The genetics of heart rate variability

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chapter 9

General discussion and future perspectives

The aim of this PhD project was to find and characterize the genetic determinants responsible for heart rate variability (HRV). For this purpose, we used various (genetic) epidemiology study designs/approaches to answer this question such as a validity study testing the suitability of the phenotype (**Chapter 2**), and studies of heritability (h^2) of HRV at rest and under stress (**Chapter 3**), a candidate gene association study (**Chapter 4**), and a genome-wide association study (GWAS) (**Chapter 5**). These studies provided evidence that genetic factors are partly responsible for HRV and, furthermore using a GWAS approach we are the first to have identified genetic loci associated with HRV.

VALIDITY: TESTING THE SUITABILITY OF THE PHENOTYPE FOR (GENETIC) EPIDEMIOLOGICAL STUDIES

A well-defined, good-quality phenotype is of importance for all epidemiological studies, but this is particularly true in genetic epidemiological studies in which a connection between the phenotype and its underlying genotype is investigated. Using a 'precise' phenotype can maximize the power to find such genes. A potentially useful and untapped resource for increasing sample size of HRV studies comprises routinely collected 10s or 20s electrocardiograms (ECGs). However, before using these ECGs we first wanted to determine the validity of these ultra-short recordings, i.e., whether they capture "actual" HRV. This was explored in **Chapter 2**. Our findings showed that recordings longer than 120s were not necessary to accurately measure the root mean square of the successive differences (RMSSD) and standard deviation of the normal-to-normal intervals (SDNN) and we showed that routine 10s recordings yielded a valid proxy for "actual" RMSSD measurements. For SDNN, we recommend that either 30s or averages of multiple 10s ECGs should be used. A longer recording length is recommended for SDNN because it reflects all the cyclic components responsible for HRV whereas RMSSD corresponds to short-term HRV changes only (i.e. high frequency HRV components) [1].

HERITABILITY: ESTIMATING THE RELATIVE INFLUENCE OF GENETIC AND ENVIRONMENTAL EFFECTS

Heritability studies set out to find the causes of variation in a particular trait in a specific population at a specific time [2]. We initiated our search for the genes responsible for HRV by establishing that HRV was indeed heritable under various conditions. In order to uncover genetic variance that could remain hidden when analyzing HRV at rest only, we also used HRV measured during mental and physical stress tests. The question of whether the genetic influence of HRV is more pronounced in subjects during stress has been addressed

before, in Europeans ([3-6] and African-Americans [7]. These studies have indicated that genetic influences on HRV are more pronounced in subjects during stress [8, 9]. However, few studies ([10-13]) have investigated HRV heritability in family data and none have used family data to disentangle the genetic and environmental factors influencing HRV at rest and during both mental and physical stress.

As such, in **Chapter 3**, we used the Oman Family Study to determine that HRV was heritable at rest (h^2 of 11% - 19%) and during mental (h^2 of 9% - 36%) and physical (h^2 of 7% - 18%) stress depending on the HRV trait measured. We extended our analyses by examining the relationship between rest and stress in HRV traits and discern the genetic variance at rest from that during stress. We used a bivariate modeling technique in our family data and found that most HRV measurements at rest and during stress are to a large extent controlled by the same genes. However, most traits also showed genetic effects that were stress test specific. These findings imply that some genes have an effect on the sympathovagal cardiac control (measured through HRV traits) at rest, whereas other genes are expressed only when these HRV phenotypes are measured in mentally and/or physically challenging conditions.

