Heritablity of cardiovascular risk factors in a real life setting

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HERITABILITY OF CARDIOVASCULAR RISK FACTORS IN A REAL LIFE SETTING

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. T. Sminia,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Psychologie en Pedagogiek
op woensdag 9 november 2005 om 15.45 uur
in het auditorium van de universiteit,
De Boelelaan 1105

door

Harmina Maria Kupper

geboren te Neede

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dr. H. Riese
dr. H. Snieder

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1

General introduction

Cardiovascular disease encompasses various pathologies in which the heart or the vasculature is inflicted, such as atherosclerosis, hypertension, myocardial infarction and heart failure. In the Netherlands, as in most westernized countries, cardiovascular disease is a leading cause of death in both women and men. In 2002, cardiovascular disease caused one third of all occurring deaths in the Netherlands (Koek, Leest, Verschuren, & Bots, 2003). The etiology of cardiovascular disease is complex, with many different factors contributing to an increased risk of developing cardiovascular disease (Brotman, Walker, Lauer, & O'Brien, 2005).

It is well-known that cardiovascular disease tends to run in families (Acton, Go, & Roseman, 2004; Rissanen, 1979; Deutscher, Ostrander, & Epstein, 1970). This familial influence on variation in cardiovascular physiology finds support in studies reporting that subjects with a parental history of CVD have an increased relative risk for developing CVD (Sesso et al., 2001), have higher blood pressure (van den Elzen et al., 2004) and demonstrate an enhanced stress response to laboratory stressors and a delayed recovery (Schneider, Jacobs, Gevirtz, & O'Connor, 2003), whereas subjects without a parental history do not. Familial influences can derive from the genetic resemblance between family members or from shared environmental influences. The importance of genetic factors in the familial clustering of cardiovascular disease is most clearly illustrated by findings from the Swedish twin register (Marenberg, Risch, Berkman, Floderus, & de Faire, 1994; Zdravkovic et al., 2004). They followed 21,004 twins, born between 1886 and 1925, for 26 years and showed that the probability of dying from coronary heart disease (CHD), when one member of the twin pair had died before the age of 55, was much higher among monozygotic twins compared to dizygotic twins. Heritability estimates for susceptibility to death from CHD in this sample were 57% and 38% for males and females, respectively.

In spite of this substantial heritability, finding the actual genes for cardiovascular disease has proven a very difficult task. It is increasingly appreciated that genetic epidemiological studies of complex diseases may benefit from the use of more narrowly defined risk factors -also called endophenotypes - over broadly defined disease phenotypes (Rice, Saccone, & Rasmussen, 2001). The model in figure 1.1 shows the biological pathways along which genes (here denoted as QTL for 'quantitative trait loci'), environmental history (indicated by ENV), and ongoing psychosocial stress (indicated by STRESS), work together to increase the risk to develop cardiovascular disease (CVD).

A substantial number of physiological risk factors for cardiovascular disease have been identified over the past decades that can be used as endophenotypes in the search for genes that influence the development of cardiovascular disease. Endophenotypes explain only a part of the disease risk, are situated more upstream towards the genetic sources, and are influenced by only a subset of genes relevant to the disease. The crucial characteristic of these genes is that they explain a larger part of the variance in the endophenotype than they do in the disease. Therefore, they are easier to find through the endophenotype than through the disease itself. Potential endophenotypes include among others reduced heart rate variability (Dekker et al., 2000; Mussalo et al., 2001; Huikuri et al., 2003), increased blood pressure (Pickering & Devereux, 1987; Verdecchia, Schillaci, Reboldi, Franklin, & Porcellati, 2001; Verdecchia, 2000), increased heart rate (Palatini & Julius, 2004), a shift from vagal to sympathetic activity (Arnlov, Ingelsson, Riserus, Andren, & Lind, 2004; Pellerin, Larrazet, Cohen, Witchitz, & Veyrat, 2003) and a dysfunctional HPA axis (Rosmond & Bjorntorp, 2000). Within the population, large individual differences in these physiological parameters exist (e.g. Cacioppo, Uchino, & Berntson, 1994b; Smyth et al., 1997; Ben Lamine et al.,

2004). Identifying the mechanisms that underlie these individual differences will contribute to a better understanding of the complex etiology of cardiovascular disease.

The main objective of this present thesis is to determine the relative contribution of genetic and environmental sources of variation to the individual differences in physiological risk factors for cardiovascular disease.

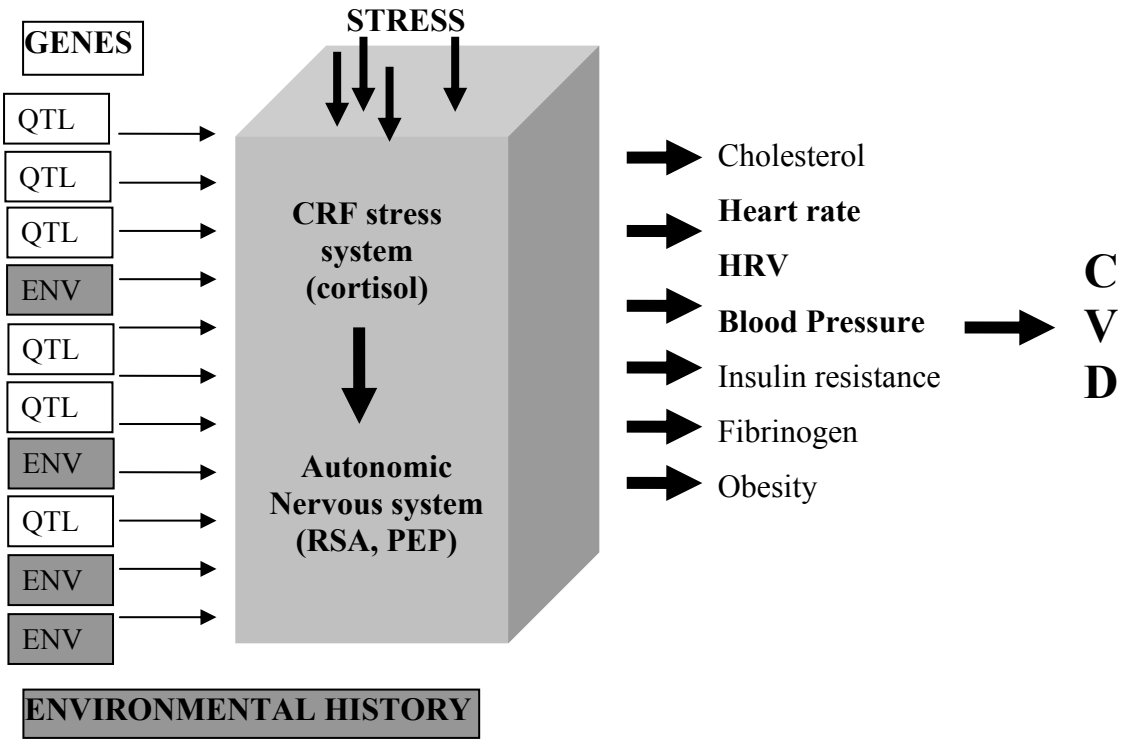


Figure 1.1 The endophenotype approach
CVD = cardiovascular disease, HRV = Heart rate variability, RSA = respiratory sinus arrhythmia, PEP = pre-ejection period, QTL = quantitative trait loci, ENV = environmental factors (e.g. prenatal, early childhood trauma, past major life events) STRESS = current psychosocial stressors (e.g. work stress, marital conflict, recent major life events).

Blood pressure

Hypertension, or an increased blood pressure, is a main risk factor for cardiovascular disease (Franklin et al., 2001; Verdecchia et al., 1998; Pickering & Devereux, 1987) that is linked to sympathetic hyperactivity (Mussalo et al., 2001). As arteries narrow, due to the build-up of atherosclerotic plaques, blood flow is restricted and blood pressure increases. With high blood pressure, the sympathetic stimulation of the heart increases, since the heart has to work harder to maintain normal circulation. This causes the heart muscle to grow, which is detrimental to proper blood flow. This downward spiral may result in heart failure and myocardial infarction.

A vast amount of twin and family studies have reported on the genetics of blood pressure and hypertension. Many laboratory studies have reported a substantial contribution of genes to the variation in blood pressure (e.g. McCaffery, Pogue-Geile, Debski, & Manuck, 1999; Vinck, Fagard, Loos, & Vlietinck, 2001; Evans et al., 2003; Snieder, Harshfield, &

Treiber, 2003; Fagard, Loos, Beunen, Derom, & Vlietinck, 2003), and have linked this variation to many regions on the genome, for example to variation in genes coding for proteins, enzymes and receptors that are associated with the renin-angiotensin aldosterone system (Brand-Herrmann et al., 2004; Castellano et al., 2003). In addition, it is well-known that environmental factors are important in the etiology of hypertension. For example, experiencing chronic stress (Matthews et al., 2004) or having increased central body fat (Allemann et al., 2001) (which actually may have both genetic and environmental origins, Cui, Hopper, & Harrap, 2002), are known to increase blood pressure, and to predict future hypertension. Only a few studies though, have based their genetic analyses on ambulatory blood pressure (Somes, Harshfield, Alpert, Goble, & Schieken, 1995; Fagard et al., 1995).

Heart rate and heart rate variability

Both a reduced heart rate variability and an elevated heart rate are caused by the withdrawal of parasympathetic control (Schwartz, et al., 1988; La Rovere, Bersano, Gnemmi, Specchia, & Schwartz, 2002) or increased sympathetic control over the heart (Palatini, & Julius, 2004) and both are independent predictors of cardiovascular morbidity and mortality (Bigger, Fleiss, Rolnitzky, & Steinman, 1993; Tsuji, et al., 1996; Dekker, et al., 1997; Nolan, J. et al., 1998; Dekker, et al., 2000; Huikuri, et al., 2003; Palatini, & Julius, 2004; Kannel, Kannel, Paffenbarger, & Cupples, 1987).

Heritability studies on resting heart rate have shown that genetic factors contribute significantly to the individual differences in heart rate (Singh, et al., 1999, Martin, 2004; Jedrusik et al., 2003). Additionally, linkage studies have identified significant regions of linkage in humans (Wilk et al., 2002; Martin et al., 2004), associated with the β1-adrenergic receptor and excitation-contraction coupling.

Genetic predisposition also plays a role in heart rate variability. Results of various studies in laboratory and clinical settings show heart rate variability to be moderately heritable (up to 39%) (Boomsma, van Baal, & Orlebeke, 1990; Sinnreich, Friedlander, Luria, Sapoznikov & Kark, 1999; Busjahn. et al., 1998; Snieder, et al., 1997; Singh, et al., 1999). Further evidence for a genetic modulation of heart rate variability comes from association studies. Heart rate variability was found to be associated with allelic variation in a common polymorphism of the gene coding for the angiotensin-converting enzyme (Thayer et al., 2003) and with variation in the acetylcholine transporter gene, that plays a role in parasympathetic acetylcholine neurotransmission (Neumann, Lawrence, Jennings, Ferrell, & Manuck, 2005).

At the same time environmental and lifestyle factors are also known to influence heart rate and heart rate variability. For example, exercise decreases heart rate and increases heart rate variability (Sandercock, Bromley, & Brodie, 2005), whereas low SES is associated with high heart rate and low heart rate variability (Steptoe A, Kunz-Ebrecht SR, Wright C et al. 2005).

Until now only short-term (< 2 hours) recordings of heart rate (variability) have been used in twin and family studies. It remains to be determined whether genetic influences play a similarly important role in heart rate and heart rate variability over prolonged periods, in a naturalistic setting.

Sympathetic control over cardiac contractility

The sympathetic nervous system plays an important role in regulating cardiac contractility (Kaye et al., 1995; Swedberg, Eneroth, Kjekshus, & Wilhelmsen, 1990). The innervation of the cardiac ventricles is almost completely of sympathetic origin (Vander, Sherman, & Luciano, 2001). Sympathetic control over contractility has been suggested to

play a vital role in the risk for left ventricular hypertrophy and heart failure (Rundqvist et al., 1997; Kaye et al., 1995; Swedberg et al., 1990). Specifically, a chronic state of sympathetic activity with chronically high contractility is thought to lead to functional down-regulation of myocardial β-receptors (El Armouche et al., 2003; Bogaert & Fraeyman, 1991; Andersson, 1986; Xiao et al., 1999). Only very few studies have reported on the genetics of left ventricular function Genetic association studies comparing the genotypes of patients with heart failure to those in non-(cardiac)patients have shown promising results for polymorphisms of the renin-angiotensin-aldosterone system and the sympathetic system (Bleumink et al., 2004). In twin and family studies the heritability estimates of echocardiographic measures of left ventricular function range between 26% and 52% (Bielen, Fagard, & Amery, 1991; Tang et al., 2000). Whole genome screens have putatively linked variation in left ventricular function to a region on chromosome 11 that codes for the cardiac myosin-binding protein (Arnett et al., 2001; Tang et al., 2002).

No studies to date have reported on the genetics of measures of sympathetic control over cardiac contractility based on impedance cardiography, like the Heather index or the pre-ejection period (PEP), although this method has been well-developed in the field of psychosomatic medicine (Sherwood et al., 1990).

Cortisol

Recently, the hypothalamus-pituitary-adrenal (HPA) axis has also been implicated in the etiology of cardiovascular disease. Cortisol, the end product of the HPA axis, is an important steroid hormone in the regulation of normal physiology. Continued or frequently repeated stressful events may deregulate HPA axis function and basal cortisol may be chronically secreted in excess. Prolonged glucocorticoid exposure may lead to hypertension (Mantero & Boscaro, 1992) and cardiovascular disease (Rosmond & Bjorntorp, 2000) because of the continued stimulation of sympathetic drive and the stimulation of the metabolism of fat cells. In addition, chronic secretion of cortisol may influence the many immunological parameters that play a role in the development of sclerotic plaques (Girod & Brotman, 2004).

Several smaller studies in adults (Wüst, Federenko, Hellhammer, & Kirschbaum, 2000a; Kirschbaum, Wüst, Faig, & Hellhammer, 1992; Young, Aggen, Prescott, & Kendler, 2000) and one large study in children (Bartels, de Geus, Kirschbaum, Sluyter, & Boomsma, 2003a) have investigated the role of genes in the determination of basal salivary cortisol levels. These studies report heritability estimates lying between 40 and 52% for several cortisol parameters. In children, the awakening period seems to be influenced most by genes. A meta-analysis performed by Bartels et al. (2003b), taking together all available cortisol studies in which a comparable design was used, found morning cortisol to be heritable for 62%. Confidence intervals of the estimate were large, however, and none of the adult samples had a sufficient size to detect small additive genetic or common environmental effects.

Ambulatory monitoring

To date, studies reporting on the impact of genetic factors on cardiovascular physiology have almost exclusively been based on laboratory data (Boomsma et al., 1990; Singh et al., 1999; Sinnreich et al., 1999; Snieder et al., 1997; Busjahn et al., 1998). Different genetic pathways may affect cardiovascular physiology assessed in short-term standardized laboratory conditions, however, compared to cardiovascular physiology recorded over prolonged periods of time in naturalistic settings. Yet, preciously little is known about the sources of individual differences in cardiovascular physiology in real life settings.

In the past decade, ambulatory monitoring has evolved from an innovative tool in fundamental research to a widely used method in fundamental as well as clinical and applied research settings. Ambulatory monitoring is a method of acquiring physiological data in subjects who are free to go about their normal daily activities, outside the confines of the laboratory or hospital environment. Since ambulatory monitoring takes place during everyday life, in the subject's own environment (naturalistic settings), such measurements have high ecological validity. A specific advantage of prolonged ambulatory monitoring over laboratory assessment of physiological parameters can be expected for the assessment of individual differences in reactivity to psychosocial stressors. In psychosomatic medicine, negative consequences of excessive reactivity are expected on cardiovascular health. Such consequences will derive from frequent exposure to realistic stressors, encountered repeatedly at home or in the work setting. It has been found that generalization of cardiovascular stress reactivity from standardized laboratory situations to actual real life situations is only moderate at best (Kamarck, Schwartz, Janicki, Shiffman, & Raynor, 2003; Doornen, Knol, Willemsen, & Geus, 1994; Gerin, Rosofsky, Pieper, & Pickering, 1994).

The advantage of ambulatory recording is not limited to stress effects. Measurement of resting baseline in the laboratory may suffer from the "white coat effect". This effect is often observed when blood pressure is measured in a hospital or laboratory setting. In these settings blood pressure is often higher than it would be when blood pressure was measured at home, because subjects tend to feel more anxious in the clinic or laboratory as compared to familiar surroundings. There is no reason to assume that this white coat effect would be limited to blood pressure only; more likely, it affects many other physiological measures as well. Directly assessing cardiovascular function in naturalistic settings, including leisure time at home and sleep, can circumvent the "white-coat" phenomenon.

Finally, and perhaps most convincingly, previous reports have suggested that ambulatory measures are better predictors for cardiovascular morbidity and mortality than laboratory or office measurements (Pickering & Devereux, 1987; Verdecchia et al., 1994; Verdecchia et al., 1998; Verdecchia, Schillaci, Reboldi, Franklin, & Porcellati, 2001) and it is likely that genetic studies based on ambulatory measurement of cardiovascular function can provide a more solid basis for future linkage and association studies.

This thesis

Twenty four hour ambulatory cardiovascular measurements were carried out in a large twin family population to estimate "ambulatory heritability" of three established risk factors (heart rate, heart rate variability and blood pressure). In addition, the present study examined the genetic contribution to ambulatory measures that reflect the activity of the three major components of autonomic function, the sympathetic and parasympathetic nervous system and the hypothalamus-pituitary-adrenal (HPA) axis.

Chapter 2 discusses the general design of the study, and will give details on the twin methodology. The advantages of the extension of the classical twin design by including the singleton siblings are discussed, including the possibility to examine a special "twin-environment". The ensuing chapters focus on the heritability of one or more of the selected physiological risk indicators for cardiovascular disease. Chapter 3 deals with the heritability of ambulatory blood pressure and focuses on the question whether exclusion of hypertensive subjects is detrimental to the estimates of heritability. Chapter 4 presents the results on the heritability of heart rate variability, using two widely used variables in cardiology, SDNN index and RMSSD. In chapter 5 the report on heart rate variability is continued by focusing on a measure that is more often used in a psychophysiological context, respiratory sinus

arrhythmia (RSA). In this chapter, a trivariate genetic analysis of respiration rate, RSA and heart period is presented. The heritability of sympathetic control of cardiac contractility is the topic of chapter 6. This study is the first to present heritability estimates for impedance-derived systolic time intervals and cardiac contractility.

In Chapter 7 the results on the heritability of basal diurnal salivary cortisol levels are presented. In the general discussion (Chapter 8), the results presented in the previous chapters are summarized and discussed in the light of existing literature.

2

General research design

The extended twin design

The purpose of the study described in this thesis was to investigate the relative influence of genetic and environmental factors on ambulatory measured physiological risk factors for cardiovascular disease. To this end, a large twin family population was approached. Comparing the similarity for a trait of identical, or monozygotic (MZ) twins (that share 100% of their segregating genes) with the similarity for that trait of fraternal, or dizygotic (DZ) twins (that share on average 50% of their segregating genes) renders information on the relative contribution of genes and environment to the variance in the trait (Falconer & Mackay, 1996). This thesis made use of an extended twin design, meaning that, in addition to the twin pair, singleton siblings were included. This adds three types of sibling pairs that share on average 50% of their segregating genes (like DZ twin pairs): an MZ twin with a singleton brother or sister, a DZ twin with a singleton brother or sister, a singleton sib with another singleton sib. The extended twin design has several advantages over a classical twin design that includes only identical and fraternal twins. Adding one or more singleton siblings increases statistical power to distinguish between genetic influences and common and unique environmental influences (Posthuma & Boomsma, 2000). Table 2.1 shows the number of families that participated, stratified by twin type and the number of additional siblings.

Table 2.1 Number of families with additional siblings

		Number of additional siblings							
		0	**1**	**2**	**3**	**4**	**5**	**6**	**Totals**
MZ	Twin pair	51	43	8	5	1	-	1	109
	Single twin	5	10	1	-	-	-	-	16
DZ	Twin pair	47	23	10	1	-	-	-	81
	Single twin	11	16	1	2	-	1	-	31
DOS	Twin pair	34	12	2	-	-	1	-	49
	Single twin	6	18	3	-	1	-	-	28
No twin			16	12	1	1	-	-	30
Total number of families		153	138	35	9	3	2	1	341

MZ = monozygotic twin, DZ = dizygotic twin, DOS = dizygotic twin of opposite sex.
In total this adds up to 816 participants eligible for the study.

When using an extended twin design however, one cannot blindly assume that means and variances of all variables are equal in singletons and twins. Twins weigh less at birth than their singleton siblings. The hypothesis that a baby with lower birth weight will have an increased risk for several chronic diseases, also called the "Barker effect of birth weight", has been confirmed in several twin and non-twin studies (Ijzerman, Stehouwer, & Boomsma, 2000; Phillips et al., 2000; Ijzerman, Stehouwer, van Weissenbruch, De Geus, & Boomsma, 2001; Fall et al., 1995; Massin, Withofs, Maeyns, & Ravet, 2001). If twins systematically differ from their singleton siblings, this would compromise the gain in power obtained by including singleton siblings in a twin family design. In that case, the parameter estimates for dizygotic twins could not be equated to those for singleton siblings. Although no twin-singleton differences were found for resting blood pressure (de Geus, Posthuma, Ijzerman, & Boomsma, 2001; Loos, Fagard, Beunen, Derom, & Vlietinck, 2001), it is not clear that such

differences will not be found in heart rate (variability) or autonomic nervous system activity. The extended twin design gives us the opportunity to specifically test the presence of twin-singleton differences and differences between the sexes for each variable measured in our twin family sample. When no differences are apparent, further genetic analyses are conducted with one correlation for the MZ group en one correlation for the DZ/sibling group.

Selection of participants

All participants were registered with the Netherlands Twin Register (NTR) and had participated in longitudinal surveys on health, lifestyle and personality. The entire study consisted of several phases: (1) the selection phase, (2) the DNA collection phase, and (3) the ambulatory monitoring phase. An overview of these phases is presented below in table 2.1.

In 1991, the NTR started a longitudinal survey study of health, lifestyle and personality among adolescent and adult twins and their family members. Addresses of twin families were obtained by asking Dutch city councils of 252 cities for addresses of twins aged 13 to 22 years. In addition, twins volunteered to register with the NTR. Every two to three years questionnaire booklets were sent out to all adolescent and adult twins and their family members who were registered with the NTR. Twins were always included, parents of twins were included in 1991, 1993 and 1995, and siblings were included from 1995 onward. The booklets contained questions on health (e.g. subjective health, presence of specific diseases) life style (e.g. smoking behavior, alcohol consumption), socio-economic status (SES), and personality (e.g. depression, anxiety scales). Depending on their year of entry in the study, participants were sent a minimum of one and a maximum of four questionnaire booklets between 1991 and 1997.

To select sibling pairs (fraternal twins, twin-sibling or sibling-sibling pairs) for a linkage study on the genetics of anxiety and depression (NETSAD), a genetic factor score of anxious depression was computed for each subject, based on a genetic multivariate analysis of the questionnaires assessing anxiety, somatic anxiety, depression, and neuroticism. This factor score gives an estimate of a subject's genetic vulnerability for anxious depression. The 12% concordant highest scoring sibling pairs and the 12% concordant lowest scoring sibling pairs in the anxious depression distribution were selected. In addition, discordant pairs were selected in which one member scored in the top 20% and the other in the bottom 20% of the distribution. If the selected sibling pairs had additional siblings with complete survey information, these were also included in the study. For a more extensive description of the selection procedure and the anxious depression factor score see Boomsma et al. (2000). Although part of the sample (at least two family members of each family) was originally selected based on extreme scores for anxiety and depression, the inclusion of additional siblings ensured that the distribution of anxious depression in the final sample was near normal, with only small kurtosis (Boomsma et al., 2000). Nonetheless, for each of the ambulatory physiological variables the relationship between the anxious depression factor score will be tested to determine whether the results were unbiased by this selection procedure.

Table 2.1 Selection of participants

Selection phase 1997	Questionnaire data N = 14.428	These subjects filled out biannual questionnaire at least once
	Selection of extreme scores on anxious depression N = 1717	**Selection criteria:** Anxious depression factor score. Selected were 12% concordant high or low scoring sibling pairs and 20% extremely discordant sibling pairs
DNA collection 1998	Response (returning buccal swab) 77.5% N = 1332 Successfully contacted (98 twice) N=1008	All members of the families of the selected sibling pairs were invited to take part in the DNA collection **Exclusions:** pregnancy (13), heart transplantation (-), pacemaker (1) and known ischemic heart disease (1), congestive heart failure (-), or diabetic neuropathy (-), presence of metal rods, screws or plates for repair of complicated bone fracture (1)
Ambulatory monitoring 1998-2003	Ambulatory monitoring, health interview Participation: 80.9% N = 816 68 subjects agreed to be tested twice	**Refusals:** 1st test: disease (2), foreign country (5), too demanding (174) Retest: pregnancy (1), foreign country (2), too demanding (27)

DNA collection

All selected participants and their parents received an invitation letter for the study and a DNA collection package. If they agreed to give a DNA sample, they were also asked to give informed consent. The buccal swabs were sent back in a prepaid envelope, using regular mail services. Next, the buccal swabs were sent to TNO (Leiden, the Netherlands) for DNA isolation.

NETAMB: Ambulatory monitoring

Of the 1332 twins and siblings that were selected and returned a DNA sample, 1008 were contacted for participation in the ambulatory monitoring study. Participants were excluded from the ambulatory recording selection in case of a pregnancy, a heart transplantation, presence of a pacemaker and known ischemic heart disease, congestive heart failure, or when subjects had metal rods in their body because of complicated bone fractures. Pregnant women were approached again half a year after delivering the baby. When refusing to participate, the reason most often stated was that participation seemed too demanding. Ambulatory monitoring took place in two data collection waves, the first taking place

between August 1998 and July 2000 and the second between October 2001 and June 2003. In total, 816 subjects were willing to participate, giving a response rate of 80.9%. These selected subjects received an invitation for the ambulatory measurements by mail, followed by a phone-call to make an appointment. They were asked to schedule the appointment on a representative (working) day. The participants were visited at home, before starting their normal daily activities. After providing informed consent, the cardiovascular monitoring devices were attached, and the procedures were explained. In addition, saliva sampling instructions were given. Instruction cards (see appendix III) for problem solving were available and telephone support could be reached during waking hours. A short health interview was held that contained questions on family history of cardiovascular disease, consumption behavior (i.e. cigarette, alcohol, and coffee consumption), exercise, health, medication, contraceptive medication, menstrual cycle, and anthropometrics. All measures taken during the visit and the ambulatory recording period are summarized in table 2.2. The total visit took about 45 minutes. The following day subjects were visited again to collect the equipment. Participants received their heart rate and blood pressure results of the monitoring period by mail.

In contrast to a laboratory experiment, there are no standardized conditions in an ambulatory study. In addition, the number of factors that influence physiology during the day is enormous. Therefore, it is necessary to obtain a detailed report of the activities the subject has engaged in during the measurement period. One very practical option, used in the present study, is to ask subjects to keep a detailed diary. Subjects were asked to keep a chronological account of their activities during the measurement day. Every 30 minutes the VU-AMS device prompted them to write down their activities, postures, social situation, location, and to give a subjective stress score to that half hour. An example of the diaries kept by the subjects is displayed in appendix I (in Dutch). At the end of the day subjects also reported on their mood state using the shortened Profile Of Mood Scale (POMS, Wald & Mellenbergh, 1990) and the next morning they filled out a short questionnaire on subjective sleep quality (Meijman, De Vries-Griever, De Vries, & Kampman, 1988). See appendix II for these questionnaires (in Dutch).

Measurement of cardiovascular function

The 24-hour electrocardiogram (ECG) and impedance cardiogram (ICG) were recorded using the Vrije Universiteit Ambulatory Monitoring System (VU-AMS46, De Geus, Willemsen, Klaver, & Van Doornen, 1995; de Geus & van Doornen, 1996; Willemsen, De Geus, Klaver, Van Doornen, & Carroll, 1996). Ambulatory systolic and diastolic blood pressure was recorded using a Spacelabs 90207 device (Redmont, Washington, USA). Figure 2.1 shows one of the subjects wearing the cardiovascular equipment. In addition, it shows the electrode configuration that was employed. The ECG and ICG signals were recorded using 6 disposable, pregelled Ag/AgCl electrodes (Conmed, NY, USA). The first ECG electrode is placed on the sternum over the first rib between the two collarbones. This electrode also serves as an ICG measuring electrode. The second ECG electrode is placed on the apex of the heart over the ninth rib at the left lateral margin of the chest approximately 3 cm under the left nipple. The third ECG electrode is a ground electrode and is placed over the right abdomen. A second ICG measuring electrode is placed over the tip of the xiphoid complex of the sternum. The ICG current electrodes are placed on the back over cervical vertebra C4 and between thorax vertebras T8-T9. Electrode resistance is kept below 10 KOhm by cleaning the skin with alcohol and rubbing.

Table 2.2 Overview of the measured variables

Ambulatory measures of the cardio-respiratory system	
Measures	**Derived variables**
Systolic blood pressure (SBP)	
Diastolic blood pressure (DBP)	
Heart period (HP)	RMSSD, SDNN index
Respiration rate (RR)	Respiratory sinus arrhythmia (RSA)
Pre-ejection period (PEP)	Systolic time ratio
Left ventricular ejection time (LVET)	Heather index (HI)
Stroke volume (SV)	Cardiac output (CO)
	Total peripheral resistance (TPR)

Anthropometric measures	
Measures	**Derived variables**
Height (q)	
Weight (q)	BMI
Waist circumference (m)	Waist-to-hip ratio
Hip circumference (m)	
Gross body movement (m)	

Hormone Measures	**Derived variables**
Salivary cortisol (at visit, 11:00h, 15:00h, 20:00h, 22:30h, awakening, 30 min. post-awakening)	Cortisol Awakening Response (CAR)

Diary & Questionnaire Measure			
Mood	**Miscellaneous**	**Health behavior**	**Health & disease**
Mood profile	Socio-economic status (SES)	Smoking	Medication use
Subjective stress score (repeated each half hour during the ambulatory recording period)		Alcohol or caffeine use Exercise Use of contraceptives	Family history of CVD Sleep quality

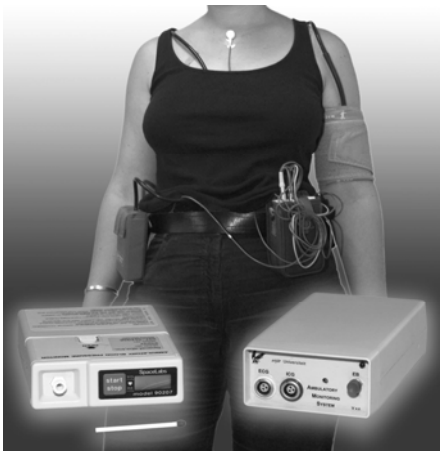
Q= derived from questionnaire, M= measured variable

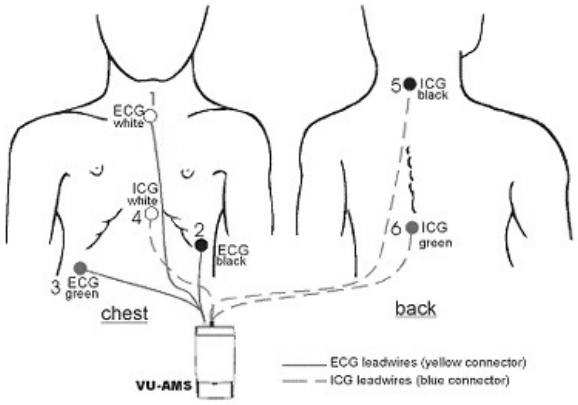
Measures from the ECG

The electrocardiogram (ECG) is used to evaluate the electrical events within the heart. The electrical current through the heart that occurs at each heart beat can be detected by recording electrodes at the surface of the skin. From the ECG a multitude of measures can be derived. The studies described in the present thesis use the ECG to measure the intervals between successive electrical pulses in the heart. The time-domain variables that are calculated based on these intervals include the mean inter-beat-interval (IBI) and the mean heart rate (HR). The beat-to-beat fluctuation in the inter-beat-interval duration is known as heart rate variability (HRV). The present study will make use of two commonly used time-domain measures of HRV derived from the ECG: the square root of the mean squared

successive differences in R-R intervals (RMSSD) and the standard deviation of the R-R intervals averaged over 5 minute periods (SDNN index).

Figure 2.1 Equipment





Left: one of the participants equipped with the ambulatory monitors, in close-up on the left the blood pressure monitor and on the right the VU-AMS ECG/ICG monitor. Right: electrode configuration for the VU-AMS monitoring device.

