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.