Having obtained evidence that genetic variants influence HRV, the next step is to locate and identify the genetic variants responsible. Traditionally, linkage analysis has been used to find the chromosomal locations of those genes relative to other co-segregating DNA sequences (anonymous markers) with known positions [14]. Consequently, we performed a multipoint linkage analysis in the OFS (see **Chapter 3** supplementary material). We observed significant linkage for HR on Chromosome 3 and suggestive linkage on Chromosomes 6, 7, and 12 for HRV phenotypes. Moreover, from our linkage plots we observed indeed that there are unique and common genes to HRV at rest and during mental and/or physical stress. For example, for SDNN and HF on Chromosome 6 we observed overlapping signals for rest, CPT and WCT, whereas for RMSSD on Chromosome 12 and LF on Chromosome 7 we observed single suggestive linkage signals (LOD $\geq$ 2) specific for WCT and rest, respectively. A significant linkage signal (LOD $\geq$ 3) for HR on Chromosome 3 was only found under mental stress (WCT). The downside of using linkage analysis for non-Mendelian, complex traits such as HRV, is that the effect sizes (or "penetrance") of the individual causal variants are too small to allow detection via co-segregation [15]. Therefore, the power to detect genes for complex traits with linkage is minimal [16] and mapping resolution is low [17], which could explain why most of our linkage results for HR and HRV in the OFS were only suggestive. A better solution for gene identification for complex traits such as HRV is to use association approaches using unrelated individuals in, for example, candidate genes studies. [18]

CANDIDATE GENE ASSOCIATION STUDY: HYPOTHESIS-BASED GENE FINDING

In **Chapter 4**, we used a candidate gene association approach to explore the genetics of HRV by investigating eight genes involved in biosynthesis, transport, breakdown, and receptor binding of acetylcholine (ACh), a parasympathetic nervous system (PNS) neurotransmitter that activates the muscarinic and nicotinic receptors. We used a tagging approach in which independent single nucleotide polymorphisms (SNPs) were selected covering the entire genes. We assessed the association of those tagging SNPs with RMSSD by a two-stage design (n = 3,429 in discovery stage and n = 3,311 in replication stage). With the exception of the choline transporter [19], these genes had not been investigated in genetic studies of HRV before.

Although, we did not find any significant associations between any of the SNPs in the eight ACh pathway genes and RMSSD, this does not detract from the merits of our study. Firstly, we had a comprehensive coverage of the genes investigated by common genetic variation using a tagging SNP approach. Secondly, a large sample size was achieved by meta-analysis of multiple cohorts in a two-stage design. Our study highlights the importance of a large sample sizes and replication. For example, a previous study by Neumann and colleagues [19] found rs333229 in the choline transporter gene *SLC5A7* to be significantly associated with HRV. This contrasts with our findings where the exact same SNP was not significantly associated to HRV at the discovery stage of our study, which was eight times larger than that of Neumann et al. [19]. This means that the previous association was most likely a chance finding caused by the small sample size, resulting in lack of power. This well-known phenomenon of non-replication in candidate gene association studies was clearly demonstrated for the first time by Ioannidis and colleagues who found that results of the first study often correlate modestly with those from subsequent research on the same association [20]. The first study often suggests a stronger effect than is seen in later studies, which is also known as the 'winners curse'.

Our negative results illustrate the challenges encountered by hypothesis-based candidate gene studies for complex phenotypes such as HRV. Different etiological mechanisms in other, yet unknown biological pathways may play a role caused by various genes, each with a small overall contribution [21]. An alternative association approach to identify genes underlying complex phenotypes is that of the genome-wide association study (GWAS). GWASs are hypothesis-free and involve interrogation of large numbers of SNPs spread across the entire genome.

META-ANALYSIS OF GENOME-WIDE ASSOCIATION STUDIES: HYPOTHESIS-FREE GENE FINDING

The GWAS design is unbiased as no prior biological knowledge and genome locations of the genetic variants involved in the trait or disease are assumed as opposed to candidate gene association studies [15]). As such, GWASs have allowed for the identification of many genetic variants responsible for part of the heritability of many complex diseases using a hypothesis-free approach [22-24]. Furthermore, GWASs in many complex diseases and phenotypes such as cardiac electrophysiology [25] and psychiatric disorders [26]) have found new associated loci that were previously unsuspected and thereby suggesting new pathophysiological hypotheses. This accentuates the power of using an unbiased genetic approach to uncover novel biology. Furthermore, in contrast to linkage studies, GWASs can be performed on unrelated subjects making it easier to achieve large sample sizes. The underlying hypothesis of GWASs lies in Lander's [27] "common disease, common variant" hypothesis, which implies that relatively common genetic variants in the population with low penetrance contribute to the genetic susceptibility to common complex traits and diseases.

