this was evident mostly in men. In women on the other hand, blunted vagal reactivity predicted increased metabolic burden 3 years later.

A limitation of the ambulatory approach of this study was that it left us with relatively small sample sizes for the measures that build on the presence of prolonged recordings at work and in leisure time. Only about half of the participants spent considerable time sitting at their work location on the testing day. This strongly reduced the number of participants available for analyses, particularly in the multiple regression models. Total numbers of complete MZ and DZ twin pairs in either Study 1 ($N_{MZpairs}$ = 43, $N_{DZ\ pairs}$ = 46) or Study 2 ($N_{MZpairs}$ = 112, $N_{DZ\ pairs}$ = 89) also did not allow us to calculate genetic correlations between the ANS variables and the various components constituting the metabolic and inflammatory risk scores to more formally test for possible pleiotropy. Full pairwise correlations in Tables 7.2 and 7.3 mitigated against the loss of participants with work time recordings, but these correlations were not corrected for clustering in families and

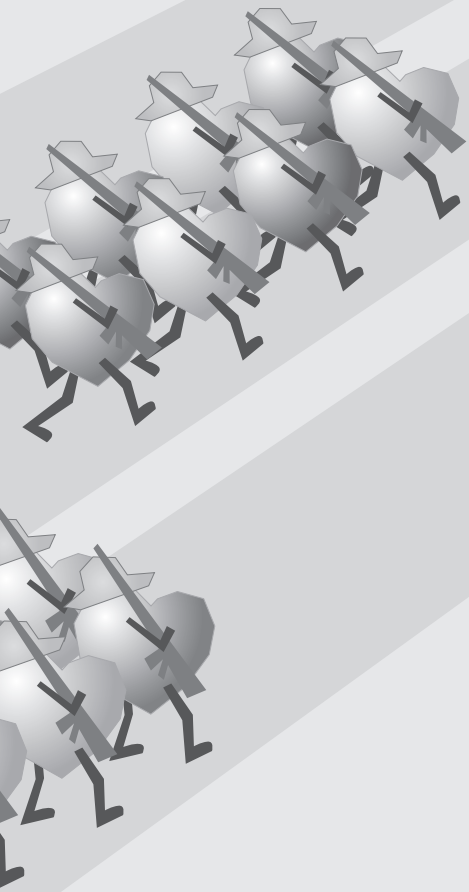
otherwise suffer from a multiple testing burden, such that formally only p-values < .0003 (=0.05/144) would survive Bonferroni correction. These limitations are balanced by some strong points, including the ambulatory design, the fact that two independent studies were done to test the association between ANS functioning and metabolic/inflammatory risk in both directions, and the extensive control for batch and other (technical) covariates on the metabolic and inflammatory parameters.

Taken together, the results from 'raw' correlations and the multivariate linear regression models largely tell the same coherent story. In real life settings, high resting heart rates together with a shift in cardiac control from parasympathetic to sympathetic dominance has adverse effects on the inflammatory and metabolic risk profiles. In contrast, an unfavorable metabolic or inflammatory risk profile does not seem to exert reciprocal detrimental effect on ANS functioning. Our finding that the causal effects flow mainly from the ANS to inflammatory and metabolic risk, rather than in reverse is in good keeping with our current biological understanding. There is solid evidence for SNS and PNS innervation of metabolically active tissues (Romijn & Fliers, 2005; Yi, La Fleur, Fliers, & Kalsbeek, 2010) and various parts of the immune system (Steinman, 2004). Together with the well-known effects of stress on the SNS and PNS, these connections can explain how, in a single swoop, behavioral states can affect three of the major players (cardiac autonomic control, metabolic and inflammatory risk) in the development of cardiovascular disease and diabetes.



Chapter 8

Summary and general discussion



Summary

The aim of this thesis was twofold: 1) to investigate the causes of individual differences in cardiac autonomic nervous system (re)activity in a healthy adult population using ambulatory recording, and 2) to investigate the consequences of these individual differences in (re)activity for the inflammatory and metabolic risk profile.

In **chapter 3**, the heritability of the three HRV measures that are currently used most in the fields of cardiology and physiology were researched in the largest 24-h ambulatory ANS dataset in twins to date. Moderate heritability estimates of about 50% were found for all three HRV measures across three different physical activity categories inventoried (sleep, sitting activities, and non-sitting light physical activity during the day). In addition, about 50% of the phenotypic covariance between any two HRV measures in this research could be explained by genetic factors. Interestingly, the genetic overlap between the three HRV measures turned out to be very high, especially between pvRSA and RMSSD and between RMSSD and SDNN, where genetic correlations were estimated at .94 and .89 in ambulatory sitting conditions. These findings provide us with the important message that HRV studies that assessed pvRSA, RMSSD or SDNN, can be safely pooled in future meta-analyses of genome-wide association studies because the genetic architecture is expected to be highly similar. A secondary goal of this study was to test whether heritability estimates were robust against confounding by RSA ceiling effects that may occur at low heart rates. Although 10.7% of the participants showed a quadratic IBI-RSA relationship, controlling for this ceiling effect did not lead to significant differences in the heritability estimates. This implicates that there is no pressing need to exclude recording periods (e.g. nighttime recording) or participants with low heart rates from genetic studies of HRV.

In **chapter 4**, we focused on the quantification of several rest and stress conditions in unstructured ambulatory recordings for future use in cardiovascular stress research. We used prolonged (24-h) ambulatory recordings of ANS activity during a representative weekday to research real-life stress reactivity. On the basis of the activity diaries filled out by the participants and the inbuilt accelerometer in the VU-AMS, two ambulatory rest (sleep, leisure) and four ambulatory stress (wake, work, work_sitting, work_peak) conditions were extracted for each participant. From these conditions several ambulatory reactivity measures were defined. The usability of these real-life reactivity measures was tested by investigating their reliability, temporal stability and heritability. We found that the ambulatory reactivity measures that were employed in this study were reliable

and showed moderate to high temporal stability over a period of three years ($0.36 < r < 0.91$ for IBI, $0.58 < r < 0.85$ for pvRSA, and $0.48 < r < 0.76$ for PEP). Almost every ambulatory autonomic reactivity measure showed significant heritability, ranging from 10 to 47%. Heritability of reactivity in daily life was largely due to new genetic variance emerging during real-life situations compared to that seen in the more relaxing 'baseline' conditions. It was concluded that real-life reactivity in ambulatory cardiac ANS data can be reliably assessed when recordings encompass a workday supplemented by periods of leisure time, preferably including sleep.

In **chapter 5**, we took a completely different approach to organize the 24-h ambulatory ANS data. Whereas in chapter 4 we sought to divide the day into homogeneous periods based on physical activity and psychosocial circumstances, as extracted from the combination of dairy self-report and the inbuilt accelerometer signals, chapter 5 used a physiological criterion to create homogeneity in the ambulatory signals analyzed. In this chapter the continuous ECG registrations were divided into several distinct heart rate bins, under the assumption that these bins represent different physiological states: low HR at 1 Hz (60 bpm), medium HR at 1.3 Hz (78 bpm), and high HR at 1.6 Hz (96 bpm) bins were defined using a beat binning algorithm. The heritability of four clinically relevant ECG repolarization (TpTe, QT, and TWA) and depolarization (QRS) parameters were estimated in the bins and compared to the heritability estimates of the same parameters obtained from a typical 10 sec resting ECG at 1.12 Hz (67 bpm) as commonly used in clinical practice. Results showed moderate to high heritability for all parameters (TpTe: 52 to 63%, QT: 34 to 69%, TWA: 55 to 72%, and QRS: 32 to 42%). Heritability estimates of the clinical resting ECG were generally lower compared to those of the binned ambulatory ECG. The difference reached significance for the resting QT interval and the TWA when compared to that obtained from the binned ambulatory ECG at the lowest frequency. A secondary goal of this study was to examine possible rate dependency of the genetic factors influencing the ECG parameters. For all parameters the genetic correlations among the different frequencies were very close to unity suggesting that the same genetic factor influences the parameters at all three heart rates. Furthermore, no significantly different heritability estimates were found for TpTe and QRS at the three heart rates. For QT, heritability significantly decreased with increasing HRs and, albeit less pronounced, a similar trend was observed for the TWA. Overall, we showed that the beat binning approach may provide better endophenotypes for genetic studies of the ECG than the classical clinical resting ECG.

Finally, we investigated the genetic overlap among the three repolarization parameters. Repolarization is strongly influenced by the SNS, and ECG parameters strongly related to repolarization might be useful as indices of cardiac SNS control (van Lien et al., 2015). We found that although the phenotypic covariance between the three repolarization parameters was mostly genetically determined, the overlap between the genetic factors influencing the three repolarization parameters was only modest, which indicates that these parameters provide unique genetic information on the repolarization phase.

In **chapter 6** and **chapter 7** we focused on the potential consequences of the individual differences in autonomic regulation as measured in chapter 4. First, in chapter 6 the heritability of a set of parameters forming another cardiovascular risk cluster, inflammation, was researched. The large biobank dataset that was available allowed for extensive genetic analyses including not only twins and siblings, but also the parents of the twins and siblings. Adding parents of twins to the design enabled us to test for the presence of assortative mating and by including data of other family members than twins, the contribution of both additive and non-additive genetic effects could be estimated without the need to remove estimation of common environmental effects. The sample size in combination with the extended twin family design furthermore ensured substantial statistical power for the estimations. We found that TNF-α, IL-6, CRP and fibrinogen showed moderate heritability (39%, 21%, 45%, and 46%, respectively). A considerable part of the genetic variation in TNF-α, CRP and fibrinogen was non-additive while heritability of IL-6 was due to additive genetic effects only. Surprisingly, the environment shared by family members was not relevant for any of the inflammatory parameters. Furthermore, with the exception of a small effect for fibrinogen, no evidence was found for spousal resemblance for any of the other pro-inflammatory markers. From this study, we conclude that a clear numerical target has been set for future genome wide screens attempting to find the actual genetic variants regulating the levels of these pro-inflammatory markers.

In the past, both a shift in autonomic balance towards sympathetic dominance and exaggerated cardiac autonomic reactivity to stress have been associated with increased cardiovascular disease risk. Previous research, mostly cross-sectional studies conducted in the laboratory, points to parallel autonomic effects on the metabolic and inflammatory profiles that are hypothesized to account in part for this risk. In **chapter 7**, the long-term bidirectional association between cardiac ANS activity and reactivity and inflammatory and metabolic risk was tested in two independent prospective studies. Metabolic and inflammatory risk scores were calculated by adding the Z-scores of several key markers

of the pro-inflammatory (TNF-α, IL-6, CRP and fibrinogen) and the metabolic (waist circumference, body mass index, glucose, triglyceride, low-density cholesterol, and high-density cholesterol levels) state. Ambulatory 24-h ANS data collection took place five years before, and five years after the biobank study. On the basis of the results from the study described in **chapter 4**, IBI, RSA, and PEP sleep and leisure resting levels and the reactivity measure (work_sitting versus leisure time activity) that suffered least from confounding by posture and/or physical activity was included. It was found that a higher resting HR during leisure time paired with increased cardiac sympathetic reactivity were associated with higher inflammatory risk five years later. In addition, higher sympathetic reactivity was associated with higher metabolic risk after five years. An unfavorable metabolic or inflammatory risk profile, in turn, had no detrimental effect on ANS functioning measured at follow-up. From this we conclude that our results are most compatible with a model that has unidirectional causal effects of resting heart rate and cardiac sympathetic reactivity on inflammatory risk and additional unidirectional effects of cardiac sympathetic reactivity on metabolic risk.

General discussion

This thesis builds on a database created during two previous data collections in twin families (ANS Study 1, wave 1 and wave 2) that used the VU-AMS system developed at the VU University two decades ago. It added to this database by conducting a second study (ANS Study 2, wave 3) with a specific focus on the association between ambulatory cardiac ANS (re)activity and cardiovascular risk factors. Due to technical improvements of the ambulatory monitoring device over time, cardiac ANS assessments for Study 2 embodied continuous recordings of the entire ECG signal (compared to R-peak registrations only for Study 1). This allowed for genetic analyses of other clinically relevant ECG components as well, such as the QT interval, the T-peak-T-end interval, QRS duration, and the T-wave amplitude. In this section, the findings will be evaluated in the light of current knowledge and directions for future research will be provided.

Individual differences in cardiac autonomic nervous system (re)activity

This thesis shows that individual differences in cardiac autonomic nervous system (re) activity in a real life setting are to a large extent caused by genetic variation, at least in a healthy adult population. These findings corroborate previous findings from ambulatory studies on twins (Busjahn et al., 1998; Kupper et al., 2004; Kupper et al., 2005; Kupper et al., 2006; Su et al., 2010) and extend them in a number of ways. First, cardiac sympathetic control has previously been operationalized by measurement of the systolic time intervals, with the PEP being the measure of first choice (de Geus et al., 2015). Recent research by our group has shown that the TWA during cardiac repolarization can be used as an additional indicator of cardiac sympathetic control (van Lien et al., 2015). Here we confirm that this measure, like the PEP, shows substantial heritability in ambulatory recordings, strengthening our confidence in the role of genetic factors in cardiac sympathetic control. Secondly, we parsed the ambulatory recordings into different conditions using two new approaches: 1) behaviorally informed conditions that can be expected to reflect states of low versus high emotional and mental engagement with the environment, and 2) physiologically informed conditions where the ECG was ensemble-averaged across fragments with similar heart rates across the day and night. In our previous studies, including chapter 3 in this thesis, conditions were created mainly by focusing on the control for posture and physical activity level.

The added value of the TWA

Although PEP is the current gold standard in clinical physiological research, PEP scoring is highly laborious especially when research moves to an epidemiological scale with ambulatory data measured across extended time periods in thousands of participants. For this reason, the TWA has recently been put forward as a valuable addition to PEP to index sympathetic nervous system activity (van Lien et al., 2015). Earlier twin studies of the TWA estimated heritability between 34 to 72% (Haarmark et al., 2011; Mutikainen et al., 2009). The broad range in these estimates was ascribed to the lead that was used to derive the TWA. The TWA in these earlier studies was mostly assessed during quiet sitting in the laboratory. In the study described in chapter 5, heritability of the TWA was estimated between 58% and 72%, depending on the heart rate at which the TWA was measured. A remaining task is to determine the genetic underpinnings of the TWA in the same content-based way as was done for the PEP in chapter 4 and test for differences in heritability as a function of sleep, leisure and work time. Furthermore, an interesting

question is to what extent the genetic factors influencing the PEP and the TWA overlap. The phenotypic correlation is significant but moderate and many different biological processes may be involved in the manifestation of cardiac contractility and ventricular repolarization. However, the PEP-TWA correlation may be due entirely to shared genetic factors which would suggest that a bivariate approach with both traits simultaneously could help detect genetic effects on cardiac SNS activity in gene finding studies.

Different ways to parse unstructured ambulatory ANS recordings into meaningful conditions

The major advantage of ANS recordings in real-life over laboratory assessments is the gain in ecological validity. Where laboratory recordings claim to capture ANS activity or responsivity to stress in general, the stress induced in the laboratory setting is actually quite artificial. Psychological challenges and mental effort experienced in real-life will correspond more closely to the chronic and repeated stress that may ultimately predispose us to adverse health conditions like cardiovascular disease. The major drawback of using real-life recordings is that they are unstructured by nature, stressful events are less demarcated and, importantly, stress during a recording on an arbitrary day is not guaranteed unless a stressful event is planned. On top of this, ambulatory measurements are more prone to confounding influences of posture, physical activity, and time of day compared to typical laboratory assessments in which measurements are typically short and recordings take place with the participants being under controlled (physical) conditions.

In this thesis, two different strategies to organize unstructured ambulatory ANS data have been explored. For one strategy, the focus was on clustering data based on the reported activities by the participants and accelerometer data obtained from the recordings (content-based, chapter 4). The other approach concerned organizing the data based on physiology and grouped ECG complexes of comparable heart rates (physiology-based, chapter 5). The latter physiology-based approach has the advantage of completely controlling for heart rate, and strongly, albeit not perfectly, reduces the confounding effects of postural change and physical activity. The disadvantage is that it cannot deal with effects of the emotional state of the subject at the time of the physiological recording. For this approach thousands of frequency-defined heart beats scattered across the entire measurement are used to constitute the different bins. As such, it is no longer possible to integrate the physiological signal with the emotion and/

or activity that the participant was exposed to at a particular time during the recording. The content-based approach fares better here. However, posture and physical activity are far less stringently controlled for as compared to the physiology-based approach. Also, effects of heart rate on ECG parameters of interest cannot be as well controlled as with the physiology-based approach. Strong heart rate effects were for instance found for the TWA (van Lien et al., 2015).

From chapter 4, we concluded that all content-based rest (sleep, evening leisure time activity) and stress conditions (wake, work, work_sitting, work_peak) performed equally well from a psychometric point of view. Control for physical activity becomes particularly manifest when studying cardiovascular reactivity since this may put constraints on the pairs of conditions that can be used to define ambulatory reactivity (i.e. only make rest-and-stress-pairs of conditions that are equal in posture and physical activity). One way to overcome the issue of confounding by postural change or physical activity in the content-based approach would be to more rigorously control for these effects. A few decades ago, Blix, Stromme, & Ursin (Blix, Stromme, & Ursin, 1974) introduced the concept 'additional heart rate' which refers to heart rate increments that can be ascribed to (non-metabolic) task demands. This requires that metabolic demands are continuously monitored in addition to heart rate. Co-registration of heart rate and oxygen consumption (VO_2) during different levels of physical activity can establish the linear slope between heart rate and VO_2. This can be used to predict the heart rate at a given VO_2. Deviations of the actual heart rate form this predicted heart rate is known as the 'additional heart rate'. This additional heart rate method could be partly extended to PEP and RSA, as cardiac SNS and PNS activity may also scale linearly with VO2, at least at low intensity physical activity.

For heart rate, this method has already proven to be successful in the laboratory (Carroll, Turner, & Rogers, 1987; Carroll, Turner, & Hellawell, 1986; Carroll, Turner, & Prasad, 1986; Sherwood, Allen, Obrist, & Langer, 1986; Stoney, Langer, & Gelling, 1986; Turner & Carroll, 1985). Since implementation of this method in prolonged ambulatory recordings is technically not possible due to the demanding requirements for VO_2 measurements, Myrtek & Brugner (Myrtek & Brugner, 1996) introduced a new method in which continuous co-registrations of the ECG and physical activity was realized by means of accelerometery. In this method, heart rate and accelerometer values at a given time point (during an event) were compared to the moving average of the previous minutes. When the difference in heart rate between these periods was at least 3 beats and the concurrent change in physical activity was minimal, an event was considered 'emotional'.