Measures from the ICG

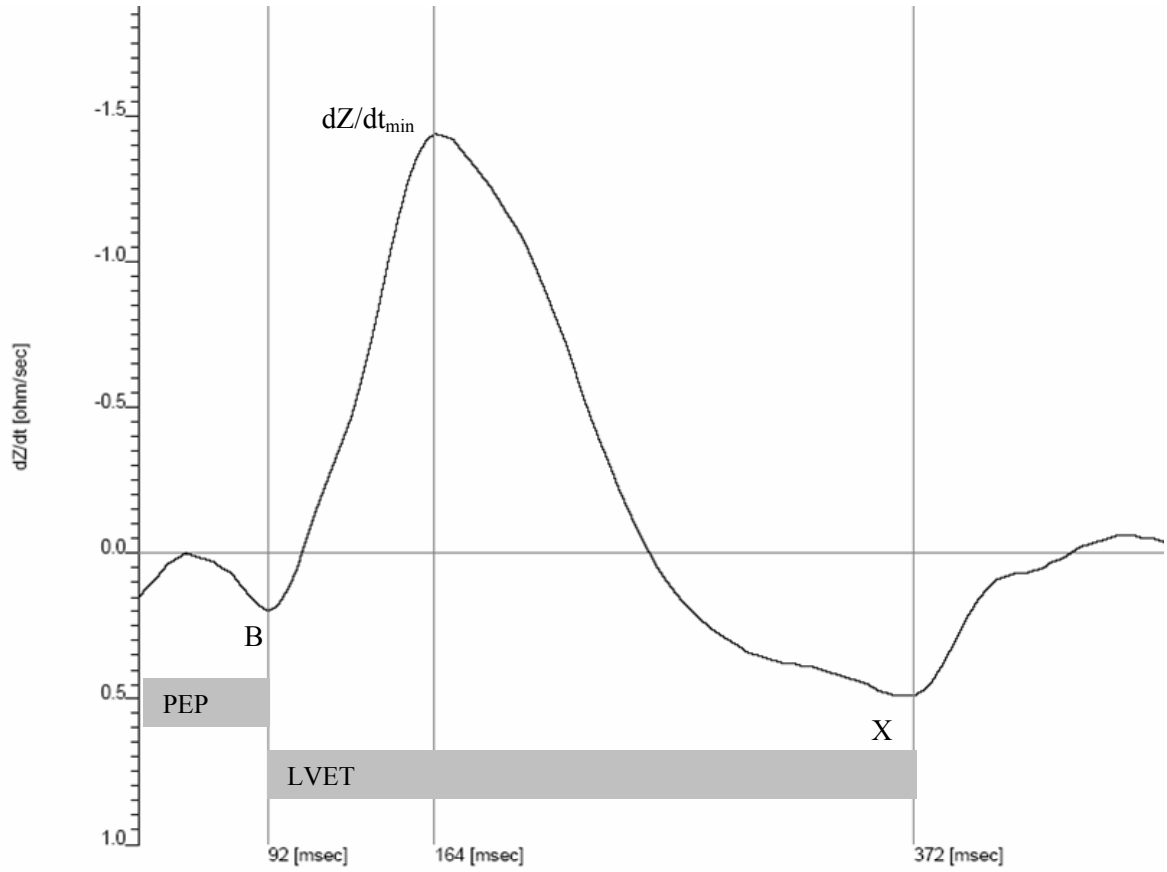
A further method to measure ambulatory cardiac activity is impedance cardiography (ICG). This technique is used to measure sympathetic and parasympathetic influences on the heart. Impedance cardiography (ICG) uses 4 electrodes at the skin surface to record the change in bio-impedance (dZ) over the thorax. The dZ signal contains three major components: high frequent impedance changes due to the ejection of blood into the aorta during systole, low frequent impedance changes due to arm and upper body movement, and, in between these frequencies, the thoracic impedance changes due to respiration.

After appropriate filtering, several measures of sympathetic and parasympathetic activation can be extracted. This present study made use of the impedance changes due to respiration to determine respiratory sinus arrhythmia (RSA), a measure of heart rate variability that is almost completely parasympathetic in origin. RSA was determined using the peak-to-trough method (Fouad, Tarazi, Ferrario, Fighaly, & Alicandri, 1984; Grossman, van Beek, & Wientjes, 1990; Grossman & Kollai, 1993), which combines respiratory time intervals and the heart period time series to obtain an RSA value at each breath. RSA is calculated as the difference between the shortest inter-beat-interval during heart rate acceleration in the inspiration phase and the longest inter-beat-interval during heart rate deceleration in the expiration phase.

The dZ/dt signal that reflects the high frequent impedance changes due to the ejection of blood into the aorta during systole can be seen in figure 2.2. Three characteristic time points are identified. First, there is the moment of the upstroke or B-point at which the ventricular valves open and the blood starts streaming into the aorta. Then the dZ/dt_{min} point follows, at which moment the change in impedance is at its minimum and the velocity of the blood is at its maximum. Finally, the incisura or X-point can be detected, at which moment the ventricular valves close again. The area under this impedance curve reflects the amount of blood that is ejected per heart beat, and is called stroke volume.

The cardiac pre-ejection period (PEP) is a systolic time interval that embodies specific sympathetic cardiac activation. PEP reflects the time interval between the onset of the electromechanical systole (Q-wave onset) and the onset of left ventricular ejection at the opening of the aortic valves (the B-point in the ICG). Pharmacological blockade studies (Schachinger, Weinbacher, Kiss, Ritz, & Langewitz, 2001; Cacioppo et al., 1994a) have shown PEP to be a reliable indicator of sympathetic inotropic control over cardiac contractility. The other systolic time interval is the left ventricular ejection time (LVET), which is used as a sympathetic chronotropic index (LVET, Thayer & Uijtdehaage, 2001). LVET is defined as the time interval between the opening (B-point) and closing (X-point) of the aortic valves. The present study will use two other measures of sympathetic control over cardiac contractility: the ratio of PEP to LVET, which is less preload dependent measure than PEP alone, and the Heather index for myocardial contractility that is more related to the cardiac output. The Heather index is defined as the level of impedance (in Ohm) at the moment of minimal change in impedance (dZ/dt_{min}) divided by the time between the R-top in the ECG and the time of the occurrence of the dZ/dt_{min} point, corrected for basal impedance.

Figure 2.2 Typical high frequency dZ/dt complex of the impedance cardiogram



Blood pressure monitoring

Systolic and diastolic blood pressures were measured during waking hours using an inflatable cuff wrapped around the upper arm on the non-dominant side, and an ambulatory monitor (Spacelabs 90207, Redmont, Washington, USA) that automatically inflated and

deflated the cuff for pressure measurement. Arm circumference was measured to choose the appropriate arm-cuff size. The Spacelabs monitor uses the oscillometric method of detection. Measurements were initiated automatically every 30 minutes during waking hours. The blood pressure recordings were read from the devices using Spacelabs software.

Data loss

In a number of subjects, either blood pressure data or AMS data were entirely missing. One AMS recording failed because of a large metal internal fixation device for repair of a complicated bone fracture. Ten AMS recordings failed due to errors in uploading the data to the computer or due to equipment failure during the recording period. Blood pressure measurements were absent for 20 participants. Three subjects were too obese to fit the largest arm cuff; one person had a muscle disease making blood pressure measurement impossible, and 16 blood pressure recordings were lost due to Spacelabs equipment failures that could not be solved by the participants.

Reasons for partial loss of signal in both ECG/ICG and blood pressure monitors were multiple: a loose or dried electrode, a wire dysfunction, non-compliance of the subjects to keep arm relaxed during a blood pressure measurement, non-compliance of subjects to solve a distress signal from any of the cardiovascular devices. In some cases, subjects did not want to wear the devices anymore and aborted the measurement. For 5 subjects both AMS and blood pressure recordings were actively discarded by ourselves. Four of these subjects had drunk excessively the evening before the measurement and for the fifth subjects no diary was present.

3

Heritability of daytime ambulatory blood pressure in an extended twin design

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Hypertension. 2005; 45, 1 : 80-85.

Abstract

The present study estimated the genetic influences on ambulatory systolic and diastolic blood pressure and on hypertensive status derived from ambulatory levels, in a family sample of 535 twins and 257 singleton siblings. This "extended twin design" was used to explicitly test the possibility that results obtained in singleton siblings are different from those obtained in twins. To examine the effects of excluding (medicated) hypertensive subjects, the genetic analyses were first performed under strict exclusion (medication and/or blood pressure > 135/85 mmHg), next without the medicated subjects and finally without any exclusion. For the latter analysis, the untreated blood pressure values in subjects taking antihypertensive medication were estimated by augmenting the observed blood pressure by the published efficacy of the specific antihypertensive medication used. No evidence was found for differential means, variances or covariances of ambulatory blood pressure in singletons compared to twins. This indicates that estimates of heritability of ambulatory blood pressure from twin studies can be generalized to the singleton population. Heritability of hypertension, defined as a mean daytime blood pressure > 135/85 mmHg or antihypertensive medication use, was 61%. Genetic contribution to ambulatory blood pressure was highest when all subjects were included (systolic: 44-57%; diastolic: 46-63%) and lowest under strict exclusion (systolic: 32-50%; diastolic: 31-55%). We conclude that exclusion of (medicated) hypertensives removes part of the true genetic variance in ambulatory blood pressure.

Introduction

A large number of twin and family studies have shown significant genetic contributions to individual differences in blood pressure (Evans et al., 2003; Boomsma, Snieder, de Geus, & van Doornen, 1998; Colletto, Cardon, & Fulker, 1993; McCaffery, Pogue-Geile, Debski, & Manuck, 1999; Snieder, Harshfield, & Treiber, 2003). Most of these studies have based their genetic analyses on conventional office blood pressure measurements. The genetics of ambulatory blood pressure (ABP) may differ, however, because it is unaffected by the 'white-coat' effect (Mancia & Parati, 2004). The added value of ABP measurements is best illustrated by studies showing that ABP is a better predictor of target organ damage (Pickering & Devereux, 1987), cardiovascular morbidity and mortality (Verdecchia et al., 2001; Verdecchia, 2000) than conventional office blood pressure.

To date, only four twin studies (Fagard et al., 1995; Somes et al., 1995; Vinck et al., 2001; Fagard et al., 2003) and one family study (Kotchen et al., 2000) reported heritability estimates for daytime or 24-hr ABP. Estimates ranged from 22% to 62% for systolic blood pressure (SBP) and from 38% to 63% for diastolic blood pressure (DBP). With the exception of Vinck et al. (2001) and Fagard et al. (2003), sample sizes for the twin analyses have been rather small, i.e. at most 66 pairs in total. Thus, there is a relative paucity of adequately powered twin studies on ambulatory measures. One way of increasing statistical power is to include singleton siblings. Such an extended twin design (Posthuma & Boomsma, 2000) further provides an optimal design to address the question whether results from twin studies on the genetics of ABP may be generalized to the singleton population, because it matches twins and singletons for familial factors like SES, diet habits and maternal behaviors during pregnancy.

Existing twin and family studies of ABP have excluded subjects taking antihypertensive medication (Fagard et al., 2003; Vinck et al., 2001), or have performed their analyses on normotensive subjects only (Fagard et al., 1995; Somes et al., 1995), thereby removing an important part of the population variance of interest (Palmer, 2003). The present study estimated the genetic influences on hypertensive status and ambulatory SBP and DBP in a large sample of twins and their singleton siblings. To examine the effects of exclusion, the genetic analyses on ABP were first performed on normotensive subjects only, secondly after exclusion of medicated hypertensive subjects, and finally without any exclusion.

Methods

Subjects

The study sample was composed of 230 monozygotic (MZ) twins (85 men), 305 dizygotic (DZ) twins (111 men) and 257 singleton siblings (98 men) from 339 families, all registered in the Netherlands Twin Register (NTR). Families were originally asked to participate in a genetic linkage study of anxious depression, which is described elsewhere (Boomsma et al., 2000). Of the 1332 offspring who returned a DNA sample (buccal swabs) for the linkage study, 1008 were successfully contacted for the cardiovascular ambulatory monitoring study, of which 192 subjects refused or were excluded. Reasons for a priori exclusion were pregnancy, heart transplantation, pacemaker and known ischemic heart disease, congestive heart failure, or diabetic neuropathy. In 24 subjects, no data were available due to equipment failures, or data were judged unreliable.

In total, 792 subjects from 339 families participated. In 132 families only the twin pair participated, in 74 families the twin pairs and 1 sibling participated, in 25 families the twin pair and two or more sibs participated. In some families only the (singleton) siblings participated (these include families in which one of the twin pair participated with one or more singleton siblings). In 45 families, one sib participated, while in 51 families, two singleton sibs participated. In the remaining 12 families between three and six sibs participated. Their average age was 31.3 (SD = 11.2) years. Zygosity of the twins was determined by DNA typing. The Ethics Committee of the Vrije Universiteit approved of the study protocol and all subjects gave written consent before entering the study. No payment was made for participation, but all subjects received an annotated review of their BP recording.

Procedures

Subjects were visited in the morning before going to work and were requested to refrain from intense physical activity both on the preceding and the ambulatory monitoring day. A Spacelabs 90207 ABP monitor (Redmont, Washington, USA) and an ambulatory ECG/ICG recorder (Vrijkotte, van Doornen, & de Geus, 2000), which includes a vertical accelerometer, were attached to the subject and their operation was explained. Arm circumference was measured to choose the appropriate arm-cuff size. Blood pressure measurements were initiated automatically every 30 minutes. Before inflating, the device gave an auditory two-tone beep to warn participants to keep their arm as still and relaxed as possible. Subjects were unable to observe their own blood pressure readings. The monitor was programmed to retake a measurement two minutes after a misreading. Every 30 (± 10) minutes subjects were prompted by an auditory beep to write down a chronological account of activity (e.g. deskwork, housekeeping, watching TV), posture (lying, sitting, standing, walking and bicycling), and location (e.g. at home, at work, at a public place). When they went to bed, participants removed the blood pressure monitor. The signal from the vertical accelerometer was combined with the diary information to check the diary entries on posture and physical activity for accuracy.

Data reduction

Previous recommendations for excluding artifacts and outliers from ambulatory recordings were followed (Berardi, Chau, Chanudet, Vilar, & Larroque, 1992). The reported times of diner and lunch, awakening and bedtime were used to compute mean SBP and DBP across all readings in the morning, afternoon, and evening. To assess the confounding of different physical activity patterns on ABP levels, we also computed the average ABP on the three periods of the day using only blood pressure values obtained during sitting activities. Applying ESH criteria, hypertension was considered present when subjects were currently on prescribed antihypertensive medication or when mean daytime ABP was higher than 135/85 mmHg (O'Brien et al., 2003).

Statistical analysis

Heritability estimates of hypertension and daytime ABP were obtained from structural equation modeling of the MZ and DZ/sib variances and covariances using the structural equation program Mx (Neale, Boker, Xie, & Maes, 2003). The ‘raw data’ option was used to allow for families of different composition in terms of the number of participating twins and siblings.

Hypertension - Heritability of hypertension was assessed using a liability-threshold model, which assumes a latent, normally distributed liability to disease that is manifest as a categorical phenotype (Falconer & Mackay, 1996). For hypertension the underlying distribution was modeled to have one threshold, which allows for two categories, affected and unaffected. Linear effects of age and sex were allowed to influence the liability threshold. Sources of variation in hypertension liability considered in the modeling were additive genetic factors (A), shared environmental factors (C), and unique environmental (E) factors. Nested submodels were compared to the full (ACE) model in order to arrive at the most parsimonious and best fitting model. The fit and parsimony of the various models was judged using likelihood ratio tests.

Ambulatory BP - Quantitative analyses of ABP were carried out in several steps. First, a series of increasingly constrained univariate biometrical genetic models were fit for each period of the day, for SBP and DBP separately, to test assumptions of homogeneity of means and variances for MZ twins, DZ twins, and singleton siblings, and the homogeneity of correlations of males versus females and of DZ twins versus singletons (Neale & Cardon, 1992; Neale et al., 2003). The resulting most parsimonious saturated model indicated to what extent we could limit the number of estimated parameters in the ensuing analyses. Next, two theoretically distinct trivariate biometric models were fit to estimate the relative contribution of genetic and environmental influences to the variance and covariance of mean SBP and DBP across the three periods of the day. The first model was an *independent pathway model*, which specifies three common factors. One for genetic (A) sources of variance, one for shared environmental sources of variance (C) and one for unique environmental (E) sources of variance, while it also allows for period-specific influences of A, C and E for all periods of the day. This model is depicted in figure 3.1a. We tested whether the ACE variance decomposition for both the common and the specific factors could be reduced to an AE or CE model. A *common pathway model* was fit next. This model is more stringent and hypothesizes that the covariation between ABP measured at the three periods of the day is determined by a single, common latent variable, called "systolic (or diastolic) blood pressure". "Systolic (or diastolic) blood pressure" itself can be influenced by A, C and E. As in the independent pathway model, there are still genetic and environmental effects specific for BP measured at each period of the day. This model can be seen in figure 3.1b. It was tested whether this more parsimonious common pathway provided a better description of the data than the independent pathway model, and whether specific genetic influences could be dismissed. To evaluate the relative fit of the various nested models we used Akaike's Information Criterion (AIC, Akaike, 1987), an index of goodness-of-fit for which a larger negative value indicates greater parsimony of the model. Significance tests of the individual path coefficients were carried out by constraining paths to zero and applying likelihood ratio tests.

The above analyses on ABP were performed thrice, in three different sets of subjects. In the normotensive set we excluded all subjects diagnosed with hypertension (ABP > 135/85) and subjects on antihypertensive medication. In the second and unmedicated set we only excluded the 29 subjects on antihypertensive medication. The third set included all available subjects. To obtain ABP values in these medicated subjects, drug-class specific treatment effect averages, obtained from a recent large systematic review of the effect of antihypertensive treatment on *ambulatory* blood pressure (Mancia & Parati, 2004) were added to the observed pressures. Unlike substitution methods, this adjustment makes no assumptions regarding the underlying reasons for treatment, and keeps the relative ranking of the treated subjects intact.

Figure 3.1a Independent Pathway Model

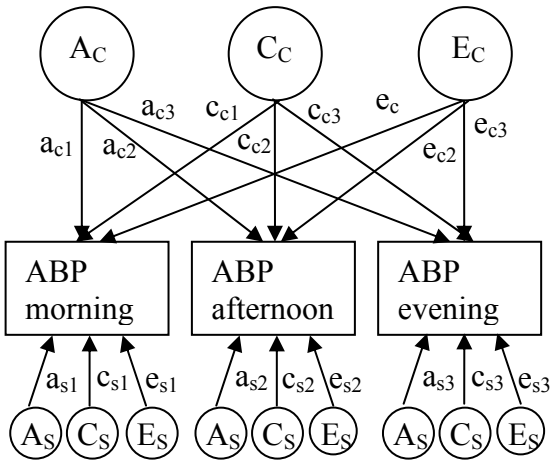
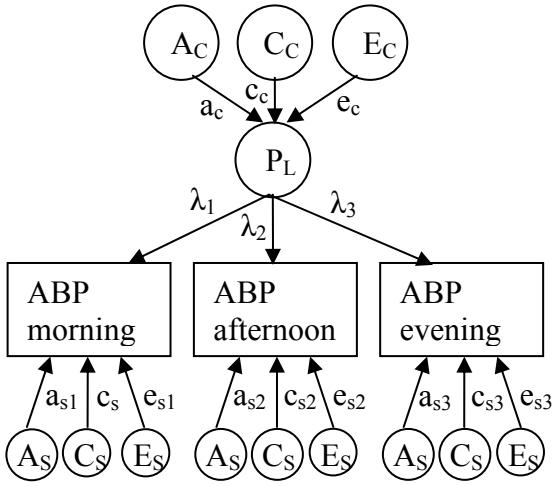


Figure 3.1b Common Pathway Model



The figure illustrates both the independent (a) and common pathway model (b). The rectangles contain the observed systolic or diastolic ABP at the three periods of the day; the circles represent the latent factors: A_C= common additive genetic factor, C_C= common shared environmental factor, E_C= common unique environmental factor, P_L= latent underlying phenotype. A_S=specific additive genetic factor, C_S= specific shared environmental factor, E_S= specific unique environmental factor. The lambda (λ) paths represent factor loadings for the three measurements (morning, afternoon, evening) on the latent daytime ambulatory blood pressure (P_L). For ease of reading, the model is shown for one family member only.

Results

On average 27 (± 4) blood pressure measurements (≈ 13.5 hours) took place during the recording period, of which on average 13 (± 5) during sitting posture (≈ 6.5 hours, which is 48% of the total monitoring period). Although the sample was previously selected based on the presence of at least two family members with extreme scores on personality questionnaires, their scores did not correlate significantly with hypertension diagnosis, or with SBP and DBP. Throughout the day, men had significantly higher SBP and DBP than women.

At all 3 periods, age was significantly correlated with SBP (.24-.33) and DBP (.31-.35). Both sex and age were kept as covariates in all further model fitting analyses.

BMI correlated significantly with both SBP (.24-.31) and DBP (.20-.25). As several studies reported a genetic covariation between BP and BMI (McCaffery, Pogue-Geile, Debski, & Manuck, 1999; Carmelli, Cardon, & Fabsitz, 1994; North et al., 2004) and since genetic variance is removed when shared genes influence both variable and covariate, we intentionally did not include BMI as covariate.

Hypertension

In our sample, 115 (14.5%) of the 792 subjects received a hypertension diagnosis based on their ambulatory recording, 29 of them receiving antihypertensive medication. Among the hypertensive were 31 monozygotic twins. The homogeneity of covariances over the sexes and between DZ twins and siblings was confirmed and no evidence for a difference in twin pair versus singleton sibling pair correlation in hypertensive risk was found. The liability threshold was higher for women compared to men, and higher for younger vs. older subjects. The sex and age-corrected tetrachorical correlations for the liability dimension were .62 for MZ twins and .29 for DZ twins, indicating genetic influences. This was confirmed by model fitting, the results of which are shown in table 3.1. Leaving out both shared environmental and genetic influences from the model (E-model) caused a large increase in χ^2, indicating a significant worsening of the fit. This shows a clear influence of *familial* factors on hypertension. Statistical power was insufficient to discriminate between genetic influences and shared environmental influences, but given the pattern of twin correlations and the AICs of the AE and CE models, it is most likely that the AE model is the preferred model. In the AE model, variance in hypertension diagnosis is for 61% explained by genetic influences.

Table 3.1 Model Fitting Results for Hypertension

Model	$\Delta\chi^2$	Δdf	p	AIC	A	C	E
ACE	-	-	-	-	61% (0-83)	0% (0-49)	39% (17-75)
AE	0	1	1.000	-2.000	61% (33-83)	-	39% (17-67)
CE	2.355	1	0.125	0.356	-	37% (18-56)	63% (44-82)
E	18.914	2	0.000	14.914	-	-	100%

Shown are delta chi-square ($\Delta\chi^2$) values and gain in df of the models and accompanying estimates for additive genetic (A) and shared (C) and unique environmental (E) influences. AIC = Akaike's Information Criterion. The 95% confidence intervals are given between parentheses.

Ambulatory BP

Table 3.2 presents the mean ABP for the three periods of the day in the normotensive, unmedicated and full set of subjects. It illustrates the impact of excluding subjects with hypertension and/or using antihypertensive medication. Although the means do not dramatically change (2% to 4.5%), strict exclusion brings about a 30% to 39% reduction in the standard deviation in comparison to the "true" population. Exclusion of medicated subjects led to much smaller changes in means (-0.5% to 0.5%) and moderate reductions in standard deviations (1 to 10.5 %).

Table 3.2 Means (SD) for ambulatory SBP and DBP at the three daily periods

Period of day	BP (mmHg)	Sex	Normotensives (645 <N<657)	Unmedicated set of subjects (747<N<759)	Full set of subjects (772<N<786)
Morning	SBP	M	129.8 (9.1)	133.5 (11.4)	134.2 (12.1)
		F	124.2 (8.3)	125.9 (10.1)	126.7 (11.3)
	DBP	M	79.3 (6.4)	82.5 (8.7)	83.2 (9.4)
		F	77.4 (6.5)	80.8 (7.5)	81.3 (8.3)
Afternoon	SBP	M	129.6 (8.1)	132.6 (10.1)	133.2 (10.9)
		F	122.9 (8.5)	125.9 (10.1)	125.3 (10.2)
	DBP	M	77.4 (6.4)	80.4 (8.2)	81.1 (8.9)
		F	77.4 (6.0)	78.6 (7.1)	79.1 (7.7)
Evening	SBP	M	129.0 (8.5)	132.1 (10.4)	132.7 (10.9)
		F	122.9 (8.1)	124.7 (10.1)	125.3 (10.8)
	DBP	M	76.0 (6.3)	79.3 (8.7)	79.9 (9.2)
		F	76.6 (6.8)	77.9 (7.8)	78.4 (8.4)

M= males, F = females

Next, the resemblance between MZ twins and between DZ twins or sibling pairs was examined by calculating age-adjusted Pearson correlations, stratified by sex, as shown in table 3.3. Throughout a larger MZ than DZ correlation is evident, suggesting the presence of additive genetic and unique environmental influences.

Table 3.3 Resemblance between MZ and DZ/sib pairs for ambulatory SBP and DBP

Period of day	Sex of pairs	SBP		DBP	
		rMZ	rDZ/sib	rMZ	rDZ/sib
Morning	M	**.72 / .60 / .68**	**.38 / .38 / .41**	**.42 / .63 / .68**	**.21 / .34 / .40**
	F	**.44 / .56 / .49**	**.13 / .23 / .27**	**.34 / .51 / .51**	**.21 / .28 / .33**
	OS		.09 / .16 / **.26**	-	.16 / .12 / .10
Afternoon	M	**.40 / .62 / .60**	**.27 / .25 / .32**	**.63 / .70 / .65**	**.38 / .39 / .43**
	F	**.59 / .68 / .64**	**.18 / .23 / .27**	**.62 / .72 / .73**	**.30 / .30 / .35**
	OS	-	.08 / .18 / **.26**	-	.19 / .14 / **.15**
Evening	M	.34 / **.49 / .59**	**.35 / .32 / .39**	.31 / **.49 / .56**	.17 / **.45** / **.43**
	F	.19 / **.35** / **.40**	**.18 / .19 / .27**	.21 / **.43 / .45**	**.18 / .21 / .27**
	OS	-	.17 / .11 / **.24**	-	.18 / .09 / **.15**

Shown are the age-corrected correlations for the normotensive set of subjects / the set excluding medicated subjects / the full set of subjects (with ABP corrected for medication). M = males, F = females, OS = opposite sex. Correlations that are significant at a .05 level are printed bold faced.

Multivariate genetic analyses - The means and variances of both SBP and DBP were equal for MZ and DZ twins, and singleton siblings. Importantly, we found no twin-singleton differences in ABP in all three sets of subjects, suggesting that results obtained in twins can be generalized to singletons.

Multivariate model fitting resulted in the preference for a model without shared environmental factors (AE model) over the full model (ACE model) for all three sets of subjects. Statistical power was sufficient to discern the AE and CE model, since quantitative

analyses have higher statistical power than the ordinal analyses performed for hypertension status. Although there was sufficient power (at $\beta = .80$, $\alpha = 0.05$) to detect effects of 23% or higher, no significant common environmental effect was found. We further tested the hypothesis that a common latent trait was underlying blood pressure at all three periods of the day (common pathway model).

Table 3.4 Heritability estimates for SBP and DBP under the common pathway model

BP	Period of day	Common influences		Specific influences
		Genetic	Environment	Environment
Normotensive set of subjects				
DBP	Morning	40% (28 to 53)	23% (13 to 35)	37% (31 to 44)
	Afternoon	55% (39 to 70)	30% (19 to 47)	15% (09 to 21)
	Evening	31% (20 to 41)	17% (10 to 28)	52% (46 to 59)
SBP	Morning	38% (24 to 51)	29% (18 to 44)	32% (27 to 38)
	Afternoon	50% (32 to 65)	38% (24 to 56)	12% (07 to 18)
	Evening	32% (19 to 44)	24% (14 to 38)	44% (38 to 50)
Unmedicated set of subjects				
DBP	Morning	52% (39 to 63)	23% (15 to 36)	24% (20 to 29)
	Afternoon	61% (46 to 73)	27% (17 to 41)	12% (07 to 14)
	Evening	43% (32 to 53)	19% (12 to 30)	37% (32 to 42)
SBP	Morning	49% (34 to 62)	30% (18 to 45)	22% (18 to 26)
	Afternoon	57% (41 to 71)	35% (22 to 51)	09% (06 to 12)
	Evening	42% (29 to 54)	26% (16 to 39)	38% (27 to 36)
Full set of subjects				
DBP	Morning	55% (43 to 65)	23% (15 to 35)	22% (18 to 26)
	Afternoon	63% (50 to 74)	27% (17 to 39)	10% (07 to 14)
	Evening	46% (35 to 56)	19% (12 to 29)	35% (30 to 39)
SBP	Morning	50% (38 to 61)	30% (20 to 42)	20% (17 to 24)
	Afternoon	57% (44 to 69)	34% (24 to 48)	09% (06 to 12)
	Evening	44% (33 to 55)	27% (18 to 38)	29% (25 to 34)

Shown are the heritability estimates for the normotensive set of subjects, the unmedicated set of subjects and the full set of subjects. The 95% confidence intervals are given between parentheses.

Indeed this model was preferred over the initial independent pathway model in all 3 sets of subjects. We found no specific genetic influences for each of the daily periods and the largest part of unique environmental influences was also common to all 3 periods. Table 3.4 shows the common pathway estimates for A, which corresponds to the heritability, and for E which corresponds to the influence of the common environmental factor. The specific E estimates represent unique environmental influences that are specific to each of the periods of the day.

The highest heritability estimates were found in the full set of subjects. In comparison to the results on the normotensive subjects heritability estimates were substantially higher (SBP: +7 to 12%, DBP: +8 to 15%). Furthermore, confidence intervals were wider for the normotensive subjects compared to the full set of subjects. An increase in heritability was already seen when unmedicated subjects were included, but only when medicated subjects were included we found non-overlapping confidence intervals with the normotensives.

Individual differences in daily physical activity on the measurement day did not confound the genetic analyses of ABP. When above analyses were repeated using BP measurements obtained during sitting activities, the results were essentially unchanged.

Discussion

Based on daytime ambulatory measurements of SBP and DBP, obtained in 792 twins and singleton siblings, the present study showed that the individual differences in hypertension status are for 61% genetically determined. Heritability estimates for ambulatory SBP and DBP were between 44-63% when no exclusion criteria were upheld. These estimates correspond well to those found in previous ambulatory and laboratory/clinical studies in another large adolescent and adult healthy twin samples (Evans et al., 2003; Fagard et al., 1995; Fagard, Loos, Beunen, Derom, & Vlietinck, 2003; Vinck, Fagard, Loos, & Vlietinck, 2001). Our study had a number of strengths in design that provide confidence in its outcome. The extended twin design increases the statistical power to distinguish between components of A, C and E compared to a design including only MZ and DZ twins (Posthuma & Boomsma, 2000). Furthermore, it allowed us to test the possibility that results obtained on singleton sibling pairs differed somehow from those obtained in twin pairs. This is important because the lower birth weight in twins might be considered to reflect an impaired fetal environment, which, according to the "Barker hypothesis", may impact on BP regulation (Law & Shiell, 1996). By comparing singletons with twins from the same family, the two comparison groups are perfectly matched for familial influences (same parents, same womb although at a different time, same family environment). Our analyses showed that MZ and DZ twins and singleton siblings did not differ from each other in means or variances on any of the ABP measures. Importantly, sibling-sibling covariance did not differ from sibling-twin or DZ-twin covariance, which strongly argues against a special twin intrauterine disadvantage with deleterious effects on adult ABP. The absence of any twin-singleton difference repeats previous findings in resting laboratory blood pressure (de Geus et al., 2001) and indicates that estimates of the heritability of ABP from twin studies are not systematically biased and can be generalized to the general population.

Exclusion of hypertensive subjects clearly distorted MZ and DZ twin correlations, as well as the variances. Excluding medicated subjects further increased the distortion, although the effect was only very minor in this population. Our results showed that restricting the sample to normotensives only, not only caused a decrease in total variance but specifically reduced heritability estimates. With this result we extend the earlier findings (Cui, Hopper, & Harrap, 2003) on conventional office BP, to prolonged BP measurements in naturalistic settings. The effect of excluding groups of subjects, on grounds of hypertension and/or medication is undesirable as the reduced heritability estimates directly lead to a loss of power in linkage studies. This effect was convincingly illustrated by Hunt et al. (Hunt et al., 2002) who showed that removing medicated subjects from the sample led to the disappearance of a QTL for conventional office SBP on chromosome 6.

The substantial heritability of office blood pressure has motivated many large scale efforts to identify hypertension predisposing genes through linkage (Province et al., 2003) approaches. Because ABP is a better predictor of target organ damage (Pickering & Devereux, 1987), cardiovascular morbidity and mortality (Verdecchia et al., 2001; Verdecchia, 2000) than office BP, these gene finding attempts may be well served by using ABP. Here we show that genetic variance of ABP is sufficiently high to justify its use in genome searches. The repeated measures structure inherent in ambulatory monitoring brings further advantages. We separated

the entire ambulatory recording into 3 daily periods, allowing for the possibility that different genetic factors would affect blood pressure regulation during leisure (evening) and work (morning, afternoon) periods. For both SBP and DBP a common factor influenced all 3 periods. There were no separate genetic factors influencing blood pressure at different periods of the day. From a gene finding point of view, the common genetic factor structure is advantageous on two accounts. The repeated measures structure increases statistical power to find genes in linkage analysis (Evans, 2002). Additionally these genes, by virtue of having a pervasive influence on SBP or DBP across all situations, will also have the largest clinical relevance.

Limitations

An important limitation to our study is the lack of nighttime recordings. We opted not to burden our subjects by asking them to continue wearing the blood pressure monitor at night. In our experience, this causes large attrition in non-patient populations. In family-based studies, the loss of a single subject is more hard-felt than in population samples, where an additional randomly drawn subject can be easily recruited without loss to the overall study design. It is possible, however, that different genetic factors come into play during the day than at night. Some indication for this possibility, although the confidence intervals of the estimates for the three daily periods were overlapping, is seen in the systematically lower heritability in the evening compared to morning and, particularly, afternoon recordings.

Our sample consisted of young Caucasian adults aged mainly between 20 and 40, with an age range between 15 to 81 years. Therefore, generalization of our results beyond the main age range or to a population of different ethnicity should be done only hesitantly. A recent family study performed an age-stratified longitudinal genetic analysis of office blood pressure and found little variation in the genetic architecture over time (Brown et al., 2003). This suggests that a common set of genes may be contributing to the observed variation in BP across a wide age range. In our sample, separating the analyses of ABP over multiple age cohorts would have compromised statistical power to detect, for instance, twin singleton differences.