Only one GWAS was performed on resting HRV phenotypes in the community based Framingham Heart Study [28]. They tested 70,987 common genetic variants for association with the ratio of low and high frequency power (LF/HF), total power (TP), and SDNN, however, none of the results attained a genome-wide significance threshold (p-value = 1×10^{-7}), which they attributed to limited power.

In order to develop a robust GWAS that can detect the small genetic effects expected to underlie a complex trait such as HRV, a large sample size is crucial. As such a meta-analysis from multiple independent GWASs is the preferred method to increase power and reduce false-positive findings [23, 29]. Accordingly, the Genetic Variance of Heart Rate Variability (V_gHRV) Consortium was formed of multiple worldwide research groups who each conducted a GWAS on SDNN, RMSSD, and/or peak valley RSA/HF (pvRSA/HF) HRV measurements. In **Chapter 5** a two-staged meta-analysis of GWASs (meta-GWAS) was performed consisting of a discovery and replication stage. Once we successfully finalized our discovery stage, which yielded 23 independent genetic variants associated with HRV at the suggestive level of $p < 1 \times 10^{-6}$, we set out to confirm these associations during our replication stage. The importance of replication is to provide convincing statistical evidence for association as well as preclude associations caused by chance findings or artefacts. Such is the case with our study, where our replication samples were drawn from different populations of the same ethnicity as those from the discovery stage, demonstrating that the original association discovered was not due to systematic biases. [30]

Moreover, since we had shown that 10s ECG recordings yielded a valid proxy for "actual" RMSSD measurements, we used digital 10s ECGs from the LifeLines cohort (n=12,101) [31-33], thereby significantly increasing the sample size of our replication stage.

By using this two-staged meta-GWAS approach in European ancestry populations we obtained strong evidence that collectively 17 SNPs for the three traits (10 for SDNN, 11 for RMSSD, and 5 for pvRSA/HF) (of which 11 were independent) in eight loci were associated with HRV. See Table 6.1. As an aside, when we compare the (modest power of) multipoint linkage analysis peaks we performed on HRV at rest in OFS [Supplementary Figure 3.1 in Chapter 3] with our eight loci found to be associated with HRV in our meta-GWAS [shown in Table 5.1 of Chapter 5] we see: (a) a suggestive linkage peaks (LOD ≥ 2) for SDNN and HF on Chromosome 6 which is the third loci associated with HRV; and (b) a suggestive peak on Chromosome 12 for RMSSD which is our second loci. Many of these SNPs and loci were also associated in Hispanic/Latino and African-American cohorts, further strengthening the evidence for these loci. Several of our hits overlapped with those previously identified in a GWAS for HR [34], but six independent SNPs in four loci were novel and uniquely associated with HRV including our most significant hit in *NDUFA11* on Chromosome 19.

However most of the genome-wide significant SNPs are not causal themselves as they are not functional, but they most likely merely tag the functional variants [35]. Post-GWAS analysis is therefore needed to translate our findings into functional/biological meaning. Such post-GWAS analyses consist of stepwise application of various bioinformatics-based approaches, including database searches and the application of so-called *in silico* tools [17, 35]. In our study, we applied a previously published [36] bioinformatics-based pipeline to all of our 17 genome wide significant SNPs. Interestingly one SNP (rs12980262 in *NDUFA11* [NADH dehydrogenase (ubiquinone) 1 alpha sub-complex, 11]) associated with SDNN was a missense (or non-synonymous) SNP, which results in an amino acid change. The lead SNPs for RMSSD (rs12974991) and pvRSA/HF (rs12974440), both intronic variants on Chromosome 19, were in perfect LD with this SNP implying that these association signals may be caused by the missense mutation. The *NDUFA11* gene encodes a subunit of the membrane-bound mitochondrial complex I. That is, it is involved in mitochondrial respiration and electron transport and is also known as B14.7 and Cl-B14.7 [37]). In bovine heart mitochondrial complex I B14.7 was identified and found to show similarity to complexes involved in protein translocation across the inner mitochondrial membrane [38]; and in humans, complex I subunit NDUFA11's cDNA was cloned and sequenced from human heart mitochondria [39]. Five years later a case report [39, 40] of patients with isolated complex I deficiency originating from a homozygosity mapping of 20 consanguineous families identified a splice-site mutation in the *NDUFA11* gene. Clinically, five phenotypes are frequently seen: severe neonatal lactic acidosis, cardiomyopathy-encephalopathy, hepatopathy-tubulopathy, leukodystrophy with macrocephaly, and Leigh's and Leigh-like syndromes which were the most common presentations of complex I deficiency at

Table 6.1: Loci and SNPs identified in this thesis that are associated with HRV.