Higher levels of physical activity were dealt with by an algorithm that is explained in detail in Myrtek (Myrtek, 2004). Although additional heart rate was succesfully captured by this method in several studies, associations with the emotional appraisal of the events have not been consistently found in the ambulatory situation (Ebner-Priemer et al., 2007; Myrtek, Aschenbrenner, & Brugner, 2005; Myrtek & Brugner, 1996). This may in part be due to the fact that postural changes, with a large impact on heart rate through venous pooling effects on stroke volume, are not taken into account in their approach. The increased precision of accelerometer data and the improved algorithms for both posture and metabolic demands detection from such signals may well make it worthwhile to revive this method.

Gene by environment interaction

The most striking pattern observed in the ambulatory setting was the increase in genetic variance during psychologically engaging events. This increase, which reflects gene-environment interaction, had already been noted in previous laboratory stress research (de Geus et al., 2007; Wang et al., 2009). Stress-specific genetic effects were generally more evident in the ambulatory setting (up to 40%) compared to those found in the standardized part of our study (4% to 11%) and previous laboratory studies (7% to 23%) (de Geus et al., 2007; Wang et al., 2009). This can probably be ascribed to the difference in the appraisal of the stressors encountered in the different studies. Whereas de Geus et al. (2007) included typical short mental stress tasks in their study, Wang et al. (2009) used tasks that more closely approached stress in real-life, and the ambulatory part of our study described in chapter 4 solely comprised of real-life work-related demands. The latter are more likely to be motivationally relevant for the individual and invoke more subjective stress. Unfortunately, we did not confirm this by adequate measurements of subjective stress, as an added set of items to the paper diary was considered to increase the already high burden of ambulatory monitoring for the subject. Current day smartphone-based ecological momentary assessment was not yet in reach when Study 1 was started. In retrospect, our data show that it is not necessary to include pre-planned stressors in the measurement protocol to induce sufficient reactivity. The choice of measurement days will depend on the research question. The inclusion of a work day with social engagement paired to mental effort seems important, but other stressors like child care or prolonged caregiving for elderly/diseased family members may also yield substantial psychophysiological reactivity.

Because the studies described in this thesis are larger than any previous study and have the added advantage of being performed under ecologically valid conditions they provide the research community with the best estimates of the genetic architecture of cardiac autonomic control to date. As it stands, we conclude that individual differences in sympathetic and parasympathetic influences on cardiac electrical activity are best explained by invoking only two sources of variance, additive genetic influences and unique environmental influences. The additive genetic variance varies between different markers, across the different times of day, and across the different pursuits of daily life, but the average of all reported ambulatory RSA heritabilities throughout this thesis was 49% and for PEP it was 38%.

The long-term association between ANS (re)activity and inflammatory and metabolic risk

Inflammatory processes and metabolic disturbances have both been associated with cardiovascular disease, and both have been associated with ANS functioning. Although evidence for a deleterious role of ANS functioning characterized by a shift in sympathovagal balance to sympathetic dominance in increased inflammatory and metabolic risk has previously been found in both directions, bidirectional influences between ANS functioning and both risk profiles has never been researched in one study before. The study described in chapter 7 was also the first to include reactivity to real-life stress, captured as work-related ANS activity versus leisure time ANS activity in the evening, as a factor.

Our results point to an unidirectional association of higher ambulatory resting heart rate paired to exaggerated cardiac sympathetic reactivity with higher inflammatory risk at follow-up. Exaggerated cardiac sympathetic reactivity did also precede higher metabolic risk measured 4.6 years later. Conversely, inflammatory and metabolic risk were not associated with ANS functioning measured 5.4 years later. With this study we show that both higher baseline heart rate and large sympathetic responsivity to work-related challenges can make one prone to develop cardiovascular problems in future. Multiple short-term stress experiences or stress that is experienced chronically may both be at the basis of these associations. Since the reactivity measure that was incorporated in this study pertains to work and recovery from work-related activity (evening leisure time), it may be noteworthy that considerable variation in the experience of work stress between individuals exists. Vrijkotte, van Doornen, & de Geus (Vrijkotte et al., 2000) studied this topic and found that individuals that experienced high imbalance at work (a combination

of high effort and low reward) had higher heart rates at work and directly after work, a higher systolic blood pressure during work and leisure time, and lower 24-h vagal tone on work days and a non-workday. Individuals that experienced high overcommitment (inability to unwind from work), on the other hand, showed a general pattern of shorter PEP during work and leisure time periods across workdays and the non-workday. In addition, smaller absolute sleep-wake differences and decreased PEP variability were observed in individuals experiencing high overcommitment, possibly pointing to down-regulation of cardiac beta-receptors by chronic cardiac SNS activity (Vrijkotte et al., 2004). These results illustrate that certain coping styles or personality profiles may give rise to a particular ANS reactivity profiles that may compromise physical health in the long run.

Future directions

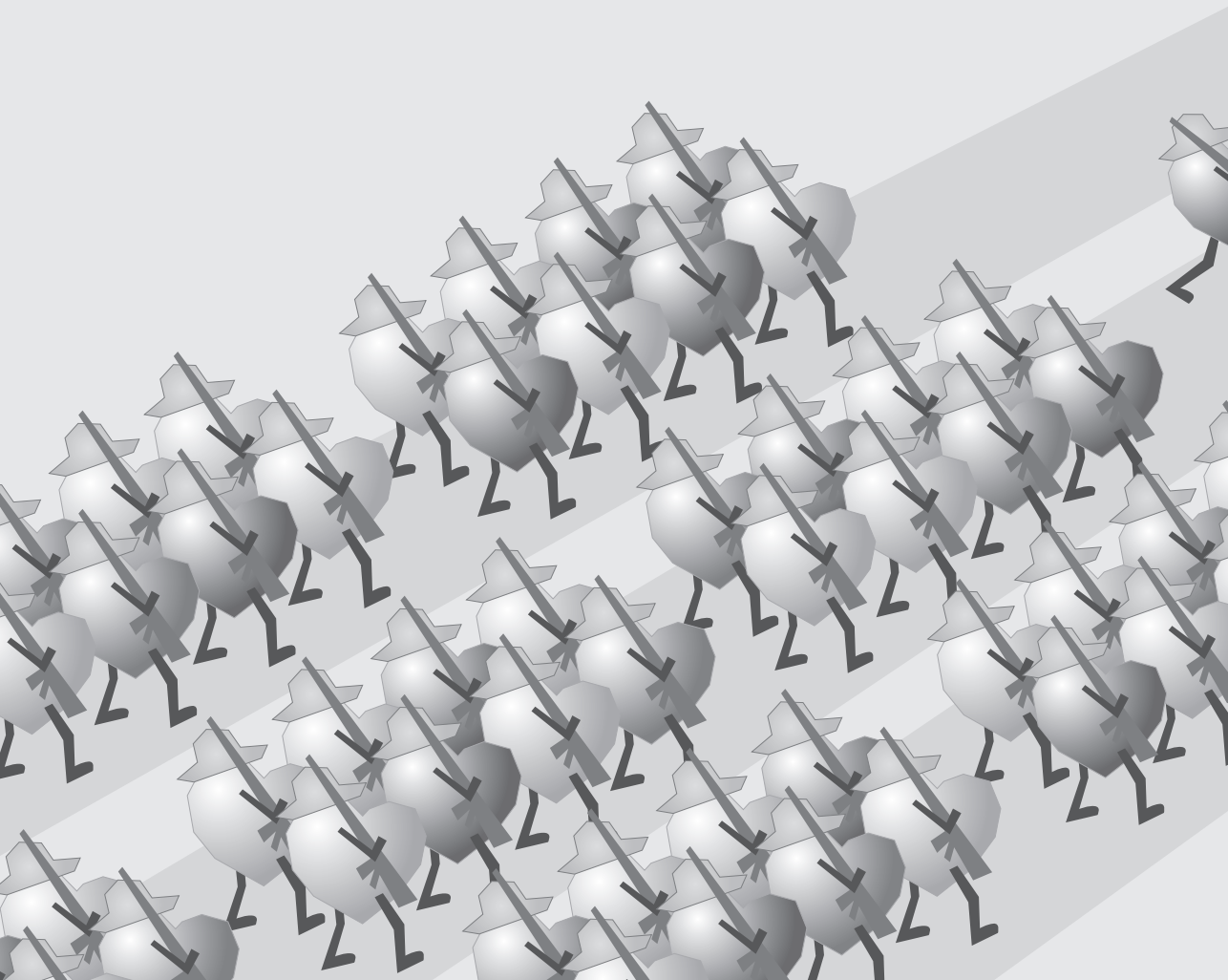
Having established that the regulation of cardiac autonomic control can largely be explained by genetic variation, it becomes important to characterize the actual causal variants responsible for this heritability. As already outlined in the introductory chapter, the first steps have been undertaken towards this aim. Many candidate gene association studies have been conducted and even a few single-cohort GWA studies. The yield of these attempts has, perhaps not surprisingly, been modest. Cardiac autonomic activity is a complex phenotype, and only a very small percentage of the total genetic variance is expected to be explained by a single genetic variant. Instead, lots of variants with very small effects may be at play. In addition, structural variation and rare variants could be of importance to ANS traits in comparable ways as they are to other traits (Eichler et al., 2010; Manolio et al., 2009). For future research, the key lies in expanding the sample sizes of the gene finding efforts, preferably by pooling across many studies in meta-analysis and by taking a hypothesis-free stance, i.e. by focusing on a GWA approach rather than a candidate gene approach. This is exactly what is currently being done by the Genetic Variance in Heart Rate Variability (V_gHRV) consortium, in an attempt to detect the genetic variants causing individual differences in resting RSA. A major concern that this consortium is facing is that the various studies to be pooled did not always use the same metric to quantify RSA. In that regard, our finding that future gene finding studies can safely pool studies that have assessed RSA and RMSSD or RMSSD and SDNN to gain power is very reassuring.

A major component missing from the current ANS data set is self-reported emotional state during the ambulatory recording. There are clear links between ANS functioning and emotion although a one-to-one mapping of emotional state on ANS remains very complex (Kreibig, 2010). Previous research found that people that have a genetic vulnerability to develop anxiety and/or depression showed more negative affect in response to stress in daily life compared to people who did not have this genetic vulnerability (Gunthert et al., 2007; Wichers et al., 2007; Wichers et al., 2008). These findings point to gene-environment interaction effects that may also be reflected in ANS functioning.

Emotional state can be assessed in several ways and at several levels. It was only until few decades ago that emotional state was solely assessed by means of classical questionnaires, the focus of which is on past experience and current beliefs built on that experience. This makes the outcomes of questionnaires inevitably prone to retrospective bias. Although this may be a good thing for some purposes, for instance in clinical practice where focus lies on the participants' beliefs or mental representations of certain experiences, it may not be a good thing in other instances when one is interested in co-occurring states of multiple modes (i.e. mood, physiology, and behavior). For this reason, emotional state is increasingly measured by Ecological Momentary Assessment (EMA) procedures that repeatedly assess e.g. mood at various (random) times of the day using tablets or smartphones (Conner & Barrett, 2012; Fahrenberg et al., 2007). The idea that two different constructs are measured by EMA and by questionnaires is emphasized even more by studies that found only moderate correlations between outcomes of real-time mood assessment and retrospective questionnaires measuring the same phenomena (Ebner-Priemer & Trull, 2009b).

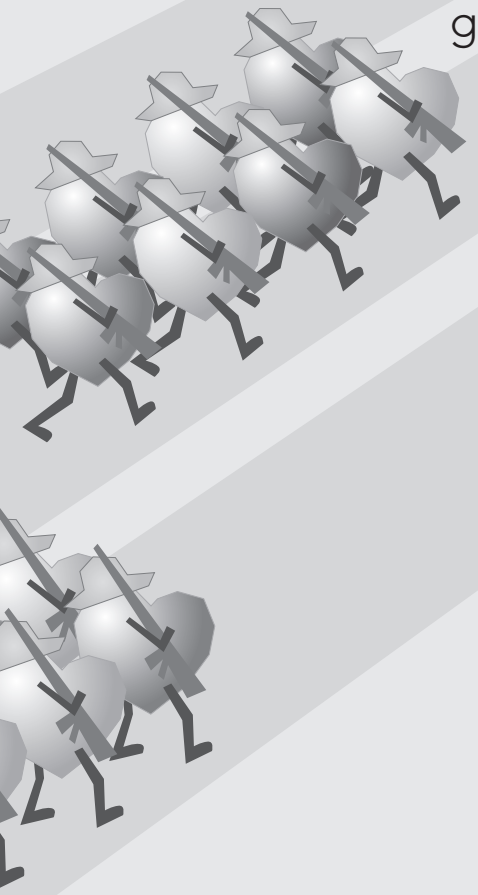
This discrepancy between momentary and recalled symptoms may also explain why research linking the physiological state to mood or personality assessed by retrospective questionnaire has been rather inconclusive (Conner & Barrett, 2012). Emotional states assessed by EMA in real-life settings may be more strongly correlated to ANS activity than questionnaire-based measures of anxiety and depression symptoms to laboratory based physiology. Support for this notion comes from studies that found ambulatory levels of cardiovascular parameters (BP, HR, HRV) to be more strongly associated to momentary self-report measures compared with the classical retrospective self-reports (Bhattacharyya, Whitehead, Rakhit, & Steptoe, 2008; Kamarck et al., 2005). In ANS Study 1 and 2 we used a paper-and-pencil diary to mainly record posture and (physical) activity at the expense of detailed emotional state measurement. The current-day more advanced assessment

methods were simply not available. We deliberately kept the protocol identical in Study 2 as we needed to balance the comparability of data across waves against the advantages of the newly available technology. Future research, however, would do well to replace the paper dairies for applications on a smart phone. Such *apps* can accommodate many types of self-reported data collection, including the assessment of momentary mood (Trull & Ebner-Priemer, 2013). Data obtained from these assessments is 'on the spot' and has great potential to help us gain more insight in the complex interplay between our ambulatory behavioral state and our ambulatory ANS functioning. This thesis clearly confirmed that ambulatory ANS functioning plays a major role in our long-term health.



Chapter 9

Nederlandse samenvatting: Oorzaken en gevolgen van individuele verschillen in ambulant gemeten autonome zenuwstelsel activiteit (dutch summary)



Hart- en vaatziekten zijn een van de belangrijkste doodsoorzaken in Westerse landen. Ze vormen een verzamelnaam voor een groep aandoeningen betreffende het hart (bijvoorbeeld hartfalen, hartritmestoornissen), de vaten (bijvoorbeeld aneurysma, slagaderverkalking), maar ook een beroerte wordt tot de hart- en vaatziekten gerekend. De ontstaanswijze van deze aandoeningen is vaak heterogeen waar veel verschillende factoren in meer of mindere mate aan bij kunnen dragen. Hierbij kan men denken aan demografische factoren als sekse en leeftijd, maar ook sociaal-economische klasse, levensstijl (roken, alcoholgebruik, voeding), psychologische factoren (angst, stress, depressie), een bepaalde genetische aanleg, en mogelijk een samenspel tussen deze factoren (Brotman et al., 2007; Brotman et al, 2005). Voordat hart- en vaatziekten tot uiting komen, zijn er vaak wel al waarschuwingssignalen of condities aanwezig die vooraf kunnen gaan aan het ontstaan van de aandoening, of die hier een bij-product van zijn. Voorbeelden hiervan zijn het metabool syndroom dat gekenmerkt wordt door obesitas, hypertensie, hyperlipidemie, en hyperglycemie (Bayturan et al., 2010), inflammatie (Danesh et al., 2008), een coagulatie/fibrinolyse imbalans (Libby & Theroux, 2005), een verminderde hartslagvariabiliteit (Dekker et al., 1997; Dekker et al., 2000), en een verhoogde hartslag (Fox et al., 2007). Al deze lichamelijke risicofactoren worden beïnvloed door de activiteit van het autonoom zenuwstelsel.

Het autonoom zenuwstelsel is verantwoordelijk voor onze homeostase en coördineert daartoe verschillende lichaamsfuncties die het lichaam beschermen tegen veranderingen in de interne of externe omgeving (bijvoorbeeld door fysieke inspanning, een verandering van lichaamshouding, voedselinname, of een bloeding). Het autonoom zenuwstelsel bestaat uit het parasympathische zenuwstelsel en het sympathische zenuwstelsel. Het sympathische zenuwstelsel wordt ook wel het 'vecht-of-vlucht' systeem genoemd; het maakt het lichaam klaar om in actie te komen. Het zorgt er onder meer voor dat de hartslag, ademhaling, hartcontractiliteit en bloeddruk omhoog gaan, dat de luchtpijptakken zich verwijden, de zweetproductie op gang komt, dat er epinefrine (een stimulerende stof) wordt vrijgegeven en dat er bloed naar de spieren wordt gestuurd. Het parasympathische zenuwstelsel daarentegen zorgt voor het behoud van het lichaam door de opname van voedingsstoffen te promoten en te zorgen dat het lichaam zich ontdoet van afvalstoffen. Deze tak van het autonoom zenuwstelsel wordt ook wel het 'rust-en-verteer' systeem genoemd. Het verlaagt de hartslag en verhoogt de hartslagvariabiliteit, de pupillen worden kleiner, en de maag en speekselklieren worden gestimuleerd. Beide takken van het autonoom zenuwstelsel origineren in het brein en projecteren naar verschillende

gebieden in het lichaam, waaronder het hart. Voor het onderzoek in dit proefschrift zijn 24-uurs opnamen van het hart gemaakt. Er zijn continue electrocardiogram (ECG) en impedantiecardiogram (ICG) signalen verzameld in meer dan 1300 tweelingen en hun familieleden. Deze metingen vonden plaats op een representatieve doordeweekse dag. Uit het ECG hebben we de hartslag bepaald. Hartslag zelf is echter het resultaat van een onbekende mix van sympathische en parasympathische invloeden op het hart. Om goed onderscheid te kunnen maken tussen deze twee systemen, hebben we naast hartslag ook hartslagvariabiliteit, als maat voor de parasympathische aansturing van het hart, berekend. Hier zijn verschillende maten voor, maar aan de basis van het concept liggen de periodieke veranderingen in de tijdsintervallen tussen opeenvolgende hartslagen. Als maat voor de sympathische aansturing van het hart hebben we de pre-ejectieperiode (PEP) bepaald en hebben we in **hoofdstuk 5** ook de T-golf amplitude (TWA) uit het ECG onder de loep genomen. De PEP is het tijdsinterval tussen de aanvang van de elektrische prikkel die het hart doet samenknijpen en het openen van de linker hartklep.