Perspectives

Genetic contribution to the variance in blood pressure is very likely to be polygenic, with only very small contributions of the individual quantitative trait loci to the final hypertensive risk. Future gene finding studies, particularly linkage studies, should therefore aim to maximize statistical power. In this regard, ABP monitoring has a number of advantages over conventional office measurements. Heritability of ABP is comparable to office blood pressure, but the genes underlying ABP are likely to have better predictive validity for target organ damage (Pickering & Devereux, 1987) and cardiovascular morbidity and mortality (Verdecchia et al., 2001; Verdecchia, 2000). Ambulatory recording has an intrinsic multivariate nature and the repeated, highly correlated blood pressure measures can be exploited to reduce measurement error and improve the estimation of the latent genetic factor. Our findings further show that a part of the genetic variance in ABP is lost when hypertensive (and/or medicated) subjects are excluded. In future linkage and association studies such exclusion should be avoided.

4

Heritability of Ambulatory Heart Rate Variability

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Abstract

Reduced heart rate variability (HRV) is a prognostic factor for cardiac disease and cardiac mortality. Understanding the sources of individual differences in HRV may increase its diagnostic use and provide new angles for preventive therapy. To date, the contribution of genetic and environmental factors to the variance in HRV has not been investigated during prolonged periods of ambulatory monitoring in a naturalistic setting.

In 772 healthy twins and singleton siblings, ambulatory ECG was recorded during 24 hours. Two time-domain measures of HRV were used: the standard deviations of all normal to normal intervals across 5-min segments (SDNN index) and the root mean square of successive differences between adjacent normal RR intervals (RMSSD). Multivariate genetic analyses across 4 periods of day (morning, afternoon, evening, night) yielded significant estimates for genetic contribution to the mean ambulatory SDNN index (ranging from 35% to 47%) and the mean ambulatory RMSSD (ranging from 40% to 48%).

Ambulatory HRV measures are highly heritable traits that can be used to support genetic association and linkage studies in their search for genetic variation influencing cardiovascular disease risk.

Introduction

Heart rate variability is a clinically relevant cardiovascular phenotype. Reduced heart rate variability is an independent predictor of cardiac disease and cardiac mortality (Dekker et al., 2000; Nolan et al., 1998; Huikuri et al., 2003; Dekker et al., 1997; Tsuji et al., 1996; Bigger et al., 1993). The major explanation for this predictive effect is that reduced heart rate variability reflects a shift in cardiac sympathovagal balance from parasympathetic to sympathetic control over the heart rhythm (Schwartz et al., 1988; La Rovere et al., 2002). Understanding the sources of individual differences in heart rate variability may increase its diagnostic use and, if these differences can be traced to genetic polymorphisms, may provide new angles for preventive therapy. The first step in the establishment of genetic contribution to a clinical phenotype is the estimation of its heritability in samples of genetically related subjects.

In laboratory studies, a significant genetic contribution to heart rate variability has been established by twin and family studies. Heritability estimates at rest range from 13-39% (Boomsma et al., 1990; Sinnreich et al., 1999; Busjahn et al., 1998; Snieder et al., 1997; Singh et al., 1999), but during exposure to various stress tasks the genetic contribution increases up to 51% (Boomsma et al., 1990). This suggests that genetic influences are more pronounced when the subject is challenged by mentally and emotionally taxing tasks. Accordingly, we hypothesize that heritability of heart rate variability measures will be even higher when recorded over prolonged periods in a naturalistic setting.

To date, the genetics of heart rate variability in such recordings have not been investigated. The purpose of the present study was to estimate the contribution of genetic and environmental factors to the variance in ambulatory measured heart rate variability, using a twin family design.

Methods

Subjects

Participants were registered with the Netherlands Twin Register (NTR). All families were selected for a genetic linkage study in search of genes influencing personality traits as described in detail elsewhere (Boomsma et al., 2000). Briefly, the families were selected to have two siblings (dizygotic twin pair, or sib-twin pair, or sib-sib pair) discordant or concordant for anxiety, neuroticism or depression. In addition to these siblings, however, all other family members have been recruited for study and the resulting distribution of anxiety, neuroticism and depression scores was near-normal with only mild kurtosis.

Of the 1332 offspring who returned a DNA sample (buccal swabs) for the linkage study, 1008 were successfully contacted for a cardiovascular ambulatory monitoring study, of which 192 refused or were excluded. Reasons for exclusion were pregnancy, heart transplantation, pacemaker and known ischemic heart disease, congestive heart failure, or diabetic neuropathy. In 14 of the remaining 816 subjects, no data were available due to equipment failures. Ten subjects showed a very noisy ECG signal and were excluded from the analyses. Subjects (20 in total) using rhythm-altering medication (n=2), and HRV-reducing antidepressants (tricyclic antidepressants (n=2) and benzodiazepines (n=10)) and antihypertensive medication (β-blockers (n=13)), or a combination of these, were excluded from the analysis. The final sample consisted of 218 monozygotic (MZ) twins (79 men), 301 dizygotic (DZ) twins (107 men) and 253 singleton siblings (97 men) from 339 families. For the majority of the twin pairs, zygosity was determined by DNA typing; in a small part (8%)

zygosity questionnaires were used. The mean age was 31.3 years (SD = 10.6) for men and 30.8 years (SD = 10.9) for women. The Ethics Committee of the Vrije Universiteit approved of the study protocol and all subjects gave written consent before entering the study. No payment was made for participation.

Study design

Subjects were visited at home on a weekday, before starting their normal daily activities. They were subjected to an interview on health status and current medication use. The Vrije Universiteit Ambulatory Monitoring System 46 (VU-AMS device, de Geus, Willemsen, Klaver, & van Doornen, 1995; de Geus & van Doornen, 1996) was attached and its operation explained. Subjects wore the VU-AMS device the entire day and night up until awakening the next morning. Every 30 (± 10) minutes the ambulatory device produced an audible alarm beep to prompt them to fill in a detailed diary. They wrote down a chronological account of activity, posture, location, presence of other persons, and amount of perceived stress during each past 30 minutes. On the following day the research assistant collected the device at home.

Heart rate variability

The VU-AMS device continuously recorded the electrocardiogram (ECG) from a six-electrode configuration. Two heart rate variability measures were extracted from the IBI time series: the standard deviations of all normal to normal intervals (SDNN) and the root mean squares of the successive differences between adjacent normal to normal intervals (RMSSD). In addition to cardiac measures, the device also recorded vertical acceleration as a proxy for gross body movement. The vertical accelerometer information was combined with the diary information to divide the entire recording into smaller fragments that were stationary with regard to physical activity and posture, e.g. within each fragment no shifts in activity/posture occurred. The fragments were never shorter than 5 minutes or longer than 1 hour. They were coded for posture (lying, sitting, standing, walking and bicycling), activity (e.g. deskwork, housekeeping, watching TV), and location (e.g. at home, at work, at a public place). SDNN was computed across all 5-minute periods that fitted in the coded fragment, effectively yielding the SDNN index. SDNN index and RMSSD were averaged over the entire fragment. Based on the reported times of diner and lunch, awakening and bedtime, mean RMSSD and SDNN index were computed across all fragments in the morning, afternoon, evening and nighttime sleep periods. In 8% of the subjects the exact time of diner, lunch, awakening or bedtime could not be extracted from either diary or body movement. For these subjects, the missing time was imputed using the mean times of these events in the rest of the sample.

Statistical analysis

Confounding - The individual differences in ambulatory heart rate variability were expected to be sensitive to three main confounders: differences in sex and age (Antelmi et al., 2004), and differences in physical activity patterns on the measurement day (Osterhues, Hanzel, Kochs, & Hombach, 1997). All analyses below were age and sex-adjusted, to control for the first two of these confounders. Because ambulatory recorded subjects may differ in their activity patterns, the potential influence of physical activity and postural changes on interindividual variance in heart rate variability measures needs to be taken into account. This was done here by calculating the twin correlations twice: once including the entire recording, containing data during all postures, and once including those fragments of the recording during which a subject was either sitting or lying.

Twin correlations - MZ twins share all their genetic material, while DZ twins and siblings share on average 50% of their segregating genes. A larger resemblance of MZ than DZ twins, or other first degree relatives, thus indicates that their larger genetic resemblance is associated with a larger phenotypic resemblance (Boomsma, Busjahn, & Peltonen, 2002). To determine the extent to which monozygotic twin pairs are more similar than dizygotic or sibling pairs, Pearson correlation coefficients were calculated per zygosity using SPSS-11 (SPSS Inc., Chicago, USA). All possible MZ and DZ/sib pairs were used.

Structural equation modeling - To answer the question to what extent genes, shared and non-shared environment contribute to the variance of SDNN index and RMSSD, biometrical genetic models were fitted to the data using the structural equation program Mx (Neale et al., 2003). First, nested univariate unconstrained models were fitted to test assumptions of the (extended) twin model. For each period of day, we tested the equality of means and variances for MZ twins, DZ twins, and singleton siblings. Likewise, we examined the presence of sex and age effects on the means and variances. In a final step, we tested for heterogeneity of correlations of males versus females and of DZ twins versus singletons.

The resulting most parsimonious unconstrained models were the ones against which the variance decomposition models were tested. The observed variance was decomposed into 3 sources: additive genetic influences (A), shared environment (C), and non-shared environment (E) following Neale and Cardon (1992). For DZ twins and sibling pairs similarity in shared environmental influences was fixed at 100% and similarity of additive genetic influences at 50%. For MZ twins similarities of additive genetic, and shared environmental influences were fixed at 100%. Non-shared environmental influences are uncorrelated in all twin and sibling pairs. After establishing the most parsimonious variance components model (ACE, AE, CE, or E) for each period of day, a full 4-variate Cholesky decomposition was used to test whether the same or different genetic and environmental factors influenced heart rate variability at each of the 4 periods of the day. A priori, we expected a single genetic factor to underlie the variance across all 4 periods for both SDNN index and RMSSD. This was tested by contrasting a full Cholesky decomposition against a genetic factor model, which allows for a common genetic factor and specific additive genetic influences at each period. It was further tested whether unique environmental influences could also be better described by such a factor structure, or that a Cholesky decomposition should be preferred.

Nested models were compared by likelihood ratio test, using twice the difference between the log-likelihoods of two models, which is asymptotically distributed as χ^2. A high χ^2 against a low gain in degrees of freedom will generate a significant p-value and denotes a worsening of the fit (related to the more parsimonious model).

All postures		MZM 70<N<78	DZM 50<N<54	MZF 131<N<138	DZF 118<N<127	DOS m 47<N<54	DOS f 57<N<65	Sib m 88<N<97	Sib f 142<N<154
SDNN index (ms)	Morning	82.3 (30.9)	78.6 (19.5)	67.8 (18.1)	67.3 (18.9)	81.4 (20.9)	72.7 (18.6)	73.9 (21.7)	69.7 (19.4)
	Afternoon	78.5 (28.5)	77.1 (19.9)	64.7 (18.6)	62.5 (16.2)	77.6 (24.3)	68.8 (19.1)	69.0 (20.8)	64.9 (19.9)
	Evening	78.7 (26.4)	83.6 (22.8)	69.7 (23.1)	63.5 (18.0)	82.1 (22.8)	68.4 (20.1)	73.5 (24.4)	66.4 (20.4)
	Night	89.0 (31.7)	98.8 (26.8)	70.8 (28.9)	70.8 (22.7)	98.2 (27.4)	80.7 (27.2)	88.1 (29.7)	73.7 (24.0)
RMSSD (ms)	Morning	41.1 (26.6)	41.5 (30.1)	31.8 (14.3)	31.8 (16.0)	41.1 (18.7)	36.7 (22.4)	36.1 (17.7)	34.2 (19.1)
	Afternoon	38.7 (22.7)	40.7 (24.5)	32.5 (15.8)	30.5 (12.6)	40.7 (19.5)	36.5 (23.9)	32.9 (14.8)	32.1 (17.9)
	Evening	39.7 (22.4)	46.5 (21.8)	40.5 (21.3)	34.0 (15.3)	46.2 (22.1)	42.2 (39.0)	40.4 (23.2)	36.7 (19.5)
	Night	57.4 (35.5)	70.8 (35.2)	58.7 (33.9)	49.1 (30.5)	72.0 (37.5)	60.9 (37.1)	59.1 (33.8)	50.7 (25.4)
Sitting and lying postures only									
SDNN index (ms)	Morning	73.5 (25.1)	73.8 (17.0)	64.4 (18.4)	63.3 (20.3)	79.4 (20.4)	67.3 (17.9)	70.7 (23.5)	65.9 (21.4)
	Afternoon	73.3 (26.0)	72.8 (18.4)	61.7 (19.0)	59.0 (16.8)	74.8 (21.7)	65.1 (20.0)	65.7 (20.4)	61.5 (20.5)
	Evening	72.6 (25.0)	77.0 (20.1)	67.0 (24.3)	60.6 (19.8)	80.1 (23.4)	62.6 (17.7)	69.0 (22.9)	63.8 (22.1)
	Night	85.4 (30.4)	97.1 (25.4)	77.5 (26.2)	69.8 (21.2)	94.7 (23.9)	78.4 (23.0)	85.0 (26.1)	72.4 (21.8)
RMSSD (ms)	Morning	43.7 (28.8)	46.0 (40.1)	35.4 (16.9)	34.6 (19.7)	43.5 (25.7)	43.5 (25.7)	38.7 (21.6)	38.4 (22.8)
	Afternoon	41.0 (26.2)	43.2 (28.8)	36.4 (19.1)	33.4 (14.3)	42.3 (25.2)	42.3 (25.2)	35.5 (17.3)	36.0 (21.6)
	Evening	40.5 (23.6)	47.1 (22.7)	45.2 (27.2)	36.5 (18.1)	46.4 (32.6)	46.4 (32.6)	41.9 (23.9)	40.2 (22.6)
	Night	58.1(36.3)	71.4 (35.8)	60. 2 (35.1)	50.1(31.0)	65.5 (35.7)	65.5 (35.7)	59.6 (33.8)	51.2 (25.7)

Table 4.1 Means (SD) of SDNN index and RMSSD for all daily periods

N=number of subjects (varies slightly per daily period), MZM=monozygotic male twins, DZM=dizygotic male twins, MZF=monozygotic female twins, DZF=dizygotic female twins, DOS=dizygotic twins of opposite sex. Sib m and sib f are male and female singletons.

Results

The valid ambulatory recording time was on average 22:13 hours (SD = 3:21 hrs), of which 51% was spent in a sitting or lying posture. Although the sample was previously selected based on the presence of at least two family members with extreme scores on personality questionnaires, their scores did not correlate significantly with both SDNN index and RMSSD at all, which points out that the results were not biased by this selection criterion. At all four periods of day SDNN index was significantly correlated with RMSSD (morning r = .83, afternoon r = .86 evening r = .87 and night r = .87). Since the RMSSD distribution was skewed at all time periods, its natural logarithm was used in all further analyses. Table 4.1 presents the untransformed means and standard deviations for SDNN index and RMSSD separately for each subject group, using heart rate variability from all postures (upper panel) or from fragments of sitting/lying only (lower panel).

Twin & sibling correlations

Table 4.2 shows the resemblance between MZ and DZ/sibling pairs for SDNN index and RMSSD. All correlations were calculated twice: once on all available data and once using only fragments where subjects were either sitting or lying. In spite of the potentially large effects of differences in posture and physical activity on heart rate variability similarity in twins and siblings, the correlations based on sitting/lying-only fragments differed only marginally from those that included the entire recording. Although the potential confounding by mixing data across different postures had little actual impact, we decided to restrict further model fitting to the most "pure" data, i.e. fragments where subjects had been sitting or lying (sleep).

Table 4.2 Age-adjusted twin/sibling correlations for SDNN index and RMSSD

		SDNN index		RMSSD*	
		rMZ	**rDZ&sib**	**rMZ**	**rDZ&sib**
Morning	Men	**.64/.58**	**.41/.37**	**.54/.50**	**.32/.27**
	Women	**.58/.45**	**.22/.18**	**.51/.45**	**.24/.23**
	OS	-	**.27**/.04	-	.11/.08
Afternoon	Men	**.66/.65**	**.36/.26**	**.53/.59**	**.27/.28**
	Women	**.57/.51**	**.21/.21**	**.49/.58**	**.17/.23**
	OS	-	**.27**/.07	-	**.26**/.13
Evening	Men	**.45/.41**	**.24**/.13	**.57/.56**	**.30/.25**
	Women	**.44/.48**	**.26/.28**	**.49/.56**	**.18/.28**
	OS	-	**.17**/.13	-	.12/.09
Night	Men	**.71/.69**	**.25/.25**	**.63/.57**	**.36/.31**
	Women	**.55/.55**	**.17/.18**	**.47/.46**	**.13/.15**
	OS	-	**.15**/.12	-	**.18/.**14

** RMSSD was ln transformed to approach normality in the data. Shown are 1) twin correlations based on the entire recording including all postures, 2) twin correlations based exclusively on the fragments of the recording where subjects were either sitting or lying. OS= opposite sex. Correlations that were significant (p<.05) are printed boldfaced.*

Structural equation modeling

First, we fitted a series of univariate unconstrained models. The means and variances of both SDNN index and RMSSD were equal for MZ and DZ twins, and singleton siblings. Importantly, equating male or female correlations or DZ correlations to correlations across any of the other sib-sib pairings (MZ twin – singleton sibling, DZ twin - singleton sibling, singleton sibling – singleton sibling) yielded no significant worsening in the fit of the model. This allowed us to reduce the number of parameters to be estimated, but also implies that the results obtained in twins can be generalized to singletons. Both RMSSD and SDNN index decreased with age in accordance with previous findings (Antelmi et al., 2004) and were higher in males at all periods of day. The sex difference for SDNN index and RMSSD repeats previous findings (Umetani, Singer, McCraty, & Atkinson, 1998) although the opposite has been found for RMSSD (Antelmi et al., 2004). In view of their effects, sex and age were retained as covariates in the final variance components analyses.

Table 4.3 Summary of the model fitting results

RMSSD*							
Model	**-2LL**	**df**	**p-value**	**Vs.**	**$\Delta\chi^2$**	**Δdf**	**AIC**
ACE [1]	15868.505	2939					
AE [2]	15868.720	2949	1.000	1	0.215	10	-19.785
CE [3]	15888.039	2949	.034	1	19.534	10	-0.466
E [4]	15941.199	2959	.000	1	72.694	20	32.694
ACE [5]	15875.789	2941	.026	1	7.284	2	3.284
AE [6]	**15875.789**	**2951**	**1.000**	**5**	**0.000**	**10**	**-20.000**
AE [7]	15907.917	2953	.000	6	32.128	2	28.128
SDNN index							
Model	**-2LL**	**df**	**p-value**	**Vs.**	**$\Delta\chi^2$**	**Δdf**	**AIC**
ACE [1]	24169.084	2874					
AE [2]	24169.375	2884	1.000	1	0.291	10	-19.709
CE [3]	24192.712	2884	.000	1	23.337	10	3.337
E [4]	24245.154	2894	.000	1	76.07	20	36.07
ACE [5]	24173.498	2876	0.110	1	4.414	2	0.414
AE [6]	**24173.498**	**2886**	**1.000**	**5**	**0.000**	**10**	**-20.000**
AE [7]	24192.774	2888	.000	6	19.276	2	15.276

*Summary of the model fitting results for RMSSD and SDNN index. *To approach normality in the data, RMSSD was ln transformed. AIC = Akaike's information criterion. Bold = best fitting model.*

1. *Multivariate ACE model in full Cholesky decomposition*
2. *Multivariate AE model in full Cholesky decomposition*
3. *Multivariate CE model in full Cholesky decomposition*
4. *Multivariate E model in full Cholesky decomposition*
5. *ACE independent pathway model for A, Cholesky decomposition for C and E*
6. *AE independent pathway model for A, Cholesky decomposition for E*
7. *AE independent pathway model for all factors*

The resulting most parsimonious unconstrained models were contrasted against different multivariate variance components models (ACE, AE, CE, and E). Only additive genetic (A) and non-shared environmental (E) sources significantly contributed to individual

variation in SDNN index and RMSSD. For the additive genetic variance the genetic factor model showed the best fit. Unique environmental variance had to be left in full Cholesky decomposition. In this final model the common genetic factor explained between 28% and 45% of the variance in SDNN index and between 32% and 48% of the variance in RMSSD (see table 4.3). Specific genetic influences on SDNN index were always present except for the afternoon and added between 2% and 12% to total heritability. Specific genetic influences on RMSSD were only present in the afternoon and during nighttime sleep and added 2% and 8% respectively to total heritability.

Discussion

Individuals characterized by low heart rate variability are at increased risk for cardiac events (Tsuji et al., 1996; Dekker et al., 2000; Nolan et al., 1998; Huikuri et al., 2003), sudden cardiac death (Bigger et al., 1993) and overall mortality (Tsuji et al., 1996; La Rovere et al., 1998). Based on prolonged measurements of heart rate variability in naturalistic settings obtained in a sample of 772 twins and singleton siblings, the present study showed that individual differences in two often used HRV measures, SDNN index (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Janszky et al., 2004) and RMSSD (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996) are to a large extent determined by additive genetic factors. For ambulatory SDNN index, heritability estimates from genetic model fitting ranged from 35% to 47%. For ambulatory RMSSD, heritability estimates ranged from 40% to 48%.

Table 4.4 Multivariate heritability estimates for SDNN index and RMSSD

	SDNN index			RMSSD*		
	Common h^2	**Specific h^2**	**Total**	**Common h^2**	**Specific h^2**	**Total**
Mo	28% (14 to 44)	7% (2 to 14)	35%	41% (27 to 53)	0% (-4 to 4)	41%
Af	36% (20 to 50)	0% (-4 to 4)	36%	46% (32 to 59)	2% (-6 to 6)	48%
Ev	45% (31 to 58)	2% (-8 to 8)	47%	48% (35 to 60)	0% (-3 to 3)	48%
Ni	31% (18 to 45)	12% (4 to 21)	43%	32% (19 to 45)	8% (2 to 15)	40%

* *RMSSD was ln transformed to approach normality in the data. Mo = morning, Af = afternoon, Ev = evening, Ni = night. Shown are the heritability estimates (h^2) for SDNN index and RMSSD. Total heritability has been specified into heritability caused by shared genetic components and heritability caused by time-specific genetic components. Between parentheses the 95% confidence intervals are presented.*

Our heritability estimates correspond well to those in a previous study using much shorter ambulatory recording periods (<4 hours). Using segregation analysis, the Kibbutzim family study (Sinnreich et al., 1999) found genetic influences to account for 45% of age and sex-corrected RMSSD. However, our estimate for SDNN index is substantially larger than the estimate from a family study based on the Framingham Heart Study and the Framingham Offspring study (Singh et al., 1999). In this latter study, SDNN was obtained rather than SDNN index. SDNN was averaged over a two-hour fragment obtained during a routine, scheduled examination at the Framingham Heart Study clinic. Genetic factors accounted for only 19% of the inter-individual variation in SDNN. A major difference in the genetic study design might account for these diverging findings. The Framingham studies used spouse and

sibling correlations to produce synthetic estimates of variance components. Because a significant spouse correlation was found, the resemblance between siblings was attributed in part to a shared household. Spouse correlation, however, may also reflect assortative mating for exercise behavior, a variable known to be associated with SDNN index (Pardo et al., 2000). It is of note that the age-corrected sibling correlations in the Framingham study (.23-.26) correspond very closely to our age-corrected sibling correlations, suggesting that, where the studies can be directly compared, they are actually very consistent.

Our study made use of an extended twin design which strongly increases statistical power to distinguish between components A, C and E compared to a design including only MZ and DZ twins (Posthuma & Boomsma, 2000). Although there was sufficient power (at β=.80, α=0.05) to detect effects of 23% or higher, no significant common environmental effect was found.

The extended twin design further allowed us to test the possibility that results obtained on singleton sibling pairs were identical to those obtained in twin pairs. This is important because the much lower birth weight in twins might be considered to reflect an impaired fetal environment, which according to the "Barker hypothesis" may influence autonomic function (IJzerman et al., 2003; Phillips et al., 2000). Monozygotic or dizygotic twins did not differ from singleton siblings in means, variances and covariances on any of the measures. The absence of any twin-singleton difference repeats previous findings in other cardiovascular risk factors (de Geus et al., 2001) and indicates that our results can safely be generalized to the population at large.

We separated the entire ambulatory recording into four periods of day, to allow for the possibility that different genetic factors would affect heart rate regulation during awake and sleeping periods, or during leisure (evening) and work (morning, afternoon) periods. Although evidence was found for separate genetic factors influencing heart rate variability at different daily periods, their contribution to the total genetic variance was marginal in comparison to the common genetic factor that influenced heart rate variability at all times of day. From a gene finding point of view, the common genetic factor structure is advantageous on at least two accounts. Using highly genetically correlated multivariate phenotypes can yield higher statistical power to find genes in linkage analysis (Allison et al., 1998). Secondly, these genes, by virtue of having a pervasive influence on heart rate variability across all situations, will also have the largest clinical relevance. This assumes that the genes causing low heart rate variability in this relatively young population remain of importance in later life. Note that we cannot exclude expression of different heart rate variability genes throughout the life span.

In conclusion, this study provides a strong confirmation that genes are important in the regulation of ambulatory heart rate variability, a clinically relevant phenotype for a wide range of cardiovascular diseases (Janszky et al., 2004; Huikuri et al., 2003; Tsuji et al., 1996; Bigger et al., 1993). The next step is to trace to actual genetic polymorphisms that influence ambulatory heart rate variability to provide new angles for preventive therapy. Already, the first whole genome screens and candidate gene studies have been initiated (Busjahn et al., 1998; Singh et al., 2002), and these initiatives should be rapidly extended. Because the power to detect genes increases with the availability of genetically correlated repeated measurements, we believe ambulatory heart rate variability to be an important asset in the search for genetic variation influencing cardiovascular disease risk.

5

A genetic analysis of ambulatory cardiorespiratory coupling

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Abstract

This study assessed the heritability of ambulatory heart period, respiratory sinus arrhythmia (RSA), and respiration rate and tested the hypothesis that the well-established correlation between these variables is determined by common genetic factors. In 780 healthy twins and siblings, 24-hr ambulatory recordings of ECG and thorax impedance were made. Genetic analyses showed considerable heritability for heart period (37%-48%), RSA (40%-55%) and respiration rate (27%-81%) at all daily periods. Significant genetic correlations were found throughout. Common genes explained large portions of the covariance between heart period and RSA and between respiration rate and RSA. During the afternoon and night, the covariance between respiration rate and RSA was completely determined by common genes. This overlap in genes can be exploited to increase the power of linkage studies to detect genetic variation influencing cardiovascular disease risk.

Introduction

The difference in heart period during the inspiratory and expiratory phases of the respiratory cycle is known as respiratory sinus arrhythmia (RSA). RSA is affected in a dose-response way by muscarinergic blockers or vagal cooling and is regarded to be a valid non-invasive index of cardiac vagal tone (Berntson et al., 1997; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Various time and frequency domain measures of RSA have become available that produce highly comparable results in both clinical and experimental settings (Grossman et al., 1990; Penttila et al., 2001; Bigger, Jr. et al., 1992; Grossman, Wilhelm, & Spoerle, 2004).

Studies in males and females in all age groups, using either patients or healthy controls, have collectively shown the existence of large individual differences in RSA (Grossman & Kollai, 1993; Ben Lamine et al., 2004). Studies examining these individual differences have demonstrated that lower values of RSA are associated with higher risk for cardiac disease (Singer et al., 1988; Bigger, Jr. et al., 1995; La Rovere et al., 1998) and hypertension (Liao et al., 1996; Mussalo et al., 2001). Reduced RSA is also associated with anxiety (Thayer, Friedman, & Borkovec, 1996; Watkins, Grossman, Krishnan, & Sherwood, 1998), posttraumatic stress disorder (Cohen et al., 1997) and, prospectively, with major depressive disorder (Rottenberg, Wilhelm, Gross, & Gotlib, 2002). The link between RSA amplitude and behavioral engagement can already be found in children of 3 months of age (Bazhenova, Plonskaia, & Porges, 2001) and children aged 5 to 6 years (Doussard-Roosevelt, Montgomery, & Porges, 2003).

In view of the potential relevance of individual differences in RSA to index future disease, insight is needed into its genetic and environmental origins. The twin design is a powerful method to do so (Boomsma et al., 2002; Neale & Cardon, 1992). A twin study compares the intra-pair resemblance for a certain trait in identical twins to the trait resemblance in fraternal twins. This allows the estimation of the relative contribution of genetic and environmental factors to variance in the trait. In addition, separate contributions can be estimated for environmental factors shared by all family members ("C") and environmental factors unique ("E") to each family member (including measurement error). Shared environment may include effects of parental socioeconomic status, diet, rearing style and parental guidance in lifestyle choices (e.g. exercise). To obtain sufficient statistical power to detect significance of such shared environmental influences, large samples of twins are needed. Power can be further increased by adding one or more non-twin siblings of the twins (Posthuma & Boomsma, 2000). This "extended twin design" was adopted in the current study, where RSA was obtained in 527 twins and 253 of their singleton siblings.

In previous twin studies, a significant genetic contribution to RSA has already been established. Heritability estimates ranged from 13% to 39% (Boomsma et al., 1990; Sinnreich et al., 1999; Snieder et al., 1997; Singh et al., 1999; Busjahn et al., 1998). The above estimates, however, were all based on the variance in RSA observed under resting conditions. Additional variance may appear under physical and psychological challenge, which is known to significantly reduce the mean value of RSA in most subjects, although large individual differences in the magnitude of RSA responsiveness are found (Houtveen, Rietveld, & De Geus, 2002; Cacioppo et al., 1994b). Using a comparable set of stressors, two large twin studies have shown a relative increase in *genetic* variance during stress (Boomsma et al., 1990; Snieder et al., 1997). Heritabilities for RSA level found at rest (24% in the adolescents, 31% in the middle-aged) increased substantially during a reaction time and mental arithmetic task (up to 51% in adolescents, and 43% in the middle-aged). This suggests that genetic influences are more pronounced when the subject is challenged by mentally and emotionally taxing tasks.

Accordingly, we hypothesize that heritability of RSA will be even higher when recorded over prolonged periods of time in a naturalistic setting since such challenges are more likely to be encountered in daily life. A first aim of the present study is to assess heritability of ambulatory measured RSA levels across an entire day and night.

Many studies have documented a significant covariance between RSA and heart period at rest and during laboratory challenges (Sahar, Shalev, & Porges, 2001; Medigue et al., 2001). Short heart periods, just as low levels of RSA, have been shown to predict cardiovascular disease (Dyer et al., 1980; Kannel, et al., 1987; Jouven, Zureik, Desnos, Guerot, & Ducimetiere, 2001). In addition, heart period is significantly shorter in subjects with psychiatric disorders (Carney et al., 1988; Austen & Wilson, 2001; Rechlin, Weis, Spitzer, & Kaschka, 1994; Lehofer et al., 1997). A straightforward interpretation of RSA and heart period covariance is that low RSA and short heart period both index low cardiac vagal tone. These correlated risk factors may further reflect a common genetic susceptibility, for instance through genes influencing parasympathetic nervous system activity. Only a single study has addressed the possibility of a genetic contribution to this association (De Geus, Boomsma, & Snieder, 2003). Common genes were indeed found to explain a large part of the association between the resting levels of heart period and RSA, although significant contribution was also found for unique environmental factors. To date, no studies have tested the contribution of genes and environment to the association of heart period and RSA under mentally challenging or naturalistic conditions.

A similar state of affairs applies to the significant covariance between RSA and respiration rate. Many studies have testified to the robustness of this association, at rest, during laboratory challenges, and during ambulatory recordings (Kollai & Kollai, 1992; de Geus et al., 1995; Snieder et al., 1997; Grossman & Kollai, 1993; Grossman et al., 2004), but only a single study has examined the source of the association in a genetically informative design (Snieder et al., 1997). Here, respiration rate-RSA covariance seemed to be due to overlap in both genetic and nonshared environmental factors contributing to these variables, both at rest and during a set of mental stress tasks. Nothing is currently known, however, about the genetics of the respiration rate-RSA covariance in naturalistic settings.

In short, the previous two bivariate twin studies on the genetics of RSA and heart period and the genetics of RSA and respiration rate were performed in a *laboratory* setting. No study has addressed the genetic architecture of the association between *ambulatory* heart period and RSA and *ambulatory* RSA and respiration rate. Furthermore, no trivariate genetic analysis of heart period, RSA and respiration rate has been performed to date, and the possible contribution of common genes to heart period and respiration rate remains to be determined. A second aim of this study, therefore, is to estimate the relative contribution of common genetic and environmental factors to the covariance between heart period, RSA and respiration rate measured in a naturalistic setting.

Methods

Subjects

Subjects were all registered in the Netherlands Twin Register (NTR). Their families were originally selected for a genetic linkage study searching for genes for depression, which is described elsewhere (Boomsma et al., 2000). Of the 1332 offspring who returned a DNA sample (buccal swabs) for the linkage study, 1008 were successfully contacted for a cardiovascular ambulatory monitoring study, of which 192 refused or were excluded. In total 816 subjects were willing to participate in cardiovascular ambulatory monitoring. A priori

reasons for exclusion were pregnancy, heart transplantation, presence of a pacemaker and known ischemic heart disease, congestive heart failure, or diabetic neuropathy. For 14 of the 816 subject recordings were unavailable due to equipment failures while 8 subjects had either a noisy ECG or a noisy thorax impedance signal, and were therefore excluded from the analysis. Fourteen subjects using rhythm-altering medication, heart rate variability-reducing antidepressants (tricyclic antidepressants and benzodiazepines), antihypertensive medication (β-blockers), or a combination of these were excluded from the analysis. The final sample consisted of 222 identical twins (80 men), 305 fraternal twins (109 men) and 253 singleton siblings (97 men) from 339 families. Mean age was 31.0 years (SD = 10.8). Zygosity of the twins was determined by DNA typing. The Ethics Committee of the Vrije Universiteit approved of the study protocol and all subjects gave written consent before entering the study. No payment was made for participation, but all subjects received an annotated review of their ambulatory ECG recording.

Ambulatory measurements

The Vrije Universiteit Ambulatory Monitoring System 46 (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; 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 lead 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 contains three major components: high frequent impedance changes due to the ejection of blood into the aorta during systole, low frequent impedance changes due to arm and upper body movement, and, in between these frequencies, the thoracic impedance changes due to respiration. Intervals of 100 seconds of the dZ signal were band-pass filtered with 0.1 and 0.4 Hz cut-offs after tapering with $(\sin(x))^2$ to yield the respiration signal.