Locus	Chr	SNP	Position (bp) Build36	Trait	*Closest Gene*	Gene synonyms	Annotation	Phenotype(s) and/or disease(s) associated, including annotations associated with variants to the closest gene[a]
1	19	rs12974991[b]	5845584	RMSSD	***NDUFA11***	*B14.7; CI-B14.7*	IN	Mitochondrial complex I deficiency, isolated NADH-CoQ reductase deficiency, malignant melanoma
		rs12974440[b]	5845386	pvRSA/HF			IN	
		rs12980262[b]	5844058	SDNN			M	
2	12	rs10842383	24663234	SDNN	*LINC00477 (C12orf67)*	*C12orf67, FAM191B, FLJ32894*	IG, HR[i]	Heart rate
				RMSSD				
				pvRSA/HF				
3	6	rs236349	36928543	SDNN	***PPIL1***	*CYPL1; hCyPX; PPIase; CGI-124*	IG	Acne(severe)
				RMSSD				
				pvRSA/HF				
4	12	rs7980799[c]	33468257	RMSSD	*SYT10*	*n/a*	IN, HR[ii]	Cholesterol, cholesterol LDL, heart rate, hip, lung cancer, malignant melanoma, stroke, vitamin K
		rs1351682[c]	33490042	pvRSA/HF			IG, HR[iii]	
		rs1384598[c]	33514166	SDNN			IG, HR[iv]	
5	7	rs4262[d]	93389364	SDNN	*GNG11*	*GNGT11*	UTR5, Q, HR[v]	Heart rate, waist-hip ratio,
				pvRSA/HF				
		rs180238[d]	93388383	RMSSD			UP, Q, HR[vi]	
6	14b	rs4899412[e]	71534015	SDNN	***RGS6***	*GAP*	IN, Q	Amyotrophic lateral sclerosis (sporadic), body height, C-reactive protein, economic and political preferences (environmentalism), hip, lung cancer, malignant melanoma, obesity (extreme), ocular physiological phenomena, schizophrenia, sleep, smoking cessation, socioeconomic factors, waist circumference, Yang-deficiency constitution
		rs2052015[e]	71556806	RMSSD				
	14c	rs2529471	71883022	SDNN			IN	
	14a	rs36423	71422955	SDNN			IG	
				RMSSD				

Table 6.1: Loci and SNPs identified in this thesis that are associated with HRV. (continued)

Locus	Chr	SNP	Position (bp) Build36	Trait	*Closest Gene*	Gene synonyms	Annotation	Phenotype(s) and/or disease(s) associated, including annotations associated with variants to the closest gene[a]
7	15a	rs2680344	71440538	SDNN	*HCN4*	*n/a*	IN, HR[vii]	Brugada syndrome, familial sick sinus syndrome, negative regulation of heart rate, positive regulation of cardiac muscle contraction, positive regulation of sodium ion transmembrane transporter activity, regulation of heart rate by cardiac conduction, regulation of membrane potential, SA node cell action potential, sick sinus syndrome 2 (autosomal dominant), voltage-gated sodium channel activity, atrial fibrillation, Brugada syndrome, heart rate, lung cancer, sinus node disease, ventricular tachycardia
	15b	rs1812835	71294557	RMSSD	***NEO1***	*HsT17534, NGN; IGDCC2; NTN1R2*	IN, Q	Body fat distribution, lung cancer
8	20	rs6123471	36273570	RMSSD	*KIAA1755*	*RP5-1054A22.3*	UTR3, HR[viii]	Basophils, heart rate, prostatic neoplasms