Onderzoeksvragen

Eerder onderzoek heeft al aangetoond dat er grote individuele verschillen bestaan in de activiteit van het autonoom zenuwstelsel in rust (Berntson et al.1994; Berntson et al., 2008; Cacioppo et al., 1994; Grossman & Kollai, 1993; Light et al., 1998; Salomon et al., 2000) en dat deze versterkt worden door stress opgewekt in een laboratorium (de Geus et al., 2007; Houtveen et al., 2002; Lucini et al., 2002; Wang et al., 2009), maar ook door aanhoudende psychosociale stress (Riese et al., 2000; Vrijkotte et al., 2004). Het is echter onduidelijk in hoeverre een laboratorium situatie een ware reflectie biedt van de werkelijke wereld, want dat is waar we uiteindelijk in geïnteresseerd zijn. Meten we wel dezelfde soort lichaamsreacties in het laboratorium als in het echte leven? Er zijn al belangrijke stappen gezet in het ambulante, of veldonderzoek, vaak nog gestructureerd met een vooraf geplande stressor en rustmeting, maar ook ongestructureerd al dan niet met inachtneming van de verschillende dagdelen. Laatstgenoemd onderzoeksterrein is een nog grotendeels onontgonnen gebied waar een grote uitdaging ligt in de volgende vraagstellingen: Hoe kunnen we het beste structuur aanbrengen in ongestructureerde 24-uurs ambulante hartmetingen? Of met andere woorden, hoe kunnen we rust en stress condities definiëren zonder dat er een vooraf geplande stressor plaatsvindt tijdens de meting? Kunnen we deze condities ook gebruiken voor toekomstig stress-onderzoek? Wat is de invloed van genen en omgeving op verschillende fysiologische risicofactoren

voor hart- en vaatziekten, gemeten in het dagelijks leven? Zien we de invloed van genen in ambulante stress-situaties toenemen ten opzichte van een rustperiode, net als in het laboratorium? Hebben de ambulante rust- en stress condities predicerende waarde voor (cardiovasculaire) gezondheid op de lange termijn? En omgekeerd, zijn bloedmarkers gerelateerd aan de metabole en inflammatoire toestand voorspellend voor ambulante autonome zenuwstelsel (re)activiteit op een later moment? Dit zijn de hoofdvragen van het onderzoek dat wordt beschreven in dit proefschrift. Voor ik de belangrijkste bevindingen van dit onderzoek zal bespreken, wil ik eerst kort ingaan op de rationale achter het tweelingonderzoek.

Tweelingonderzoek

Het onderzoek beschreven in dit proefschrift is uitgevoerd bij tweelingen en hun familieleden die ingeschreven staan bij het Nederlands Tweelingen Register (NTR). Door onderzoek bij tweelingen uit te voeren kunnen we uitzoeken in hoeverre een bepaalde eigenschap wordt bepaald door genen en in welke mate door de omgeving. Aan de basis van het klassieke tweelingonderzoek ligt de wetenschap dat eeneiige (monozygote, of MZ) tweelingen nagenoeg 100% van hun genetische materiaal met elkaar delen en twee-eiige (dizygote, of DZ) tweelingen 50%. Door bepaalde eigenschappen van grote groepen MZ en DZ tweelingen met elkaar te vergelijken, kunnen we erachter komen of MZ tweelingen meer op elkaar lijken dan DZ tweelingen wat betreft de gemeten eigenschap. Als dat zo is, dan wijst dit erop dat genen (deels) van invloed zijn op het tot uiting komen van de eigenschap. Lijken de tweelingen meer op elkaar dan op basis van genetische invloeden kan worden verklaard, dan duidt dit op invloeden van een gedeelde omgeving. Hierbij kan gedacht worden aan de dagelijkse leefomgeving die gedeeld wordt tussen de tweelingen die hen op elkaar kan doen lijken, bijvoorbeeld het gezin. Als laatste is er nog de rol van de unieke omgeving, zoals het woord uniek al impliceert gaat dit om factoren die uniek zijn voor het individu. Met andere woorden omgevingsinvloeden die niet gedeeld worden door familieleden, zoals eigen vrienden. De erfelijkheid wordt voorts berekend door de proportie van de variatie die aan genetische factoren toegeschreven kan worden te delen door de totale variatie die de eigenschap laat zien.

Het klassieke tweelingmodel kan nog verder uitgebreid worden door naast tweelingen ook familieleden van tweelingen in het onderzoek te betrekken (bijvoorbeeld broers, zussen, ouders, of partners). Dit wordt dan ook een ‘uitgebreide tweelingstudie’ genoemd. De voornaamste reden waarom dit gedaan wordt is om de studie krachtiger

te maken zodat we beter onderscheid kunnen maken tussen de genetische en omgevingsinvloeden die gedeeld worden binnen een familie. Een uitbreiding van het klassieke model met ouders of partners maakt het ook mogelijk nog andere effecten te onderzoeken zoals gelijkenissen tussen partners (is er partnerselectie of gaan partners over de tijd meer op elkaar lijken doordat ze een omgeving delen) of culturele transmissie (Boomsma et al., 2002; Falconer & Mackay, 1996).

Naast een uitbreiding van het tweelingmodel met meer familieleden, kan het model ook uitgebreid worden wat betreft het aantal eigenschappen dat gemodelleerd wordt. Dit is interessant wanneer men wil onderzoeken op welke manier de eigenschappen met elkaar samenhangen. Zoals ook in dit proefschrift gedaan wordt kan dat bijvoorbeeld toegepast worden wanneer men geïnteresseerd is in de vraag in hoeverre dezelfde genen betrokken zijn bij verschillende maten voor eenzelfde eigenschap (hartslagvariabiliteit), of wanneer men de genetische samenhang en/of of het optreden van nieuwe genetische invloeden over herhaalde metingen wil onderzoeken (metingen van dezelfde eigenschap op verschillende hartslagniveaus, of tijdens rust en stress momenten).

Erfelijkheid van de parasympathische aansturing van het hart

In **hoofdstuk 3** hebben we de erfelijkheid van de drie meest gebruikte maten voor hartslagvariabiliteit onderzocht in de grootste dataset met 24-uurs ambulante metingen tot nu toe (RMSSD, SDNN, en pvRSA). De erfelijkheid in deze studie werd geschat rond de 50%. Hiermee bevestigen we eerdere tweelingstudies, maar vonden daarbij ook dat de schattingen van gelijke orde waren voor alle drie parameters over drie verschillende condities (hartslagvariabiliteit gemeten tijdens slaap, uitsluitend zittende activiteiten overdag, en tijdens lichte niet-zittende fysieke activiteit overdag). Met deze studie toonden we ook aan dat ongeveer 50% van de samenhang tussen de drie maten voor hartslagvariabiliteit door genetische factoren verklaard kan worden. Ook vonden we dat de genetische overlap, of correlatie, tussen de drie hartslagvariabiliteit maten erg hoog was. Dit betekent dat nagenoeg dezelfde genen ten grondslag liggen aan de verschillende hartslagvariabiliteit maten en dit heeft belangrijke implicaties voor toekomstig onderzoek dat tracht de daadwerkelijke genen te vinden die hierbij betrokken zijn. Voor dit type onderzoek zijn enorm grote datasets nodig. Een trend op dit vlak is dat onderzoeksgroepen over de hele wereld steeds vaker de krachten bundelen door datasets samen te voegen om zo tot grotere aantallen te komen en meta-analyses te doen over deze samengevoegde datasets. De drie hartslagvariabiliteitmaten die we in

deze studie hebben onderzocht kunnen nu dus ook met een gerust hart samengevoegd worden omdat we verwachten dat de genetische architectuur vrijwel gelijk is.

Erfelijkheid van de sympathische aansturing van het hart

Om op niet-invasieve manier de sympathische aansturing van het hart te meten wordt vooral gebruikt gemaakt van systole tijdsintervallen, met de PEP als eerste keuze (de Geus et al., 2015). In **hoofdstuk 4** wordt de erfelijkheid van de PEP geschat tussen de 22 en 45%. Recent onderzoek door onze groep heeft echter laten zien dat de TWA tijdens de repolarisatie fase goed gebruikt kan worden als extra indicator van de sympathische aansturing van het hart (van Lien et al., 2015). In **hoofdstuk 5** van dit proefschrift laten we zien dat deze maat gemiddeld tot hoog erfelijk is (58 tot 72%). Dat deze maat, net als de PEP significante erfelijkheid laat zien, geeft dat extra ondersteuning aan het idee dat de sympathische aansturing van het hart onderhevig is aan genetische invloeden.

Manieren om de ongestructureerde ambulante hartmetingen in betekenisvolle condities op te delen

In **hoofdstuk 4** hebben we met behulp van de activiteitendagboeken die de deelnemers gedurende de meetdag bijhielden en de bewegingsmeter die ingebouwd is in de VU-AMS hartmeter, twee ambulante rust- en vier ambulante stresscondities geëxtraheerd voor elke deelnemer. Voor de rustcondities hebben we de gemiddelde hartactiviteit tijdens slaap en in de vrije tijd berekend. De eerste stressconditie was de gemiddelde hartactiviteit over de gehele wakkere periode. Als tweede hebben we de gemiddelde activiteit tijdens een werkdag berekend. Deze laatste conditie hebben we ook nog een keer berekend maar dan alleen voor zittende activiteiten op het werk. Als laatste hebben we nog de periodes met de hoogste hartslag tijdens zittende activiteiten op het werk geselecteerd die optelden tot minstens een half uur en daar de mediaan van genomen. Op basis van deze condities hebben we verschillende maten voor ambulante reactiviteit samengesteld die berekend werden door het absolute verschil tussen de rust- en stressconditie te berekenen voor de verschillende autonome maten (hartslag, hartslagvariabiliteit en de PEP).

De bruikbaarheid van deze maten als zijnde 'real-life' reactiviteitsmaten hebben we getest door de betrouwbaarheid, temporele stabiliteit en de erfelijkheid te onderzoeken. We vonden dat de maten betrouwbaar gemeten konden worden en een gemiddelde tot hoge temporele stabiliteit lieten zien over een periode van drie jaar. Deze temporele

stabiliteit konden we in kaart brengen omdat een deel van de deelnemers twee keer mee heeft gedaan met het onderzoek. Hierdoor konden we de hartprestatie in de verschillende condities correleren met de hartprestatie in dezelfde condities drie en een half jaar later. Tot slot vonden we dat de meeste ambulante reactiviteit maten erfelijk waren; de schattingen lagen tussen de 16 en 47%. Het bleek dat de erfelijkheid van reactiviteit gemeten in het dagelijks leven grotendeels verklaard kon worden doordat er nieuwe genetische variatie in het spel kwam bij een hogere ten opzichte van een lagere mate van interactie met de psychosociale omgeving. De conclusie van deze studie is dan ook dat reactiviteit in het dagelijks leven goed en betrouwbaar gemeten kan worden en dat hiermee andere genetische informatie wordt blootgelegd dan die wordt gevonden tijdens rustmetingen in het laboratorium of standaard klinische ECG metingen.

In **hoofdstuk 5** hebben we een andere benadering gekozen om de 24-uurs metingen te organiseren. In plaats van de dag op te delen in homogene periodes gebaseerd op fysieke activiteit en psychosociale omstandigheden, gebruikten we voor deze studie een fysiologisch criterium om homogeniteit te creëren in de ambulante signalen. In deze studie richtten we ons volledig op geselecteerde delen van het 24-uurs ECG zodanig dat alle ECGs werden gemiddeld van hartslagen met eenzelfde duur. De aanname was dat alle hartslagen met eenzelfde duur (dat wil zeggen bij eenzelfde hartfrequentie) eenzelfde fysiologische toestand representeren. Er werden drie toestanden geselecteerd: hartslagen bij een lage hartfrequentie (1 Hz, 60 slagen per minuut), hartslagen bij een gemiddelde frequentie (1.3 Hz, 78 slagen per minuut), en hartslagen bij een hoge frequentie (1.6 Hz, 96 slagen per minuut). Hiervoor werd een speciaal 'beat binning' algoritme ontwikkeld. Vervolgens hebben we de erfelijkheid van vier klinisch relevante repolarisatie (TpTe, QT, en TWA) en depolarisatie (QRS) parameters geschat per frequentie.

We hebben deze schattingen vergeleken met de erfelijkheidsschattingen van dezelfde parameters onttrokken uit een typisch 10 seconde durend rust-ECG op 1.12 Hz (67 slagen per minuut), zoals vaak gebruikt wordt in de klinische praktijk. We vonden een gemiddelde tot hoge erfelijkheid voor alle parameters (TpTe: 52 tot 63%, QT: 34 tot 69%, TWA: 55 tot 72%, en QRS: 32 tot 42%). De erfelijkheidsschattingen voor het klinische rust-ECG waren in het algemeen lager dan die gebaseerd op het frequentiespecifieke ambulante ECG. Dit verschil was zelfs significant voor QT en TWA wanneer het rust-ECG vergeleken werd met het gemiddelde ECG in de laagste hartfrequentie. Ook hebben we de genetische correlatie tussen de verschillende hartslagfrequenties binnen elke parameter onderzocht. We vonden dat deze correlatie erg hoog was, wat suggereert

dat de genetische factoren die een rol spelen bij grotere fysieke activiteit niet significant anders zijn dan de genen die actief zijn tijdens rustigere periodes gekenmerkt door een lagere hartfrequentie. Voor QT en TWA nam de erfelijkheidsschatting echter wel af met een toenemende hartslag. Deze resultaten impliceren dat het goed mogelijk is ongestructureerde ECG metingen te organiseren op basis van hartslagfrequentie. Daarbij toont dit onderzoek aan dat de parameters vastgesteld door middel van deze benadering misschien zelfs beter gebruikt kunnen worden voor genetische studies dan het typische klinische rust-ECG.

Tot slot hebben we in dit zelfde onderzoek nog de genetische overlap tussen de verschillende repolarisatieparameters onderzocht. Alhoewel de correlatie tussen de verschillende repolarisatieparameters vooral door genen verklaard kon worden, was de overlap tussen de genetische factoren die de parameters beïnvloedden niet zo groot. Dit suggereert dat elke onderzochte repolarisatieparameter unieke genetische informatie met zich meedraagt met betrekking tot de repolarisatiefase en dat er informatie wordt gemist wanneer slechts een of een deel van deze parameters onder de loep wordt genomen.

Erfelijkheid van inflammatoire markers

In **hoofdstuk 6** hebben we de erfelijkheid van een ander cardiovasculair risicocluster onderzocht: inflammatie. De inflammatoire respons wordt in gang gezet door de pro-inflammatoire cytokines Tumor Necrosis Factor-alpha (TNF-α) en Interleukin(IL)-1 (Tracey, 2002). De inflammatoire cascade wordt verder gestimuleerd door de productie van IL-6 welke op haar beurt de acute fase respons stimuleert. Deze respons wordt gekenmerkt door de synthese van C-Reactive Protein (CRP) en fibrinogeen (Gabay, 2006; Gabay & Kushner, 1999; Packard & Libby, 2008). Eerder onderzoek heeft aangetoond dat verhoogde TNF-α, IL-6, CRP en fibrinogeen niveaus geassocieerd zijn met onder meer een toegenomen risico op hart- en vaatziekten (Cesari et al., 2003; Danesh et al., 2008; Danesh et al., 2004; Humphries et al., 2007; Libby & Theroux, 2005; Packard & Libby, 2008; Woods et al., 2000). Eerder onderzoek heeft ook aangetoond dat de manifestatie van deze markers deels door genetische factoren verklaard kan worden. Echter, de variatie in de erfelijkheidsschattingen die tot op heden zijn gevonden is erg groot. Daarbij zijn de schattingen, met een paar uitzonderingen daargelaten, gebaseerd op relatief kleine groepen (~ 400) tweelingen (de Lange, et al., 2001; de Lange et al., 2006; de Maat et al., 2004; Grunnet et al., 2006; Jermendy et al., 2011; MacGregor et al., 2004; Rahman et al.,

2009; Reed et al., 1994; Sas et al., 2012; Su et al., 2008; Su et al., 2009a; Su et al., 2009b; Wessel et al., 2007; Wang et al., 2011; Worns et al., 2006). Voor deze studie hebben we gebruik gemaakt van data die verzameld is tijdens een grote biobank studie, uitgevoerd door het NTR. Aan dit project hebben naast tweelingen en hun broers en zussen ook andere familieleden, waaronder de ouders van tweelingen, deelgenomen.

Het toevoegen van familieleden van tweelingen aan het onderzoek, maakt het mogelijk verder te differentiëren tussen additief genetische en non-additief genetische effecten en daarbij ook de gedeelde omgeving mee te modeleren (in het klassieke tweeling model dient op basis van de tweelingcorrelaties gekozen te worden tussen het modeleren van ofwel additief genetische effecten ofwel de gedeelde omgeving). Het toevoegen van ouders aan een tweelingstudie, maakt het verder mogelijk ook de mogelijke effecten van partnerselectie te onderzoeken. De immuun-parameters die we hebben onderzocht waren de cytokines TNF-α en IL-6 en de acute fase reactanten CRP en fibrinogeen. In totaal hebben er 3,534 tweelingen, 1,568 broers en zussen, en 2,227 ouders van tweelingen uit 3,095 families meegenomen in de analyses voor deze studie. Dit maakt deze studie het meest omvangrijk in zijn soort tot nu toe.

We vonden dat de pro-inflammatoire markers gemiddeld erfelijk waren, schattingen kwamen uit op 39%, 21%, 45%, en 46% voor TNF-α, IL-6, CRP en fibrinogeen, respectievelijk. Daarbij vonden we dat een substantieel deel van de genetische variatie in TNF-α, CRP en fibrinogeen non-additief van aard was terwijl de erfelijkheid van IL-6 volledig toe te schrijven was aan additief genetische effecten. Invloeden van een gedeelde omgeving bleken voor geen enkele immuunmarker significant. Ook vonden we geen bewijs dat partnerselectie een prominente rol speelt in de manifestatie van de onderzochte markers. We concluderen dat deze grote studie duidelijke erfelijkheidsschattingen neerzet welke gebruikt kunnen worden als referentie voor toekomstige genoom-wijde associatie studies die trachten de daadwerkelijke genen te vinden die verantwoordelijk zijn voor de manifestatie van deze markers.