VU-AMS software (www.psy.vu.nl/vu-ams) was used to display the recorded heart period time series as a cardiotachogram together with the respiration signal. Suspected erroneous R wave detection (e.g. due to ectopic beats) was automatically tagged for deletion. The starting points of inspiration and expiration were automatically scored for each breath, but interactive visual inspection allowed correction of erroneous respiration scoring or the selective removal of noisy signal fragments. From the corrected signal, mean heart period, respiration rate and RSA were computed. RSA was determined using the peak-to-trough method (Grossman et al., 1990; Fouad et al., 1984; Grossman & Kollai, 1993), which combines respiratory time intervals and the heart period time series to obtain an RSA value at each breath. This method yields highly comparable results to frequency domain based methods (Grossman et al., 1990) and has the advantage of additionally providing the respiratory frequency. RSA was computed for each breath as the difference between the shortest heart period during heart rate acceleration in the inspirational phase (which was made to include 1000 milliseconds from the following expiration to account for phase shifts) and the longest heart period during deceleration in the expirational phase (including 1000 milliseconds from the following expiratory pause/ inspirational phase). When no phase-related acceleration or deceleration was found, the breath was assigned a RSA score of zero.

Procedure

Subjects 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. Subjects were instructed to wear the device the entire day and night up until awakening the next morning. Written 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. Subjects were asked to keep a detailed diary. Every 30 (± 10) minutes the device produced an audible alarm beep to prompt them to write down a chronological account of activity, posture, location, social situation, and amount of perceived stress during each past 30 minutes. On the following day the researcher collected the device at home.

Statistical analysis

Data reduction - In addition to the cardio-respiratory measures, the VU-AMS device also recorded vertical acceleration. Accelerometer output was averaged every 30 seconds, and used as a proxy for gross body movement. Time-stamped information from the diary about activity and posture was combined with an interactive graphical display of body movement as a function of time, which made it possible to accurately specify the exact start and end times of the changes in activity or posture that the subjects had reported in the diary. We divided the entire recording into smaller intervals that were completely stationary with regard to physical activity and posture. Each interval was coded for posture (lying, sitting, standing, walking and bicycling), activity (e.g. deskwork, housekeeping, watching TV), and location (e.g. at home, at work, at a public place). The coded intervals were never shorter than 5 minutes or longer than 1 hour. Based upon the reported times of dinner and lunch, awakening and bedtime, 4 averages were computed over all coded intervals available for each of the 4 periods of the day: morning, afternoon, evening and nighttime. Averages were computed once using all posture data and once using only those intervals when subjects were sitting or lying. In 8% of the subjects the exact time of dinner, lunch, awakening or bedtime could not be extracted from either diary or body movement. For these subjects, the missing time was imputed using the mean times of these events in the rest of the sample.

Confounding variables - Individual differences in the ambulatory physiological variables were expected to be sensitive to three main confounding variables: differences in age, sex (De Meersman, 1993; Umetani et al., 1998), and physical activity patterns (Sacknoff, Gleim, Stachenfeld, & Coplan, 1994; Osterhues et al., 1997). To control for the first two of these variables, all correlation coefficients were age-adjusted, and calculated separately for the sexes. In model fitting analysis it was specifically tested whether the effects of sex and age on the means and variances could be dismissed from the model. To examine the influence of possible individual differences in physical activity patterns, analyses were performed twice; once using the averages based on all postures and once using the averages based on the data during which a subject was sitting or lying.

Twin correlations - To determine to what extent monozygotic twins are more similar than dizygotic twins or singleton siblings Pearson correlation coefficients were calculated for all groups, using SPSS software (SPSS Inc., Chicago, USA). In the computation of these correlations, fraternal twin pairs and singleton siblings were regarded as a single group, since these pairs all share on average 50% of their genetic material (this assumption was tested in the model fitting). All possible fraternal twin pairs or sib pairs that could be formed within a family were used.

Structural equation modeling - In order to answer the question to what extent genes, common environment and unique environment contribute to the variances and covariances between the three variables (heart period, RSA and respiration rate), a biometrical genetic model was fitted to the observed data using the structural equation program Mx (Neale et al., 2003). First, a series of unconstrained models was fitted to test the equality of means and variances for identical twins, fraternal twins, and singleton siblings. We then examined the presence of sex effects on the means and variances. Next, the significance of a linear regression of age on the three variables was tested. Lastly, 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.

The main questions were addressed in various nested trivariate (heart period, RSA and respiration rate) variance components models. In a twin study, the observed variance can be decomposed in four possible latent sources of variance: additive genetic effects (A), non-additive genetic effects (D), shared environment (C), and non-shared environment (E) following Neale and Cardon (1992). However, in a design that includes identical twins, fraternal twins and sib 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 choose to model A, C and E. For identical twins, fraternal twins and sibling pairs alike, similarity in shared environmental influences was fixed at 100%. Similarity of additive genetic influences was fixed at 50% for siblings and fraternal twins and at 100% for identical twins. Per definition, there was no similarity in the non-shared environmental influences.

For each of the four periods of day, a full trivariate ACE model in Cholesky decomposition (Neale & Cardon, 1992) was tested against the nested more parsimonious AE, CE or E models. The resulting best fitting model was used to further test the source of the observed covariance between the three variables. Specifically we tested the comparative fit of models with the genetic or environmental correlations set to zero, and of models with genetic or environmental correlations set to 1, i.e. models in which a single genetic or environmental factor influenced heart period, RSA, and respiration rate.

Throughout, nested models were compared using the likelihood ratio test. 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 (i.e., larger negative) values fit better than models with higher values. The final best fitting most parsimonious model was used to estimate the genetic and environmental contribution to the covariance between heart period, respiration rate and RSA.

Table 5 .1 Means (SD) and correlations for heart period, RSA and respiration rate

	Morning	Afternoon	Evening	Night
Heart period (ms)				
Men	847 (126)	811 (121)	854 (122)	1058 (139)
Women	764 (101)	752 (94)	793 (104)	937 (109)
lnRSA (ln(ms))				
Men	3.8 (0.4)	3.7 (0.5)	3.7 (0.5)	3.9 (0.5)
Women	3.9 (0.5)	3.8 (0.5)	3.9 (0.5)	4.0 (0.5)
Respiration rate (breath/minute)				
Men	16.3 (1.4)	16.7 (1.4)	17.5 (1.6)	15.4 (2.0)
Women	16.3 (1.3)	16.8 (1.3)	17.4 (1.6)	16.1 (2.0)
Heart period & lnRSA correlation				
Men	**.48****	**.52****	**.48****	**.46****
Women	**.47****	**.48****	**.47****	**.43****
Respiration rate & lnRSA correlation				
Men	**-.26****	**-.23****	**-.26****	**-.32****
Women	**-.21****	**-.14***	**-.23****	**-.28****

*Shown are means (SD) and correlations for each period of day, separately for the sexes. ** $p < .01$, * $p < .05$.*

Results

The average duration of valid measurement was 22:13 hrs (sd = 3:21 hrs). RSA was skewed at all daily periods and its natural logarithm was used in all further analyses. Figure 5.1 gives scatterplots of heart period and lnRSA and of lnRSA and respiration rate. Complete measurements during sitting activities were obtained in 689 subjects for all four periods of the day, in 91 subjects one or more periods were missing. Since part of the sample (two in each family) was originally selected for a study in search for genes influencing anxious depression, Pearson correlation coefficients were computed between a summary score of anxious depression (see (Boomsma et al., 2000) for more details) and our ambulatory variables. No meaningful association was found suggesting that the sample selection was unbiased.

Repeated measures ANOVA showed a significant between-subject effect of sex for heart period ($F(1,688) = 112.6$, $p < .001$), lnRSA ($F(1,688) = 7.8$, $p < .01$) and respiration rate ($F(1,688) = 4.3$, $p < 0.05$). Male heart period, lnRSA and respiration rate were lower than female heart period, lnRSA and respiration rate at all periods of day. A significant effect of the daily period was found for heart period ($F(3,686) = 1263.8$, $p < .001$), lnRSA ($F(3,686) = 55.4$, $p < .001$) and respiration rate ($F(3,686) = 203.9$, $p < .001$). This was entirely due to a decrease in respiration rate together with an increase in heart period and lnRSA during sleep in contrast to all three waking periods. Means and standard deviations of the variables are presented separately for each daily period and per sex in table 5.1.

Table 5.2 Age-adjusted twin and sibling correlations for heart period, RSA and respiration rate

		Respiration rate		lnRSA		Heart period	
		MZ	**DZ/sib**	**MZ**	**DZ/sib**	**MZ**	**DZ/sib**
Morning	Corr.	**.21**	**.15**	**.46**	**.21**	**.60**	**.31**
	N pairs	90	573	91	573	91	573
Afternoon	Corr.	**.42**	**.22**	**.51**	**.24**	**.55**	**.34**
	N pairs	90	570	91	570	92	570
Evening	Corr.	**.50**	**.21**	**.58**	**.24**	**.46**	**.28**
	N pairs	89	589	90	589	90	589
Night	Corr.	**.83**	**.50**	**.61**	**.27**	**.62**	**.38**
	N pairs	87	543	88	543	88	543

Correlations that were significant (p<.05) are printed boldfaced. MZ = identical twin pairs, DZ/sib = fraternal twin or sibling pairs N pairs = number of pairs of subjects.

Twin & sibling correlations

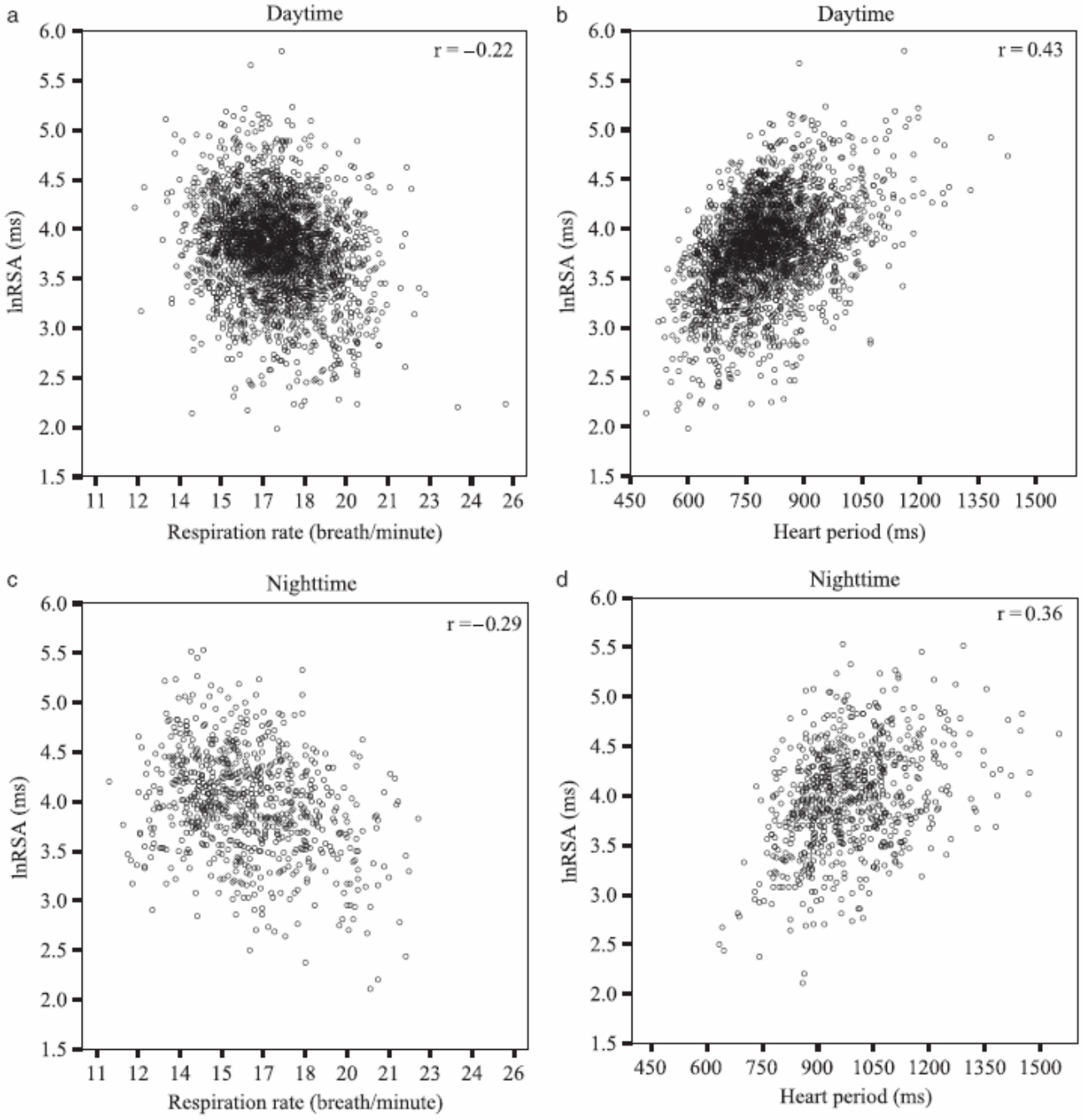
Correlations were computed twice. For the waking periods (morning, afternoon, evening) data were used 1) from the entire recording period, that is including different levels of physical activity and multiple postures, or 2) from periods with sitting activities only. In spite of the potential confounding effects of individual differences in the frequency of postural changes and physical activity, virtually identical patterns of twin and sibling correlations were obtained when either all data were used or sitting only data. To avoid possible interpretations based on movement artifact, however, we confined further analyses to intervals which involved a constant sitting posture (which comprised 51% of the total recording time).

Table 5.2 shows the resemblance between identical twins and fraternal twins/sibling pairs for heart period, lnRSA and respiration rate. It is immediately obvious from the systematically larger identical twin than fraternal twin/sib correlations that genetic factors influence all three variables. The increase in identical twin correlation for respiration rate at night further suggests higher heritability during sleep.

Structural equation modeling

Several assumptions of the extended twin design were tested in a series of unconstrained models. All morning, afternoon, evening and nighttime means of heart period, lnRSA and respiration rate could be set equal for identical twins, fraternal twins and siblings without a significant loss of fit of the model. Dropping the linear regression effect of age or sex on all variable means, however, significantly worsened the fit. The variances were homogeneous across zygosity and across sex. Likewise, testing for heterogeneity in the correlations across sex within zygosity showed that, in all cases, the correlations were homogeneous across males and females.

Figure 5.1 Scatter plots for daytime and nighttime



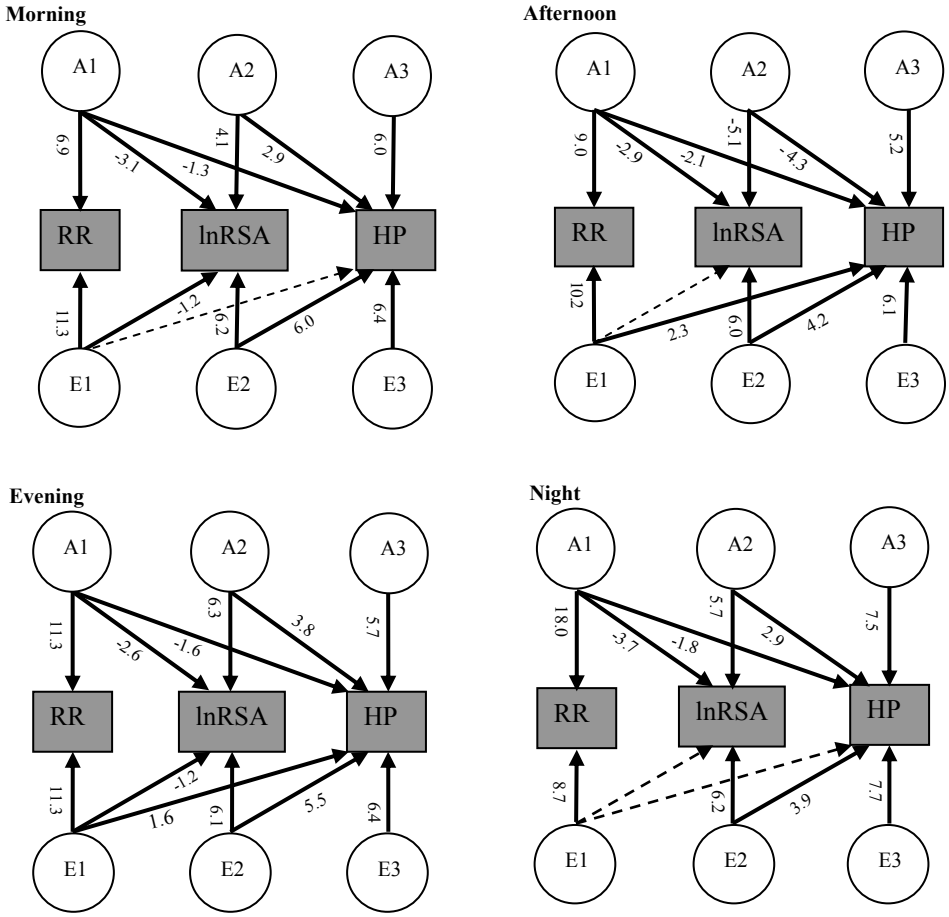
Plots are collapsed over sex, visualizing the relation between lnRSA and respiration rate and between lnRSA and heart period respectively. HP= heart period, RR= respiration rate.

Importantly, equating fraternal twin correlations to correlations across any of the other sib-sib pairings (identical twin - singleton sibling, fraternal twin - singleton sibling, singleton sibling - singleton sibling) produced no significant worsening in the fit of the model. Although the main aim here was to reduce the number of parameters to be estimated, note that these results imply that the estimates obtained in twins can be generalized to singletons.
The resulting most parsimonious model (no sex or twin-singleton effects on variances or sib-pair covariances) was used to test four separate trivariate variance decomposition models, for each period of day (morning, afternoon, evening, and night). For all three variables the shared environmental component (C) could be removed without a significant loss in fit (see table 5.3). This resulted in models that included only the additive genetic and unique environmental variance components (AE model). From this AE model we could not dismiss any of the genetic correlations between respiration rate, lnRSA, and heart period. The full three factor model also had to be maintained for most of the unique environmental influence, with a few notable exceptions, visualized as the non-significant paths in figure 5.2. A striking reduction in the importance of the environmental influences was seen at night, when both the covariance between respiration rate and heart period and between respiration rate and lnRSA became entirely genetic in origin. The final best fitting models for each of the periods of day are shown in figure 5.2.

Heritability is sum of the genetic variance due to factors A1 to A3 divided by the total variance (see footnote figure 5.2 for a computational example). Table 5.4 gives the heritability estimates for respiration rate, lnRSA and heart period under the best fitting models (boldfaced rows in table 5.3). Heritability estimates for respiration rate were all significant, but varied greatly over the four daily periods. During waking hours, heritability ranged between 27% and 50%. At night, however, it increased up to 81%. To investigate whether it is a decrease of environmental variance or an increase of genetic variance that causes the heritability estimate to increase so dramatically at night, we examined the changes in absolute variances. We found that variance attributed to genetic effects increased much more over the four periods of day compared to the decrease found in the variance ascribed to unique environmental effects. As can be seen in figure 5.2 the increase in genetic variance from evening to night for respiration rate is 196.3 ($=18.0^2$-11.3^2), while the decrease in environmental variance is 52.0 ($=8.7^2 - 11.3^2$). Thus, the increased heritability for respiration rate during the night is caused mainly by increased genetic variance in respiration rate during the night.

Heritability estimates for lnRSA ranged from 40% in the morning to 55% in the evening. As can be calculated from the path coefficients, the contribution of genes shared with respiration rate (genetic factor A1) to the heritability of lnRSA was between 8% (evening) and 16% (night). Since it has been suggested that RSA corrected for respiration rate would be a better predictor than uncorrected RSA (Grossman et al., 2004), we did an additional genetic analysis for respiratory-corrected RSA. These heritability estimates were 38%, 47%, 46% and 46% for the daily periods respectively, which is lower than the estimates for uncorrected RSA in the multivariate analyses.

Figure 5.2 The most parsimonious decomposition of variances and covariances for respiration rate (RR), lnRSA and heart period (HP)



For each of the daily periods, factor A1 represents the genetic influences on respiration rate, which are partly shared with lnRSA and heart period. Factor A2 represents the genetic influences on lnRSA, which are partly shared with heart period, but unshared with respiration rate. Factor A3 represents the remaining genetic influences on heart period that are unshared with respiration rate and lnRSA. Notation for the unique environmental factors (E1 through E3) follows analogous reasoning. The numbers next to the paths represent the unstandardized path coefficients. Non-significant paths are indicated by dotted lines. Heritability can be computed by standardizing these coefficients following path tracing rules. For example, heritability of morning lnRSA is

$$\frac{\text{Summed genetic variance}}{\text{Total variance}} = \frac{((-3.1)^2 + (4.1)^2)}{((-3.1)^2 + (4.1)^2 + (-1.2)^2 + (6.2)^2)}.$$

Table 5.3 Summary of the model fitting results

Morning

Model	**Versus**	**Δdf**	**χ^2**	**p**	**AIC**
ACE					
AE	ACE	6	3.644	0.725	-8.356
CE	ACE	6	11.128	0.085	-0.872
E	ACE	12	80.956	0.000	56.956
Reduced AE[1]	**AE**	**7**	**4.295**	**0.420**	**-9.705**

Afternoon

Model	**Versus**	**df**	**χ^2**	**p**	**AIC**
ACE	-	-	-	-	-
AE	ACE	6	1.963	0.923	-10.037
CE	ACE	6	15.354	0.018	3.354
E	ACE	12	89.743	0.000	65.743
Reduced AE[2]	**AE**	**7**	**3.171**	**0.272**	**-10.829**

Evening

Model	**Versus**	**Δdf**	**χ^2**	**p**	**AIC**
ACE					
AE	**ACE**	**6**	**1.51**	**0.959**	**-10.49**
CE	ACE	6	23.93	0.001	11.93
E	ACE	12	120.773	0.000	96.773

Night

Model	**Versus**	**Δdf**	**χ^2**	**p**	**AIC**
ACE					
AE	ACE	6	4.164	0.654	-7.836
CE	ACE	6	40.584	0.000	28.584
E	ACE	12	222.107	0.000	198.107
Reduced AE[3]	**AE**	**8**	**5.606**	**0.486**	**-10.394**

Summary of the model comparison results for each daily period.
[1] no rE between respiration rate and heart period, [2] no rE between respiration rate and lnRSA, [3] no rE between respiration rate and lnRSA and between respiration rate and heart period. Shown are fit statistics for models with additive genetic, common environmental and unique environmental (ACE) influences, additive genetic and unique environmental (AE) influences, common and unique environmental (CE) influences and unique environmental (E) influences. The full models include all genetic (rA) and unique environmental (rE) correlations between respiration rate, lnRSA and heart period. All associations between the variables have been tested for significance, only the best fitting most parsimonious model is presented. Boldfaced rows represent the model that fitted the data best. Low p values denote significant worsening of fit. AIC = Akaike's Information Criterion.

Heritability estimates for heart period ranged from 37% in the morning to 48% at night. The contribution to this heritability of genes that are shared between respiration rate and heart period was between 1% (morning) and 4% (afternoon). The contribution of genes shared only with lnRSA (genetic factor A2), which may reflect genes affecting cardiac vagal control, was between 6% (night) and 17% (afternoon). This leaves a substantial unique

genetic influence (genetic factor A3) on heart period, which may reflect genetic effects on the intrinsic pacemaker frequency and the sympathetic nervous system.

Table 5.5 shows the standardized covariances (correlations) between respiration rate, lnRSA and heart period (upper off-diagonal elements), as well as the standardized genetic contributions to the covariance between these three variables (lower off-diagonal elements). The genetic contribution to the covariance between two variables is calculated as the ratio of the genetic covariance (which is the genetic correlation between two variables and the product of the squared roots of the two heritabilities) to the total observed covariance. The remaining part of all covariances is explained by unique environmental factors. The correlation between morning lnRSA and respiration rate, for instance, is -0.23, with genes contributing 62% to this covariance and unique environment 38%.

Table 5.4 Heritability estimates for respiration rate, RSA and heart period

	Respiration rate	**lnRSA**	**Heart period**
h^2 morning	27% (12 to 41)	40% (27 to 54)	37% (24 to 50)
h^2 afternoon	44% (28 to 57)	49% (34 to 59)	45% (28 to 54)
h^2 evening	50% (37 to 61)	55% (43 to 65)	40% (24 to 53)
h^2 night	81% (74 to 86)	54% (41 to 64)	48% (26 to 68)

Shown are the heritability estimates (h^2) for respiration rate, RSA and heart period, under the best fitting models. The 95% confidence intervals are presented in parentheses. All values are significant at a .05 level.

lnRSA and heart period covaried significantly at all four periods, with correlation coefficients ranging between .35 and .45. The contribution of common genetic factors to the heart period-lnRSA covariance between the measures varied between 30% and 52%. The correlations between respiration rate and lnRSA were all significant and ranged between -.17 and -.24. The contribution of common genetic factors to the respiration rate-lnRSA covariance varied between 62% and 100%. The correlation between respiration rate and heart period was only significant at night (-.19). All of this respiration rate-heart period covariance was explained by genetic factors.

Discussion

This study assessed the heritability of ambulatory heart period, RSA and respiration rate in 339 families and tested the hypothesis that the covariance between these variables is caused by common genetic factors. Our study had a number of strengths in design that provide confidence in its outcome. The extended twin design increases statistical power to distinguish between genetic and common and unique environmental influences compared to a design that includes only identical and fraternal twins (Posthuma & Boomsma, 2000). Furthermore, it allowed us to show that results obtained in singleton siblings do not differ from those obtained in twins. The absence of any twin-singleton difference replicates previous findings in other cardiovascular risk factors (de Geus et al., 2001) and indicates that our heritability estimates can safely be generalized to the population at large.

Table 5.5a-d Standardized covariances between respiration rate, lnRSA and heart period and the genetic contributions to these covariances

Morning

	Respiration rate	**lnRSA**	**Heart period**
Respiration rate	**-**	***-.23***	*-.07*
lnRSA	**62% (34 to 85)**	**-**	***.42***
Heart period	**100%**	**30% (10 to 48)**	**-**

Afternoon

	Respiration rate	**lnRSA**	**Heart period**
Respiration rate	**-**	***-.17***	*.02*
lnRSA	**100%**	**-**	***.45***
Heart period	**45% (13 to 72)**	**52% (32 to 66)**	**-**

Evening

	Respiration rate	**lnRSA**	**Heart period**
Respiration rate	**-**	***-.24***	*.01*
lnRSA	**69% (37 to 96)**	**-**	***.42***
Heart period	**49% (3 to 95)**	**47% (28 to 63)**	**-**

Night

	Respiration rate	**lnRSA**	**Heart period**
Respiration rate	**-**	***-.27***	***-.19***
lnRSA	**100%**	**-**	***.35***
Heart period	**100%**	**49% (27 to 68)**	**-**

Significance at a .05 level is indicated in bold. The upper-diagonal elements report the standardized phenotypic covariances between the variables (=correlations), while the lower-diagonal elements show the genetic contributions (%) to these covariance. The 95% confidence intervals are presented in parentheses. Note that a non-significant correlation between the variables can still have significant genetic contributions, if the genetic and unique environmental correlations are of opposite sign, as was the case for daytime correlations between heart period and respiration rate.

The current study estimated the heritability of RSA to lie between 40% and 55%. This agrees with a previous twin study, in which RSA heritability (based on spectral power in the heart period time series) was estimated at 39% across a short 30 minute recording interval in 141 twin pairs (Busjahn et al., 1998). On the other hand, using a sib-parent design, Singh and colleagues (1999) found that genetic factors accounted for only 16% of the interindividual variation in high frequency power. In that study, however, high frequency power was obtained from a two hour interval recorded during a routine examination at the Framingham Heart Study clinic, which is more similar to a laboratory, rather than ambulatory, recording. Other laboratory twin studies have also reported more modest heritability estimates for resting RSA, varying from 19% to 31% (Boomsma et al., 1990; Snieder et al., 1997).

When RSA is measured during stressful tasks, however, heritability increases to reach levels comparable to those found in the current ambulatory study (Snieder et al., 1997).This pattern of results is in keeping with the idea that under conditions of challenge or in naturalistic settings other (or more) aspects of cardiovascular regulation come into play, allowing genetic differences between individuals to become more pronounced (See also Boomsma et al., 1998). However, it is unclear whether the effects of the same genetic factors

that operate at rest are amplified by stress, or whether new genetic factors emerge during stress. Heritability estimates do not tell us *which* genes are contributing *in what way*. It may well be that different genetic pathways operate to affect resting RSA measured in a standardized setting compared to ambulatory RSA recorded over prolonged periods of time in a naturalistic setting.

Indeed, it is possible that genes that cause *low* RSA at rest act to cause *high* RSA under ambulatory conditions. This could explain the apparent contradiction in two studies that have examined the genetic basis of RSA by a candidate gene approach. An ambulatory study by Busjahn et al. (1998) in Caucasian twins, found that subjects homozygous (DD) for an insertion/deletion polymorphism in intron 16 of the angiotensin-converting enzyme gene had significantly higher levels of ambulatory RSA. The opposite finding was found, however, using laboratory recordings in African American subjects (Thayer et al., 2003). Although ethnicity was invoked as a plausible explanation for the discrepancy in results, contribution of the difference in ambulatory versus laboratory design cannot be excluded.

In the present study, heritability of ambulatory heart period is estimated between 37% and 48%. Previous twin studies that assessed the heritability of ambulatory heart period (Fagard et al., 2003; Jedrusik et al., 2003) estimated heritability of the 24 hour mean of heart period to be slightly higher, between 51% and 70%. When we compare all estimates from ambulatory studies (including our own) to the published twin data on resting laboratory values, we find no evidence that the heritability of heart period increases in ambulatory recordings over laboratory recordings as it did for RSA. Laboratory studies in adult twins have reported heritabilities of resting heart period to lie between 50% and 77% (Ditto, 1993; Russell, Law, Sholinsky, & Fabsitz, 1998; Li et al., 2001; Fagard et al., 2003). It should again be noted that the same heritability may arise from different genetic pathways influencing heart period in different measurement settings.

In the present study the genetic contribution to the variance in daytime respiration rate was between 27% and 50%. Only one previous study reported on the heritability of respiration rate. In that study respiration rate was measured under rest and stress conditions in a laboratory setting (Snieder et al., 1997). The genetic component of variance was estimated around 62% for the resting condition and between 51 and 60% for stress conditions. The lower estimates in our study compared to Snieder et al. (1997) may reflect the fact that during the laboratory experiment there was no talking. Talking influences respiration and increases the environmental component of the variance decomposition to the detriment of the genetic component. Interestingly, in the current study a strong increase in the heritability of respiration rate was seen at night, suggesting that respiration rate is more under genetic control during sleep. Neurobiologically, this makes good sense. During sleep respiratory frequency will most purely reflect intrinsic rhythmogenesis by the brainstem and some aspects of respiratory rhythmogenesis may even be entirely specific to sleep (Mackiewicz & Pack, 2003). Indeed, heritability increased mainly because of an increase in total genetic variance, suggesting that new genetic variation is being expressed during sleep. This clearly demonstrates the advantage of ambulatory recording over resting recordings, in particular the added value of nighttime recordings.

Genetic contributions to the covariances

RSA was significantly correlated to respiration rate and heart period. About 6% of the between-subject variance in RSA was explained by respiration rate, which is comparable to the association found in many laboratory studies (Houtveen et al., 2002; Grossman & Kollai, 1993; Ritz, Thons, & Dahme, 2001). The association between ambulatory RSA and heart

period was stronger, with RSA explaining about 25% of the between-subject variance in heart period. This is again comparable to what is found in laboratory settings (Sahar et al., 2001; De Geus et al., 2003; Medigue et al., 2001). A significant association between heart period and respiration rate was found at night only and this association was completely due to genetic effects. It should be noted that our focus was on between-subject covariance only. Much higher correlations between respiration rate and RSA and RSA and heart period are found in a pure within-subject analysis of ambulatory recordings (Grossman et al., 2004).

In agreement with our hypothesis, significant genetic correlations between heart period, RSA and respiration rate were found at all periods of the day. This suggests that common genes influence individual variation in respiration rate, RSA and heart period. The genetic correlations are not unity, though, suggesting that additional unique genes contribute to each of the variables. It is important to note here that our results do not reveal the exact causal chain of events that give rise to the common genetic factor. The common genetic factor may act entirely through one of the traits, i.e. they may influence respiration rate, which influences RSA, which in turn influences heart period. It may also primarily affect heart period, which in turn affects respiration rate and RSA, possibly through baroreflex action on respiratory generator neurons. Finally, the common genetic factor may reflect pleiotropic genes that influence each of the traits, but without any causal effects of the traits on each other. Pleiotropic genes simultaneously affecting between-subject variance in respiration rate, RSA, and heart period may be found at different levels. They may reflect individual differences in the general state of arousal, for instance as part of neuroticism or depression (Carney et al., 2001) that would affect both respiratory drive and tonic vagal drive. A pleiotropic genetic basis of respiration rate, RSA and heart period may also derive from genes affecting general aspects of neurotransmission (e.g. serotonergic receptors or secondary signaling proteins) in either limbic or brainstem areas involved in cardiovascular and respiratory control (Severson, Wang, Pieribone, Dohle, & Richerson, 2003; Richerson, 2004).

It has been suggested that RSA is a more valid index of between-subject differences in cardiac vagal control when individual differences in respiratory behavior are taken into account (Grossman & Kollai, 1993; Grossman et al., 2004) and that the validity of heart rate variability measures as predictors for cardiovascular disease could potentially improve from a correction for respiration rate. If, however, the genetic correlation between respiration rate and RSA shown in our analyses derives from pleiotropic genes, a correction for respiration rate could actually remove individual differences in cardiac vagal control. Our genetic analysis of the respiratory-corrected RSA showed heritability to decrease substantially compared to the estimates based on uncorrected RSA. Resolving the nature of the common genetic factor causing respiration rate and RSA to covary is an important future mission, but for now, removing genetic variance shared by RSA and respiration rate by using residualized scores seems unfounded. We suggest that future studies employ a multivariate design retaining both respiration rate and RSA as predictor variables.