LEGEND:
Chr: chromosome, SNP: single nucleotide polymorphism, bp: base pairs, IN: intron variant; M: missense; IG: intergenic variant; UP: upstream gene variant 2KB; UTR5: 5' UTR variant; UTR3: 3' UTR variant, Q: associated with an eQTL, HR: HRV SNPs that are in pairwise LD with identified loci associated with heart rate (HR) from den Hoed, M. et al. (2013).
Pairwise LD of SNPs between HRV and HR: I r2=1 between rs10842383 and rs17287293 [HR], iisame SNP, iiir2=0.782 between rs1351682 and rs7980799 [HR], ivr2=0.695 between rs1384598 and rs7980799[HR], vr2=0.570 between rs4262 and rs180242[HR], vir2=0.893 betweenrs180238 and rs180242[HR], viir2=0.505 between rs2680344 and rs4489968, viiir2=1 between rs6123471 and rs6127471[HR]
a Phenotype(s) and disease(s) associated with gene or gene variants were given by www.ensemble.org
b These SNPs are all in complete LD (r2=1)
c r2=0.782 between rs7980799 and rs1351682; r2=0.695 between rs7980799 and rs1384598; r2=0.903 between rs1351682 and rs1384598
d r2=0.600 between rs4262 and rs180238
e r2=0.237 between rs4899412 and rs2052015
- No phenotype or disease is known to be directly associated with gene, n/a not applicable
Bold genes are from loci uniquely associated to HRV (i.e. not overlapping with loci associated with HR)

a young age. Leigh's syndrome is a neurodegenerative disorder characterized by a wide variety of abnormalities from severe neurological problems to a near absence of abnormalities, most frequently affecting the CNS and manifesting as a progressive decline in CNS functions [41].

Regarding the other HRV-associated SNPs, one SNP (rs36928543, an intergenic variant) on Chromosome 6 which was associated with all three HRV traits was close to the Peptidylprolyl isomerase (cyclophilin)-like 1 (*PPIL1*) gene. *PPIL1* is a member in the cyclophilin family [37]. PPIL1 is recruited by Ski interaction protein (SKIP), into the spliceosome which enables the active site of PPIL1 to remain open in the PPIL1-SKIP complex and enables molecular chaperones to facilitate the folding of other proteins in the spliceosomes [42, 43]. Furthermore, *PPIL1* has been reported to be one of the key driver genes, as a subset of the 'antigen' superset, which was strongly associated to coronary artery disease SNPs based on an integrative genomic study that combined association signals from a GWAS, a tissue-specific eQTL dataset and data-driven regulatory networks [44].

The regulator of G protein signaling 6 (*RGS6*) gene on Chromosome 14 was found to be linked to three independent signals for SDNN and RMSSD. The large *RGS6* gene encodes a member of the regulator of G protein signaling (RGS) family of proteins and may modulate neuronal, cardiovascular and lymphocytic activities, and cancer risk [37]. In normal conditions the vagal nerve is activated by numerous reflex control pathways such as arterial baroreceptors, and regulated by G protein-coupled receptors (GPCRs). GPCR effector response is regulated by RGS proteins, which act as GTPase-activating proteins (GAPs) for G_{α} by accelerating their intrinsic GTPase activity. This then ends both G_{α}- and $G_{\beta\gamma}$-mediated cellular signaling. The role of RGS6 comes into play as a critical negative regulator of M2R signaling in the sinoatrial (SA) node of the heart [45] thereby functioning to set the parasympathetic "tone" by acting as the "brake" on vagal stimulation of the heart [46] (Figure 6.1). This was evidenced by observing that the loss of RGS6 provoked exaggerated bradycardia in response to carbachol, a synthetic choline ester drug that mimics the effect of ACh on both muscarinic and nicotinic receptors [47], in mice and isolate perfused hearts showing that this response was not dependent on effects of RGS6 in tissues beyond the heart. In addition, the loss of RGS6 enhanced the effect of carbachol on inhibition of action potential firing in SA node cells [45]. The results of our GWAS confirm the importance of RGS6 for the PNS in *humans*. This is reinforced by Posokhova et al [48] who described an increase in HRV in four subjects heterozygous for loss of function variants in RGS6 that introduced amino acid changes to different domains of the RGS6 protein.