Wederkerige associatie tussen sympathische en parasympatische (re)activiteit en de inflammatoire en metabole risicoprofielen?

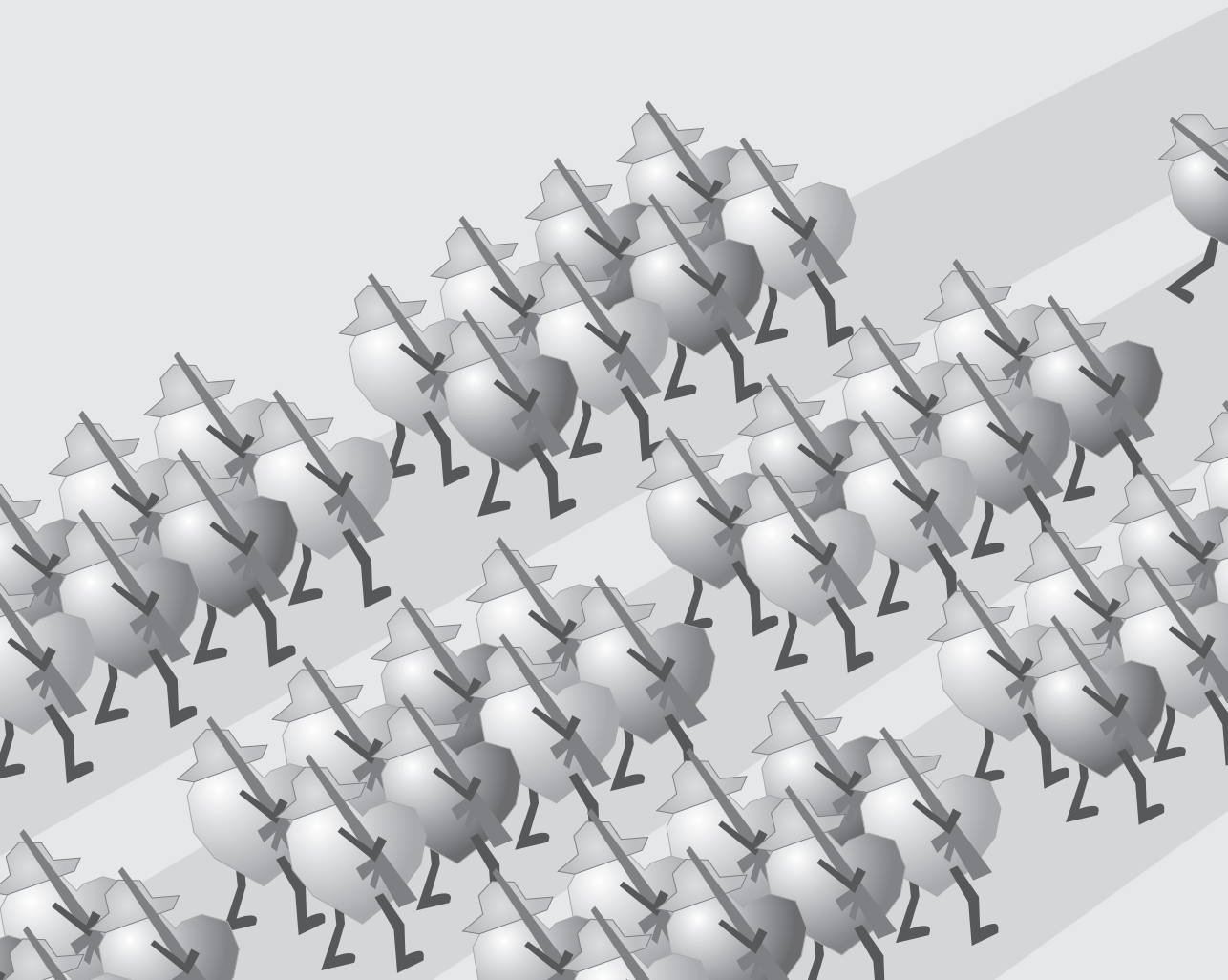
In **hoofdstuk 7** onderzochten we de lange termijn relatie tussen sympathische en parasympathische zenuwstelsel (re)activiteit en de inflammatoire en metabole risicoprofielen. In het verleden zijn zowel een verschuiving in autonome balans van sympathische dominantie en een overdreven autonome reactiviteit op stress geassocieerd met een

verhoogd cardiovasculair risico. Eerder onderzoek wijst op parallele autonome effecten op de metabole en inflammatoire risicoprofielen die deels verantwoordelijk zijn voor dit risico. Deze eerdere studies waren echter vaak cross-sectioneel van aard en uitgevoerd in het laboratorium of in een kliniek. In het onderzoek beschreven in dit hoofdstuk testen we de bidirectionele lange termijn associatie tussen autonome zenuwstelsel activiteit en reactiviteit en inflammatoir en metabool risico in twee onafhankelijke prospectieve studies. We hebben inflammatoire en metabole risiscoscores berekend door de Z-scores van een aantal van de belangrijkste markers van de pro-inflammatoire (TNF-α, IL-6, CRP en fibrinogeen) en metabole (middelomtrek, body mass index, glucose, triglyceride, low-density cholesterol, en high-density cholesterol levels) toestand bij elkaar op te tellen. Deze markers zijn bepaald tijdens de eerder genoemde biobank studie. De dataverzameling van de 24-uurs ambulante autonome zenuwstelsel activiteit vond vijf jaar voor en vijf jaar na de biobank studie plaats. Op basis van de resultaten beschreven in **hoofdstuk 4**, hebben we de hartslag, RSA, en PEP tijdens slaap en in de vrije tijd als rustlevels meegenomen, samen met de reactiviteitsmaat (werk_zittend – vrije tijd) die het minst onderhevig was aan vertekening door houding en/of fysieke activiteit. We vonden dat een hogere rusthartslag in de vrije tijd gekoppeld met een verhoogde sympathische reactiviteit geassocieerd waren met hoger inflammatoir risico vijf jaar later. Ook werd gevonden dat een hogere sympathische reactiviteit geassocieerd was met een verhoogd metabool risico vijf jaar later. Een ongunstig metabool of inflammatoir profiel had omgekeerd geen nadelig effect op autonome zenuwstelsel (re)activiteit vijf jaar later. Uit deze studie concluderen we dat er slechts unidirectionele causale effecten zijn van een verhoogde rusthartslag en sympathische reactiviteit op inflammatoir risico. Ook zijn er slechts unidirectionele effecten van sympathische reactiviteit op metabool risico.

Conclusies

De studies die beschreven staan in dit proefschrift zetten duidelijke numerieke erfelijkheidsschattingen neer wat betreft de regulatie van autonome controle over het hart gemeten in het dagelijks leven. Gebleken is dat individuele verschillen in sympathische en parasympathische invloeden op de elektrische activiteit van het hart het beste verklaard kunnen worden door twee typen variantie: additieve genetische invloeden en unieke omgevingsinvloeden. De additieve genetische variantie varieert voor de verschillende markers, over de verschillende dagdelen, en over de verschillende bezigheden in het dagelijks leven. We hebben aangetoond dat er verschillende manieren zijn om data

van ongestructureerde ambulante hartregistraties dusdanig te organiseren zodat deze gebruikt kunnen worden voor (klinisch) psychofysiologisch onderzoek. We vonden dat genetische invloeden over het algemeen toenemen tijdens stress en dit lijkt vooral te komen doordat er andere genen actief worden bij lichaamsreacties op psychosociale gebeurtenissen in het dagelijks leven. Dit patroon wordt ook wel gezien bij reacties op typische stress taken die vaak in laboratoria worden gebruikt, maar niet altijd en veel minder sterk. Tot slot is met dit proefschrift aangetoond dat het functioneren van het autonoom zenuwstelsel ook op de lange termijn een belangrijke rol speelt in onze gezondheid.



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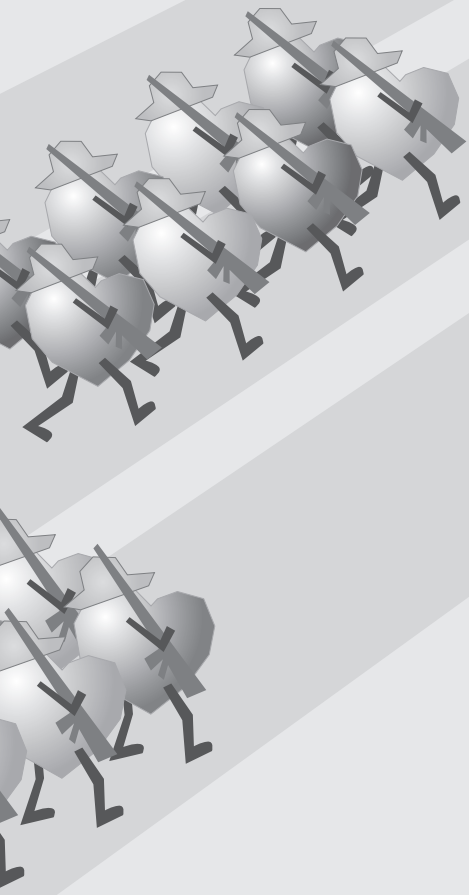

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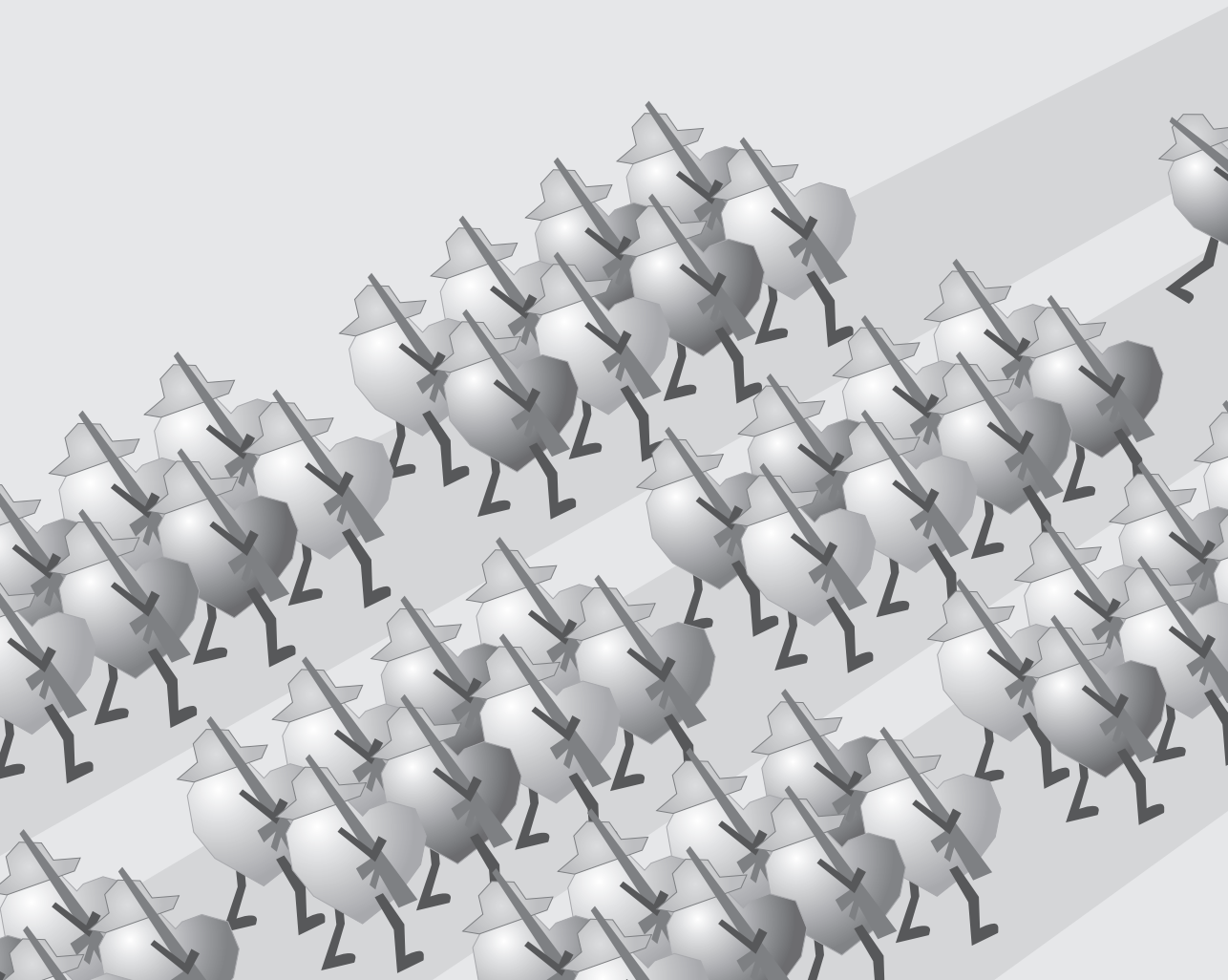
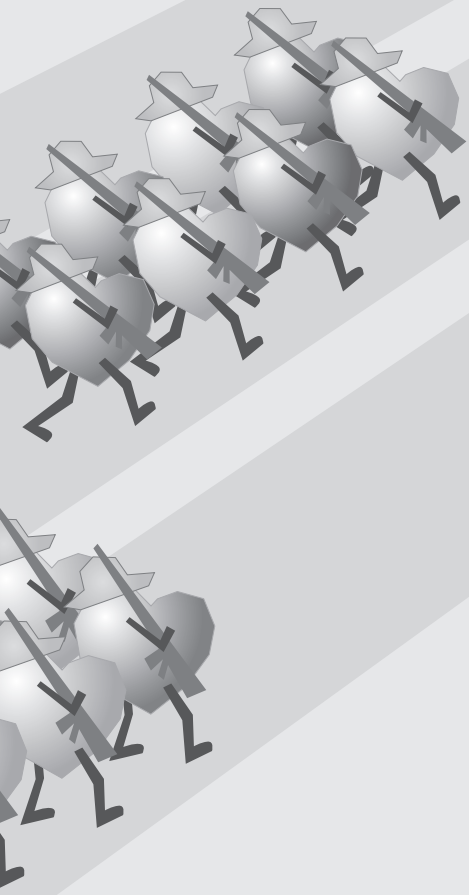


Appendices



Appendix I

Invitation letter and brochure

VU VRIJE UNIVERSITEIT AMSTERDAM

NEDERLANDS TWEELINGEN REGISTER (NTR)

DATUM	ONS KENMERK	UW BRIEF VAN	UW KENMERK
	NETAMB3-082010/b1		
E-MAIL	**TELEFOON**	**FAX**	**BIJLAGE(N)**
m.neijts@.vu.nl	020 5982537	020 598 8832	Informatiefolder

Betreft: Uitnodiging Familieonderzoek naar lichamelijke en geestelijke gezondheid

Geachte heer, mevrouw …….,

U ontvangt deze brief omdat wij u graag willen uitnodigen voor bovengenoemd onderzoek. In de afgelopen jaren hebben u en een aantal van uw familieleden meegedaan met (vragenlijst)onderzoek van het Nederlands Tweelingen Register (NTR). Uw medewerking hieraan was van zeer groot belang.

Het onderzoek waar wij u nu voor uitnodigen is in 1997 begonnen en inmiddels hebben reeds 800 tweelingen en hun familieleden hieraan meegewerkt. Bij dit onderzoek zullen wij u thuis bezoeken om een korte vragenlijst met u door te nemen en u een hartactiemeter om te doen. Hiermee zal uw hartactie gedurende 24 uur gemeten worden. Verder zal de onderzoeker uw bloeddruk meten. Ook wordt u gevraagd een aantal maal op een watje te kauwen voor de verzameling van speekselhormonen. Meer informatie over het onderzoek en de metingen, vindt u in de bijgaande folder.

Binnen één tot twee weken nadat u deze brief heeft ontvangen, zullen wij telefonisch contact met u opnemen om verdere uitleg te geven en om een afspraak met u te maken. Uw persoonlijke hartslaggegevens met een korte toelichting worden u kort na de metingen toegestuurd. Wij hopen op uw medewerking.

Met vriendelijke groet, namens het onderzoeksteam,

Drs. Melanie Neijts
Dr. Gonneke Willemsen
Prof. dr. Eco de Geus

FACULTEIT DER PSYCHOLOGIE EN PEDAGOGIEK
Biologische Psychologie

WWW.FPP.VU.NL

BEZOEKADRES
Van der Boechorststraat 1
Kamer 2B-35
1081 BT Amsterdam

POSTADRES
Van der Boechorststraat 1
1081 BT Amsterdam

Informatie

Familieonderzoek naar lichamelijke en geestelijke gezondheid

Achtergrond

In 1991 is in Nederland een langlopend tweeling-familieonderzoek gestart naar de geestelijke en lichamelijke gezondheid. Bij dit onderzoek wordt gebruik gemaakt van het Nederlands Tweelingen Register (NTR) dat in 1987 is opgericht aan de Vrije Universiteit in Amsterdam. Met behulp van vragenlijsten en soms ook interviews wordt informatie verzameld over de geestelijke gezondheid. De lichamelijke gezondheid wordt gemeten door bloed- en speekselonderzoek, door bloeddrukonderzoek, of door onderzoek waarbij de hartfunctie wordt gemeten met elektrodes op de borst. De aansturing van het hart is rechtstreeks van belang voor hart- en vaatziekten. Daarnaast zijn er aanwijzingen dat de aansturing van het hart gekoppeld is aan het immuunsysteem. In dit onderzoek willen wij de aansturing van het hart verder onderzoeken en daarvoor vragen wij uw medewerking.

Resultaten tot nu toe

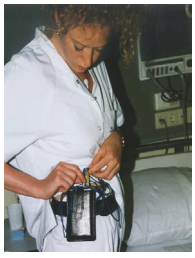
Wij hebben al kunnen aantonen dat veel aspecten van de lichamelijke gezondheid deels bepaald worden door familiale aanleg. Voor een groot deel komt dat omdat familieleden hun erfelijk materiaal delen. Bij bloeddruk, hartslag, cholesterol en de bloedsuikerspiegel blijkt deze erfelijke invloed zelfs van zeer groot belang. Het afgelopen jaar zijn variaties in een aantal genen gevonden die deze erfelijke invloeden verklaren. Deze genen zijn mede dankzij onderzoek van het NTR opgespoord. Dit helpt ons verder om te begrijpen hoe het mechanisme van erfelijke invloed op hart- en vaatziekten werkt.