Our results showed that RSA and heart period share another set of genes that are independent of respiration rate (factor A2 in figure 5.2). A putative explanation for the additional genetic factor A2 in figure 5.2 is that it represents respiration-independent gene effects on central vagal tone or the efficacy of muscarinergic effects on the sinoatrial node. Decreases in cardiac vagal control are well-known to increase heart period as well as decrease RSA (Berntson et al., 1994; Adinoff, Mefford, Waxman, & Linnoila, 1992). The significant genetic contribution to the covariance between heart period and RSA concurs with a previous investigation in a young and middle-aged twin cohort (virtually non-overlapping with the present cohort) in which a common genetic factor contributed 54% and 70% respectively to

the association between heart period and RSA (De Geus et al., 2003). In view of the clinical importance of cardiac vagal control in cardiology (Tsuji et al., 1996; Bigger, Jr. et al., 1995), finding genes influencing both heart period and RSA is an important objective.

The power to detect such genes, particularly in linkage studies, is known to increase when a multivariate approach is used (Amos, de Andrade, & Zhu, 2001; Evans, 2002). This could be done using repeated measures of the same variable as well as using different, but genetically correlated, variables. The latter multivariate strategy has already successfully been applied in whole genome scans for blood pressure that added body mass index as a second phenotype (Turner, Kardia, Boerwinkle, & Andrade, 2004). Prolonged ambulatory recording of respiration rate, RSA and heart period has an inherent repeated measure structure and the three variables are highly genetically correlated. Measurement in a naturalistic setting has the additional advantage of providing a window on the function of the autonomic nervous system in the context of repeated and ecologically valid stressors. We conclude therefore, that ambulatory measurement of cardiovascular and respiratory signals can help shorten the search for the actual genetic polymorphisms that influence cardiovascular disease risk.

6

Heritability of indices of cardiac contractility in ambulatory recordings

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Abstract

Sympathetic control of cardiac contractility plays an important role in the risk for heart failure, but little is known about the relative contribution of genetic factors to individual variation in cardiac contractility. In a naturalistic setting, the extent of sympathetic inotropic control will vary with the demands of daily activities. Therefore, the genetic architecture of cardiac contractility may change across the day, or from awake to sleep periods. To establish heritability of cardiac contractility in a naturalistic setting, we recorded 24-hour ambulatory ECG and thorax impedance signals in 755 healthy adult twins and their singleton siblings. Three indices of cardiac contractility were derived: the pre-ejection period (PEP), the ratio of PEP to the left ventricular ejection time (PEP/LVET ratio), and the Heather index (HI). Multivariate genetic analyses across four periods of day (morning, afternoon, evening, night) yielded significant estimates for genetic contribution to PEP (48% to 62%). Heritability estimates for PEP/LVET ratio and HI ranged between 35% and 58% and between 38% and 50% respectively. Contractility during the three waking periods was largely influenced by genetic factors that were common to the entire 24 hour period. During sleep additional genetic influences emerged that accounted for 8% and 20% respectively of the night-time heritability for PEP and the Heather index. In conclusion, impedance-derived measures of sympathetic control of cardiac contractility show substantial heritability at all periods of the day and during sleep.

Introduction

Heart failure is a complex, progressive, and highly prevalent disorder that accounts for a large part of cardiac morbidity and mortality (Haldeman, Croft, Giles, & Rashidee, 1999; Koek et al., 2003; Hunt et al., 2001). In heart failure, there is a decrease in stroke volume that results from systolic dysfunction (loss of intrinsic inotropy), diastolic dysfunction (decreased ventricular compliance), or a combination of the two. Chronically high sympathetic nervous system activity plays a crucial role in the clinical progression of heart failure and may directly contribute to its pathophysiology (Kaye et al., 1995; Rundqvist, Elam, Bergmann-Sverrisdottir, Eisenhofer, & Friberg, 1997; Swedberg et al., 1990). This suggests that the well-known genetic component in the susceptibility to heart failure (Arnett, de Las, & Broeckel, 2004; Bleumink et al., 2004) may derive in part from individual differences in sympathetic inotropic control.

There is an unfortunate lack of information, however, on the influence of hereditary factors on indices of sympathetic control of cardiac contractility, such as the pre-ejection period (PEP), the ratio of PEP to the left ventricular ejection time (LVET), and the Heather index (HI). Moreover, no previous study has investigated sympathetic control of cardiac contractility over prolonged periods of time in a naturalistic setting. Since heart failure is a complex disorder that may be caused by many genetic and environmental influences or an interaction of these, such analyses may provide new angles for future genetic linkage and association studies. In this report, impedance cardiography (Cybulski, Miskiewicz, Szulc, Torbicki, & Pasierski, 1993; Sherwood et al., 1990) was used to measure the systolic time intervals (PEP, LVET) and the HI across a 24-hour period in a large group of healthy twin families. An extended twin design was used to estimate the heritability of these indices of ambulatory sympathetic control of cardiac contractility.

Methods

Subjects

All participants were registered in the Netherlands Twin Register. Their families were originally selected for a genetic linkage study searching for genes influencing anxiety and depression, which is described elsewhere (Boomsma et al., 2000; Middeldorp et al., in press). Of the first 1332 offspring who returned a DNA sample (buccal swabs) for the linkage study, 1008 were successfully contacted for a cardiovascular ambulatory monitoring study. Of these, 192 subjects refused or were excluded based on our a priori reasons for exclusion (pregnancy, heart transplantation, presence of a pacemaker and known ischemic heart disease, congestive heart failure, or diabetic neuropathy). A final 816 subjects were eligible and willing to participate in cardiovascular ambulatory monitoring. Data from 34 participants on cardioactive medication (including all β-blockers) were discarded. For 14 additional subjects recordings were unavailable due to equipment failures, while 13 subjects had either a noisy ECG or a noisy thorax impedance signal, and were therefore excluded from the analysis. The final sample consisted of 215 identical twins (77 men), 296 fraternal twins (107 men) and 244 singleton siblings (94 men) from 339 families. Mean age was 30.6 years (SD=10.4). Zygosity of the twins was determined by DNA typing. The Ethics Committee of the Vrije Universiteit approved the study protocol and all subjects gave written consent before entering the study. No payment was made for participation, but all subjects received an annotated review of their ambulatory ECG and blood pressure recordings.

Procedure

Subjects were visited at home, before starting their normal daily activities. During a short interview, information on health status, current and past medication use and family history of cardiovascular disease was obtained. The VU-AMS ambulatory ECG/ICG monitor was attached together with an ambulatory blood pressure monitor (Spacelabs) and their operation was explained. Subjects were instructed to wear both devices the entire day and night up until awakening the next morning. Written 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. Subjects were asked to keep a detailed diary. Every 30 (±10) minutes the ambulatory device produced an audible alarm beep to prompt them to write down a chronological account of activity, posture, location, social situation, and amount of perceived stress during each past 30 minutes. On the following day, the researcher collected all equipment at home.

Impedance cardiography

The VU-AMS (version 4.6) measures the ambulatory electrocardiogram (ECG), the thorax impedance (Z_0), the changes in impedance (ΔZ) and the impedance cardiogram (ICG) continuously from a six-electrode configuration (de Geus & van Doornen, 1996; Riese et al., 2003; Willemsen et al., 1996). In addition, it measures vertical acceleration, which is used as a proxy for gross body movement. Each R-wave in the ECG is automatically detected. At the R-wave peak a millisecond counter is read out and reset to obtain the IBI time series, which is stored continuously. To measure thorax impedance the VU-AMS device uses a 4-spot electrode technique. Thoracic impedance was 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 render basal impedance (Z). Filtering Z at 0.1 Hz (high pass) supplied the ΔZ signal, which in turn was filtered at 30.0 Hz (high pass) to obtain dZ/dt, the high frequent impedance changes due to the ejection of blood into the aorta during systole. A Z_0 value was determined by averaging Z samples at the R-wave. ΔZ and dZ/dt were led to an A/D converter of the microprocessor, and digitized at 10 and 250 Hz respectively. The ECG and ICG signal of each 60-second period was ensemble averaged with reference to the R-wave (Muzi et al., 1985). This assembled dZ/dt waveform will be referred to as a "60-second ensemble average". The described procedure reduces the impact of single-beat fluctuations in the impedance signal through respiration and slow thorax movement. Systolic time intervals scored in the resulting 60-sec ensemble-averaged ICG correspond very closely to the mean systolic time intervals obtained over the (reliable) single-beat ICG waves in that same minute (Muzi et al., 1985; Boomsma, de Vries, & Orlebeke, 1989; Kelsey & Guethlein, 1990; Kelsey et al., 1998). Off-line processing of the ensemble averages of the DZ/dt signal allows the computation of systolic time intervals and volumetric measures.

Data reduction

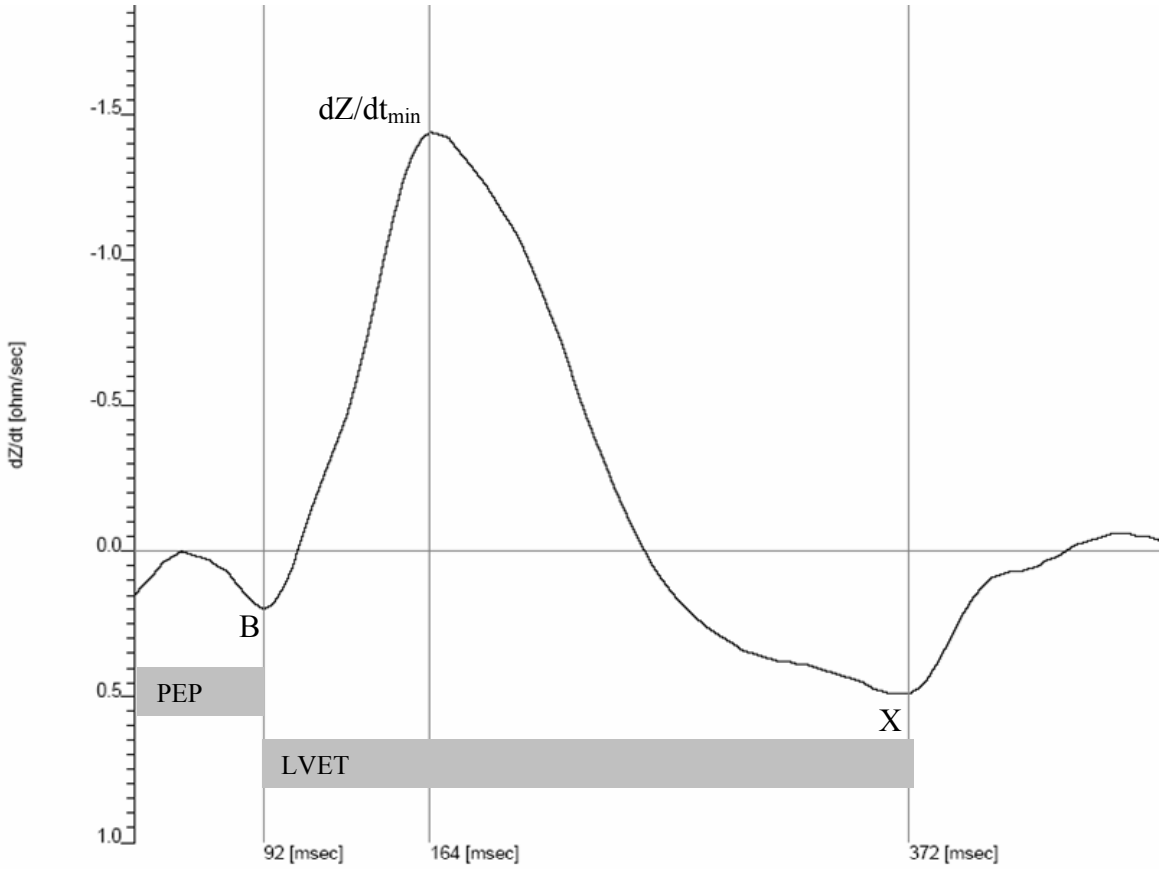
Using the diary entries in combination with the vertical accelerometer signal and the heart rate, the entire recording was divided into periods that were defined by posture, activity, location and social situation. To reduce the amount of visual inspection needed, the same ensemble averaging strategy used to obtain 60-second averages from single-beat waveforms was applied to obtain large-scale ensemble averages across these periods. A previous ambulatory study by Riese et al. (2003) showed that such a large-scale ensemble averaging validly recaptured the information in the original 60-second ensemble averages while substantially reducing the total amount of visual inspection needed. In view of the confounding effects of changes in posture on systolic time interval based indices of contractility, during the daytime recordings we carefully selected only periods when subjects were sitting.

ICG waveform scoring

Systolic time intervals and the dZ/dt_{min} were manually scored with a VU-AMS interactive software program (www.psy.vu.nl/vu-ams) that graphically displayed both the 60-second ensemble averages and the large-scale ensemble averages of the dZ/dt signal. Noise was reduced by deleting noisy 60-second ensemble averages from the large-scale ensemble averages. For every large-scale ensemble average three time points were scored: the upstroke, or B-point, the dZ/dt_{min} point and the incisura or X-point (see Figure 6.1). Large-scale ensemble averages were deleted when there was too much noise to be able to detect the three ICG waveform characteristics with certainty, or when dZ/dt_{min} was positive.

Left ventricular ejection time (LVET) was defined as the time between the opening and closing of the aortic valves, in other words the B-X time interval. The PEP was defined as the time interval between the onset of the electromechanical systole (Q-wave onset) and the onset of left ventricular ejection at the opening of the aortic valves. The VU-AMS impedance cardiograph uses the R-B time interval instead of the Q-B time interval to estimate PEP (Sherwood et al., 1990). Previous studies have shown that there is little intra- and interindividual variability in the Q-R time interval (Willemsen et al., 1996) and that the R-wave yields more reliable PEP estimates (Berntson, Lozano, Chen, & Cacioppo, 2004). We added a fixed Q-R interval of 48 ms to this abbreviated PEP to allow easy comparison with Q-wave based PEP. This also allowed us to compute the ratio of PEP/LVET, proposed as an index of myocardial contractility less dependent on preload (Weissler, Harris, & Schoenfeld, 1968). The Heather Index of myocardial contractility, corrected for Z_0 (HI; units/s^2), was calculated as the ratio of dZ/dt_{min} to the R-Z interval, divided by Z_0 (Kelsey & Guethlein, 1990; Sherwood et al., 1990). A reduced PEP and increased values of PEP/LVET ratio and HI all signal increased inotropic control, i.e. larger sympathetic drive to the left ventricle.

Figure 6.1 Large-scale ensemble average



Indicated are the characteristic B-point or upstroke, the dZ/dtmin point or the top of the waveform, and the X-point or incisura. PEP = pre-ejection period (R-B time interval), LVET = left ventricular ejection time (B-X interval).

Structural equation modeling

To estimate to what extent genes, shared environment and unshared environment contribute to the variance in sympathetic control of cardiac contractility, biometrical genetic models were fit to the data on PEP, PEP/LVET ratio and HI with the use of the structural equation program Mx (Neale et al., 2003). Quantitative analyses were carried out in several steps. First, a series of increasingly constrained univariate models were fit for each period of the day and for each variable separately, to test the assumptions of genetic modeling with an extended twin design. The homogeneity of means and variances for MZ twins, DZ twins, and singleton siblings and for males and females were tested first. Next, homogeneity of correlations of male and female pairs and of DZ twins and sibling pairs was tested. The resulting most parsimonious unconstrained models indicated to what extent the number of estimated parameters could be limited in the subsequent analyses and these most parsimonious models were the ones against which the variance decomposition models were tested.

In general, the observed variance can be decomposed in four possible latent sources of variance (Boomsma et al., 2002). The two environmental sources are environmental effects that are shared by members of a family (C), and environmental effects that are unique to each member of a family (E). Two kinds of genetic effects are distinguished: additive genetic

effects (A) and non-additive genetic effects. Non-additive genetic effects include dominance effects (D) and epistasis. Dominance describes the interaction between alleles at the same locus and epistasis describes the interaction of alleles at different loci (Neale & Cardon, 1992). 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 choose the models that were most appropriate. For MZ, DZ twins and sibling pairs, similarity in shared environmental influences was fixed at 100%. For same-sex first-degree relatives, similarity of additive genetic influences was fixed at 50% and similarity for dominance genetic influences was fixed at 25%. For MZ twins, similarities of additive and dominance genetic influences were fixed at 100%. Unshared environmental influences are uncorrelated in all twin and sibling pairs. To reduce complexity of genetic modeling, we discarded data from 7 singleton siblings (1 male, 6 female) in a few families with more than four additional singleton siblings. To evaluate quantitative sex differences in the genetic architecture, parameter estimates were allowed to differ between males and females. Because phenotypic correlations were close to zero for opposite sex pairs for PEP and PEP/LVET ratio, genetic correlations in opposite-sex first degree relatives were constrained at zero and the log likelihood of this model was compared to the model in which additive and dominant genetic correlations were fixed at 0.5 and 0.25, respectively.

After establishing the most parsimonious variance components model (ACE or ADE, AE, CE, or E) for each daily period, we used a full four-variate Cholesky decomposition to test whether the same or different genetic and environmental factors influenced cardiac contractility at each of the four periods of the day (morning, afternoon, evening and night). The Cholesky decomposition imposes a structure of stratification in several shared latent factors. In the case of a four-variate analysis, there is a main factor that loads on all variables, followed by a second factor that loads on all but the first variable, followed by a third latent factor that loads on the final two variables. The final factor only loads on the last variable. In the full model all variance components (A, D/C and E) are structured this way. A priori, we expected a single genetic factor to underlie the variance throughout the day and night. This was tested by contrasting a full Cholesky decomposition against a genetic one factor model (independent pathway model) for each variable. The independent pathway model specifies three common factors. One for genetic (A) sources of variance, one for shared environmental sources of variance (C) or for dominance genetic effects (D), whichever is modeled, and one for unique environmental (E) sources of variance, while it also allows for period-specific influences of A, C/D and E for all periods of the day.

Akaike's Information Criterion (AIC) (Akaike, 1987) was used to evaluate the relative fit of the various nested (and non-nested) models, which is an index of goodness-of-fit for which a larger negative value indicates greater parsimony of the model. Significance tests of the individual path coefficients were carried out by constraining paths to zero and applying likelihood ratio tests.

Table 6.1 Means (SD) for pre-ejection period (PEP), left ventricular ejection time (LVET), PEP/LVET ratio, and the Heather index (HI) for each period of day

	N	PEP (ms)	LVET (ms)	PEP/LVET	HI (Ω/ms^2)
Morning					
Men	265	96.4 (14.9)	292.4 (34.1)	.34 (.07)	-.52 (.17)
Women	457	99.6 (18.5)	289.0 (35.7)	.35 (.09)	-.63 (.20)
Afternoon					
Men	271	95.9 (14.2)	284.6 (32.7)	.34 (.07)	-.58 (.18)
Women	468	98.2 (17.1)	285.0 (33.9)	.35 (.09)	-.66 (.21)
Evening					
Men	264	95.7 (13.6)	293.2 (33.6)	.33 (.07)	-.60 (.21)
Women	456	98.0 (16.2)	297.0 (35.2)	.34 (.08)	-.66 (.21)
Night					
Men	289	105.4 (15.4)	334.7 (26.6)	.32 (.06)	-.48 (.15)
Women	505	104.8 (15.3)	331.5 (27.2)	.32 (.06)	-.54 (.16)

N=number of subjects with complete data

Results

General descriptive statistics

On average, the ambulatory monitoring period had a duration of 21 hours and 20 minutes (± 4:14h), which included an average of 43 (±12) large-scale ensemble averages of the dZ/dt signal. Of these, 50.5% were recorded either during sitting or lying posture. Means for PEP, PEP/LVET ratio and HI for all periods that subjects were sitting or lying are presented in Table 6.1.

Although the sample was previously selected based on the presence of at least 2 family members with low or high scores on personality traits related to the risk for anxiety and depressive disorder, these traits did not correlate significantly with our indices of cardiac contractility.

We first tested the need to include two possible confounders, body mass index (BMI), and age in the genetic models. BMI had no effect on the means of any of the variables. Nighttime PEP was significantly shorter with increasing age, but otherwise no age effect was found. For all periods of day we found that with increasing age the PEP/LVET ratio significantly decreased. For HI, a significant influence of age (lower with increasing age during the day and higher with increasing age at night) on the means of all daily periods was found. Consequently, age was entered as a covariate in the genetic model fitting.

Table 6.2 Twin correlations for pre-ejection period (PEP), left ventricular ejection time (LVET), PEP/LVET ratio, and the Heather index (HI) for each period of day

		PEP		PEP/LVET		HI	
		rMZ	**rDZ/sib**	**rMZ**	**rDZ/sib**	**rMZ**	**rDZ/sib**
	Men	**.71**	**.38**	**.63**	**.28**	**.56**	**.32**
Morning	Women	**.72**	**.23**	**.62**	**.25**	**.55**	**.40**
	OS	**-**	.01	**-**	-.03	**-**	**.17**
	Men	**.70**	**.42**	**.64**	**.41**	**.45**	**.26**
Afternoon	Women	**.73**	**.24**	**.64**	**.19**	**.43**	**.34**
	OS	**-**	-.01	**-**	-.09	-	**.12**
	Men	**.69**	**.32**	**.80**	**.31**	**.59**	**.12**
Evening	Women	**.64**	**.21**	**.48**	**.22**	**.56**	**.34**
	OS	**-**	-.05	**-**	-.07	**-**	**.24**
	Men	**.70**	**.25**	**.62**	**.22**	**.56**	.08
Night	Women	**.46**	**.13**	**.50**	**.20**	**.35**	**.14**
	OS	**-**	-.08	**-**	-.07	**-**	.02

PEP/LVET, HI, and nighttime PEP twin correlations were corrected for influences of age on the mean. OS = opposite sex.

The resemblance between MZ twins and between DZ twins and/or sibling pairs was examined by calculating Pearson correlations (age-adjusted for nighttime PEP, PEP/LVET and HI), stratified by sex, as shown in Table 6.2. Throughout, a larger MZ than DZ/sib correlation was evident, suggesting the presence of additive genetic and unique environmental influences. For PEP and the PEP/LVET ratio the majority of MZ twin correlations were more than twice as large as the DZ correlations, indicating the possible presence of dominance genetic effects. For these two variables an ADE model was fitted. In addition, the opposite sex correlations for these variables were near zero suggesting that different genes may be acting in males and females. The HI correlations suggest an ACE model as the best model to start with.

For HI the means and variances were equal for MZ twins, DZ twins and singleton siblings. Mean HI was lower in females but there were no sex-effects on the variances and covariances. For PEP and PEP/LVET male and female means could be set equal. Variances of PEP and PEP/LVET ratio, however, significantly differed between males and females. The variance decomposition model fitting therefore employed a scalar sex limitation (Neale & Cardon, 1992) to account for these differences. Within the male and female group, MZ, DZ and singleton sibling variances could be constrained to be equal. For all variables we were able to reduce the covariance structure of all daily periods to one MZ correlation and one DZ/sibling correlation for same-sex twin and sib pairs. For DZ twins and sibling pairs of opposite sex the correlation could be constrained at zero without a significant loss of fit for PEP and PEP/LVET ratio, but not for HI. In all further genetic model fitting, the genetic correlation for the opposite sex pairs was constrained to zero for PEP and PEP/LVET ratio. For Heather index, the correlation between additive genetic effects was fixed at its theoretical value of 0.5.

Genetic analyses

Univariate genetic analysis showed that shared environment was of no importance for Heather index and that dominance genetic effects could be dismissed from the models for

PEP and PEP/LVET ratio. For all three variables, a model including only additive genetic factors and unique environment (AE model) was preferred over all other possible models (ACE/ADE, CE or E). To test the hypothesis that a single genetic factor underlies the genetic variation throughout the day, one factor genetic models were compared with the fit of an AE Cholesky decomposition.

Table 6.3 Multivariate model fitting results for pre-ejection period (PEP), left ventricular ejection time (LVET), PEP/LVET ratio, and the Heather index (HI)

	Model	Vs.	$\Delta\chi^2$	Δdf	p-value	AIC
	PEP					
1	ADE Chol.					
2	AE Chol.	1	9.035	10	.529	-10.965
3	E Chol.	2	72.737	10	.000	52.737
4	AE Ind.	2	2.975	2	.226	-1.025
5	**AE Ind. reduced [1]**	**4**	**4.063**	**3**	**.255**	**-1.937**
6	AE Ind. reduced [2]	5	5.463	1	.019	3.463
	PEP/LVET ratio					
1	ADE Chol.					
2	AE Chol.	1	6.283	10	0.791	-13.717
3	E Chol.	2	57.534	10	.000	37.534
4	AE Ind.	2	4.777	2	.092	0.777
5	AE Ind. reduced [1]	4	0.103	3	.991	-5.897
6	**AE Ind. reduced [2]**	**5**	**3.08**	**1**	**.079**	**1.08**
	HI					
1	ACE Chol.					
2	AE Chol.	1	2.851	10	0.985	-17.149
3	CE Chol.	1	11.138	10	0.347	-8.862
5	E Chol.	2	93.488	10	0.000	53.488
6	AE Ind.	2	6.666	2	0.036	2.666
7	**AE Ind. reduced [1]**	**6**	**1.06**	**3**	**0.787**	**-4.94**
8	AE Ind. reduced [2]	7	21.358	1	0.000	19.358

The most parsimonious model is printed bold faced. AIC = Akaike's information criterion; Chol. = Cholesky decomposition;
[1] Independent pathway model without specific genetic influences during waking period.
[2] Independent pathway model without specific genetic influences throughout the 24 hour period.

The multivariate model fitting results are presented in table 6.3. For PEP, PEP/LVET ratio and HI alike, one genetic factor was responsible for the genetic influences on the individual variation throughout the 24-hour recording. Heritability estimates for the final most parsimonious models are presented in table 6.4. Although all variables showed a decrease in heritability during the evening and a further decrease during the night, these were not significant. For PEP and HI, additional specific genetic factors were present, but only during the night, and accounted for 8% and 20% of the variance respectively. The remainder of the variance was accounted for by unique environmental influences.

Table 6.4 Heritability estimates for pre-ejection period (PEP), left ventricular ejection time (LVET), PEP/LVET ratio, and the Heather index (HI)

	PEP		
	Common h^2	**Specific h^2**	**Total h^2**
Morning	62% (49-72)	-	62%
Afternoon	62% (48-72)	-	62%
Evening	55% (41-66)	-	55%
Night	40% (27-52)	08% (01-15)	48%
	PEP/LVET ratio		
	Common h^2	**Specific h^2**	**Total h^2**
Morning	58% (43-69)	-	58%
Afternoon	56% (41-68)	-	56%
Evening	48% (32-61)	-	48%
Night	35% (19-51)	-	35%
	HI		
	Common h^2	**Specific h^2**	**Total h^2**
Morning	50% (37-60)	-	50%
Afternoon	45% (32-57)	-	45%
Evening	38% (24-52)	-	38%
Night	21% (10-33)	20% (11-28)	41%

Age was regressed from the mean for PEP, PEP/LVET ratio and HI. For HI sex was an additional covariate. The 95% confidence intervals for each of the estimates are given in parentheses. h^2 = heritability estimate.

Discussion

Based on prolonged measurements in naturalistic settings obtained in a sample of 755 healthy adult twins and singleton siblings, the present study showed that individual differences in impedance-based measures of sympathetic control of cardiac contractility are substantially determined by additive genetic factors with heritability estimates varying between 35% and 62%.

Daytime generally is associated with relative sympathetic dominance while nighttime is characterized by parasympathetic dominance (Buijs et al., 2003a; Carrington, Walsh, Stambas, Kleiman, & Trinder, 2003; Hilton, Umali, Czeisler, Wyatt, & Shea, 2000). To allow for the possibility that different genetic factors would affect sympathetic control of the contractility of the heart during waking and sleeping hours or during leisure (evening) and work (morning, afternoon) periods the entire ambulatory impedance recording was split into four periods of day. Total genetic influence on variance in cardiac sympathetic control was found to be higher during daytime than during the evening and lowest during the night. In spite of the lower heritability, multivariate analysis showed that, at night, new genetic variance emerged for PEP and the HI. This is in keeping with studies in rodents on diurnal variation in gene-expression in the heart (Martino et al., 2004; Young, Razeghi, Cedars, Guthrie, & Taegtmeyer, 2001). These studies found variation in diurnal gene-expression to be driven in part by the central circadian pacemaker, but also by changes from light to dark phases. The presence of the night-specific genetic influences on our cardiac contractility indices supports the proposition by Young (2003) that the presence of night-specific gene-expression in the hearts of rodents may be extrapolated to humans.

The sympathetic nervous system has long been suggested to play a vital role in the risk for left ventricular hypertrophy and heart failure (Rundqvist et al., 1997; Kaye et al., 1995; Swedberg et al., 1990). Therefore, subjects with a genetic make-up that gives rise to an increased β-adrenergic drive to the left ventricle, evident in a shorter PEP and lower PEP/LVET ratio and an increased HI value, may be at larger risk to develop heart failure than subjects with lower inotropic drive. Specifically, a chronic state of sympathetic hyperactivity is thought to enhance age-related functional down-regulation of myocardial β-receptors (El Armouche, Zolk, Rau, & Eschenhagen, 2003; Bogaert & Fraeyman, 1991; Andersson, 1986; Xiao, Cheng, Zhou, Kuschel, & Lakatta, 1999). Down-regulation in the face of chronically enlarged sympathetic inotropic drive might initially constitute a cardioprotective compensatory response, but in the long run will negatively affect cardiac output (Communal & Colucci, 2005; Engelhardt, Hein, Wiesmann, & Lohse, 1999; Iwase et al., 1996). The resulting reduction in cardiac output is initially compensated by a further increase in cardiac sympathetic drive, supported by increased activity level of the renin-angiotensin system. In the disease phase, the effectiveness of the additional sympathetic activity is further reduced and net contractility is lowered. Indeed, studies employing impedance cardiography have shown that hearts with reduced left ventricular function show an increase in PEP/LVET ratio, and a severity-dependent decrease in HI (Fuller, 1994). In addition, the failing heart has been associated with a prolonged PEP and a decrease in LVET (Weissler et al., 1968; Ahmed, Levinson, Schwartz, & Ettinger, 1972).

Genes influencing cardiac inotropic drive may interact with genetic susceptibility in other domains. Three previous studies have reported heritabilities of left ventricular structure and diastolic filling, using a twin or sibling design. A study in healthy black twins showed that both genetic and environmental factors play a role in the individual variation in left ventricular mass (Harshfield, Grim, Hwang, Savage, & Anderson, 1990). A study in young adult twins showed that genes accounted for 43% and 26% of the variation in respectively early and late left ventricular filling velocity (Bielen et al., 1991), while a more recent study reported heritabilities of 50% and 52% for these measures of diastolic filling (Tang et al., 2000). Finally, two recent linkage studies implicated a role for genes coding for the cardiac myosin-binding protein in a region on chromosome 11 in measures of left ventricular contractility, filling and structure (Arnett et al., 2001; Tang et al., 2002).

Not surprisingly, systolic time intervals and contractility measures derived from impedance cardiography are of increasing importance in the diagnosis and management of patients suffering from heart failure (Parrott, Burnham, Quale, & Lewis, 2004; Strobeck & Silver, 2004; Ventura, Pranulis, Young, & Smart, 2000; Rasmussen, Sorensen, & Kann, 1975). Here, we suggest a further use of these indices, namely as endophenotypes in the search for genetic susceptibility causing high sympathetic inotropic drive at a young premorbid age. Genetic variation in the three indices of cardiac contractility throughout the day and night was largely explained by a common set of genes. This common genetic factor structure is advantageous for gene-finding on at least two accounts. First, using highly genetically correlated multivariate phenotypes provides higher statistical power to find genes in linkage analysis (Allison et al., 1998). Secondly, these genes, by virtue of having a pervasive influence on cardiac contractility across all situations, will also have the largest clinical relevance. We conclude, therefore, that these ambulatory impedance-derived indices of cardiac contractility provide a useful target for future gene finding studies targeting the risk for heart failure.

7

Familial influences on basal salivary cortisol in an adult population

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Abstract

To understand the underlying genetic and environmental sources of individual variation in basal cortisol levels, we collected salivary cortisol at awakening and at six fixed time points during the day in adult twins and their singleton siblings. Reported time of awakening was verified with heart rate and body movement recordings. Cortisol data were available for 199 MZ twins, 272 DZ twins and 229 singleton siblings from 309 twin families. No differences in cortisol means and variances were found between twins and singleton siblings. Additionally, the correlations for DZ twins and siblings were not significantly different, indicating generalizability of twin study results to the general population. Genetic model fitting showed heritability for cortisol levels during the awakening period (34% for cortisol level at awakening and 32% for cortisol level at 30 minutes after awakening) but not for cortisol levels later during the day. The current study shows that, while cortisol levels in the awakening period are influenced by genetic factors, cortisol levels throughout most of the day are not heritable, indicating that future gene finding studies for basal cortisol should focus on the first hour post-awakening.

Introduction

Cortisol is an important steroid hormone in the regulation of normal physiology. It is the end product of the hypothalamus-pituitary adrenal (HPA) axis. In response to disturbance of homeostasis due to physical or psychological influences, corticotrophin releasing factor (CRF) is expressed in the paraventricular nucleus of the hypothalamus and acts to stimulate the secretion of adrenocorticotrophic hormone (ACTH) in the pituitary. ACTH travels to the adrenals, where it stimulates the production of cortisol in the outer layer of the adrenal cortex. By a negative feedback mechanism, cortisol inhibits the production of both ACTH and CRF, thereby inhibiting its own secretion. Under influence of the central nervous system about 10 to 15 well-defined ACTH driven pulses of cortisol are secreted over 24 hours, resulting in cortisol's well-known circadian rhythm that is characterized by peak levels in the early morning and a nadir around midnight. Normally, stress-induced secretion is superimposed on the basal circadian rhythm. When the HPA axis is deregulated however, for example by continued or frequently repeated stress challenges, basal cortisol may be chronically secreted in excess, with potentially harmful effects. Prolonged glucocorticoid exposure may lead to muscle atrophy, decreased sensitivity to insulin, hyperlipidemia, hypercholesterolemia, impairment of growth (Meaney et al., 1991), osteoporosis (Adachi, 2001), immune destabilization (Bateman, Singh, Kral, & Solomon, 1989), hypertension and cardiovascular disease (Mantero & Boscaro, 1992; Girod & Brotman, 2004). In addition, deregulated HPA axis activity is a predictor for diabetes and stroke (Rosmond & Bjorntorp, 2000).