Finally, on Chromosome 15, two independent intronic HRV-associated SNPs were located in the hyperpolarization activated cyclic nucleotide gated potassium channel 4 (*HCN4*) gene (rs2680344 for SDNN) and neogenin 1 (*NEO1*) gene (rs1812835 for RMSSD). The *HCN4* gene has also been found to be associated with resting HR [34] and is a known pacemaker gene. More interestingly and novel is the association of *NEO1* (a.k.a: *HsT16534*,

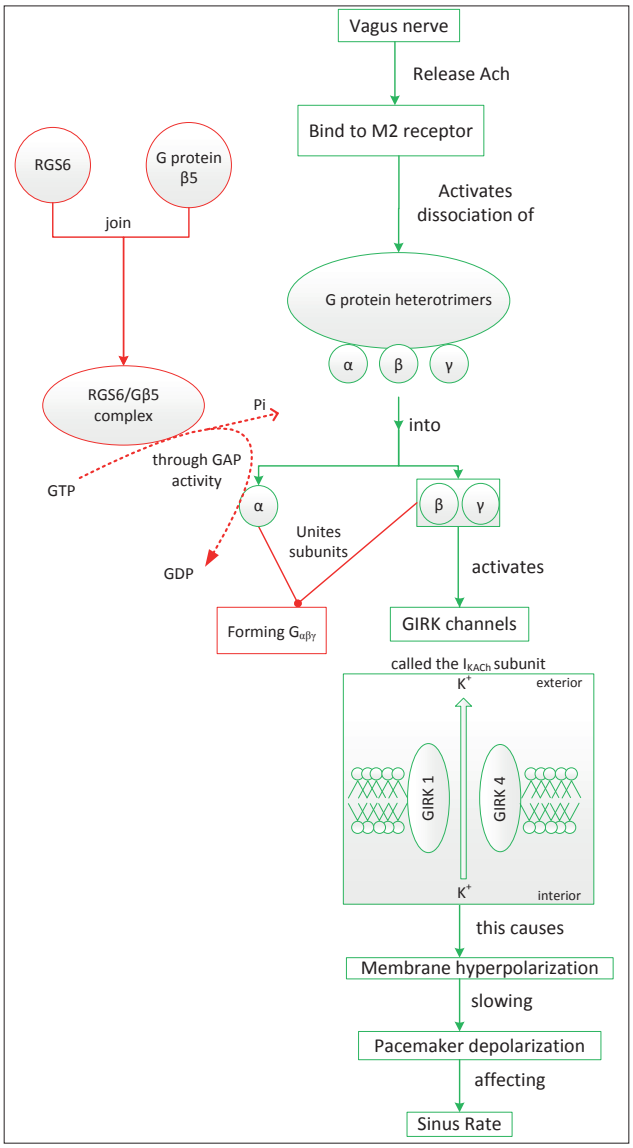

Figure 6.1: A schematic representation of the inhibition of PNS activity on the sinoatrial node by RGS6. Green coloured pathway: The vagus nerve releases acetylcholine (ACh) which binds to the muscarinic (M2) receptor. This activates the dissociation of the inactive G protein heterotrimer, which is composed of three subunits (α, β, and γ) also known as $G_{\alpha\beta\gamma}$, into two components namely an α and a $G_{\beta\gamma}$ unit. The $G_{\beta\gamma}$ unit then interacts and activates the G protein-gated inwardly rectifying potassium (GIRK) channel, which is a *muscarinic potassium channel* (I_{KACh}) composed of GIRK1 and GIRK4 subunits. This allows for potassium ions (K^+) to permeate outwardly, which results in a membrane hyperpolarization, which then slows the pacemaker depolarization subsequently slowing down the sinus rate. Red coloured pathway: RGS6 binds with G-protein β5 to create the RGS6/Gβ5 dimer complex, which activates GTPase activating proteins (GAPs) - a regulatory protein that hydrolyses guanosine triphosphate (GTP), breaking a phospate bond (Pi) to make guanosine diphosphate (GDP) - on the G-protein α. This creates the joining of the three G protein subunits (α, β, and γ) to form the inactive $G_{\alpha\beta\gamma}$ heterotrimer. [46,105]