Waarom bent u belangrijk voor dit nieuwe onderzoek?

Tussen 2004 en 2008 heeft u meegedaan aan onderzoek van het NTR, waarbij u thuis bent bezocht door onze medewerkers, die bloed bij u hebben afgenomen. In het nieuwe onderzoek willen we de gegevens uit dit eerdere onderzoek koppelen aan metingen van bloeddruk en hartfunctie. Omdat u deel uitmaakt van een tweelingfamilie kunnen we het totale belang van erfelijke aanleg op bloeddruk en hartfunctie in kaart brengen. Daarvoor wordt gebruik gemaakt van de metingen bij eeneiige en twee-eiige tweelingen en hun broers of zussen. Voor dit deel van het onderzoek wordt gekeken naar de overeenkomsten tussen familieleden.
Waarschijnlijk heeft u in het verleden ook aan het vragenlijstonderzoek van het NTR meegedaan. Daarin werd gevraagd naar stressvolle gebeurtenissen, gevoelens van angst en depressie maar ook geluk en tevredenheid. In dit nieuwe onderzoek zullen we ook kijken in welke mate het effect van erfelijke aanleg afhangt van uw geestelijke gezondheid, nu en in het verleden.

Wat houdt het onderzoek in?

U hoeft voor het onderzoek niet naar de VU te komen, wij komen naar u. Enkele dagen voor het thuisbezoek krijgt u van ons een vragenlijst en materiaal voor het afnemen van speeksel thuisgestuurd. Op de dag van het onderzoek komen we u 's ochtends thuis bezoeken op een datum en tijd die u het beste schikt. Het bezoek neemt 30-40 minuten van uw tijd in beslag. U krijgt dan een hartmeter om. Via zeven meetelektroden, die met plakkertjes op de rug en borst bevestigd zijn, worden de aansturing en de samentrekkingskracht van het hart gemeten. Wij stellen u een aantal vragen over uw gezondheid. Tevens meten we de bloeddruk in rust en tijdens een kort computertestje. Daarna vragen we u om de hartmeter gedurende de verdere dag en de nacht om te houden tot aan de volgende morgen. De meter is daarvoor speciaal ontworpen aan de Vrije Universiteit. Hij is klein, kan onder de kleding worden gedragen en levert weinig hinder op bij dagelijkse werkzaamheden.



De VU AMS hartactiemeter bij een verpleegkundige tijdens het werk

Omdat de hartactie kan veranderen als gevolg van veranderingen in activiteit, houding en stemming, vragen wij u om tijdens de meetdag een dagboekje bij te houden. De ochtend van de meetdag, vlak voor het slapen gaan en de ochtend erna neemt u wat speeksel af. Dit gebeurt door even op een watje te kauwen dat u daarna in een bijgeleverd buisje doet. De volgende dag kunt u zelf de hartmeter afdoen en deze samen met de buisjes in een retourenvelop terugsturen. Binnen enkele weken na het onderzoek ontvangt u een overzicht van uw hartslaggegevens.

Eerder hebben al ruim 800 personen uit families, ingeschreven bij het Nederlands Tweelingen Register, meegedaan aan dit soort onderzoek. In totaal zullen ongeveer 1300 mensen aan dit onderzoek deelnemen. Door hun èn uw medewerking krijgen we meer kennis over het verband tussen lichamelijke en geestelijke gezondheid en over de reden waarom mensen daarin van

elkaar verschillen. We hopen dat die kennis zal bijdragen aan een betere gezondheid van iedereen.

Vertrouwelijkheid

Alle persoonlijke gegevens worden strikt vertrouwelijk behandeld en gecodeerd verwerkt bij een wetenschappelijke rapportage.

Vrijwilligheid van deelname

Deelname is vrijwillig en u kunt zich op elk moment, ook na ondertekening van het toestemmingsformulier, zonder opgave van redenen uit het onderzoek terugtrekken.

Meer informatie

Voor praktische informatie kunt u te allen tijde contact opnemen met de uitvoerend onderzoeker: drs. Melanie Neijts.
Verdere informatie over de wetenschappelijke achtergrond van de studie wordt desgevraagd verstrekt door: prof. dr. Eco de Geus of dr. Gonneke Willemsen.
Wilt u informatie over dit onderzoek inwinnen bij een onafhankelijk arts, dan is dr. Richard Ijzerman, tel. 020-4440533, bereid uw vragen te beantwoorden.

Dit onderzoek is door de Medisch Ethische Toetsingscommissie van het VU Medisch Centrum aangeduid als een onderzoek zonder risico, waarvoor een vrijstelling van verzekering is afgegeven.

Vrije Universiteit Amsterdam
Biologische Psychologie
T.a.v. drs. M. Neijts
Van der Boechorststraat 1
1081 BT Amsterdam
Tel. 020 5988787

U kunt ook een kijkje nemen op onze website: http://www.tweelingenregister.org onder "Onderzoek."

Appendix II

Confirmation letter

VU VRIJE UNIVERSITEIT AMSTERDAM

NEDERLANDS TWEELINGEN REGISTER (NTR)

DATUM	ONS KENMERK	UW BRIEF VAN	UW KENMERK
	NETAMB3-062010/b2		
E-MAIL	**TELEFOON**	**FAX**	**BIJLAGE(N)**
m.neijts@vu.nl	020 5982537	020 598 8832	3

Betreft: Bevestiging deelname familieonderzoek naar lichamelijke en geestelijke gezondheid

Geachte heer, mevrouw ……..,

Hierbij sturen wij de bevestiging van de afspraak die we telefonisch hebben gemaakt voor de ambulante meting. De afspraak zal plaatsvinden op:

Datum: ……..…… **Tijd:** ……..
(Het bezoek zal ongeveer 45 minuten duren)

Bij deze brief is het volgende gevoegd:

1. Een toestemmingsformulier. Dit formulier wordt gebruikt om uw deelname aan de studie te bevestigen. Wilt u het invullen zodat de onderzoeker het mee kan nemen na het bezoek?
2. Een vragenlijst. Het zou fijn zijn als u deze ingevuld hebt voor wij bij u langskomen zodat wij deze meteen mee terug kunnen nemen. Het duurt ongeveer 15 minuten om deze in te vullen.
3. Materialen voor de speekselverzameling. Er moeten 2 verzamelingen op de ochtend vóór het bezoek worden gedaan. Wij hebben hiervoor twee buisjes met watjes en een instructie meegestuurd. Leest u deze de avond van tevoren alstublieft goed door.

Omdat hartslag en bloeddruk beïnvloed worden door inspanning en alcoholgebruik, willen wij u vragen om de dag voor de meting niet te sporten en de avond voor de meting geen of slechts weinig alcohol te gebruiken. Als u medicijnen (inclusief de pil) gebruikt, wilt u deze dan klaarleggen voor wij bij u langskomen?
Wij komen met de auto naar u toe en doen ons uiterste best om op het afgesproken tijdstip bij u aanwezig te zijn. Mocht dit door files of om een andere reden niet mogelijk zijn, dan laten wij u dit vanzelfsprekend zo spoedig mogelijk weten. Mocht u zelf onverhoopt verhinderd zijn op de afgesproken datum, zou u ons dit dan zo snel mogelijk willen laten weten? Wij zijn te bereiken op 020-5982537 of 06-50698826. Bedankt voor uw deelname! Wij zien ernaar uit u te bezoeken.

Met vriendelijke groet,
Melanie Neijts

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WWW.FPP.VU.NL

BEZOEKADRES
Van der Boechorststraat 1
Kamer 2B-35
1081 BT Amsterdam

POSTADRES
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1081 BT Amsterdam

Appendix III

Informed consent

Toestemmingsformulier (Informed Consent)

Familieonderzoek naar lichamelijke en geestelijke gezondheid

- Ik bevestig dat ik de informatiefolder voor de proefpersoon (kenmerk NTRAMB3-082010) heb gelezen en ik begrijp de informatie. Ik heb voldoende tijd gehad om over mijn deelname na te denken en ben in de gelegenheid geweest om vragen te stellen. Deze vragen zijn naar tevredenheid beantwoord.
- Ik geef toestemming voor deelname aan bovengenoemd medisch-wetenschappelijk onderzoek.
- Ik weet dat mijn deelname geheel vrijwillig is en dat ik mijn toestemming op ieder moment kan intrekken zonder dat ik daarvoor een reden hoef op te geven. Mijn gegevens zullen dan, als ik dat wil, worden vernietigd.
- Ik geef toestemming om de gegevens te verwerken voor doeleinden zoals beschreven in de informatiefolder met kenmerk NTRAMB3-082010.
- Ik ga ermee akkoord dat de gegevens uit het huidige onderzoek gekoppeld mogen worden aan gegevens die eerder van mij verzameld zijn, zoals erfelijk materiaal, resultaten van de bloedmonsters en/of de vragenlijsten.

Voornaam ..... **man / vrouw***

Achternaam .....

Adres .....

.....

Telefoonnr .....

Geboortedatum .....

Handtekening **Datum**

.....

* Doorhalen wat niet van toepassing is

Appendix IV

Instructions saliva collection

Speekselverzameling

Er zijn in totaal 5 momenten waarop speekselverzamelingen plaatsvinden. De eerste 2 speekselverzamelingen vinden plaats op de ochtend dat de onderzoeker bij u langskomt. U krijgt daarom alvast een instructie en een setje wattenrolletjes meegestuurd.

Het is de bedoeling dat u ***direct na het wakker worden, terwijl u nog in bed ligt, èn 30 minuten daarna*** speeksel verzamelt. Wilt u de avond van te voren de buisjes bij uw bed, bijvoorbeeld op het nachtkastje, klaarleggen? Wij willen u verder vragen in het onderstaande schema in te vullen hoe laat u het speeksel hebt verzameld. Dit is belangrijk omdat de hoeveelheid van het hormoon ook afhankelijk is van het tijdstip op de dag.

Het is belangrijk dat u een half uur van tevoren niet eet of drinkt. Een glas water mag wel. Wij willen u dus ook vragen te wachten met het ontbijt tot u de 2 speekselverzamelingen hebt gedaan. Verder is het belangrijk dat u niet vlak voor de speekselverzameling uw tanden poetst (zo vermijdt u dat het speeksel met bloed wordt vermengd). In de ruimte voor opmerkingen kunt u aangeven als er iets niet helemaal goed is gegaan of als zich misschien een uitzonderlijk voorval heeft voorgedaan.

Instructie

- U draait de dop van de buis en haalt het wattenrolletje eruit.
- U legt het wattenrolletje in uw mond gedurende 1 minuut. Het is belangrijk dat het wattenrolletje ***goed doordrenkt*** wordt. U kunt dit bevorderen door niet te slikken en licht op het wattenrolletje te kauwen.
- Stop het wattenrolletje na 1 minuut terug in het plastic hulsje in de juiste buis en draai de dop erop.
- Vul in onderstaand schema de tijd van de speekselverzameling in.

Buisje	Verzamelmoment	Tijd
1. Ontwaken	In bed, direct na het wakker worden, op de dag dat de onderzoeker bij u langskomt	….. uur ….. min
2. Ontwaken +30	30 minuten na het wakker worden	….. uur ….. min

Opmerkingen

Op de buisjes staat aangegeven welke u eerst gebruikt (1. Ontwaken) en welke daarna (2. Ontwaken + 30). Er wordt een reserve buisje meegestuurd, mocht er iets misgaan. De onderzoeker zal de buisjes na het bezoek meenemen.

Appendix V

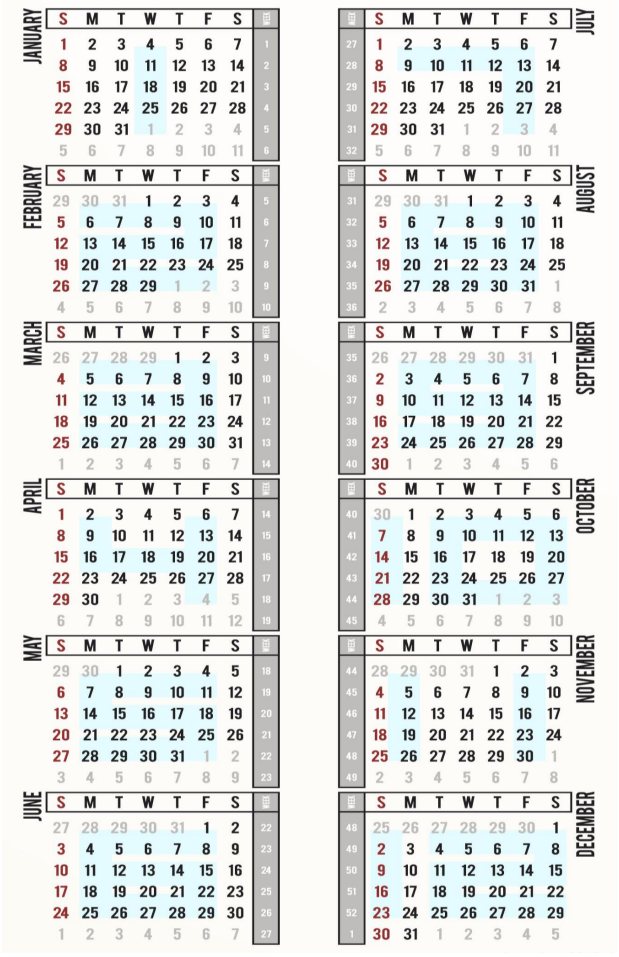
Health and lifestyle interview

Interview

Familieonderzoek naar lichamelijke en geestelijke gezondheid

- In te vullen door de testleider -

2012



JANUARY	S	M	T	W	T	F	S	WEEK
	1	2	3	4	5	6	7	1
	8	9	10	11	12	13	14	2
	15	16	17	18	19	20	21	3
	22	23	24	25	26	27	28	4
	29	30	31	1	2	3	4	5
	5	6	7	8	9	10	11	6

FEBRUARY	S	M	T	W	T	F	S	WEEK
	29	30	31	1	2	3	4	5
	5	6	7	8	9	10	11	6
	12	13	14	15	16	17	18	7
	19	20	21	22	23	24	25	8
	26	27	28	29	1	2	3	9
	4	5	6	7	8	9	10	10

MARCH	S	M	T	W	T	F	S	WEEK
	26	27	28	29	1	2	3	9
	4	5	6	7	8	9	10	10
	11	12	13	14	15	16	17	11
	18	19	20	21	22	23	24	12
	25	26	27	28	29	30	31	13
	1	2	3	4	5	6	7	14

APRIL	S	M	T	W	T	F	S	WEEK
	1	2	3	4	5	6	7	14
	8	9	10	11	12	13	14	15
	15	16	17	18	19	20	21	16
	22	23	24	25	26	27	28	17
	29	30	1	2	3	4	5	18
	6	7	8	9	10	11	12	19

MAY	S	M	T	W	T	F	S	WEEK
	29	30	1	2	3	4	5	18
	6	7	8	9	10	11	12	19
	13	14	15	16	17	18	19	20
	20	21	22	23	24	25	26	21
	27	28	29	30	31	1	2	22
	3	4	5	6	7	8	9	23

JUNE	S	M	T	W	T	F	S	WEEK
	27	28	29	30	31	1	2	22
	3	4	5	6	7	8	9	23
	10	11	12	13	14	15	16	24
	17	18	19	20	21	22	23	25
	24	25	26	27	28	29	30	26
	1	2	3	4	5	6	7	27

WEEK	S	M	T	W	T	F	S	JULY
27	1	2	3	4	5	6	7	
28	8	9	10	11	12	13	14	
29	15	16	17	18	19	20	21	
30	22	23	24	25	26	27	28	
31	29	30	31	1	2	3	4	
32	5	6	7	8	9	10	11	

WEEK	S	M	T	W	T	F	S	AUGUST
31	29	30	31	1	2	3	4	
32	5	6	7	8	9	10	11	
33	12	13	14	15	16	17	18	
34	19	20	21	22	23	24	25	
35	26	27	28	29	30	31	1	
36	2	3	4	5	6	7	8	

WEEK	S	M	T	W	T	F	S	SEPTEMBER
35	26	27	28	29	30	31	1	
36	2	3	4	5	6	7	8	
37	9	10	11	12	13	14	15	
38	16	17	18	19	20	21	22	
39	23	24	25	26	27	28	29	
40	30	1	2	3	4	5	6	

WEEK	S	M	T	W	T	F	S	OCTOBER
40	30	1	2	3	4	5	6	
41	7	8	9	10	11	12	13	
42	14	15	16	17	18	19	20	
43	21	22	23	24	25	26	27	
44	28	29	30	31	1	2	3	
45	4	5	6	7	8	9	10	

WEEK	S	M	T	W	T	F	S	NOVEMBER
44	28	29	30	31	1	2	3	
45	4	5	6	7	8	9	10	
46	11	12	13	14	15	16	17	
47	18	19	20	21	22	23	24	
48	25	26	27	28	29	30	1	
49	2	3	4	5	6	7	8	

WEEK	S	M	T	W	T	F	S	DECEMBER
48	25	26	27	28	29	30	1	
49	2	3	4	5	6	7	8	
50	9	10	11	12	13	14	15	
51	16	17	18	19	20	21	22	
52	23	24	25	26	27	28	29	
1	30	31	1	2	3	4	5	

Meten van taille en heupomtrek

Algemeen

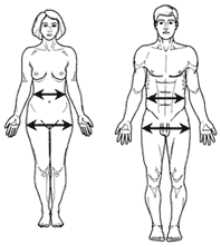
We gebruiken een centimeter van 1,5 meter lang. De centimeter wordt om het lichaam getrokken, zodat deze goed aansluit (niet te los en niet te strak). De taille omtrek wordt op de blote huid gemeten. De heupomtrek over de broek. Zorg dat de centimeter parallel loopt op buik- en rugzijde. Omtrek is opgeschreven op een halve cm nauwkeurig.

Taille

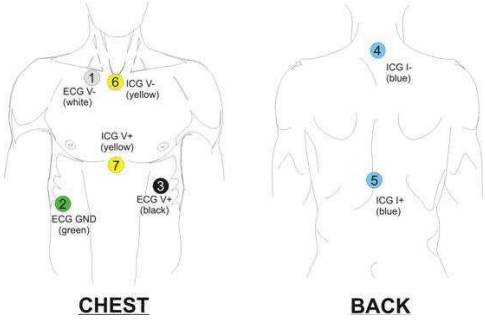
De taille wordt gemeten op het smalste punt van het middel. Dit punt ligt net boven de navel.