Large individual differences exist in the diurnal levels of cortisol (Smyth et al., 1997) and a possible source of this variation is genetic makeup. Several twin studies have been conducted to determine the influence of genetic and environmental factors on basal cortisol (for a review see Bartels, van den Berg, Sluyter, Boomsma, & de Geus, 2003b). The majority has focused on one basal cortisol sample during the morning hours (07:45 h – 09:00 h, not related to awakening), and only two studies in adults (Linkowski et al., 1993; Wüst et al., 2000a) and one study in children (Bartels et al., 2003a) report on the heritability of basal cortisol collected during an entire day. Linkowski and coworkers determined cortisol in blood samples taken every 15 minutes for 24 hours in 21 twin pairs. Genes influenced the timing of the nocturnal nadir and the proportion of overall temporal variability associated with pulsatility. No genetic effects were detected for the 24-hour mean and the timing of the morning acrophase (Linkowski et al., 1993). Wüst et al. collected eight saliva samples from awakening until 20:00 h in 104 twin pairs and reported significant genetic control (40% and 48%) for the different measures of the early morning acrophase, while cortisol variation during the rest of the day was predominantly under shared and non-shared environmental control (Wüst et al., 2000a). The study by Bartels and colleagues in 216 children of the age of 12 (Bartels et al., 2003a) showed a similar genetic pattern as the adult twin study by Wüst et al. (2000a) with significant genetic influences on cortisol levels an hour post-awakening (about 57%), and only environmental influences for the afternoon and evening levels of cortisol.

These studies indicate that genes influence the cortisol levels in the early morning, no matter what age, but not during the rest of the day. The present study increased the power to detect influences of genetic and common environmental factors by increasing the sample size and by adding the singleton siblings of the twins to the design (Posthuma & Boomsma, 2000). This extended twin design has the additional advantage that it allows testing of the assumption that twin results can be generalized to singletons.

Methods

Subjects

Subjects were registered with the Netherlands Twin Register (NTR) and were originally selected for a genetic linkage study for anxious depression (Boomsma et al., 2000; Middeldorp et al., in press). Briefly, families were selected when two siblings (dizygotic twin pair, sib-twin pair, or sib-sib pair) were extremely discordant or concordant for anxious depression. In addition to the sibling pair, all registered family members were recruited for the study. The resulting distribution of anxiety, neuroticism and depression scores was near-normal with only mild kurtosis. Of the first 1332 offspring who returned a DNA sample (buccal swabs) for the linkage study, 1008 were successfully contacted for a cardiovascular and hormonal ambulatory monitoring study. Of these, 192 refused participation or were excluded. Exclusion criteria were pregnancy, heart transplantation, pacemaker and known ischemic heart disease, congestive heart failure, or diabetic neuropathy. As the collection of saliva was added to the study protocol after the study started, a further 98 subjects did not participate, leaving a total of 718 subjects who took part in the cortisol collection. For 18 subjects the cortisol data had to be discarded because they used corticosteroid medication (12 subjects), or had unreliable profiles (6 subjects) due to working a night shift or uncertainty about the sample order. The final study sample consisted of 700 subjects, including 199 MZ twins (75 males), 272 DZ twins (94 males) and 229 singleton siblings (88 males), who were tested in two data collection waves. For 153 families only the twin was included, while for 144 families 1 or 2 additional siblings were included. For the remainder of the families between 3 and 6 additional siblings were included. Zygosity of the twins was determined by DNA typing. The Ethics Committee of the Vrije Universiteit approved of the study protocol and all procedures were carried out with adequate understanding and written consent of the subjects.

General procedure

Subjects were requested to refrain from intense physical exercise on both the preceding and the ambulatory monitoring days. Subjects were visited at home early in the morning, before starting their normal activities. They were subjected to an interview on health status and received instructions on the saliva sampling for cortisol assessment. In addition, a 24-hour electrocardiogram (ECG) and impedance cardiogram (ICG) recording was made using the Vrije Universiteit – Ambulatory Monitoring System (VU-AMS) ambulatory monitor (de Geus et al., 1995; de Geus & van Doornen, 1996) that includes a vertical accelerometer. Furthermore, every 30 minutes blood pressure was recorded using a Spacelabs 90207 ambulatory blood pressure monitor (Redmont, Washington, USA). Results on cardiovascular measures have been published elsewhere (Kupper et al., 2004; Kupper et al., 2005a; Kupper et al., 2005b). Subjects wore the VU-AMS monitor the entire day and night until after awakening the next morning. In a chronological diary subjects recorded the actual times saliva collection took place, and indicated any deviations from the instructions.

Saliva collection

Saliva sampling was performed using Salivette® sampling devices (Sarstedt, Rommelsdorf, Germany). Subjects were instructed to chew gently on the polyester swab for 45 seconds to obtain the desired amount of saliva. They were asked to refrain from brushing their teeth and consuming food and drinks from half an hour before saliva sampling. The first sample was collected in the presence of the researcher, at the start of the measurement day.

Instructions were to take the next samples at 11:00 h, 15:00 h, 20:00 h, 22:30 h (or prior to going to bed, when earlier), upon awakening the next morning (preferably while still in bed), and 30 minutes post-awakening. This last sample was only available for the 428 subjects who participated in a second data collection wave.

Cortisol analysis

All samples were stored frozen at the laboratory at a temperature of –25°C. Cortisol concentration was determined in Düsseldorf, Germany, in two batches. In the first batch, consisting of the samples of 272 subjects from 166 families, cortisol concentration was determined by a time-resolved immunoassay with fluorescence detection (DELFIA, see Dressendorfer, Kirschbaum, Rohde, Stahl, & Strasburger, 1992). Intra and inter assay variability of this method were less than 10% and 12% respectively. In the samples of the 428 subjects (from 128 families) of the second batch, cortisol concentration was determined using a commercial competitive chemiluminiscence immunoassay (LIA, IBL Hamburg, www.ibl-hamburg.com). Intra and inter assay variability of this method were less than 7.7% and 11.5%, respectively.

Measures & outlier detection

In addition to the seven diurnal cortisol samples, we computed the cortisol awakening response (CAR) by subtracting the cortisol concentration at awakening from the cortisol concentration 30 minutes later. When cortisol concentration reached values more than three times the standard deviation above or below the mean for that sampling time, the sample was discarded (this happened in 1% of all samples).

Statistical analysis

Confounders - In the past decades, a multitude of research has been published on factors influencing cortisol. The main factors implicated are age, gender, smoking, mood, bodily composition, contraceptive pills, sleep duration, sleep quality, and awakening time, although many studies report contradictory results (Deuschle et al., 1997; Knutsson et al., 1997; Wüst et al., 2000b; Ukkola et al., 2001). Therefore, we decided to test all of these potential confounders for their influence on basal diurnal cortisol using regression analyses in SPSS (SPSS Inc., Chicago, USA). The effects of sex, age, current mood state, (as measured by the POMS, Wald & Mellenbergh, 1990), body mass index (BMI), sleep quality (assessed by the Groningen Sleep Quality Scale, Meijman et al., 1988), reported sleep duration, current habitual smoking status (yes/no), and oral contraceptives use on the cortisol samples were tested. Since two methods (DELFIA and LIA) were used to determine the cortisol concentration in saliva, we also treated the type of immunoassay as a possible confounder in our genetic analyses.

Genetic modeling - To answer the question to which extent genes, common environment and non-shared environment contribute to the variance of basal cortisol, a biometrical genetic model was fitted to the observed data using the structural equation modeling program Mx (Neale et al., 2003). A series of unconstrained models was fitted to test the assumptions of the extended twin model. In this series, we first tested the equality of means and variances for MZ twins, DZ twins, and singleton siblings and examined the presence of sex effects on the means and variances. Then we tested whether cortisol determination method significantly affected the means. Finally, we tested for heterogeneity of correlations of males versus females and of DZ twins versus singletons. The resulting most parsimonious saturated model

indicated to which extent we could limit the specification of the genetic models and provided correlations for the MZ group and the DZ/sibling group.

In a twin study, the observed variance can be decomposed in four possible latent sources of variance. The two environmental sources are environmental effects that are shared by members of a family (C), and environmental effects that are unique to each member of a family (E). Two kinds of genetic effects are distinguished: additive genetic effects (A) and non-additive genetic effects. Non-additive genetic effects include dominance effects (D) and epistasis. Dominance describes the interaction between alleles at the same locus (Neale & Cardon, 1992). 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 choose which model was more appropriate. For MZ, DZ twins and sibling pairs alike, similarity in shared environmental influences was fixed at 100%. Similarity of additive genetic influences was fixed at 50% for siblings and DZ twins and at 100% for MZ twins. In the case of dominance (when the MZ correlation is more than twice the DZ correlation) similarity of dominant genetic influences was fixed at 25% for siblings and DZ twins and at 100% for MZ twins. Per definition, there is no similarity in the non-shared environmental influences for all three types of sibling pairings.

For each of the cortisol samples, a full univariate ACE or ADE model (Neale & Cardon, 1992) was tested against the nested more parsimonious AE, CE or E models. The resulting best fitting model indicated how much of the variance is attributed to genetic influences and how much is attributed to environmental influences. Throughout, nested models were compared using the likelihood ratio test. To determine whether shared genetic influences would underlie the two cortisol levels of the awakening period, a bivariate full ADE model in Cholesky decomposition was tested against more parsimonious models (AE and E models). The Cholesky decomposition imposes a structure of stratification in several shared latent factors. In the case of our bivariate analysis, there is a main factor of global importance that loads on both variables, followed by a second factor that loads only on the last variable. In the full model all variance components (A, D and E) are structured this way. Significance of the individual path coefficients was tested by constraining paths to zero and comparing the fit with likelihood ratio tests. In the bivariate model, the heritability of the CAR was also estimated. Because of the design of the model, CAR heritability reflects the remaining genetic influence on the difference in cortisol levels between the two samples, after removing the heritability for the two individual mean cortisol levels. Akaike's Information Criterion (Akaike, 1987) was calculated for each of the univariate and bivariate models. AIC offers a quick approach to judging the fit of nested models and models that are not nested, like an AE and CE model. Those with lower (i.e. larger negative) values fit better than models with higher values.

Results

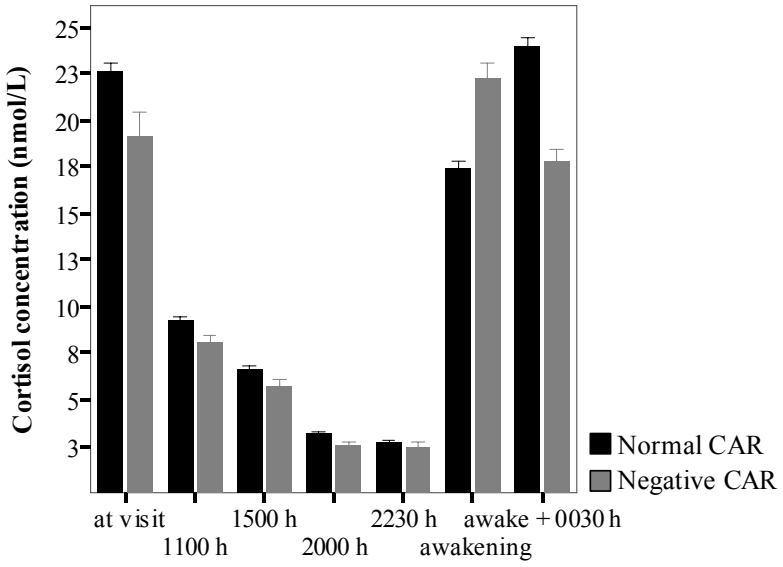
Descriptive statistics

Of the 700 subjects that participated in the saliva collection for cortisol determination, 587 had a complete diurnal profile of six samples (of these, 376 subjects participated in the second data collection wave, and provided seven samples). For 10 subjects less than four samples were present. Subjects complied moderately well with the instructed sampling times. Between 74% and 85% of the time-bound samples (11:00 h, 15:00 h, 20:00 h and 22:30 h) were reported to be taken within 15 minutes of the requested sampling time. Of the samples

outside the 30 minute window, the majority was taken later than the requested sampling time (1-3% was taken more than 15 minutes earlier, 21%-25% was taken more than 15 minutes later, and 14%-17% was taken more than 30 minutes later than the required sampling time).

Looking at the individual profiles, most subjects (89%) showed the well-known diurnal rhythm of cortisol. For 77 subjects for whom both early morning samples were present, the cortisol awakening response was negative. Figure 7.1 shows the diurnal cortisol profile for those with a normal awakening response (black bars) and those with a negative response (gray bars). It is possible that these subjects showing a negative response really do have a deviant response to awakening. However, an alternative explanation might be that subjects woke up earlier than they reported, and that their data therefore represent the down stroke of the morning acrophase. To test this alternative explanation, we exploited the fact that we had simultaneous recordings of heart rate and body movement on the sampling days. For 59 of the 77 subjects an ECG/motility recording of the early morning hours was available. For 18 subjects ECG/motility data were missing due to signal loss in the middle of the night. We identified an earlier awakening moment than reported in 80% of the subjects with available ECG/motility data. Figure 7.2 shows the combined ECG/motility signal for one subject with a discrepancy between his actual awakening time and his reported awakening/sampling time. The time difference between actual awakening and reported awakening in this example was 35 minutes (range = 10 min – 02:15 h), which suggests that the negative awakening response is an artifact caused by sampling after the actual awakening response occurred. To determine whether these results on earlier awakening are also found in the group with a normal awakening response, a random sample of 77 subjects was drawn from those with normal awakening responses.

Figure 7.1 Cortisol diurnal profiles for normal and negative awakening response groups



Represented cortisol means are corrected for method of cortisol determination. Error bars represent the standard error. CAR: cortisol awakening response.

For eight of these randomly drawn subjects ECG/motility data were missing due to signal loss in the middle of the night. In 87% of the subjects with available ECG/motility data the reported awakening time corresponded with the actual awakening as judged from the ECG and body movement recordings. These observations indicate that waking up earlier than reported was indeed responsible for the majority of the apparent negative cortisol awakening responses. Because the awakening response was assessed incorrectly in these subjects that showed a negative CAR, their awakening response samples were excluded from further analyses.

Table 7.1 shows the descriptive statistics for the cortisol samples. A clear circadian rhythm can be observed. The average cortisol concentrations at the seven sampling times were significantly different from each other and moderately correlated (between .08 and .47), with the exception of the awakening and 30 minutes post-awakening samples which were highly correlated (r=.65, p=.000).

Table 7.1 Descriptive statistics & twin correlations for diurnal cortisol

Sample	N	Mean sampling time (sd)	Mean cortisol (sd) in nmol/L	MZ correlation	DZ/sib correlation
At visit	667	8:35 h (1:07 h)	20.87 (10.49)	.33 (.13-.49)	.16 (.05-.26)
11:00 h	655	11:11 h (0:30 h)	8.69 (4.07)	.11 (.00-.34)	.09 (.00-.19)
15:00 h	675	15:18 h (0:42 h)	6.14 (3.07)	.08 (.00-.28)	.13 (.03-.23)
20:00 h	638	20:12 h (0:31 h)	2.85 (1.60)	.20 (.00-.42)	.18 (.08-.29)
22:30 h	649	22:39 h (0:28 h)	2.37 (1.95)	.00 (.00-.18)	.19 (.09-.29)
Awakening	563	7:16 h (0:51 h)	15.68 (7.36)	*.52 (.26-.68)*	*.12 (.00-.24)*
Awake + 0:30 h	319	7:45 h (0:58 h)	23.91 (8.23)	*.41 (.00-.67)*	*.14 (.00-.30)*

N = number of subjects sd = standard deviation CAR = cortisol awakening response
Means were corrected for cortisol determination method. For the correlations the 95% confidence intervals are given in parentheses. Italic: variables for which an ADE instead of an ACE model is fitted.

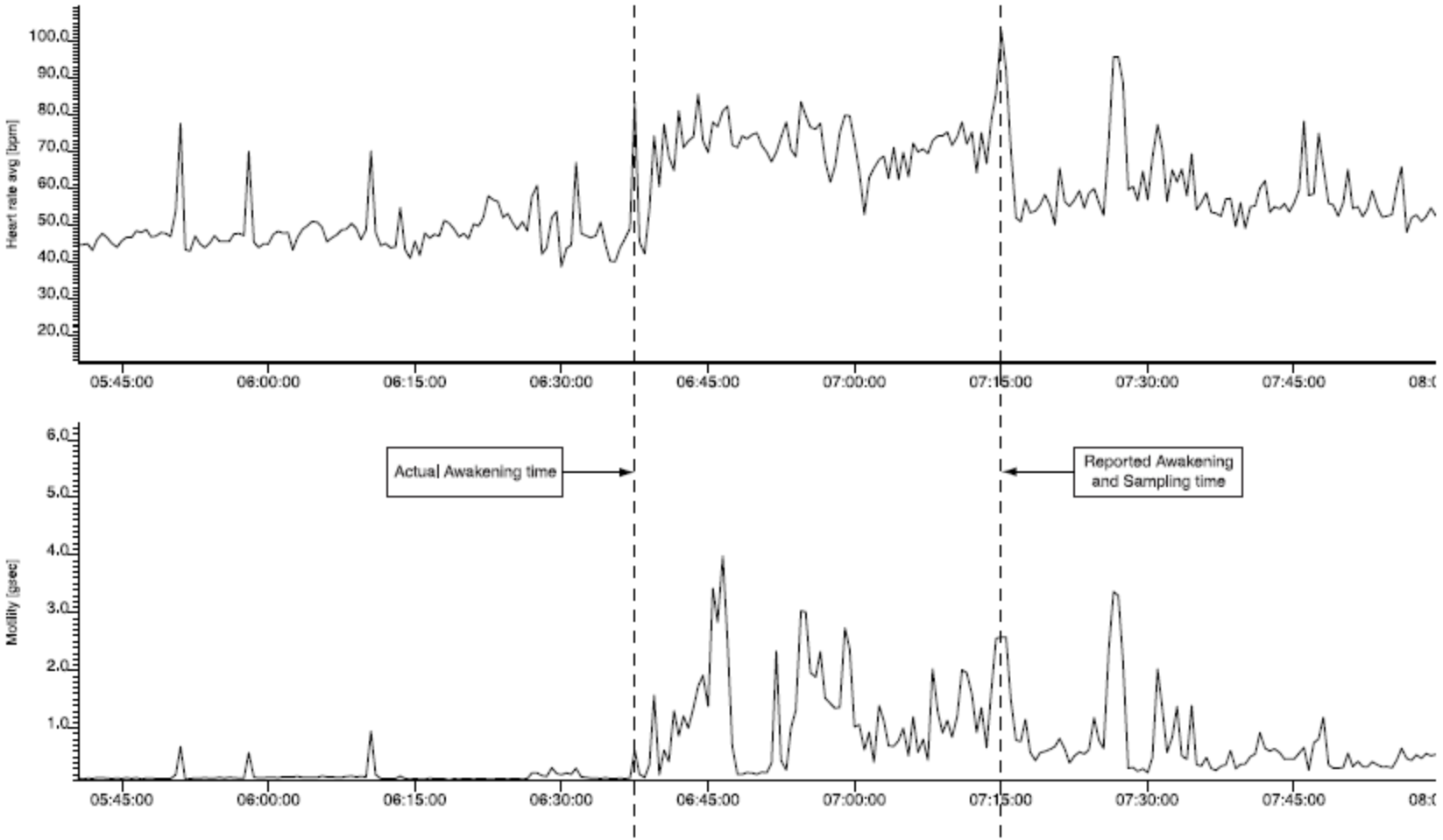
Confounders

When examining the association between the cortisol concentration at the time points and the potential confounding variables (sex, age, current mood state, BMI, sleep quality, sleep duration, current habitual smoking status (yes/no), and oral contraceptives use), no association reached the .01 significance level.

Genetic model fitting

Model fitting showed that the means and variances for MZ twins, DZ twins, and singleton siblings could be constrained to be equal. Male correlations were equal to female correlations and correlations were not significantly different between DZ twins and singletons. These results indicate that DZ twins and siblings may be treated as individuals from one group in further analyses. There was a significant mean effect of the method used to determine the cortisol concentration. Mean cortisol levels were higher in the second group of participants (when LIA was used) than in the first group of participants (when DELFIA was used). We therefore kept type of immunoassay as a covariate on the mean and the variance in the variance decomposition models. Estimation of the effect of type of immunoassay on the variance was performed following Purcell's gene-interaction model (Purcell, 2002).

Figure 7.2 Example of an ECG/motility signal accompanying a negative cortisol awakening response



The twin correlations from the final, most parsimonious model are presented in the final two columns of table 7.1. There were minimal differences in MZ and DZ/sib correlations during most of the day. Based on the pattern of correlations, substantial familiar influences are only present for the visit sample and the early morning measures of cortisol. In case of the two early morning measures (awakening, 30 min post-awakening) MZ correlations were more than twice as high as the DZ/sib correlations, suggesting the presence of dominance genetic effects. Therefore, we fitted ADE models for these measures. We continued model fitting with the most parsimonious saturated model, i.e. a model with equal means, variances and two correlations, including type of immunoassay as a covariate. Table 7.2 summarizes the model fitting results for all univariate variance decomposition models. The accompanying univariate model estimates and 95% confidence intervals are shown in Table 7.3.

Univariate genetic analyses - For both the awakening sample and the 30 min post-awakening sample, dismissing the dominance genetic effect did not cause a significant worsening of fit. In the AE model, additive genetic factors accounted for 33% of the variance in cortisol levels at awakening, while non-shared environmental influences accounted for the remaining 67% of the variance. For the cortisol concentration at 30 minutes post-awakening, genetic factors explained 34% of the variance, while non-shared environmental factors explained 66% of the variance.

For the daytime samples at 11:00 h and 15:00 h, leaving out both shared environmental and genetic influences from the model (E model) did not cause a significant increase in χ^2. This means that the cortisol concentration at these time points is completely determined by non-shared environmental factors. The 20:00 h and the 22:30 h sample showed a significant worsening of fit when both genetic and common environmental factors were left out of the model, indicating that there is an influence of familial factors on cortisol levels at these time points. Statistical power, however, was insufficient to discriminate between genetic influences and shared environmental influences, since the estimates for A and C were quite small (<22%).

For the first sample, taken during the visit of the researcher, the pattern of twin correlations and the estimates in the full ACE model indicate that the AE model is the most likely model, although statistical power is insufficient to discriminate between genetic and shared environmental factors. In the AE model, variance in cortisol concentration is for 29% explained by genetic influences.

Bivariate genetic analysis - To determine whether the same genes underlie the individual differences in the two cortisol samples of the awakening period (awakening and 30 minutes post-awakening), they were analyzed in a bivariate analysis, for which the initial ADE model is illustrated in figure 7.3. The dominance genetic factor could be dismissed from the bivariate model without a significant loss of fit, thereby reducing it to a model including only additive genetic and non-shared environmental influences (AE model).

Table 7.2. Summary of the univariate model fitting results

Model	-2LL	df	Δ χ^2*	Δ df	p-value	AIC
At visit						
ACE	2029.880	661				
AE	**2029.880**	**662**	**0.000**	**1**	**1.000**	**-2.000**
CE	**2032.287**	**662**	**2.407**	**1**	**0.121**	**0.407**
E	2045.544	663	15.664	2	0.000	11.664
11:00 h						
ACE	1299.591	649				
AE	1299.817	650	0.226	1	0.635	-1.774
CE	1299.591	650	0.000	1	1.000	-2.000
E	**1302.269**	**651**	**2.678**	**2**	**0.262**	**-1.322**
15:00 h						
ACE	1131.653	669				
AE	1132.588	670	0.935	1	0.334	-1.065
CE	1131.653	670	0.000	1	1.000	-2.000
E	**1135.770**	**671**	**4.117**	**2**	**0.128**	**0.117**
20:00 h						
ACE	754.749	632				
AE	**755.23**	**633**	**0.481**	**1**	**0.488**	**-1.519**
CE	**754.765**	**633**	**0.016**	**1**	**0.899**	**-1.984**
E	764.198	634	9.449	2	0.009	5.449
22:30 h						
ACE	3906.054	641				
AE	**3907.763**	**642**	**1.709**	**1**	**0.191**	**-0.291**
CE	**3906.054**	**642**	**0**	**1**	**1.000**	**-2.000**
E	3911.967	643	5.913	2	0.052	1.913
Awakening						
ADE	1451.323	557				
AE	**1451.323**	**558**	**0**	**1**	**1.000**	**-2.000**
E	1460.687	559	9.364	2	0.002	5.364
Awake + 0030 h						
ADE	814.871	314				
AE	**815.609**	**315**	**0.738**	**1**	**0.390**	**-1.262**
E	820.233	316	5.362	2	0.032	1.362

*-2LL: twice the negative log likelihood, df: degrees of freedom, AIC: Akaike's Information criterion. When the increase in χ^2 (=Δ χ^2) is not significant (p>.05), the most restrictive model is accepted. * Fitted against less restrictive model; bold indicate(s) the most parsimonious model(s).*

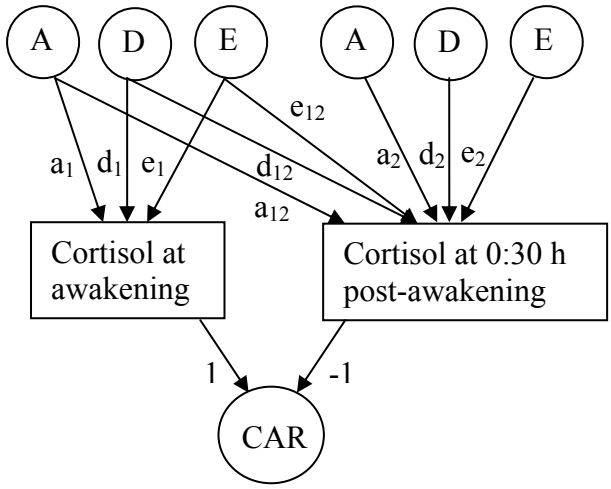
Table 7.3 Variance component estimates for each of the 7 diurnal samples

Sample	A	C	E
At visit			
ACE	.29 (.00-.43)	.00 (.00-.22)	.71 (.57-.89)
AE	**.29 (.14-.43)**	**-**	**.71 (.57-.86)**
CE	**-**	**.16 (.07-.26)**	**.84 (.84-.93)**
E	-	-	1.00
11:00 h			
ACE	.00 (.00-.29)	.08 (.00-.18)	.92 (.71-1.00)
AE	.13 (.00-.29)	-	.87 (.71-1.00)
CE	-	.08 (.00-.18)	.92 (.82-1.00)
E	**-**	**-**	**1.00**
15:00 h			
ACE	.00 (.00-.26)	.09 (.00-.19)	.91 (.74-1.00)
AE	.13 (.00-.28)	-	.87 (.72-1.00)
CE	-	.09 (.00-.19)	.91 (.81-1.00)
E	**-**	**-**	**1.00**
20:00 h			
ACE	.04 (.00-.36)	.12 (.00-.23)	.84 (.64-.95)
AE	**.22 (.07-.37)**	**-**	**.78 (.63-.93)**
CE	**-**	**.14 (.05-.23)**	**.86 (.77-.95)**
E	-	-	1.00
22:30 h			
ACE	.00 (.00-.28)	.11 (.00-.21)	.89 (.72-.98)
AE	**.16 (.00-.32)**	**-**	**.84 (.68-.99)**
CE	**-**	**.11 (.02-.21)**	**.89 (.79-.98)**
E	-	-	1.00
Sample	**A**	**D**	**E**
Awakening			
ADE	.33 (.10-.53)	.00 (.00-.16)	.67 (.47-.89)
AE	**.33 (.12-.53)**	**-**	**.67 (.47-.89)**
E	-	-	1.00
Awake + 00:30 h			
ADE	.05 (.00-.59)	.44 (.00-.72)	.51 (.28-.94)
AE	**.34 (.03-.61)**	**-**	**.66 (.39-.97)**
E	-	-	1.00

-: not available, since this estimate was set to zero. A: additive genetic variance component; D: Dominance genetic variance component; C: common environmental variance component; E: Non-shared environmental variance component. For each estimate the 95% confidence interval is given in parentheses.

Figure 7.3 Path diagram of the bivariate model for cortisol during the early morning period

A: additive genetic component, D: dominance genetic component, E: non-shared environmental component, CAR: cortisol awakening response. Letters along the paths represent path coefficients. Subtracting the cortisol concentration at awakening from the concentration 30 minutes later is established by setting the path coefficients originating from



the two measured concentrations (and pointing towards CAR) to 1 (awakening) and -1 (30 minutes post-awakening). The heritability for CAR in the AE model is computed following the formula:

$$\frac{(a_1)^2 + (a_2)^2 + (a_{12})^2 - (2 * a_1 * a_{12})}{((a_1)^2 + (a_2)^2 + (a_{12})^2 - (2 * a_1 * a_{12})) + ((e_1)^2 + (e_2)^2 + (e_{12})^2 - (2 * e_1 * e_{12}))}$$

Next, we tested whether both awakening samples are influenced by a single common genetic component, or whether additional genes come into play 30 minutes post-awakening. Dropping genetic effects unique for cortisol levels at 30 minutes post-awakening (path a_2 in figure 7.3) did not significantly worsen the statistical fit of the model, which indicates that one common genetic component influenced cortisol concentration at both sampling times. The genetic correlation (*r*G) therefore is 1.00 in this most parsimonious model. Path coefficients from this common genetic factor to cortisol at awakening and cortisol 30 minutes post-awakening were similar at .52. We further tested whether non-shared environmental effects on awakening cortisol levels also influenced cortisol levels 30 minutes later. Setting the appropriate path (path e_{12} in figure 7.3) to zero significantly reduced the fit of the model, indicating that a significant amount of non-shared environment influences both cortisol levels at awakening and at 30 minutes post-awakening. In the most parsimonious model, additive genetic components accounted for 34% of the variance of cortisol at awakening and for 32% of the variance of cortisol levels 30 minutes post-awakening. In this present model, the heritability of the CAR was also estimated. The common genetic factor may influence the second variable in a different degree than the first variable, even though all influence-exerting genes are shared. In that case, this is reflected in a difference in path coefficients, and a significant heritability for CAR. However, model fitting showed that there were no additional genetic factors influencing the CAR when heritability for the two mean cortisol levels is taken into account. Table 7.4 shows the variance decomposition results of the bivariate analysis.

Table 7.5 presents the accompanying estimates of genetic and environmental influences under the best fitting model.

Table 7.4 Bivariate model fitting results for cortisol in the early morning period

Model	-2LL	df	$\Delta\chi^2$	Δdf	Vs.	p-value	AIC
ADE	2112.305	868					
AE	2113.148	871	0.843	3	ADE	0.839	-5.157
Reduced AE[1]	**2113.861**	**872**	**0.713**	**1**	**AE**	**0.398**	**-1.287**
Reduced AE[2]	2135.244	872	22.096	1	AE	0.000	20.096
E	2127.868	874	14.72	2	AE	0.002	10.72

-2LL: twice the negative log likelihood, df: degrees of freedom, AIC: Akaike's Information criterion. When the increase in χ^2 (=$\Delta\chi^2$) is not significant (p>.05), the most restrictive model is accepted. ***Bold*** *indicates the most parsimonious model.*

[1] No non-shared additive genetic component for cortisol 30 minutes post-awakening

[2] No non-shared environmental correlation between cortisol at awakening and cortisol 30 minutes post-awakening

Discussion

To understand the underlying sources of individual variation in basal cortisol levels throughout the day, we analyzed cortisol data collected at seven fixed time points during the day in adult twins and their singleton siblings from 310 twin families. Results showed that the early morning cortisol concentrations were under considerable genetic control (34-32%), while later daytime samples (11:00 h – 22:30 h) were predominantly under environmental control.

Table 7.5 Variance component estimates for the cortisol measures of the early morning period

Sample	A	D	E
Awakening	.34 (.13-.53)	-	.66 (.47-.87)
Awakening + 00:30 h	.32 (.05-.59)	-	.68 (.41-.95)
CAR	.00 (.00-.18)	-	1.00 (.82-1.00)

The estimates for the best fitting model (AE reduced[1]) are presented in this table. The 95% confidence intervals are given in parentheses.

Our finding that the early morning cortisol concentration is under genetic control concurs in part with previous findings. Wüst et al. (2000a) reported significant heritabilities (40% and 48%) for the mean increase and area under the curve of the cortisol awakening response, but not for the awakening sample.

Bartels et al. (2003a) showed that genetic factors influenced the awakening sample for 22-24%, and the morning sample taken an hour after awakening for 56-59%. Our current results confirm the presence of significant genetic contributions to the variance of both cortisol levels at awakening and at 30 minutes post-awakening.

The twin correlations for the early morning samples (awakening and 30 minutes post-awakening) indicated that dominance genetic effects might influence cortisol levels in the early morning period but model fitting showed that the more parsimonious AE model was preferred over the ADE model. It should be noted that the statistical power to reliably detect

genetic dominance effects is small (Posthuma & Boomsma, 2000). For the first morning sample (taken during the researcher's visit), the MZ correlation was exactly twice the DZ correlation and no genetic dominance effects were suggested. Model fitting showed that a model including familial factors, most likely genetic, was the preferred model. In contrast to Wüst et al. (2000a), we did not find a genetic influence for the cortisol awakening response (CAR). Our bivariate analysis showed that the genetic influence on cortisol levels at awakening and at 30 minutes post-awakening completely overlapped. As a result, no additional heritability for the CAR was found.