Heupomtrek

De heupomtrek wordt gemeten op het breedste punt van de heupen. Als hulpmiddel: dit is het punt waar je de heupkom goed kunt voelen.



Plakken VU-AMS elektrodes



Identificatie door proefleider

Voornaam? Sekse? M / V

Geboortedatum?

□ Testleider: Checken informed consent / speekselverzameling 1 en 2 + instructie met ingevulde tijden / vragenlijst 1 / supplement

□ Testleider: Voorstellen + uitleg metingen

□ Testleider: Navraag medicatie: zie medicatiedoosjes

Merknaam	Substantienaam	Dosis / hoe vaak?	Reden
……..	……..	……..	……..
……..	……..	……..	……..
……..	……..	……..	……..
……..	……..	……..	……..

□ Assistent: Aansluiten VU-AMS staand: noteren serienummer VU-AMS op dagboekje + tijdstip start (= einde) 24-uurs meting in DB

□ Assistent: Vragen lengte, meten gewicht, omvang middel en heupomvang

Lengte …….. cm

Gewicht …….. kg

Omvang middel …….. cm

Heupomvang …….. cm

□ Interview, rustig zitten

Inventarisatie dag / beroep

1. Wat voor dag is het?
□ Vrije dag
□ Werkdag (ook indien u huisvrouw / -man bent)
□ U heeft momenteel geen werk
□ Anders, namelijk ……..

2. Wat voor soort werk doet u momenteel? (Meerdere antwoorden mogelijk)
□ fulltime betaald werk: meer dan 32 uur per week, specificeer …….
□ parttime betaald werk: 12-32 uur per week, specificeer …….
□ parttime betaald werk: minder dan 12 uur per week, specificeer …….
□ scholier / student
□ werkeloos, sinds …….. (jaartal)
□ huisman / huisvrouw, sinds …….. (jaartal)
□ arbeidsongeschikt …….. (jaartal)
□ anders, namelijk …….

IN GEVAL VAN WERK

3. Werkt u in ploegendiensten? Zo ja, hoe zien uw diensten er uit?
- ☐ Nee, geen ploegendiensten
- ☐ Ja, ik werk in ploegendiensten. Toelichting: .....

4. Hoeveelste werkdag in een rij is dit?

5. Wat is uw beroep? (Gedetailleerd weergeven, ook: leidinggevende functie of niet?)

6. Heeft u een zelfstandig beroep?
- ☐ Ja, geheel zelfstandig: eigen bedrijf of vrij beroep
- ☐ Gedeeltelijk zelfstandig, toelichting: .....
- ☐ Niet zelfstandig / loondienst
- ☐ Vrijwilligerswerk
- ☐ Nooit gewerkt

7. Wat is uw hoogst genoten opleiding welke u met een diploma hebt afgerond?

Hoe lang duurde deze opleiding? ..... jaar

En betrof dit:
- ☐ Lager onderwijs
- ☐ Middelbaar onderwijs (mavo, lbo, vmbo)
- ☐ Hoger middelbaar onderwijs (havo / vwo, mbo)
- ☐ Hoger onderwijs (universiteit, hoger niet-universitair, hbo, BaMa)
- ☐ Anders, namelijk: .....

Gezondheid

8. Voelt u zich gezond?
- ☐ Ja, ik voel me gezond > *volgende vraag overslaan*
- ☐ Normaal wel, maar vandaag niet
- ☐ Nee, ik voel me de laatste tijd niet gezond

9. Kunt u aangeven waarom u zich niet gezond voelt?

Inspanning

10. Heeft u zich gisteren zwaar fysiek ingespannen?

Sport en beweging

11. Doet u regelmatig aan sport of lichaamsbeweging?
- □ Nee > *alleen volgende vraag overslaan*
- □ Ja

12. Zo ja, welke sport(en) beoefent u? En kunt u per sport aangeven hoe lang en hoe vaak u deze sport gemiddeld beoefent?

Naam sport	Aantal jaar	Aantal maanden per jaar	Aantal keer per week	Gemiddelde tijd per keer
……	……..	……..	……	…… min
……	……..	……..	……	…… min
……	……..	……..	……	…… min
……	……..	……..	……	…… min

13. Hoeveel fietst u in een normale week? …….. uur en …….. minuten per week

14. Hoeveel wandelt u in een normale week? …….. uur en …….. minuten per week

Roken

15. Heeft u ooit gerookt?
- □ Nee > *sectie roken kan worden overgeslagen*
- □ Een paar keer om te proberen > *sectie roken kan worden overgeslagen*
- □ Ja

16. Hoeveel jaar rookt of rookte u in totaal? …….. jaar

17. Hoe vaak rookt u nu?
- □ Ik ben gestopt met roken sinds …….. (mm/jjjj)
- □ Ik rook 1 keer per week of minder
- □ Ik rook meerdere keren per week, niet elke dag
- □ Ik rook 1 of meerdere malen per dag

18. Hoeveel keer heeft u serieus geprobeerd met roken te stoppen? …….. keer

19. Wat rookt of rookte u?
- □ Sigaretten en shag, eventueel samen met sigaren, pijptabak etc.
- □ Uitsluitend sigaren of pijptabak > *sectie roken kan verder worden overgeslagen*

VOOR ROKERS EN EX-ROKERS

20. Hoeveel sigaretten rookt(e) u gemiddeld per dag? ..... sigaretten per dag

Alcohol

21. Hoe vaak drinkt u een alcoholische drank? (Inclusief de keren dat u slechts een kleine hoeveelheid, bijvoorbeeld een paar slokjes, drinkt).

- □ Ik drink geen alcohol
- □ 1 keer per jaar of minder
- □ Aantal keren per jaar
- □ Ongeveer 1 keer per maand
- □ 1 keer per week
- □ Aantal keren per week
- □ Dagelijks

22. Hoeveel glazen alcohol drinkt u gemiddeld per week (inclusief weekend)?

- □ Minder dan 1 glas
- □ 1-5 glazen per week
- □ 6-10 glazen per week
- □ 11-15 glazen per week
- □ 16-20 glazen per week
- □ 21-40 glazen per week
- □ Meer dan 40 glazen per week

Nachtrust

23. Hoe laat bent u vanochtend opgestaan? ..... uur ..... minuten

24. Is dit ook uw normale tijd van opstaan?

- □ Ja
- □ Nee, normaal sta ik op om ..... uur ..... minuten

Familie- / gezinssituatie

25. Hebt u nu een duurzame relatie?

- □ Nee
- □ Ja, niet samenwonend
- □ Ja, samenwonend / gehuwd

26. Hoe lang hebt u een relatie met uw partner? ..... jaar en ..... maanden

27. Bent u eerder getrouwd geweest / hebt u eerder een duurzame relatie gehad (samengewoond / getrouwd)?

- □ Nee
- □ Ja, deze verbintenis is geëindigd in een scheiding / verbroken sinds (jaartal) .....
- □ Ja, deze verbintenis is geëindigd door overlijden partner, sinds (jaartal) .....
- □ Ja, anders, namelijk .....

28. Hebt u kinderen? Zo ja, hoeveel?
- □ Nee
- □ Ja, ik heb ……. biologische kinderen
- □ Ja, ik heb ……. niet-biologische kinderen

29. Wilt u voor uw kinderen aangeven wat hun geboortedatum en geslacht is?

30. Hoeveel kinderen wonen er op dit moment bij u thuis?

Biologisch / niet-biologisch	Geboortedatum	Geslacht	Thuiswonend ja / nee
……	…… - …… - ……..	……	……
……	…… - …… - ……..	……	……
……	…… - …… - ……..	……	……
……	…… - …… - ……..	……	……

ALLEEN VOOR VROUWEN

Menstruatie

31. Enkele vragen met betrekking tot de menstruele cyclus.
Is uw menstruele cyclus regelmatig?
- □ Ja
- □ Nee onregelmatig
- □ Menopauze
- □ Anders, nl. ……

32. Wat is gemiddeld het aantal dagen tussen twee menstruaties? ……. dagen

33. Wat was de eerste dag van uw laatste menstruatie? …….. (dd/mm/jjjj)

34. Gebruikt u anticonceptie?
- □ Ja, nl. …… (pil / spiraaltje / pessarium / injecties / etc.)
  Van het merk: ……..
- □ Nee

IN GEVAL VAN MENOPAUZE

35. Komt de onregelmatigheid van uw menstruatie mogelijk door het begin van de menopauze?
- □ Ja
- □ Nee

36. Hoe lang geleden was uw laatste menstruatie? ……. jaar en …….. maanden

37. Weet u de eerste dag van uw laatste menstruatie nog? Zo ja, Welke dag was dat?
- □ Ja, dat was …….. (dd/mm/jjjj)
- □ Nee

38. Gebruikt u hormoon vervangende middelen? Zo ja, welke medicatie gebruikt u?
- □ Ja, nl. ……
- □ Nee

- □ Invullen vragenlijst 2
- □ Stroop / Serial Subtraction

Bloeddruk	SBP	DBP	HR		Tijd (uu:mm)
Rust 1				**Start vragenlijst 2**	
Rust 2				**Eind vragenlijst 2**	
Stroop				**Start Stroop**	
SS				**Eind SS**	

Toelichten

- □ Folder overheid
- □ Dagboekje
- □ Retour sturen spullen

Meenemen

- □ Getekende Informed Consent
- □ Cortisol samples 1 en 2
- □ Instructie speekselverzameling met ingevulde tijden van verzameling 1 en 2
- □ Vragenlijst 1 (met supplement) en 2
- □ Handtekening op formulier proefpersoonvergoeding

Opmerkingen

Stroop
Volgorde van de juiste kleuren

Nr	Kleur	Fout
1	Blauw	
2	Geel	
3	Groen	
4	Geel	
5	Rood	
6	Blauw	
7	Rood	
8	Geel	
9	Rood	
10	Blauw	
11	Groen	
12	Geel	
13	Groen	
14	Groen	
15	Rood	
16	Groen	
17	Blauw	
18	Groen	
19	Geel	
20	Geel	
21	Rood	
22	Rood	
23	Geel	
24	Blauw	
25	Groen	
26	Rood	
27	Blauw	
28	Groen	
29	Rood	
30	Geel	
31	Groen	
32	Groen	
33	Geel	
34	Groen	
35	Geel	
36	Blauw	
37	Blauw	
38	**Groen**	
39	Rood	
40	Geel	
41	Rood	
42	Groen	
43	Geel	
44	Rood	
45	Groen	
46	Rood	
47	Blauw	
48	Groen	
49	Blauw	
50	Groen	
51	Geel	
52	Groen	
53	Groen	
54	Groen	
55	Rood	
56	Geel	
57	Groen	
58	Groen	
59	Geel	
60	Groen	
61	Geel	
62	Geel	
63	Groen	
64	Geel	
65	Rood	
66	Blauw	
67	Rood	
68	Geel	
69	Rood	
70	Blauw	
71	Groen	
72	Geel	
73	Groen	
74	Groen	
75	Blauw	
76	Geel	
77	Groen	
78	Geel	
79	Rood	
80	Blauw	
81	Rood	
82	Geel	
83	Rood	
84	Blauw	
85	Groen	
86	Geel	
87	Groen	
88	Groen	
89	Rood	
90	Groen	
91	Blauw	
92	Groen	
93	Geel	
94	Geel	
95	Rood	
96	Rood	
97	Geel	
98	Blauw	
99	Groen	

Aantal fouten: .....

Serial Subtraction
2 min terugtellen in stappen van 7 vanaf 1263

Nr	Count	Fout
1	1256	
2	1249	
3	1242	
4	1235	
5	1228	
6	1221	
7	1214	
8	1207	
9	1200	
10	1193	
11	1186	
12	1179	
13	1172	
14	1165	
15	1158	
16	1151	
17	1144	
18	1137	
19	1130	
20	1123	
21	1116	
22	1109	
23	1102	
24	1095	
25	1088	
26	1081	
27	1074	
28	1067	
29	1060	
30	1053	
31	1046	
32	1039	
33	1032	
34	1025	
35	1018	
36	1011	
37	1004	
38	997	
39	990	
40	983	
41	976	
42	969	
43	962	
44	955	
45	948	
46	941	
47	934	
48	927	
49	920	
50	913	
51	906	
52	899	
53	892	
54	885	
55	878	
56	871	
57	864	
58	857	
59	850	
60	843	
61	836	
62	829	
63	822	
64	815	
65	808	
66	801	
67	794	
68	787	
69	780	
70	773	
71	766	
72	759	
73	752	
74	745	
75	738	
76	731	
77	724	
78	717	
79	710	
80	703	
81	696	
82	689	
83	682	
84	675	
85	668	
86	661	
87	654	
88	647	
89	640	
90	633	
91	626	
92	619	
93	612	
94	605	
95	598	

Aantal fouten: .....

Appendix VI

Activity diary

Dagboekje

Familieonderzoek naar lichamelijke en geestelijke gezondheid

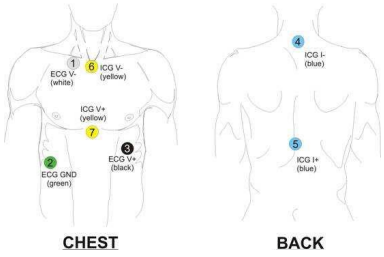
VRIJE UNIVERSITEIT
NEDERLANDS TWEELINGEN REGISTER

STICKER

1. Wat wordt er van u verwacht?

De hartactiemeter

U krijgt de meter 's ochtends aan het begin van de (werk)dag bevestigd. Als de meter eenmaal gestart is, hoeft u er verder niets aan te doen; u kunt uw dagelijkse activiteiten gewoon verrichten. Graag zouden wij zien dat u gedurende de meetdag geen extreem fysieke inspanning verricht en niet teveel alcohol drinkt. Gelieve de hartactiemeter 24 uur, dag en nacht, om te houden. Wanneer de hartactiemeter is aangesloten, knippert er een groen lampje.
Als het lampje langzaam knippert (2-3 maal per 10 seconden), is alles in orde en wordt de hartactie gemeten. Als het lampje snel knippert, is dit meestal een teken dat een van de elektrodes heeft losgelaten. In dat geval, willen wij u vragen de elektrodes te controleren en opnieuw vast te maken.



Invullen van het activiteitendagboek

De gemeten biologische signalen (zoals de hartslag) worden beïnvloed door lichamelijke activiteit en lichaamshouding. Om de gegevens goed te kunnen interpreteren is het noodzakelijk een nauwkeurig beeld te hebben van uw activiteiten. Daarom vragen we u tijdens de ambulante metingen ***elk uur*** een activiteitendagboek in te vullen.

1. U vult eerst in het dagboekje de begin- en eindtijd in.
2. Vervolgens vult u, op volgorde van tijd, in wat u sinds de vorige keer hebt gedaan. Van belang hierbij is dat u naast het soort activiteit ook de verandering in houding vermeldt. Als u bijvoorbeeld eerst aan tafel hebt gezeten en vervolgens een tijdje hebt gestaan, schrijft u dit dan op. Ook willen wij graag weten of u in gezelschap van mensen was of alleen. Probeer het dagboek elk uur in te vullen.

Voorbeeld dagboekje

Tijd **Van** *7* **uur** *15* **min** **Tot** *8* **uur** *15* **min**	**Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)**

Kastje werd omgehangen, gestaan en gegeten (thuis). Onderzoeker was 7.30 weg, rondgelopen, ontbijt klaargezet. Alleen ontbeten, zittend (thuis). Daarna beetje opgeruimd en katten eten gegeven. Om 8 uur naar buiten met vuilnis (lopen en staan). Daarna ongeveer 20 minuten naar het werk gefietst.

Tijd Van *8* uur *15* min Tot *9* uur *15* min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)

Aangekomen op werk, computer aan gezet (zittend), koffie gehaald (gelopen), gekletst met collega's (staand). 9.00 uur aan het werk. PC werk (alleen) zittend.

Tijd Van *9* uur *15* min Tot *10* uur *15* min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)

Vergadering met collega's zittend, hevige discussies (op werk). Daarna zitten achter PC (alleen). 2 keer opgestaan om naar printer te lopen

2. Dag 1	
Ochtend	**Ontbijt … uur … min**
Tijd **Van ….. uur …min** **Tot ….. uur …min**	**Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)**
Tijd Van ….. uur …min Tot ….. uur …min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)
Tijd Van ….. uur …min Tot ….. uur …min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)

Tijd **Van** **….. uur …min** **Tot** **….. uur …min**	**Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)**
Tijd Van ….. uur …min Tot ….. uur …min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)
Tijd Van ….. uur …min Tot ….. uur …min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)

Middag	**Lunch … uur … min**
Tijd **Van ….. uur …min** **Tot ….. uur …min**	**Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)**
Tijd Van ….. uur …min Tot ….. uur …min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)
Tijd Van ….. uur …min Tot ….. uur …min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)

Tijd **Van ….. uur …min** **Tot ….. uur …min**	**Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)**
Tijd Van ….. uur …min Tot ….. uur …min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)
Tijd Van ….. uur …min Tot ….. uur …min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)

Avond	**Avondeten … uur … min**
Tijd **Van ….. uur …min** **Tot ….. uur …min**	**Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)**
Tijd Van ….. uur …min Tot ….. uur …min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)
Tijd Van ….. uur …min Tot ….. uur …min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)

Vanavond om 22.30uur, of daarvoor als u eerder gaat slapen, gaat u weer speeksel verzamelen. Wilt u ervoor zorgen dat u een half uur van tevoren niets meer eet of drinkt (behalve een glas water)? Vergeet u niet in te vullen hoe laat u het speeksel verzamelt?

Tijd **Van ….. uur …min** **Tot ….. uur …min**	**Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)**
Tijd Van ….. uur …min Tot ….. uur …min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)
Tijd Van ….. uur …min Tot ….. uur …min	Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner, etc.), locatie (werk, thuis, etc.)

Speekselverzameling 3 (3. Avond)	**22.30 uur òf voor het slapen gaan als dit eerder is**	**Werkelijke tijd:** **….. uur ….. min**

Wilt u voor u gaat slapen nog de POMS stemmingsvragenlijst op de volgende pagina invullen?

En denkt u er aan de 2 laatste buisjes voor de speekselverzameling alvast op uw nachtkastje klaar te leggen voor morgenvroeg, als u wakker wordt?

POMS Stemmingsvragenlijst

De woorden in de lijst hieronder beschrijven stemmingen of gevoelstoestanden. Lees ieder woord nauwkeurig en kruis dan aan ***hoe u zich vandaag voelde***. Denk niet te lang na over uw antwoord. Het gaat om uw eerste indruk. Er bestaan geen foute antwoorden. Elk antwoord is goed, als het maar uw eigen stemming weergeeft. Sla geen woord over.

	Helemaal niet	Een beetje	Enigszins	Nogal	Heel erg		Helemaal niet	Een beetje	Enigszins	Nogal	Heel erg
1. Neerslachtig	□	□	□	□	□	20. Ongelukkig	□	□	□	□	□
2. Slecht gehumeurd	□	□	□	□	□	21. Woedend	□	□	□	□	□
3. Uitgeput	□	□	□	□	□	22. Lusteloos	□	□	□	□	□
4. Actief	□	□	□	□	□	23. Vol energie	□	□	□	□	□
5. Zenuwachtig	□	□	□	□	□	24. Rusteloos	□	□	□	□	□
6. Hulpeloos	□	□	□	□	□	25. Onwaardig	□	□	□	□	□
7. Geërgerd	□	□	□	□	□	26. Knorrig	□	□	□	□	□
8. Helder	□	□	□	□	□	27. Doodop	□	□	□	□	□
9. Paniekerig	□	□	□	□	□	28. Schuldig	□	□	□	□	□
10. Droevig	□	□	□	□	□	29. Opgeruimd	□	□	□	□	□
11. Vriendelijk	□	□	□	□	□	30. Angstig	□	□	□	□	□
12. Opstandig	□	□	□	□	□	31. Droefgeestig	□	□	□	□	□
13. Vermoeid	□	□	□	□	□	32. Kwaad	□	□	□	□	□
14. Levendig	□	□	□	□	□	33. Afgemat	□	□	□	□	□
15. Gespannen	□	□	□	□	□	34. Onzeker	□	□	□	□	□
16. Eenzaam	□	□	□	□	□	35. Wanhopig	□	□	□	□	□
17. Bezorgd	□	□	□	□	□	36. Behulpzaam	□	□	□	□	□
18. Verbitterd	□	□	□	□	□	37. Ontmoedigd	□	□	□	□	□
19. Aan het eind van mijn krachten	□	□	□	□	□	38. Mopperend	□	□	□	□	□

3. Dag 2

Ochtend

Wilt u als u wakker wordt, terwijl u nog in bed ligt, speekselverzameling 4 doen? En vult u dan in onderstaand schema in hoe laat u dit heeft gedaan?

Speekselverzameling 4 (4. Ontwaken)	**Wakker worden (in bed)**	**Werkelijke tijd:** **….. uur ….. min**

Om de hartactiemeter uit te zetten, houdt u het knopje bovenaan het kastje 5 seconden ingedrukt. Daarna mag u de hartactiemeter afdoen en de batterijen er uit halen. Over 30 minuten vragen we u voor de laatste keer speeksel te verzamelen. Denkt u er aan dat u in de tussentijd niets eet of drinkt (behalve een glas water)? Wacht u alstublieft ook nog even met het poetsen van uw tanden? U mag ondertussen de laatste vragen invullen. Ze staan hieronder.

Slaapkwaliteit

Hieronder volgen 15 uitspraken over de kwaliteit van uw slaap. Het gaat er om dat u aangeeft of de uitspraak van toepassing is op uw slaap ***zoals die de afgelopen nacht was***. De uitspraken lijken soms op elkaar, maar zijn nooit hetzelfde. Beantwoord alstublieft elke vraag.

	Ja	**Nee**
1. Ik heb geen oog dicht gedaan	□	□
2. Ik had, nadat ik wakker geworden was, moeite weer in slaap te vallen	□	□
3. Ik ben tijdens de slaapperiode opgestaan	□	□
4. Ik vind dat ik heel slecht geslapen heb	□	□
5. Ik sliep makkelijk in	□	□
6. Ik sliep niet langer dan 5 uur	□	□
7. Ik lag langer dan een half uur wakker	□	□
8. Ik ben meerdere malen wakker geworden	□	□
9. Ik lag erg te woelen	□	□
10. Ik vind dat ik goed geslapen heb	□	□
11. Ik heb naar mijn gevoel maar een paar uur geslapen	□	□
12. Ik had, nadat ik was opgestaan, een moe gevoel	□	□
13. Ik ben naar mijn gevoel slaap tekort gekomen	□	□
14. Ik voelde me, nadat ik was opgestaan, goed uitgerust	□	□
15. Heeft u vannacht slechter geslapen als gevolg van de hartslagmetingen?	□	□
16. Hoe laat ging u naar bed?	uur min	
17. Hoe laat stond u op?	uur min	

Speekselverzameling 5 (5. Ontwaken + 30)	**30 min na het wakker worden**	**Werkelijke tijd:** **….. uur ….. min**

Ontbijt … uur … min

4. Einde van het onderzoek

Het onderzoek is afgelopen! Wij willen u zeer hartelijk bedanken voor uw medewerking en inzet. Als laatste willen wij u vragen onderstaande spullen naar ons terug te sturen. U heeft daarvoor een grote bubbeltjes-envelop gekregen waar het goede adres al op staat. Let u goed op dat u alles in de envelop doet:

- Hartactiemeter
- Elektroden
- Beschermhoes hartactiemeter + draagriem
- Batterijen
- 3 buisjes met wattenrolletjes van de speekselverzameling
- Dagboekje

Als u de vragenlijsten nog niet heeft meegegeven aan de onderzoeker:

- Vragenlijst 1
- Vragenlijst 2

Een postzegel plakken is niet nodig, dit betaalt de VU (u stuurt de spullen terug naar ons antwoordnummer). Helaas is de envelop net wat te dik om door de brievenbus te kunnen. Wij willen u daarom vriendelijk verzoeken deze:

- Af te geven / in de postbak te deponeren bij een postkantoor bij u in de buurt
- TNT Post is tegenwoordig ook steeds vaker gevestigd in winkels als supermarkten, boekhandels, tabakszaken en drogisterijen. Vaak kunt u ook daar een postbak of -zak vinden waar u grotere pakketten kunt posten
- Indien mogelijk, kunt u de envelop afgeven bij de postafdeling van uw werk

U krijgt van ons binnen enkele weken, nadat wij uw gegevens hebben verwerkt, een uitdraai van uw hartslag en bloeddruk thuisgestuurd. Als dank voor uw deelname, sturen wij een cadeaubon van EUR 10,- mee.

Als u nog vragen of opmerkingen heeft, kunt u deze op de volgende pagina kwijt.

Naam onderzoeker / contactpersoon: .....

Telefoonnummer: 020-5982537 òf 06-50698826

Appendix VII

Thank you letter and annotated review of the recording

VU VRIJE UNIVERSITEIT AMSTERDAM

NEDERLANDS TWEELINGEN REGISTER (NTR)

DATUM	ONS KENMERK	UW BRIEF VAN	UW KENMERK
	NETAMB3-062010/b5		

E-MAIL	TELEFOON	FAX	BIJLAGE(N)
m.neijts@vu.nl	020 5982537	020 598 8832	uitdraai hartslag cadeaubon

Geachte heer , mevrouw,

Wij zijn onlangs bij u op bezoek geweest in het kader van het familieonderzoek naar lichamelijke en geestelijke gezondheid. Tijdens dat bezoek hebben wij uw bloeddruk gemeten en kreeg u een hartactiemeter om, die u tot de volgende ochtend omhield. Zoals beloofd ontvangt u van ons hierbij een uitdraai van uw resultaten met een korte toelichting.

Als blijk van dank voor uw deelname voegen wij tevens een cadeaubon van EUR 10,- bij.

Via de NTR website wwww.tweelingenregister.org kunt u op de hoogte blijven van de ontwikkelingen binnen dit onderzoek. De knop “Onderzoek” aan de linkerkant van de site brengt u op een pagina waarop ook dit onderzoek vermeld wordt.

Wij willen u bij deze hartelijk danken voor uw bijdrage aan het onderzoek van het Nederlands Tweelingen Register.

Met vriendelijke groet, namens het onderzoeksteam,

Drs. Melanie Neijts
Prof. dr. Eco de Geus
Dr. Gonneke Willemsen

FACULTEIT DER PSYCHOLOGIE EN PEDAGOGIEK
Biologische Psychologie

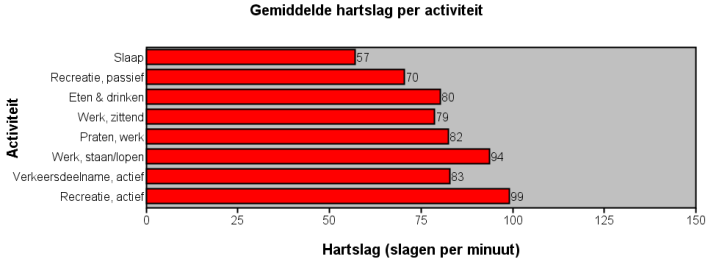
WWW.FPP.VU.NL

BEZOEKADRES
Van der Boechorststraat 1
Kamer 2B-35
1081 BT Amsterdam

POSTADRES
Van der Boechorststraat 1
1081 BT Amsterdam

Uw persoonlijke gegevens

De hartactiemeter die u tijdens ons bezoek om kreeg en dat u tot de volgende ochtend omhield, registreerde ondermeer uw hartslag en de hoeveelheid lichaamsbeweging.
Door de bewegingsregistratie te combineren met de gegevens uit uw dagboekje konden we de gemiddelde hartslag tijdens verschillende bezigheden bepalen. Hieronder ziet u deze gegevens weergegeven. Aan de linkerkant ziet u de verschillende bezigheden gedurende de meetperiode. In het balkje daarnaast ziet u uw gemiddelde hartslag voor iedere bezigheid. Deze wordt tevens aangegeven door de lengte van de balk.



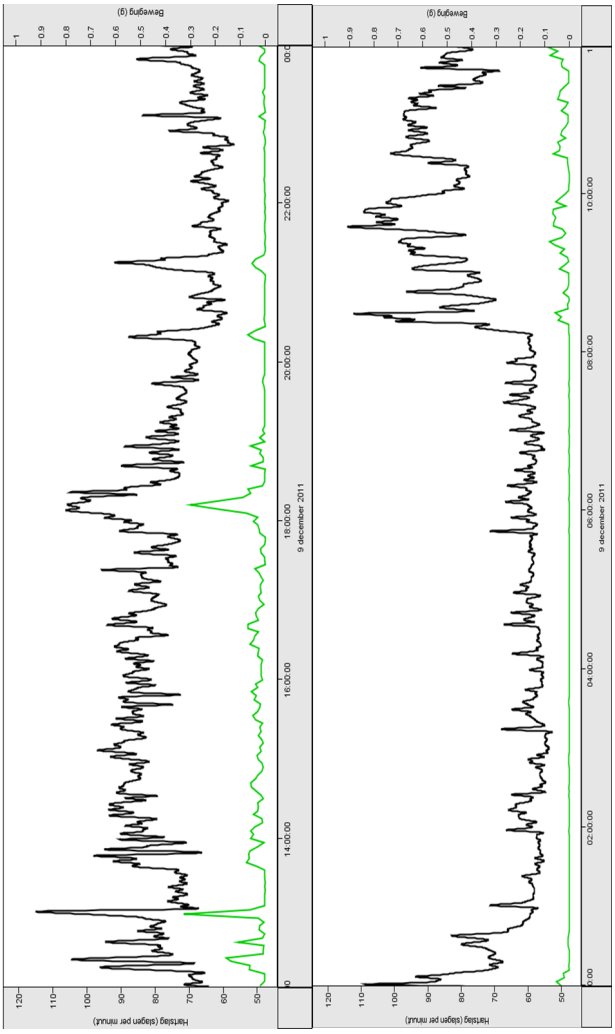
Een uitleg van de categorieën kunt u vinden op onze website www.tweelingenregister.org door via de knop 'Onderzoek' aan de linkerkant naar de lijst met NTR onderzoeken te gaan. Onder 'TITEL ONDERZOEK' ziet u deze studie, 'Familieonderzoek naar lichamelijke en geestelijke gezondheid,' staan.

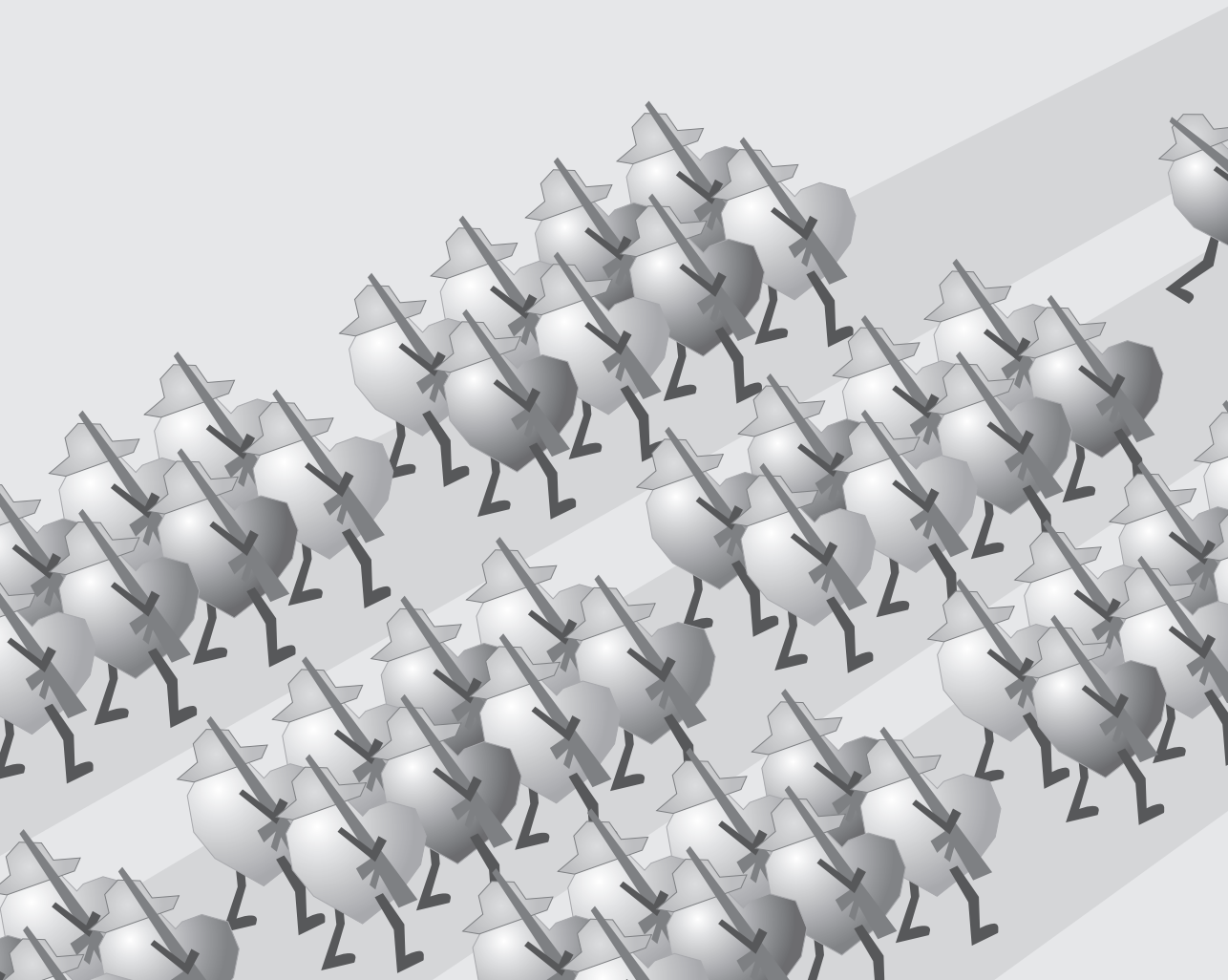
Op de achterzijde vindt u een grafiek die uw hartslag en beweging weergeeft over de gehele periode dat u de hartactiemeter om had. Bovenaan in de figuur staan de gegevens voor de eerste helft van de meetperiode, onderaan de gegevens voor de tweede helft van de meetperiode.

Naast de hartactiviteit hebben wij tijdens het bezoek een aantal keer uw bloeddruk gemeten. Bloeddruk wordt altijd weergegeven door twee waarden: de bovendruk en de onderdruk. Deze waarden worden uitgedrukt in millimeters kwikdruk (mm Hg).

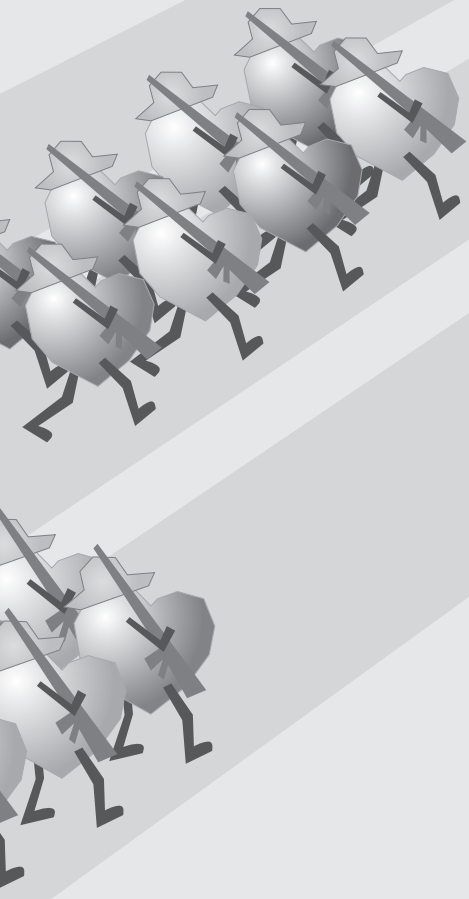
Uw gemiddelde bloeddruk over de metingen was **... /... mm Hg**.

Een bloeddruk wordt als verhoogd beschouwd als de bovendruk hoger is dan 160 mm Hg en/of de onderdruk hoger is dan 95 mm Hg.