Our results indicate that the variation in daytime cortisol levels, in particular during the late morning and afternoon, is predominantly influenced by non-shared environmental factors. The large impact of non-shared environmental factors on cortisol levels from late morning to evening agrees with the notion that cortisol is secreted as a reaction to disturbance in the homeostatic equilibrium. Whether shared environmental factors play an additional role remains unclear. Like Bartels et al. (2003a), the study lacked power to discriminate between the AE and CE model for some of the sampling times, although the power in our study was larger than in all previous studies. The extended twin design employed in the current study increases statistical power to distinguish between the components A, C and E compared to a design including only MZ and DZ twins, giving it a statistical power sufficient to reliably detect familial effects larger than 35%. For effects smaller than 35% over 1500 subjects are needed (Posthuma & Boomsma, 2000).

Recently, several studies reported on the effect of birth weight on cortisol concentrations (Phillips et al., 2000; Kajantie et al., 2002; Kajantie et al., 2004). The lower birth weight in twins may impact, according to the "Barker hypothesis", on HPA axis activity (Phillips et al., 2000). In the present study, we were able to test whether the results obtained in twins differed from those in singleton siblings. By comparing singleton siblings with twins from the same family, the two comparison groups are perfectly matched for familial influences (same parents, different intrauterine circumstances, same family environment). Our analyses showed that MZ and DZ twins and singleton siblings did not differ from each other in means or variances on any of the basal diurnal cortisol samples. Importantly, sibling-sibling covariance did not differ from sibling-twin or DZ-twin covariance, which strongly argues against a special twin intrauterine disadvantage with deleterious effects on adult cortisol concentrations. The lower birth weight in twins therefore does not seem to be a sign of diminished growth in the womb, but seems a natural adaptation to a twin pregnancy. The absence of any twin-singleton difference further indicates that estimates of the heritability of cortisol from twin studies generalize to the population at large.

The availability of electrocardiogram and movement registration allowed us to check whether the time the awakening sample was taken corresponded with the real awakening time. Our results indicated that the observed negative awakening response in a subset of subjects was most likely due to an earlier awakening time than reported, resulting in an earlier acrophase than assumed based on the reported sampling times. These results suggests we should be careful when dealing with deviant cortisol awakening responses, as a decreased or altered response might as well be an artifact due to an earlier awakening (Desir et al., 1981; Smyth et al., 1997; Kunz-Ebrecht, Kirschbaum, Marmot, & Steptoe, 2004). The same care should be taken when examining cortisol levels at other times throughout the day. These observations should encourage future studies to very carefully check whether the first morning samples are taken at the moment subjects actually awoke, not when they were about to get up. A way of checking the compliance of subjects is by an electronic monitoring device attached to the salivette that accurately time stamps the moment the salivette was used

(Kudielka, Broderick, & Kirschbaum, 2003). Although this device is very helpful in the precise determination of the sampling times, it cannot detect that a subject awakes, but does not take the awakening sample until later on. Additional ECG and/or motility recording of the night and early morning hours can provide the true awakening time.

An explanation for the varying genetic influences found for basal cortisol may lie in the difference in the role of the HPA axis and cortisol in the early morning hours and during the day. In the early morning hours, the biological clock of our body, the suprachiasmatic nucleus, prepares the body for the upcoming period of activity by an anticipatory rise in among others heart rate and cortisol (Buijs, van Eden, Goncharuk, & Kalsbeek, 2003b). As shown by our and other results, genetic factors control the absolute values of cortisol levels in this awakening period. During daytime, the main objective of the HPA axis is to maintain homeostasis within the body. Therefore, the absolute daytime levels of cortisol will be controlled by environmental feedback. This overshadows any genetic influence on the individual differences in cortisol during the day.

Up until now gene finding studies for basal cortisol have focused on either baseline cortisol measured anytime between 8:00 h and 17:00 h (e.g. Baghai et al., 2002; Keavney et al., 2005), or at total diurnal cortisol (e.g. Rosmond, Chagnon, Bouchard, & Bjorntorp, 2001). The current study shows that, while cortisol levels in the awakening period are influenced by genetic factors, cortisol levels throughout most of the day are not heritable; indicating that future gene finding studies for basal cortisol should exclusively focus on the awakening period.

8

General summary and discussion

The research in this thesis was inspired by the well-known familial clustering of cardiovascular diseases and was designed to make a contribution to the ultimate goal of elucidating the genetic pathways to this medical condition, which is one of the most important sources of morbidity and mortality in Western societies, i.e. in 2002, one third of the deaths in the Netherlands were caused by cardiovascular disease (CVD) (Koek et al., 2003). This final chapter will give a summary of this study's results, and will then discuss the results in the light of existing literature.

Heritability of physiological risk factors for CVD

Only a small part of the pathologies encompassed under the name cardiovascular disease are caused by a single mutation in the genetic material, following Mendelian patterns of transmission. Some examples of these rare conditions include the thrombosis-causing Factor V ^{Leiden} (Kaykcoglu et al., 2005), and several kinds of Mendelian hypertension, like Liddle's syndrome and hyperaldosteronism (New, Geller, Fallo, & Wilson, 2005). Non-Mendelian cardiovascular diseases, however, are much more common, and a wide variety of them exists. The underlying quantitative variation in susceptibility to these varieties of cardiovascular disease is influenced by multiple common genes, with small individual contributions and by multiple environmental factors, as well as gene-gene and gene-environment interactions. Although many linkage and association studies have been carried out to search for genes causing complex diseases the past decade, only few have led to identification of an actual causal locus (Iliadou & Snieder, 2004). There are several ways to enhance detection probability, for example by increasing sample size, or by selecting subjects from the extreme ends of the data distribution. In addition, it is increasingly common to use cardiovascular risk factors in genetic analyses instead of the diagnosis of CVD. Since these endophenotypes lie closer to the genes in the biological pathway, their variance is expected to be explained by fewer genes, and therefore the probability to detect individual causal genes might be larger.

Before proceeding with linkage studies with these endophenotypes, this thesis took the first crucial first step of establishing meaningful genetic contribution to the cardiovascular endophenotypes. Using figure 1.1 as a guideline (see introductory chapter) we focused on the risk factors listed in table 8.1. Some of these factors had been studied before in adult twin studies (HR, BP, RSA) others had been almost completely neglected so far (cortisol, PEP, RR). Importantly, none had been measured in naturalistic settings before, whilst taking appropriate account of their sensitivity to circadian rhythms and the continuous changes in mental, emotional, and physical load that characterize such settings.

Overall, substantial contribution of genetic factors to the variance in ambulatory physiological risk factors was found. An overview is presented in table 8.1. In the following sections, results for every assessed risk factor will be summarized shortly.

Table 8.1 Summary of the heritability of cardiovascular risk factors

Cardiovascular risk factors	Measures	Heritability across waking periods	Heritability during sleep
Heart rate variability	SDNN index	35-47 %	43%
	RMSSD	41-48 %	40%
Blood pressure	SBP	46-63%	-
	DBP	44-57%	-
Heart rate	HP	37-45 %	48%
Respiratory sinus arrhythmia (parasympathetic tone)	RSA	40-55 %	54%
Cardiac contractility (sympathetic tone)	PEP	55-62 %	48%
	PEP/LVET ratio	48-58 %	35%
	HI	38-50 %	41%
HPA axis activity	Cortisol	0-34%	

Blood pressure

Hypertension is a main risk factor for cardiac disease, stroke and renal disease (Franklin et al., 2001; Verdecchia et al., 1998; Pickering & Devereux, 1987) that is linked to sympathetic hyperactivity (Mussalo et al., 2001; Neumann, Ligtenberg, Klein, Koomans, & Blankestijn, 2004). Ambulatory blood pressure is a well-established measure that is often used in diagnosing hypertension. Chapter three presented estimates for genetic influence on hypertensive status and on ambulatory systolic (SBP) and diastolic blood pressure (DBP). Hypertension diagnosis was heritable for 61%, while SBP and DBP heritability ranged between 44 and 63%.

A further important goal in this analysis was to examine the effects of exclusion on the heritability estimates. We hypothesized that exclusion of (medicated) hypertensive subjects would be detrimental to blood pressure heritability estimates. To test this, all genetic analyses on ambulatory blood pressure were first performed on normotensive subjects only, secondly after exclusion of medicated hypertensive subjects, and finally without any exclusion. In this final analysis, including both normotensive and (medicated) hypertensive subjects, a subject-specific blood pressure correction was carried out that was based on the antihypertensive medication subjects were taking at the moment of measurement. Comparing the results of these three analyses, it is evident that exclusion of hypertensive and medicated subjects alters twin correlations and causes a decrease in heritability. This makes sense, since including subjects with a daytime blood pressure in the hypertensive regions increases variance. In addition, from a genetic point of view these subjects probably are the most interesting ones. Based on the evidence provided by these results, future linkage and association studies should be encouraged to include hypertensive subjects in their studies and to apply a medication specific blood pressure correction for those on antihypertensive medication.

Heart rate variability

Vagal activation of the heart decreases heart rate and increases heart rate variability and has important protecting effects on the heart. For example, it protects the heart from arrhythmic events (Brooks, Verrier, & Lown, 1981) and atherosclerosis of the coronary artery (Beere, Glagov, & Zarins, 1984).

There is substantial individual variation in heart rate variability levels (Huikuri et al., 1990; Abdel-Rahman, Merrill, & Wooles, 1994; Ben Lamine et al., 2004). In chapter four and

five of this thesis we hypothesized that genes contribute considerably to this variance. Time-domain measures of heart rate variability were used to determine the influence of genetic components on the individual differences in heart rate variability. Chapter four reported on SDNN index and RMSSD. These two short-term heart rate variability measures reflect vagal control over the heart. Indeed, it was found that individual differences in heart rate variability can for a large part be attributed to genetic influences, as revealed by the heritability estimates, which varied between 35% and 48%.

Chapter five presented the heritability results of the third heart rate variability measure, RSA, which specifically represents the natural cycle of arrhythmia that occurs through the influence of breathing on the flow of vagal impulses to the sinoatrial node. RSA was found to be heritable in a considerable degree (35% -55%) and the magnitude of this genetic influence was in congruence with the other two indices for heart rate variability. In addition this chapter also presented heritability estimates for heart rate (37-48%) and respiration rate (27-81%). Although the multivariate analyses including respiration, RSA and heart rate were carried out separately for each period of day, the results were highly comparable. Heritabilities of RSA and heart rate increased moderately with the progression of the day. Individual differences in respiration rate could almost be fully attributed to genetic factors during nighttime.

In contrast to the SDNN index or RMSSD, the RSA measure has not often been employed in cardiology. Nevertheless, several previous studies have pointed out that RSA is the more valuable diagnostic tool to assess parasympathetic control over the heart (Hrushesky et al., 1991; Moser et al., 1994). Our results show good congruence in heritability estimates between the measures SDNN, RMSSD, and RSA. This supports the notion that besides SDNN and RMSSD, RSA might be a valuable addition to genetic research in cardiology.

Parasympathetic contribution to cardiorespiratory coupling

The question whether RSA is a more valid index of between-subjects differences in cardiac vagal tone when individual differences in respiratory behavior are taken into account, is a much debated issue in the field of psychophysiology. It has even been suggested that the validity of RSA as a predictor for cardiovascular disease could potentially benefit from a correction for respiration rate. Results from a previous genetic laboratory study, however, already suggested that the well-established association between respiration rate and RSA might be substantially due to an overlap in genes contributing to these variables (Snieder et al., 1997). Chapter five extends this finding to ambulatory settings. A trivariate analysis including ambulatory respiration rate, RSA and heart rate, was performed to determine the genetic and environmental contributions to the covariances between these three variables. The study showed the presence of a single common genetic factor shared between respiration rate, RSA and heart period, possibly representing genes affecting general aspects of neurotransmission in either limbic or brainstem areas involved in cardiovascular and respiratory control (Bradley et al., 2002; Severson et al., 2003; Richerson, 2004). A second genetic factor was identified that is shared between RSA and heart period only. The pleiotropic genes influencing both RSA and heart rate might be involved in parasympathetic activation of the heart, e.g. genes playing a role in potassium signal transmission in the sinoatrial node (Gehrmann et al., 2002).

Finally, our results showed the presence of a third factor that influenced heart period only, but none of the others. This final genetic factor represents all genetic influences on heart rate that cannot be attributed to either respiration or the parasympathetic nervous system. For example, such genetic influences might involve genes that influence the regulation of β-adrenergic receptors (Koch, 2004).

Besides the finding that RSA and respiration rate share a common set of genes, we found in an additional analysis that the heritability estimates from the analysis in which RSA was corrected for respiration rate were substantially lower than the estimates that resulted from the analysis on uncorrected RSA. It was concluded that the use of residualized scores of RSA in genetic studies is unfounded because this removes genetic variance shared by respiration rate and RSA.

Sympathetic control over cardiac contractility

Impedance-derived measures of sympathetic cardiac control have not been used in genetic studies that investigate etiological factors of cardiovascular disease. Chapter six of this thesis is the first report on the heritability of cardiac contractility, and does so in an ambulatory design. Substantial heritability was found for all contractility indices: the pre-ejection period (48-62%), the PEP/LVET ratio (40-58%), and the Heather index of cardiac contractility (38-50%). These results suggest that genetic variation is a principal determinant of sympathetic inotropic drive. This has important consequences for the risk for left ventricular hypertrophy and heart failure where the sympathetic nervous system has been suggested to play a vital role (Rundqvist et al., 1997; Kaye et al., 1995; Swedberg et al., 1990). Subjects with a genetic make-up that gives rise to an increased β-adrenergic drive to the left ventricle, evident in a shorter PEP and lower PEP/LVET ratio and an increased HI value, may be at larger risk to develop heart failure than subjects with lower inotropic drive. Specifically, a chronic state of sympathetic hyperactivity is thought to enhance age-related functional down-regulation of myocardial β-receptors (El Armouche et al., 2003; Bogaert & Fraeyman, 1991; Andersson, 1986; Xiao et al., 1999). Down-regulation in the face of chronically enlarged sympathetic inotropic drive might initially constitute a cardioprotective compensatory response, but in the long run will negatively affect cardiac output (Communal & Colucci, 2005; Engelhardt et al., 1999; Iwase et al., 1996). The resulting reduction in cardiac output is initially compensated by a further increase in cardiac sympathetic drive, supported by increased activity level of the renin-angiotensin system. In the disease phase, the effectiveness of the additional sympathetic activity is further reduced and net contractility is lowered. Indeed, studies employing impedance cardiography have shown that hearts with reduced left ventricular function show an increase in PEP/LVET ratio, and a severity-dependent decrease in HI (Fuller, 1994). In addition, the failing heart has been associated with a prolonged PEP and a decrease in LVET (Weissler et al., 1968; Ahmed et al., 1972).

Cortisol

Chapter seven of the present thesis investigated the underlying sources of individual variation in basal daytime cortisol levels for seven fixed time points during the day. Recently, cortisol levels in the morning were found to be an independent risk factor for cardiovascular disease and diabetes (Rosmond & Bjorntorp, 2000). Our results showed that only the early morning cortisol levels (at awakening and 30 minutes later) were under the control of one genetic factor (34-32%). Levels of cortisol later during the day (11:00h-22:30h) were predominantly influenced by environmental components. It is our conclusion that, when investigating the biological mechanism underlying the relation between cortisol levels and cardiovascular disease, the early morning period is the most important period to collect cortisol.

A stern requirement for obtaining reliable results is to assure good compliance of the participants with the salivary sampling procedure. Participants do not always take the samples at the indicated times. Results described in the present thesis show that participants have

trouble identifying the correct moment of awakening. Because the samples that are used to define the awakening response, are taken relative to the awakening time, imprecision of subjective estimates of awakening can have rather severe repercussions. As an unexpected bonus, the practice of salivary sampling with simultaneous motility and heart rate monitoring (in this thesis by the VU-AMS device) greatly improves the reliability of the former.

Diurnal patterns in sympathovagal balance

The well-known diurnal pattern of the sympathovagal balance, with sympathetic dominance during the day shifting to parasympathetic dominance during the night (Burgess, Trinder, Kim, & Luke, 1997; van Eekelen, Houtveen, & Kerkhof, 2004), is mirrored in the heritability estimates of the study's parasympathetic and sympathetic indices. While the heritability of heart rate variability (parasympathetic) stays stable at night compared to daytime, the heritability of pre-ejection period (sympathetic) decreases. Taken together, the diurnal pattern in sympathovagal balance is in congruence with the finding that increased vagal activity at night is predominantly a function of sleep-onset, while the decreased sympathetic activity, reflected by an increase in PEP, is predominantly influenced by circadian factors (Carrington et al., 2003).

For parasympathetic activity small (8% for RMSSD and 12% for SDNN index) sleep-specific genetic effects were found. For sympathetic activity, new genetic influences emerged during the night, too. For the Heather index, these nighttime-specific genetic influences were even as large as the influence of the common genetic factor influencing all periods of day (20%). In animal models, metabolic gene expression in the heart shows considerable diurnal variation (Takekida, Yan, Maywood, Hastings, & Okamura, 2000; Martino et al., 2004) which may have external origins, such as diurnal variation in sympathetic nervous system activity driven by the suprachiasmatic nucleus (Buijs et al., 2003a), but may also be affected by factors intrinsic to the heart, such as endogenous transcriptional regulators and timekeeping genes (Young et al., 2001). Our results suggest that separate sources of genetic variation in sympathetic activity during wake and sleep periods also exist in humans.

Comorbidity of depression and cardiovascular disease

Families were selected for participation based on the requirement that at least two members of a family scored extremely discordant or concordant on a factor score that indicated genetic vulnerability for anxious depression. Because of the recruitment of additional siblings in the selected families, independent of their anxious depression scores, the distribution of the factor score approximated the normal distribution found in the population at large (Boomsma et al., 2000). To test whether the sample could be considered unselected for the physiological cardiovascular risk factors, the degree of their association to the anxious depression vulnerability score used in the original selection was computed. Throughout, very small non-significant correlations were found, suggesting that the sample was not biased for the risk factors examined.

Previous studies have pointed towards an association of anxiety, depression and negative emotions with cardiovascular morbidity and mortality (e.g. Gottlieb et al., 2004; Rudisch & Nemeroff, 2003) and have shown an overlap in alterations in cardiac autonomic tone in both disorders (Carney, Freedland, Miller, & Jaffe, 2002). Most of these studies used a patient-versus-control-design, whereas we ascertained mostly healthy subjects from a population based register (twinning occurs randomly across many potential confounders like, for instance, SES). Only a limited amount of subjects that participated in the ambulatory

monitoring study actually had a lifetime diagnosis of clinical depression. In total, 688 of our 816 subjects participated in a clinical interview (CIDI) that was described in the thesis of van den Berg (2002). Only 104 of the interviewed subjects qualified for a lifetime diagnosis of clinical depression (diagnosed according to the DSM-IV manual) which was mostly due to a single mild episode rather than recurrent severe depression.

Finally, most of the large epidemiological trials that find comorbidity between depression and cardiovascular disease risk, generally used subjects aged substantially older than the twins and singletons in our sample. Clearly, the absence of an association between physiological cardiovascular risk factors and anxious depression in our young, premorbid sample does not exclude the possibility that, over time, genes and environment will interact increasingly to influence both symptoms of depression and symptoms of cardiovascular disease. A better insight in these longitudinal gene-environment interactions may be needed to further our in understanding of the comorbidity of cardiovascular disease and depression.

Twin-singleton differences

In twin research it is important to know whether the used variables are influenced by twin-specific effects. The extended twin design that was employed in the present thesis provided an optimal case-control match for the twins: a non-twin sibling that is raised in the same household, by the same parents. They even have shared the same womb with the twins, although alone and not at the same time. Using such a matched twin-singleton design is ideal to test the assumption that results on twins generalize to the general population. This is particularly relevant in view of Barker's "fetal origins hypothesis" that states that cardiovascular disease and non-insulin dependent diabetes originate through adaptations the fetus makes when it is undernourished. These adaptations may permanently change the structure and function of the body, and include the slowing of growth, but also may be of cardiovascular, metabolic or endocrine origin (Barker, 1999a; Barker, 1999b; IJzerman et al., 2003).

Research on the Barker hypothesis, usually operationalizes fetal undernourishment by birth weight. In general, twins have a much lower birth weight than singleton siblings. In the NETAMB sample, for example, twins were on average 921.2 grams lighter than the singletons. In spite of this difference in birth weight, for all our physiological variables (heart rate variability, respiration rate, heart rate, blood pressure, systolic time intervals, cardiac contractility and cortisol) the monozygotic or dizygotic twins did not differ from singleton siblings in means, variances and covariances of any of the measured variables. Previous studies also have found no twin-singleton differences for several cardiovascular risk factors (Akiyama et al., 1999; Andrew et al., 2001; de Geus et al., 2001), personality, depression, and emotional behavior (Kendler, Martin, Heath, & Eaves, 1995; Johnson, Krueger, Bouchard, & McGue, 2002; Moilanen et al., 1999). This evidence strongly indicates the absence of special twin intrauterine disadvantages with potentially deleterious health effects. The lower weight of twins is not a sign of diminished growth in the womb caused by disadvantageous intrauterine influences, but a natural adaptation to a twin pregnancy (Blickstein, 2004). Several studies have indicated that twins slow down their growth rate early in gestation, possibly during the first trimester (Leveno, Santos-Ramos, Duenhoelter, Reisch, & Whalley, 1979; Liu & Blair, 2002). Across twin pairs, the Barker association does hold as well as it does across singletons. Within the twin population, twins with *relatively* lower birth weights are at more risk than twins with relatively higher birth weights (Ijzerman et al., 2001). The lower *absolute* birth weight of twins compared to a singleton population does not, however, reflect the kind of impaired fetal environment relevant to the Barker hypothesis.

Age-dependent gene-expression

It is commonly appreciated that the association between cardiovascular risk factors and subsequent cardiovascular pathology may change with age (e.g. Franklin et al., 2001). Several studies have reported on age-dependent heritability of cardiovascular risk factors (body mass index, systolic blood pressure and cholesterol). Almost all originate from the Framingham Heart Study. Their study population is large enough to examine age cohorts, and participants have been measured repeatedly over the past decades. In age-stratified longitudinal genetic analyses of systolic blood pressure, body mass index and cholesterol, Framingham researchers found little genetic variation over time (Brown et al., 2003; Mathias et al., 2003). These reports suggest that there is a stable genetic influence on the observed variation in these cardiovascular risk factors over decades. However, it does not rule out the possibility that different genes affect these risk factors at different ages.

We should, therefore, keep in mind that the NETAMB sample consisted of adults (males and females) who were for the majority between 20 and 40 years of age. Generalization of the results of this thesis beyond the main age range should be done cautiously. The amount of genetic influence on, e.g. heart rate variability or PEP/LVET ratio may change as our subjects grow older, but also the kind of genes that are expressed may be age-dependent (Volkova, Garg, Dick, & Boheler, 2005). In addition, we cannot be sure that the genes that are causing low heart rate variability or an elevated PEP/LVET ratio in the present are the same genes that cause low heart rate variability or an elevated PEP/LVET ratio later in life.

Directions for future research

Pleiotropy

To date, studies on the genetic epidemiology of cardiovascular risk factors have chosen to model the risk factors separately most of the time. This approach was largely repeated in this thesis. In chapter five of the present thesis, however, it could be shown that heart rate and RSA are influenced by a shared genetic factor. The question arises whether the heritability of some (or all?) of our physiological risk factors also derives from common genetic factors. Previous research, that modeled independent risk factors for cardiovascular disease in multivariate genetic analyses, further supports this notion of genetic pleiotropy. Juo et al. (2004) showed that obesity and the thickness of the carotid intima-media may be affected by common genetic factors. In addition, a shared genetic component was reported for plasma cholesterol, systolic blood pressure, and body weight (Havill & Mahaney, 2003).
Future multivariate genetic analyses on all of our cardiovascular risk factors (reduced to one daytime and one sleep time value) will provide more insight into the existence of a common genetic susceptibility to develop cardiovascular disease.

Gene finding

The final step will be to identify the actual genes involved in the heritability of the physiological risk factors at hand. This can be done through linkage or association studies (Vink & Boomsma, 2002). In this regard, ambulatory data has a large advantage over conventional laboratory data. First, since ambulatory monitoring takes place during everyday life, in the subject's own environment such measurements have high ecological validity. Possible negative consequences of excessive reactivity on cardiovascular health will derive from frequent exposure to stress during daily life. Therefore, assessing cardiovascular function in naturalistic settings during daily life makes good sense. Secondly, due to the intrinsic multivariate nature with highly correlated repeated observations, measurement error can be reduced and estimation of the latent genetic factor improved (Allison et al., 1998). In short, ambulatory monitoring of heart rate variability, blood pressure, heart rate, respiratory sinus arrhythmia, cardiac contractility, and cortisol can help us identify the genes influencing cardiovascular disease risk.

Nederlandse Samenvatting

(Dutch summary)

De erfelijkheid van risicofactoren voor hart- en vaatziekten
Gemeten in het dagelijkse leven

Introductie

Hart-en vaatziekten zijn een belangrijke doodsoorzaak in Nederland. In 2002 was een derde van de sterfgevallen hieraan toe te schrijven (Koek et al., 2003). Hart- en vaatziekten is een verzamelnaam voor een verscheidenheid aan aandoeningen. Voorbeelden zijn herseninfarct, hartinfarct, angina pectoris en hartfalen. De oorzaken voor het ontstaan en het verloop van hart- en vaatziekten zijn complex, en vele factoren dragen hieraan bij. Zo is het duidelijk dat in sommige families hart-en vaatziekten vaker voorkomen dan in andere. Deze familiale component zou verklaard kunnen worden door een genetische aanleg, maar zou ook voort kunnen komen uit bepaalde omgevingselementen, zoals leefgewoonten. Dit proefschrift beschrijft de resultaten van een grootschalig genetisch epidemiologisch onderzoek naar hart- en vaatziekten. Het doel van dit onderzoek was het in kaart brengen van de relatieve invloed van genetische en omgevingsfactoren op een aantal belangrijke risicofactoren voor hart- en vaatziekten.

Het hart en de bloedvaten worden aangestuurd door het autonome zenuwstelsel. Het autonome zenuwstelsel heeft als taak de basale processen in de organen te reguleren die nodig zijn voor het normale functioneren van het lichaam. Het autonome zenuwstelsel bestaat uit twee takken: het parasympathische en het sympathische zenuwstelsel. Het parasympathische systeem heeft een remmend effect op het hart. Dat wil zeggen dat het de hartslag verlaagt, de hartslagvariabiliteit verhoogt en de bloeddruk verlaagt. Het sympathische systeem werkt tegenovergesteld en zorgt in het algemeen voor een verhoging van de hartslag en de bloeddruk. Gedurende de slaap is het parasympathische zenuwstelsel dominant in de aansturing van het hart en de vaten. Overdag tijdens de wakkere periode is de balans tussen het sympathische en parasympathische zenuwstelsel afhankelijk van de mentale en fysieke belasting. In rustige situaties heeft het parasympathische zenuwstelsel de overhand, terwijl bij fysieke belasting, zoals sporten, of mentale belasting, zoals sterke emoties, het sympathische zenuwstelsel actief wordt.

Er bestaan verschillende maten die de activiteit van het sympathische en parasympathische zenuwstelsel weergeven. Hartslagvariabiliteit is voornamelijk parasympathisch van oorsprong, en veelgebruikte maten van hartslagvariabiliteit zoals SDNN-index, RMSSD en RSA kunnen worden gezien als indicatoren van de parasympathische sturing van het hart. De samenknijpkracht van het hart is voornamelijk sympathisch van oorsprong en maten voor de samenknijpkracht van het hart, ofwel ‘contractiliteit’, zoals de PEP en de Heather index kunnen worden gezien als indicatoren van de sympathische sturing van het hart. Het risico op hart- en vaatziekten wordt gekenmerkt door een algemene verschuiving van de balans tussen de activiteit van het parasympathische en sympathische zenuwstelsel in de richting van sympathische dominantie. Hierbij is er sprake van een verlaagde hartslagvariabiliteit en een verhoogde bloeddruk en hartslag. Dit zijn alledrie onafhankelijke voorspellers van het krijgen van of sterven aan hart- en vaatziekten. Het vinden van genen voor deze indicatoren van parasympathische of sympathische activiteit kan ons dus meer kennis verschaffen over het uiteindelijke ziekteproces.

Tot nu toe zijn de meeste studies naar de invloed van genen op het hart- en vaatstelsel gedaan door gegevens te verzamelen in een laboratoriumsituatie. Het is echter aannemelijk dat de genetische processen die een rol spelen bij het functioneren in het dagelijkse leven niet allemaal tot uiting komen in een kunstmatige laboratoriumomgeving. Daarom is er in dit onderzoek voor gekozen om de erfelijkheid van de risicofactoren te onderzoeken in 'ambulant' gemeten gegevens. Ambulant meten is een methode waarbij met behulp van kleine draagbare meetapparatuur biologische gegevens verzameld worden bij proefpersonen die vrijelijk, in hun eigen omgeving hun normale dagelijkse bezigheden uitvoeren.

Onderzoek bij tweelingfamilies

Om dit onderzoek uit te voeren, hebben we een grote groep volwassen tweelingen benaderd voor deelname. Het vergelijken van de mate van overeenkomst voor een eigenschap tussen eeneiige (MZ) tweelingen, die dezelfde genen hebben, en de mate van overeenkomst tussen twee-eiige (DZ) tweelingen, die gemiddeld 50% dezelfde genen hebben, geeft informatie over de relatieve bijdrage van genetische en omgevingsinvloeden aan de individuele verschillen voor deze eigenschap. Als MZ tweelingen veel vaker op elkaar lijken dan DZ tweelingen is de eigenschap in grotere mate erfelijk bepaald. Naast de tweeling, hebben we ook de eventueel aanwezige enkelgeboren broers en zussen gevraagd voor deelname. Dit onderzoeksdesign wordt ook wel een *uitgebreid tweelingdesign (extended twin design)* genoemd. Zo'n design heeft voordelen ten opzichte van een klassiek tweelingdesign waaraan alleen maar tweelingen meedoen. Zo wordt het vermogen (*power*) om onderscheid te maken tussen genetische en omgevingsfactoren die gedeeld worden binnen een familie, groter door toevoeging van één of meerdere enkelgeboren broers of zussen.
In totaal werd bij 816 proefpersonen met behulp van ambulante meetkastjes de bloeddruk gedurende de dag gemeten en een 24-uurs hartregistratie gemaakt. De opname had bij voorkeur plaats op een werkdag van de deelnemer, in zijn of haar dagelijkse omgeving. Op gezette tijden gedurende de meetdag verzamelden de proefpersonen speeksel, voor de bepaling van de concentratie van het bijnierschorshormoon cortisol. Om de enorme hoeveelheid gegevens die een 24-uursmeting met zich meebrengt, te reduceren, hebben we op basis van de zelfrapportage van houdingen en activiteiten in het dagboek van de proefpersonen en het bewegingssignaal uit ambulante hartmeter voor iedere deelnemer de meting opgedeeld in stukken waarin de houding van de proefpersoon gelijk bleef. Ook berekenden we gemiddeldes over dagdelen, gebaseerd op de door de proefpersoon gerapporteerde eet-, bed- en opstatijden. Voor elk van de gemeten risicofactoren maakten we steeds twee sets, één met de gemiddeldes van de gegevens in alle houdingen gedurende een dagdeel, en één met alleen de gemiddeldes gemeten tijdens een zittende houding gedurende een dagdeel. In de volgende paragrafen worden de resultaten voor de vier belangrijkste groepen risicofactoren samengevat. In het kort geeft tabel 1 een overzicht van alle resultaten.

Bloeddruk

Elke 30 minuten werd gedurende de periode dat de proefpersoon wakker was de bloeddruk gemeten. De resultaten, beschreven in hoofdstuk 3, gaven aan dat 14.5% van de onderzoekspopulatie een te hoge bloeddruk had (een bovendruk hoger dan 135 mm kwikdruk, en/of een onderdruk hoger dan 85 mm kwikdruk, gemiddeld over de gehele dag). Mannen bleken een grotere kans te hebben om boven deze drempelwaarde te zitten, en die kans steeg

met leeftijd. Genetische analyse van de gegevens liet zien dat familiaire factoren, zoals genen en de gemeenschappelijke omgeving binnen een gezin, heel belangrijk zijn voor het hebben van een te hoge bloeddruk. Genetische invloeden verklaarden 61% van het verschil tussen mensen in het risico op hoge bloeddruk. Verder onderzochten we of gedurende de hele dag (ochtend, middag en avond) dezelfde genen een rol speelden of dat tijdens een werkperiode (ochtend, middag) andere genen een invloed uitoefenden dan tijdens een ontspanningsperiode (avond). We vonden dat over de dag heen één overkoepelende genetische factor de variatie in bloeddruk beïnvloedt. Verder testten we de hypothese dat de erfelijkheid van de bloeddruk zou worden onderschat als we de mensen met een te hoge bloeddruk of bloeddrukmedicatie uit de analyse weg zouden laten, een gebruikelijke handelwijze in veel studies naar de bloeddruk. De invloed van de algemene genetische factor op de bovendruk werd geschat op 44 tot 57% bij het includeren van mensen met hoge bloeddruk en/of bloeddrukmedicatie en daalde met 7 tot 12% door het eruit laten van deze groep. De erfelijkheid van de onderdruk werd geschat op 46 tot 63%, en deze schattingen daalden met 8 tot 15% door weglating van de gegevens van de personen met hoge bloeddruk en/of bloeddrukmedicatie. Weglaten van personen met een te hoge bloeddruk en/of bloeddrukmedicatie is in genetische analyses dus niet verstandig, omdat de rol van de genen dan wordt onderschat.

Tabel 1 Samenvatting van de erfelijkheidsschattingen voor de gemeten cardiovasculaire risicofactoren

Risicofactor	Maten	Erfelijkheid overdag	Erfelijkheid slaap
HPA-as activiteit	Cortisol	0-34%	-
Hartslagvariabiliteit	SDNN index	35-47 %	43%
	RMSSD	41-48 %	40%
Bloeddruk	SBP	46-63%	-
	DBP	44-57%	-
Hartslag	HP	37-45 %	48%
Hartslagvariabiliteit (parasympathische hartsturing)	RSA	40-55 %	54%
Hartcontractiliteit (sympathische hartsturing)	PEP	55-62 %	48%
	PEP/LVET ratio	48-58 %	35%
	HI	38-50 %	41%

Hartslagvariabiliteit

Normaal gesproken laat de hartslag een periodieke variatie zien in het tijdsinterval tussen opeenvolgende hartslagen. Dit fenomeen wordt ook wel hartslagvariabiliteit genoemd, en wordt gebruikt als maat voor parasympathische sturing van het hart. Een verlaagde hartslagvariabiliteit is een onafhankelijke voorspeller voor het ontstaan van, en het sterven door hart- en vaatziekten. Daarom is het belangrijk te weten hoe genen de variatie in hartslagvariabiliteit beïnvloeden. In hoofdstuk 4 en 5 werden drie maten van hartslagvariabiliteit behandeld. Hoofdstuk 4 ging over SDNN-index en RMSSD. Deze twee maten worden afgeleid uit het electrocardiogram, en worden veel in de cardiologische praktijk gebruikt. Het is dus erg relevant om van deze maten te weten te komen hoe erfelijk ze zijn, als ze gemeten zijn in een natuurlijke omgeving. We vonden dat één overkoepelende genetische

factor verantwoordelijk was voor een substantieel deel van de individuele verschillen in hartslagvariabiliteit over de gehele meetperiode (ochtend, middag, avond en nacht). Een samenvatting van de schattingen is te vinden in tabel 1. Gedurende de nacht vonden we een kleine additionele nacht-specifieke genetische factor (8-12%). Voor SDNN-index vonden we ook nog een kleine ochtendspecifieke genetische factor (7%).
Hoofdstuk 5 beschreef een analyse waarin gelijktijdig werd gekeken naar de relatie tussen ademhaling, RSA en hartslag. Daarbij werd niet alleen voor elk van deze variabelen de erfelijkheid bepaald, maar kon tevens worden vastgesteld of het onderlinge verband tussen deze maten ook een erfelijke component heeft. Anders gezegd, werd er bepaald of deze maten deels door dezelfde genetische factoren werden beïnvloed. RSA is een speciale maat voor hartslagvariabiliteit waarbij uitsluitend gekeken wordt naar de ademhalingsafhankelijke variabiliteit in de hartslag. Tijdens inademing gaat de hartslag sneller, terwijl tijdens uitademing de hartslag iets vertraagt. Meer dan de eerder genoemde maten voor hartslagvariabiliteit, geeft de RSA de activiteit van het parasympatische zenuwstelsel weer. RSA blijkt sterk erfelijk bepaald (zie tabel 1). Tevens werd gevonden dat de genetische factoren die de RSA beïnvloeden voor een deel (8-16%) overlappen met de genetische factoren die ademfrequentie beïnvloeden en voor een deel (6-17%) overlappen met de genetische factoren die de hartslag beïnvloeden.. Voor toekomstige genetische studies betekenen deze resultaten dat RSA niet zomaar gecorrigeerd kan worden voor ademfrequentie of hartslag, omdat dan ten onrechte informatie over de erfelijkheid van RSA weggegooid wordt.

Hartcontractiliteit
De linker hartkamer wordt vrijwel alleen maar door sympathische zenuwen aangestuurd. Het sympathische zenuwstelsel heeft dan ook een dominante rol in de regulatie van de contractiliteit (samenknijpkracht) van het hart. Om meer te weten te komen over de invloed van erfelijke factoren op de sympathische sturing van het hart hebben we gedurende de ambulante meting een impedantiecardiogram opgenomen. Het impedantiecardiogram wordt gemaakt met behulp van vier electroden op de borstkas. De gemeten wisselstroomweerstand (impedantie) geeft de veranderingen in bloedvolume in de borstkas weer en daarmee indirect het slagvolume van het hart. In combinatie met het electrocardiogram kunnen we de tijd bepalen tussen het begin van de electrische impuls in de ‘pacemaker’cellen en het openen van de linker hartklep. Dit tijdsinterval, dat ook wel de pre-ejectie periode (PEP) wordt genoemd, is een indicator van de sympathische sturing van het hart. In hoofdstuk 6 rapporteerden we de resultaten van de genetische analyse van deze gegevens. We gebruikten drie maten voor de samenknijpkracht van het hart (PEP, PEP/LVET ratio en de Heather index). Deze maten werden voor een groot deel beïnvloed door genen (35-62%). Weer was er één overkoepelende genetische component, die over de hele 24-uursperiode van invloed was. De invloed van de genen nam wat af gedurende de nacht. In de slaap kwamen wel specifieke genetische invloeden te voorschijn die overdag niet zichtbaar waren. Deze hadden een geringe, maar significante (8-20%) invloed. De erfelijkheidsschattingen voor sympathische sturing van de knijpkracht van het hart staan in tabel 1. Het potentiële belang van de genen voor sympatische zenuwstelselactiviteit is groot. Te hevige of langdurige sympathische activiteit in reactie op allerlei fysieke en mentale prikkels wordt verantwoordelijk gehouden voor het risico op hoge bloeddruk, aderverkalking, suikerziekte en hartfalen.

Cortisol

Cortisol is een steroïd hormoon dat wordt uitgescheiden uit de bijnierschors, met een belangrijke rol in allerlei lichaamsprocessen zoals de regulatie van vaatvernauwing, het suikergehalte en het afweersysteem. Cortisol is het eindproduct van een zeer nauwkeurig afgestelde hormonale cascade die begint in de hersenen en via de hypothalamus en de hypofyse eindigt in de bijnierschors, met terugkoppeling van cortisol op elk van deze tussenstations. In hoofdstuk 7 werd beschreven hoe op 7 tijdspunten gedurende de meetperiode speeksel werd verzameld waarin later de basale concentratie cortisol werd gemeten.
De resultaten lieten zien dat het cortisolniveau vroeg in de ochtend (meteen na het wakker worden, en een half uur daarna) voor een deel erfelijk wordt bepaald (32-34%), maar dat later op de dag erfelijke factoren weinig invloed hebben op het cortisolniveau. Een mogelijke verklaring voor de variërende genetische invloed die we vonden, kan liggen in de veranderende rol die deze hormonale cascade speelt in de ochtenduren en gedurende de rest van de dag. In de vroege ochtenduren bereidt de biologische klok in ons lichaam (de suprachiasmatische nucleus (SCN) in de hersenen) ons voor op de komende periode van verhoogde mentale en fysieke activiteit. De SCN doet dit door gedurende korte tijd de aanmaak van cortisol sterk te stimuleren en daarmee een aantal systemen ‘op scherp’ te zetten. Onze resultaten laten zien dat genen het cortisolniveau gedurende deze periode beïnvloeden. Gedurende de dag is de taak van cortisol vooral om lichamelijke processen weer terug in balans te brengen (‘homeostase’) als deze door allerlei prikkels van buiten af zijn verstoord. Het cortisolniveau gedurende de dag zal dus voornamelijk bepaald worden door de feedback die het systeem krijgt uit de omgeving en inderdaad vinden wij dat na 11 uur ‘s ochtends omgevingsfactoren de belangrijkste bijdrage leveren aan individuele verschillen in cortisol.

Verschillen tussen tweelingen en enkelgeborenen?

Tweelingen hebben over het algemeen een veel lager geboortegewicht dan enkelgeborenen (in ons onderzoek scheelde het 921.2 gram). Dit lagere geboortegewicht zou schadelijk kunnen zijn voor de tweelingen, als de Barker hypothese voor deze groep zou gelden. De Barker hypothese (“foetal origin hypothesis”) stelt dat aanpassingen die de foetus maakt als het ondervoed wordt, permanente veranderingen in het functioneren van het lichaam bewerkstelligt, en dat deze veranderingen hart- en vaatziekten en diabetes veroorzaken. Meestal wordt het geboortegewicht gebruikt als maat voor foetale ondervoeding. Omdat in onze studie zowel tweelingen als enkelgeborenen meededen, konden we bepalen of de tweelingen significant verschilden van de enkelgeborenen wat betreft hun fysiologisch functioneren. Wij hebben voor al de zojuist besproken fysiologische variabelen onderzocht of de gemiddeldes, de varianties en de covarianties van de tweelinggroep significant verschilden van die van de groep met enkelgeboren broers en zussen van deze tweelingen. Voor geen enkele variabele bleken tweelingen te verschillen van enkelgeborenen. Dit sterkt het idee dat het lagere geboortegewicht in tweelingen geen teken is van verminderde groei, maar een natuurlijke aanpassing aan de tweelingzwangerschap betreft.

Conclusie

Samenvattend kan worden geconcludeerd dat alle bovenstaande risicofactoren voor hart- en vaatziekten in grote mate worden beïnvloed door genetische factoren. Een belangrijk verschil van dit proefschrift met veel voorgaande studies is dat die risicofactoren ambulant werden gemeten, dat wil zeggen tijdens een normale werkdag in de eigen natuurlijke omgeving van de proefpersonen. Een dergelijke ambulante meting heeft een hoge betrouwbaarheid omdat er veel herhalingen van hetzelfde type meting worden gedaan. Ook zal de voorspellende waarde voor de meeste ziekteprocessen hoger zijn dan die van metingen in een laboratoriumsituatie omdat immers veel dichter bij de dagelijkse werkelijkheid wordt gemeten.
Deze ambulant gemeten risicofactoren zijn daarmee een veelbelovende aanwinst voor het koppelings- en associatieonderzoek waarmee de genen voor hart- en vaatziekten hopelijk op korte termijn kunnen worden opgespoord.

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Appendix I

Diary

WAT WORDT ER VAN U VERWACHT?

De hartactiemeter en de bloeddrukmeter

U krijgt de meters 's ochtends aan het begin van de werkdag bevestigd. Als de meters eenmaal gestart zijn hoeft u er verder niets aan te doen; u kunt uw dagelijkse activiteiten gewoon verrichten. De bloeddrukband wordt om het half uur automatisch opgepompt voor een meting en de hartactiemeter registreert de hartslag continu. Het kan gebeuren dat een bloeddrukmeting mislukt door teveel beweging van de arm. De meter blaast dan nog een keer op. Daarom is het van belang om tijdens een meting (duurt ongeveer 20 sec.) uw arm zo stil en ontspannen mogelijk te houden. De bloeddrukmeter mag u 's avonds vlak voor het naar bed gaan, afdoen. **Vergeet hem niet uit te zetten!** Graag zouden wij zien dat gedurende de meetdag u geen extreem fysieke inspanning verricht en niet teveel alcohol drinkt. De hartactie meter het liefst om houden totdat de onderzoeker er de volgende dag weer is.

Invullen van het activiteitendagboek

De gemeten biologische signalen (zoals hartslag en bloeddruk) 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 een activiteitendagboek bij te houden. U wordt om het half uur gewaarschuwd door middel van een langdurige pieptoon (uit te zetten door op het rode knopje op de hartactiemeter te drukken) dat het tijd is om het dagboek in te vullen (tijdens de uren waarin u slaapt zal de meter niet piepen). Na de pieptoon vult u eerst in het dagboek de begin- en eindtijd in. U kunt het beste de tijd die op de bloeddrukmeter staat gebruiken. Vervolgens vult u, op volgorde van tijd, in wat u sinds de vorige piep gedaan heeft. Van belang hierbij is dat u naast de 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. Vul ook de 4-punts schaal in, waarop u aangeeft hoe stressvol het afgelopen half uur voor u was. Probeer het dagboek elk half uur in te vullen. Mocht u de pieptoon niet hebben gehoord dan kunt u het dagboek alsnog bij de volgende piep bijwerken.

Afdoen van de meters

Als u wilt gaan douchen, moet u de meters afdoen. Op de instructiekaarten staat hoe dit in zijn werk gaat. Om de hartactie meter af te doen trekt u eerst de pluggen uit de aansluiting (het waarschuwingssignaal is nu niet erg en u kunt het stoppen door op de rode knop te drukken) en daarna maakt u de meetdraadjes los van de elektrodes. De elektrodes laat u (ook onder de douche) gewoon zitten. Bij het aansluiten sluit u de draadjes weer aan op de elektrodes (let op de nummers, zie instructiekaart) en steekt u de pluggen weer in de aansluiting.

Om de bloeddrukmeter af te doen zet u deze op 'OFF', maakt u de slang los van de aansluiting op de bloeddrukmeter en trekt u tenslotte de band los van de arm. Leg de meters op een veilige plaats weg. Bij het aansluiten van de bloeddrukmeter bevestigt u de band weer om uw arm (zie instructiekaart, dit is het makkelijkst met de hulp van iemand anders). Daarna maakt u de slang weer vast aan de bloeddrukmeter en zet deze weer op 'ON'.

Overdag:

- **elk half uur dagboekje invullen**
- **speekselverzameling op gezette tijden en schema invullen**

's Avonds:

- **stemmingsvragenlijst en resterende vragen invullen**
- **speekselverzameling op gezette tijden en schema invullen**
- **bloeddrukmeter op 'OFF' zetten en afdoen**
- **elk half uur dagboekje invullen**

's Nachts:

- **hartactiemeter omhouden**

De volgende morgen:

- **speekselverzameling op de gezette tijden en schema invullen**
- **hartactiemeter zo lang mogelijk laten meten, bij voorkeur tot onderzoeker er weer is**
- **slaapkwaliteitslijst invullen**
- **elk half uur dagboekje invullen**

Bij een alarmpiep de elektrodes controleren en opnieuw vastmaken

Schema voor de speekselverzameling
Een half uur voor speekselverzameling niets eten of drinken (behalve water)

Verzamelingsmoment	**Werkelijke tijd**	**Opmerkingen…**
Als onderzoeker er is		
11.00 uur		
15.00 uur		
20.00 uur		
22.30 uur of bedtijd		Vul de stemmingsvragenlijst in en de resterende vragen naast de stemmingslijst in
Wakker worden		
30 minuten na wakker worden		Vul de slaap kwaliteitslijst in

Ontbijt dag 1: **……** **uur**

Lunch dag 1: **……** **uur**

Avondeten dag 1: **……** **uur**

Ontbijt dag 2: **……....** **uur**

De speekselverzameling met de wattenrolletjes
De eerste speekselverzameling vindt plaats waar de onderzoeker bij is. De volgende speekselverzamelingen vinden op vastgelegde tijden plaats. Het is de bedoeling dat u steeds de exacte tijd invult waarop u elke keer speeksel verzamelt, dit omdat de hoeveelheid hormoon ook afhankelijk is van het tijdstip op de dag. In het Speekselverzamelingsschema hiernaast kunt u dit invullen. De 2 speekselverzamelingen op de vroege ochtend van dag 2 moet bij voorkeur geheel worden gedaan zonder dat u tussendoor het ontbijt nuttigt. U kunt gerust
een glas water drinken. Het is van groot belang een half uur voor het nemen van elke speekselverzameling niets te eten, of caffeïne of alcohol te nuttigen. In de ruimte voor opmerkingen kunt u bijvoorbeeld aangeven als er iets niet helemaal goed is gegaan of als zich mischienn een uitzonderlijk voorval heeft voorgedaan. Het is van belang dat u niet vlak voor de speekselverzameling uw tanden poetst (zo vermijdt u dat het speeksel met bloed vermengd wordt).

Instructie speekselverzameling:
Op de aangegeven tijdstippen pakt u het buisje met het juiste nummer. U draait de dop van de buis en haalt het wattenrolletje eruit. Dan legt u het wattenrolletje in uw mond gedurende 1 minuut. Door de gaatjes in het plastic hulsje wordt speeksel in het wattenrolletje opgenomen. 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 terug in de genummerde buis en draai de dop erop. Schema achterin dagboekje invullen Bewaar de gebruikte genummerde buizen in een donkere bij voorkeur koele ruimte (bijvoorbeeld koelkast). De onderzoeker zal de speekselbuisjes tegelijkertijd met de hartactie- en bloeddrukmeters ophalen op een afgesproken tijdstip.

De tijden waarop de speekselverzameling plaats vindt:
1) als de onderzoeker er is
2) 11.00 uur 's ochtends
3) 15.00 uur 's middags
4) 20.00 uur 's avonds
5) 22.30 uur 's avonds of voor 't slapen gaan, als dit eerder is
6) direct na het wakker worden
7) 30 minuten na het wakker worden
De buisjes zijn genummerd zoals boven aangegeven.

DATUM: 17 /08 /200 1 VOORBEELD 1 VAN EEN INGEVULD DAGBOEK.

TIJD: van 7.15 tot 7.45	*Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner etc.), locatie (werk, thuis etc.)*
Kastjes werden omgehangen door onderzoeker, thuis, *Zitten en staan, kop koffie gedronken* Mate van ervaren stress (laag) 1 (2) 3 4 (hoog)	
TIJD: van 7.45 tot 8.15	Vul in: activiteit, houding, aanwezigheid andere personen *(collega, partner etc.), locatie (werk, thuis etc.)*
Alleen in huis, beetje opgeruimd, katten eten gegeven *Om 8 uur naar buiten met vuilnis* *(lopen en staan)* *Daarna ongeveer 20 minuten naar werk fietsen* Mate van ervaren stress (laag) (1) 2 3 4 (hoog)	
TIJD: van 8.15 tot 8.45	*Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner etc.), locatie (werk, thuis etc.)*
Kletsen met collega's computer aangezet Mate van ervaren stress (laag) (1) 2 3 4 (hoog)	
TIJD: van 8.45 tot 9.15	*Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner etc.), locatie (werk, thuis etc.)*
Kletsen met collega's staand (op kantoor) *9.00 uur aan het werk* *PC werk (alleen) zittend* Mate van ervaren stress (laag) (1) 2 3 4 (hoog)	

DATUM: __/__/200___

TIJD: van ______ tot ______	*Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner etc.), locatie (werk, thuis etc.)*
Mate van ervaren stress (laag) 1 2 3 4 (hoog)	
TIJD: van ______ tot ______	*Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner etc.), locatie (werk, thuis etc.)*
Mate van ervaren stress (laag) 1 2 3 4 (hoog)	
TIJD: van ______ tot ______	*Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner etc.), locatie (werk, thuis etc.)*
Mate van ervaren stress (laag) 1 2 3 4 (hoog)	
TIJD: van ______ tot ______	*Vul in: activiteit, houding, aanwezigheid andere personen (collega, partner etc.), locatie (werk, thuis etc.)*
Mate van ervaren stress (laag) 1 2 3 4 (hoog)	

Appendix II

POMS and sleep quality questionnaires

Slaapkwaliteitslijst

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

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

POMS Stemmingsvragenlijst

Hieronder vindt u een lijst met woorden. Deze woorden beschrijven stemmingen of gevoelstoestanden. Lees ieder woord nauwkeurig en kruis dan een cijfer aan rechts van het woord, welke het beste weergeeft 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. De cijfers betekenen hetvolgende:

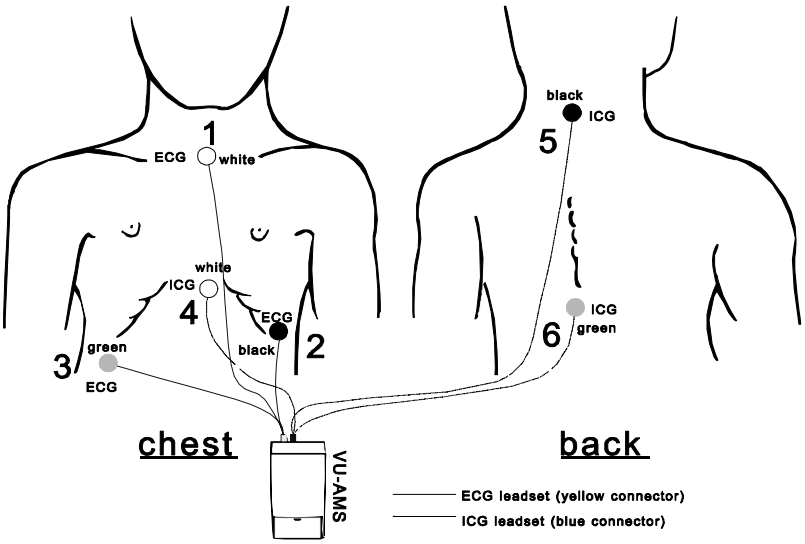
0 = helemaal niet
1 = een beetje
2 = enigszins
3 = nogal
4 = heel erg

1. Neerslachtig	0	1	2	3	4	21. Woedend	0	1	2	3	4
2. Slecht gehumeurd	0	1	2	3	4	22. Lusteloos	0	1	2	3	4
3. Uitgeput	0	1	2	3	4	23. Vol energie	0	1	2	3	4
4. Actief	0	1	2	3	4	24. Rusteloos	0	1	2	3	4
5. Zenuwachtig	0	1	2	3	4	25. Onwaardig	0	1	2	3	4
6. Hulpeloos	0	1	2	3	4	26. Knorrig	0	1	2	3	4
7. Geërgerd	0	1	2	3	4	27. Doodop	0	1	2	3	4
8. Helder	0	1	2	3	4	28. Schuldig	0	1	2	3	4
9. Paniekerig	0	1	2	3	4	29. Opgeruimd	0	1	2	3	4
10. Droevig	0	1	2	3	4	30. Angstig	0	1	2	3	4
11. Vriendelijk	0	1	2	3	4	31. Droefgeestig	0	1	2	3	4
12. Opstandig	0	1	2	3	4	32. Kwaad	0	1	2	3	4
13. Vermoeid	0	1	2	3	4	33. Afgemat	0	1	2	3	4
14. Levendig	0	1	2	3	4	34. Onzeker	0	1	2	3	4
15. Gespannen	0	1	2	3	4	35. Wanhopig	0	1	2	3	4
16. Eenzaam	0	1	2	3	4	36. Behulpzaam	0	1	2	3	4
17. Bezorgd	0	1	2	3	4	37. Ontmoedigd	0	1	2	3	4
18. Verbitterd	0	1	2	3	4	38. Mopperend	0	1	2	3	4
19. Aan het eind van mijn krachten	0	1	2	3	4						
20. Ongelukkig	0	1	2	3	4						

Appendix III

Instruction cards VU-AMS device and Spacelabs BP monitor

Short Manual VU-AMS device



Attachment of the VU-AMS device:

1. Attachment of the electrodes

Clean the skin at the 6 positions indicated in the figure. Rub the skin firmly with an alcohol soaked tissue or, if alcohol is not available, use a clean dry tissue. Attach an electrode by pressing the plastic brim of the electrode and subsequently pushing the metal stud at the center of the electrode firmly, to properly spread the contact gel.

ECG:	1.	Above the sternum, between the collar bones
	2.	Under the left breast, 4 cm (1.5") under the nipple.
	3.	At the right, between the lower two ribs.
ICG:	4.	At the sternum, where the ribs meet.
	5.	At the back, on the spine, at least 3 cm (1") above electrode 1.
	6.	At the back, on the spine, at least 3 cm (1") below electrode 4.

2. Attachment of the lead wires

Attach the lead wires to the electrodes according to the color coding in the figure above:

ECG leadset (with the yellow connector):	Position 1: white
	Position 2: black
	Position 3: green
ICG leadset (with the blue connector):	Position 4: white
	Position 5: black
	Position 6: green

Be sure to keep the leadwire connectors aside when you redress; they must be plugged into the VU-AMS device.

3. Attachment of the device

Put the VU-AMS device in its carrier bag with its connector side up. Fasten the device with the Velcro strap in the bag and gird it on with the VU-AMS belt (if its more convenient, you can also use your own belt). Make sure the device is fixed in a vertical position. Use the additional shoulder strap if necessary.

Do not forget to fill in your diary after the beep

Starting the measurement

The VU-AMS device is always standby. Measurement will (re-)start after you plug in the 2 lead wire connectors. The 'yellow' ECG leadset has to be plugged in the yellow socket marked 'ECG'. The 'blue' ICG leadset has to be plugged in the blue socket marked 'ICG'. If plugged in correctly the VU-AMS device should start beeping in the rhythm of your heart beat for a few seconds, and subsequently followed by a single long beep. This long beep signifies a successful start of a new measurement.

Marking an event

A red button is placed on top of the VU-AMS device next to the 2 lead wire connectors. To mark a special event, push this red button. Pushing it will be confirmed by a short beep. Make a note of the event in your diary.

Stopping the measurement

If you want to stop the measurement temporarily (e.g. for taking a shower) unplug the connectors from the VU-AMS device. After this, snap off the leadwires. The VU-AMS device will react with the 'alert beep'. You can silence the 'alert beep' by pressing the red button. The electrodes are waterproof and need not be removed. To restart the measurement, simply follow the instructions above starting at 'Attachment of the leadwires'.

Still working?

To check if the VU-AMS device is still working, push the red button for 5 seconds. If the measurement is still going on you will hear beeping in the rhythm of your heart beat for a few seconds.

Something's going wrong...

- You hear a double pitch beep (the 'alert beep').

Diagnosis: One of the electrodes is loose or one of the connectors is out of its socket.
Solution: Check the electrodes and connectors for proper connection. While checking you can silence the 'alert beep' by pushing the red button.

- There are no heart beat beeps after you have (re-)attached the leadwires.

Diagnosis: Electrodes are not placed at the right spot or the connectors are not plugged in the right sockets or not deep enough into their sockets.
Solution: Attach the electrodes at the right positions and connect the plugs properly.

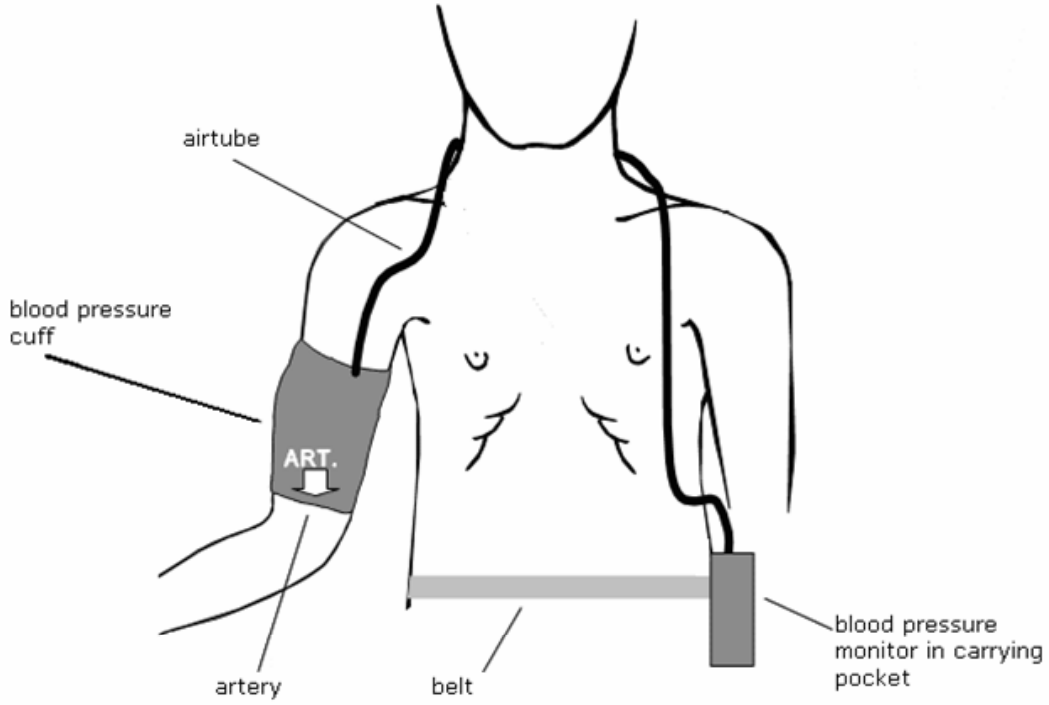
- An electrode gets loose or a plug is pulled out by accident.

Solution: No worries. Just attach the electrode again (use a spare one if necessary) or put the plug back into the socket. You will hear the beep signaling your heart beat for a few seconds and the measurement will be continued.

- Otherwise, for online help dial the number printed on your diary.

Never remove the battery, all data will be lost

Short Manual Spacelabs Blood Pressure Monitor



Fitting the monitor

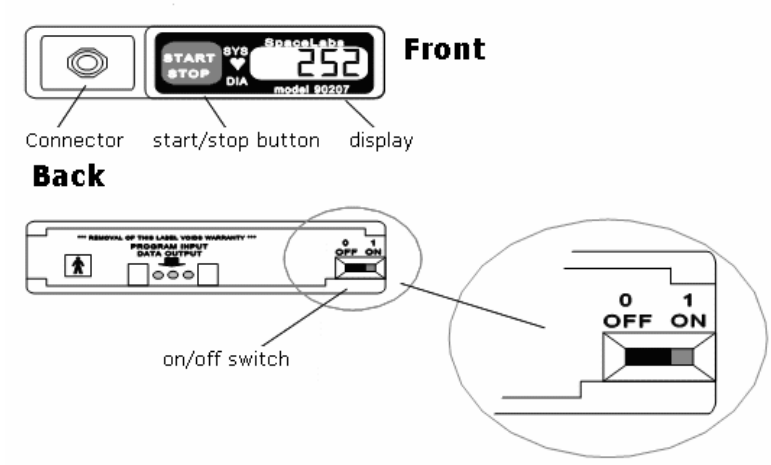
- Attach the carrying pocker for the blood pressure monitor to the belt. Tie the belt around your waist and wear the carrying pocket on your right side* (see figure above).
- Fit the cuff on your **left arm***, such that the word 'ART.' is situated on the inside of the arm, the arrow is pointing towards the inside of your elbow and there is 2.5 cm between the cuff the inside of the elbow. Make sure that the cuff is tight enough, so that it does not shift down your arm.
- Then let the airtube hang around your shoulders, and attach the tube to the connector on the front side of the blood pressure monitor, by screwing it on.

Starting the blood pressure measurements

To start the blood pressure measurements, you will have to shift the on/off switch to 'ON'. (The on.off switch is situated on the backside of the monitor).The digital display now gives the time. As long as the monitor stays on, every half hour there will bee a blood pressure measurement.

* If you are lefthanded, then wear the cuff on the right side and the carrying pocket on your rightside.

Avoid sharp or hot objects near the cuff and the airtube.



Turning the monitor off.

Short interruption: to postpone a measurement, e.g. if you cannot relax your arm, you can push the on.off button once. The measurement stops, but after 3 minutes a new measurement is automatically started.

Longterm interruption: to interupt blood pressure monitoring for longer periods, e.g when showering or going to bed, switch the monitor off, using the on/off button, remove the cuff, belt and carrying pocket. Staring the measurment again? Push the on/off button once.

At night: At night, remove monitor and cuff as is described above.

There's something wrong...

- Measurement is repeated every three minutes

Diagnosis: measurements fail because of too much movement or a cuff that is too losely fit. This automatically triggers the repeated measurements.

Solution: a. Keep your arm as still and relaxed as possible during a measurement.
b. Check if the cuff if fit tightly.

- No measurements, although you did not swtch the monitor off yourself.

Diagnosis: Cause may be that the airtube has become detached from either cuff or monitor, or that accidently you switched the on/off button to 'off'.

Solution: Check whether airtube is connected correctly to cuff and monitor and that the on/off switch is 'on'.

- Display shows a code such as EC00 or LLL.

Diagnosis: code has to be addressed by the researcher

Solution: Switch off blood pressure monitor, remove both cuff and monitor. Put monitor back in the bag that came with all equipment.

- Other poblems? Call number in diary

NB: Just before every measurement the monitor counts down from 5. The display wil show this: 5555, 4444, 3333, 2222, 1111. Then, the measurement starts. During the measurement you will see ' -----' This is normal procedure for the monitor.

Do not forget to switch the monitor OFF when you take off the cuff.

List of frequently used abbreviations

A	Additive genetic factor
ABP	Ambulatory blood pressure
ACE	Angiotensin converting enzyme
AIC	Akaike's information criterion
BMI	Body mass index
BP	Blood pressure
C	Common/Shared environmental factor
CAR	Cortisol awakening response
Corr	Correlation
CVD	Cardiovascular disease
D	Dominance genetic factor
DBP	Diastolic blood pressure
DZ	Dizygotic
dZ/dt	Change in impedance
DOS	Dizygotic twin pair of opposite sex
E	Unique/Non-shared environmental factor
ECG	Electrocardiogram
F	Female
GR	Glucocorticoid receptor
HI	Heather index
HP	Heart period
HPA axis	Hypothalamus pituitary adrenal axis
HR	Heart rate
HRV	Heart rate variability
HVZ	Hart- en vaatziekten
IBI	Inter-beat-interval
ICG	Impedance cardiogram
LVET	Left ventricular ejection time
M	Male
MZ	Monozygotic
NETAMB	Netherlands twin family ambulatory study
NTR	Netherlands twin register
OS	Opposite sex
P	p-value
PEP	Pre-ejection period
QTL	Quantitative trait locus
RMSSD	Root mean square of successive differences
RR	Respiration rate
RSA	Respiratory sinus arrhythmia
SBP	Systolic blood pressure
SDNN index	Standard deviation of normal-to-normal intervals indexed over 5 min periods
SES	Socio-economic status
VU-AMS	Vrije Universiteit Ambulatory Monitoring System
Z	Impedance (in Ohm)

List of Publications

Papers

Boomsma D.I., Vink J.M., van Beijsterveldt T.C., de Geus E.J., Beem A.L., Mulder E.J., Derks E.M., Riese H., Willemsen G.A., Bartels M., van den Berg M., **Kupper N.H.M.**, Polderman T.J., Posthuma D., Rietveld M.J., Stubbe J.H., Knol L.I., Stroet T., van Baal G.C.. Netherlands Twin Register: a focus on longitudinal research. *Twin Res*. 2002; 5: 401-406.

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Hottenga, J.J., Boomsma, D.I., **Kupper, N.H.M.,** Posthuma, D., Snieder, H., Willemsen, G., de Geus, E.J.C.. Heritability and stability of resting blood pressure, *Twin research. in press.*

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Book Chapters

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