Beweging (g)
Hartslag (slagen per minuut)
9 december 2011
Beweging (g)
Hartslag (slagen per minuut)
9 december 2011

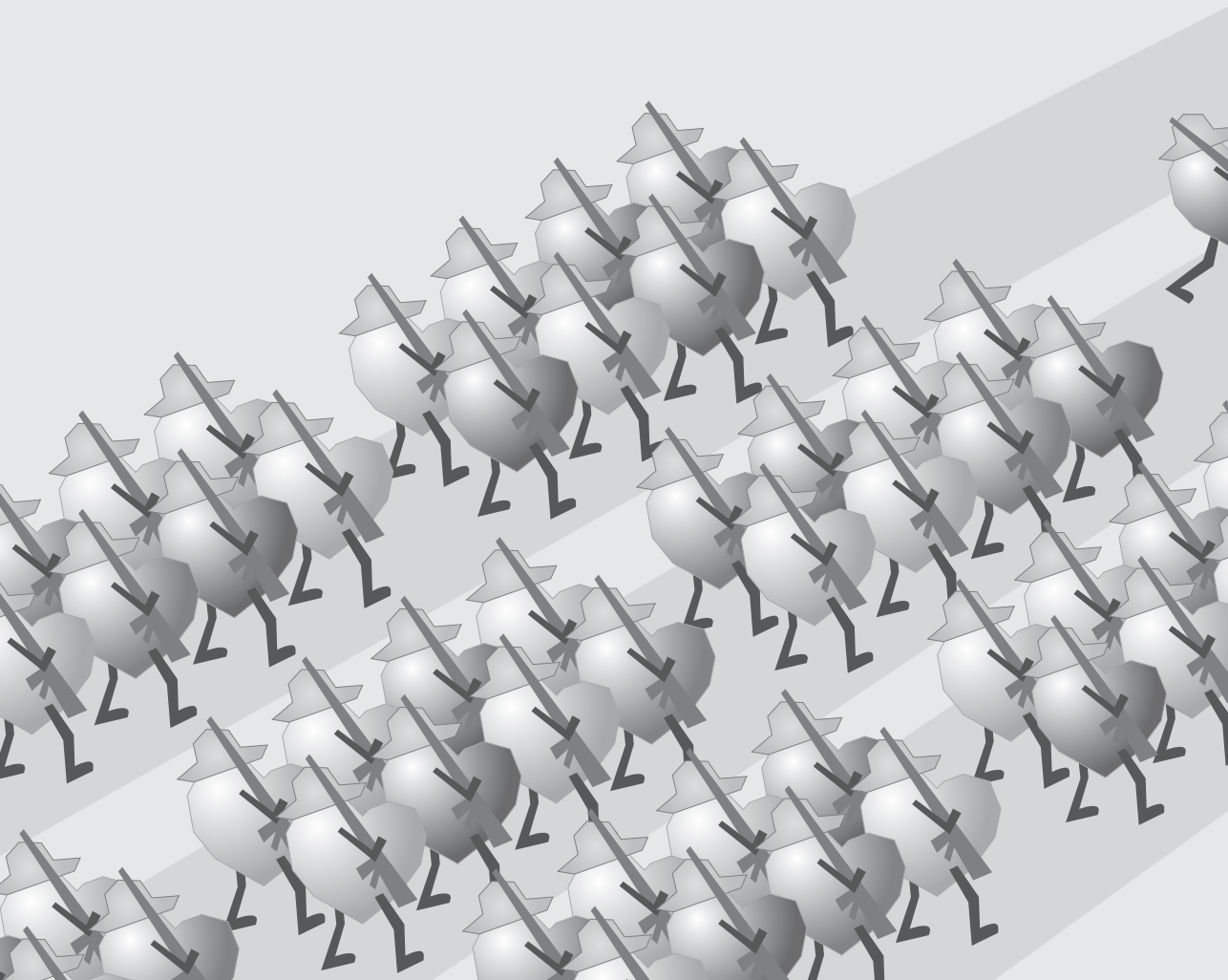


List of abbreviations

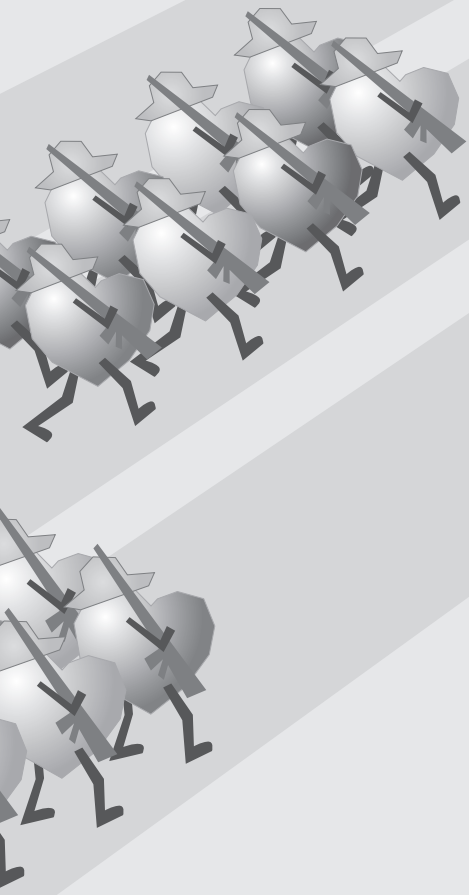


-2LL	Minus twice the logarithm of the likelihood
ACh	Acetylcholine
AIC	Akaike's information criterion
ANS	Autonomic nervous system
BMI	Body mass index
BRS	Baroreflex sensitivity
CN	Cranial nerve
CRP	C-reactive protein
CVD	Cardiovascular disease
DOS	Dizygotic opposite-sex
DZ	Dizygotic
dZ/dt	Change in thoracic impedance
E	Epinephrine
ECG	Electrocardiogram
EF	Ejection fraction
GSKL	Groningse slaap kwaliteit lijst
GWAS	Genome wide association study
HDL-cholesterol	High-density lipoprotein cholesterol
HF	High frequency
HR	Heart rate
HRV	Heart rate variability
IBI	Interbeat interval
ICG	Impedance cardiogram
IML	Intermediolateral
IL-6	Interleukin-6
LDL-cholesterol	Low-density lipoprotein cholesterol
LF	Low frequency
LF/HF	The ratio of low to high frequency power
MSNA	Muscle sympathetic nerve activity
MZ	Monozygotic
NE	Norepinephrine
nsSCR	Nonspecific skin conductance responses
NTR	Netherlands twin register
NTS	Nucleus of the solitary tract

PEP	Pre-ejection period
POMS	Profile of mood states
PNS	Parasympathetic nervous system
pvRSA	Respiratory sinus arrhythmia derived by peak-valley estimation
RMSSD	Root mean square of differences between valid, successive interbeat intervals
RSA	Respiratory sinus arrhythmia
RVLM	Rostral ventrolateral medulla
SA	Sinoatrial
SBP	Systolic blood pressure
SCL	Skin conductance level
SCR	Skin conductance response
SDNN	Standard deviation of all valid interbeat intervals
SEM	Structural equation modeling
SES	Socioeconomic status
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
SVR	Systemic Vascular Resistance
TNF-α	Tumor necrosis factor-alpha
TP	Total power
TWA	T-wave amplitude
ULF	Ultra low frequency
VLF	Very low frequency
VU-AMS	VU university ambulatory monitoring system
Z	Thoracic impedance



List of publications



De Geus, E.J.C, van Lien, R., **Neijts, M.**, & Willemsen, G. (2015). Genetics of Autonomic Nervous System Activity (pp. 357-390). In: Canli, T. (ed), *The Oxford Handbook of Molecular Psychology*, Oxford University Press: London.

Hodkinson, E. C., **Neijts, M.**, Sadrieh, A., Baumert, M., Rajesh, N., Subbiah, R. N., Hayward, C. S., Boomsma, D. I., Willemsen, G., Vandenberg, J. I., Hill, A. P., & de Geus, E. J. C. A novel approach to Holter data analysis improves phenotyping and increases the precision of genetic analysis of ECG biomarkers. *Under revision*.

Neijts, M., van Dongen, J., Kluft, C., Boomsma, D. I., Willemsen, G., & de Geus, E. J. C. (2013). Genetic architecture of the pro-inflammatory state in an extended twin-family design. *Twin Research and Human genetics*, *16*, 931-940.

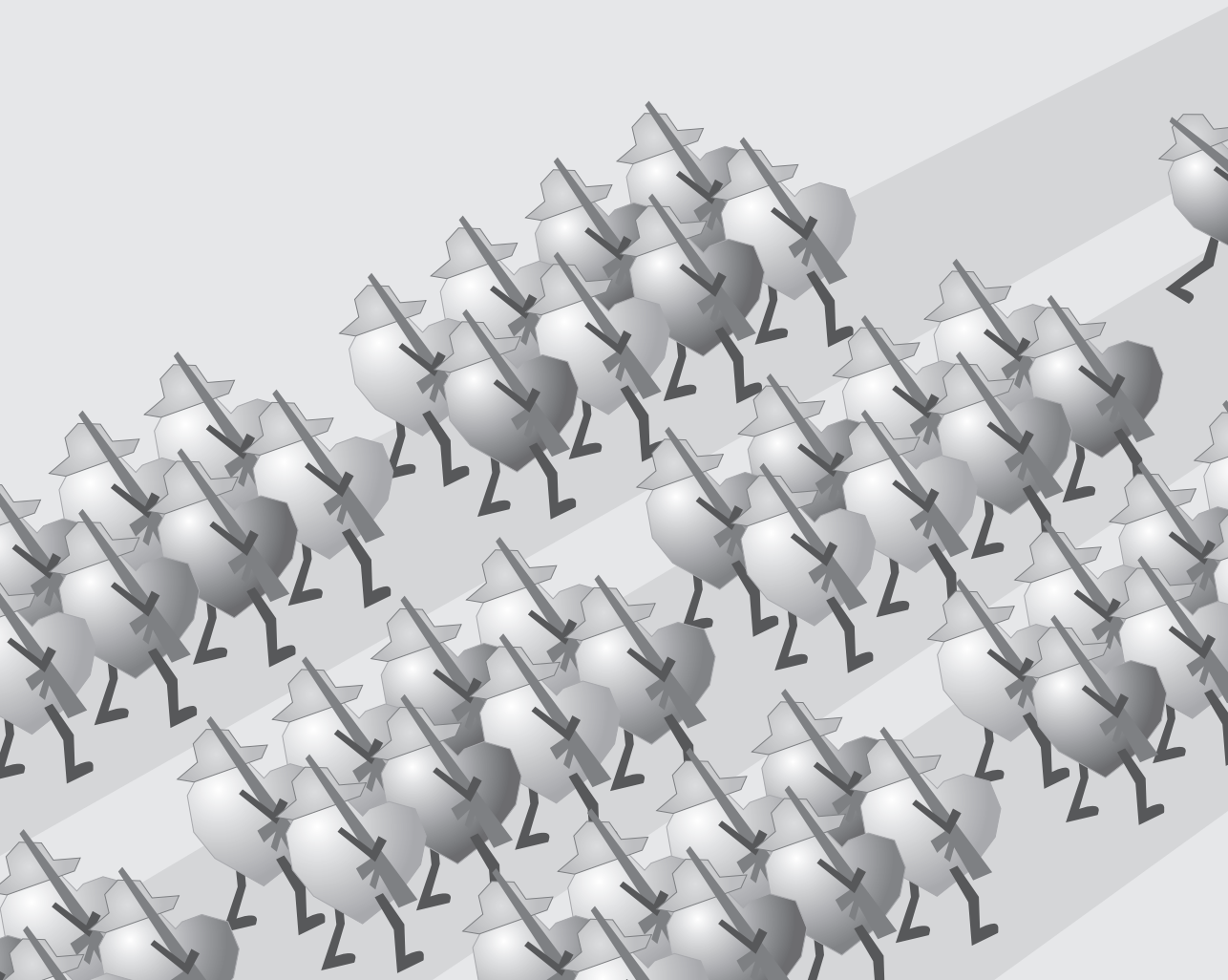
Neijts, M., van Lien, R., Kupper, N., Boomsma, D.I., Willemsen, G., & de Geus, E. J. C. Heritability and temporal stability of ambulatory autonomic stress reactivity in unstructured 24-h recordings. *Accepted for publication in Psychosomatic Medicine*.

Neijts, M., van Lien, R., Kupper, N., Boomsma, D.I., Willemsen, G., & de Geus, E.J.C. (2014). Heritability of cardiac vagal control in 24-h heart rate variability recordings: Influence of Ceiling effects at low heart rates. *Psychophysiology*, *51*, 1023-1036.

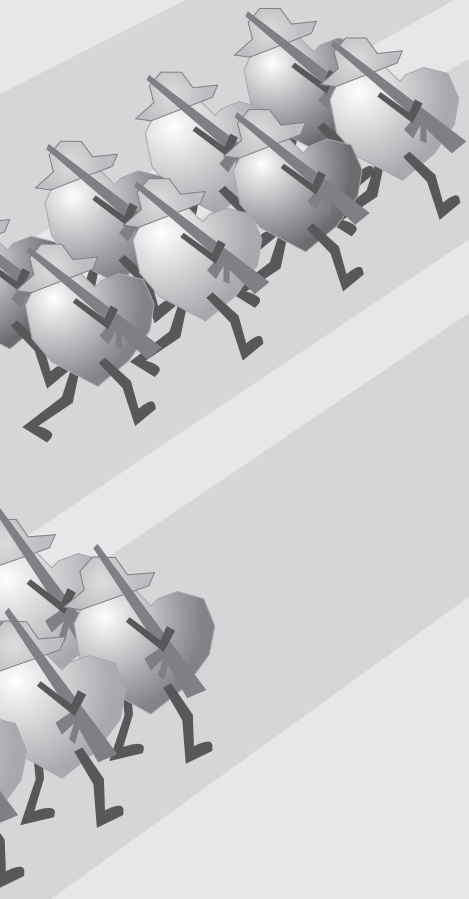
Neijts, M., Willemsen, G., Kluft, C., van Lien, R., Boomsma, D.I., & de Geus, E.J.C. Reciprocal association between ambulatory autonomic nervous system functioning and pro-inflammatory and metabolic risk profiles. *Submitted.*

Van Lien, R., **Neijts, M.**, Willemsen, G., & de Geus, E.J.C. (2015). Ambulatory measurement of the ECG T-wave amplitude. *Psychophysiology*, *52*, 225-237.

Willemsen, G., Vink, J. M., Abdellaoui, A., den Braber, A., van Beek, J. H. D. A., Draisma, H. H. M., van Dongen, J., van 't Ent, D., Geels, L. M., van Lien, R., Ligthart, L., Kattenberg, M., Mbarek, H., de Moor, M. H. M., **Neijts, M.**, Pool, R., Stroo, N., Kluft, C., Suchiman, E. D., Slagboom, P.E., de Geus, E. J. C., & Boomsma, D. I. (2013). The adult Netherlands twin register: twenty five years of survey and biological data collection. *Twin Research and Human genetics, 16,* 271-281.



Dankwoord



Klaar! Althans, voor zover dit mogelijk is binnen de zich voortdurend aan ontwikkeling onderhevig zijnde wetenschap. Maar, dit project is nu afgesloten en wat was het een groot project en wat waren er veel mensen bij betrokken! Graag zou ik dit proefschrift dan ook willen besluiten de mensen te bedanken die mee hebben geholpen aan de totstandkoming ervan.

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Ik wil de leden van de leescommissie bedanken voor het lezen en beoordelen van mijn proefschrift: prof. dr. Huizink, dr. Ijzerman, dr. Kupper, prof. dr. Snieder, en dr. Tiemeier.

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voor Psychophysiology te assisteren. Er was altijd leven in de brouwerij en we hebben samen heel wat afgelachen. Dit stokje werd vorig jaar overgedragen aan Ineke. Het is bewonderenswaardig met hoeveel gemak jij dit overgenomen hebt, naast dat je het zo druk hebt met de dataverzameling voor je eigen project. Respect!

Ik wil graag nog bedanken mijn onderzoeksassistenten Lena en Rianne; wat waren we een goed team en wat hebben we samen veel meegemaakt in die twee jaar dataverzameling. Door weer en wind en in alle vroegte hebben we bijna 600 huisbezoeken afgelegd, gewapend met een tray red bull, flessen bar le duc citroen en een doos napoleons. Ook wil ik graag alle stagiaires bedanken die mee hebben geholpen aan dit project: Amber, Elisabeth, Eline, Hester, Kirsten en Naomi. Door jullie inzet hebben we nog even extra gas kunnen geven.

De TD: Cor, Evert, Jarik, Rob, Daan en Menaka. Ik weet niet wat ik zonder jullie had gemoeten. Jullie stonden altijd met en raad en daad voor ons klaar als we tegen software of hardware problemen aanliepen. Samenwerken met jullie was een feest door jullie enorme drive en gastvrijheid; ook de keukendeur stond altijd open voor een lekker kopje koffie met koek wat na die vroege ritten zeer welkom was! Rene, Marwin en Marco: bedankt voor jullie fantastische ICT support. Jullie kwamen altijd direct voor me in actie als er problemen waren, zelfs in de tijd van de rode computers. Klasse!

Jenny en Laili, wat ontzettend fijn dat jullie mijn paranimfen willen zijn. Jenny, jou heb ik leren kennen bij BioPsy. We zijn in eerste instantie min of meer meteen aan elkaar gekoppeld toen ik twee maanden na aanvang van mijn aanstelling een kamer in Boulder met je ging delen. Gelukkig bleek dat wel een goede match; ik heb je leren kennen als een lief en oprecht persoon, barstensvol met leuke ideeën. Ik vind het fijn dat jij mij bij wilt staan! Laili, wij kennen elkaar van onze studie Psychologie en hebben lief en leed met elkaar gedeeld. Ik vind het heel speciaal dat ik ook dit met jou kan delen. Dan wil ik nog bedanken mijn andere lieve Maastricht-vrienden Michiel, Casper en Tessa. Allemaal verschillende kanten op gegaan, letterlijk en figuurlijk, maar ik geniet nog steeds van onze get togethers. En natuurlijk Aafke, jouw steun betekent heel veel voor mij. Ik vind het heel bijzonder dat we zo veel levensfases later nog steeds zo betrokken zijn bij elkaar.

Als laatste mijn familie en in het bijzonder mijn vader Peter en broertje Bram. Dank voor jullie onvoorwaardelijke steun en geloof in mij. Mama, wat had ik dit graag ook met jou willen delen, ik weet zeker dat je het fantastisch had gevonden! En tot slot, Dennis.. ik ben heel blij dat ik jou heb leren kennen en wil je bedanken voor alle liefde, vertrouwen en steun. Ik hoop dat we samen nog veel mooie momenten gaan beleven!