IGDCC2, *NGN* or *NTN1R2* [49]). Neogenin 1 is a multifunctional transmembrane receptor closely related to the immunoglobulin (Ig) superfamily and is a netrin receptor [50], which, due to its 50% shared amino acid identity with the deleted in colon cancer (DCC) receptor, is thought to mediate axon-guidance functions of netrin [50, 51]. Netrin provides migrational cues in the developing CNS [52]. Neogenin in chicken embryo (which shares 86% with the predicted amino acid sequence of human NGN [aka NEO1] [51]) was suggested to play a role in early neurite out-growth and projection [53]. It is expressed in the posterior portion of the cerebellum where new neurons grow during its development [54], and is a regulator of axonal guidance in the nervous system [55]. Knockdowns of neogenin activity in zebrafish embryos suggested a role of neogenin in determining cell polarity and/or migrational directionality in neural tube formation and somitogenesis [56]. However, neogenin gene knockout in mice showed no effect on axon guidance [57]. Thus, genetic variation in *NEO1* may change neurogenesis and affect the autonomic nervous system, as measured by HRV. This could be further investigated in model systems such as drosophila and zebrafish.

We next looked up the 17 HRV SNPs in the Netherlands Study of Depression and Anxiety (NESDA) [58] and the Netherlands Twin Registry (NTR) [59] expression quantitative trait loci (eQTL) database [60, 61]. By using eQTL analysis we hoped to provide further insight into the regulatory function of HRV-associated SNPs by studying their effect on expression of nearby genes (*cis* eQTLs). Four SNPs were shown to be eQTLs influencing *GNG11* (rs180238 and rs4262), *RGS6* (rs4899412), and *NEO1* (rs1812835) and these four SNPs were in high LD ($r^2 > 0.74$) with the top eQTLs for these genes.

In addition to the *in silico* sequencing, eQTL mapping, and annotation of our SNPs during the post-GWAS stage, we conducted a gene network analysis followed by a functional enrichment analysis of our network genes to identify enrichment of gene ontology (GO) terms [36]. Each GO annotation had a computed false discovery rate (FDR) where enrichment was considered as significant for a FDR ≤ 0.01 and suggestive for 0.01 < FDR < 0.1. These analyses showed that genes near the HRV-associated SNPs were broadly related to two significantly enriched biological processes, namely: (a) cellular signaling and response (e.g. to glucagon) and (b) metabolic processes in the cell (e.g. glycosylation).

WHAT DOES THIS ALL MEAN?

Based on the novel findings reported in this thesis, our most important conclusions are:

- 10s ECG recordings are a valid proxy for "actual" RMSSD measurements and therefore can be used as a resource for future (genetic) epidemiological HRV studies.
- HRV is heritable at rest and during mental and physical stress. Although most of the genetic effects on HRV are shared between rest and stress as indicated by large ge-

netic correlations, part of the genetic influences uniquely emerges during mentally and physically challenging conditions, since the genetic correlations between rest and stress for most HRV traits are still significantly smaller than 1.

- A large and comprehensive candidate gene study which tested the association between all common variants in eight key genes of the ACh pathway could not identify that any of these common genetic variants were associated with RMSSD. This study elucidates the limitations of *a priori* knowledge needed by candidate gene studies.
- With our hypothesis-free meta-GWAS, we are the first to identify 11 independent SNPs in eight loci significantly ($p < 5x10^{-8}$) associated with HRV. Of these, six independent SNPs in four loci (*NDUFA11*, *PPIL1*, *RGS6,* and *NEO1*) were novel and uniquely associated with HRV, i.e., they did not overlap with previously identified loci for resting HR.

FUTURE PERSPECTIVES

In this thesis we are the first to identify genetic loci robustly associated with HRV. However, some important issues remain to be studied and/or explored.

HapMap vs. 1000 Genomes

The International HapMap Project aimed to characterize genetic variants of different individuals from three major ethnic groups in samples from 270 people, thereby providing information on patterns of mostly common variation across the genome [62] (http://hapmap.ncbi.nlm.nih.gov). In contrast, the 1000 Genome Project (1000G) [63] (hyperlink: http://www.1000genomes.org/) is based on sequencing DNA from entire genomes of 2,500 individuals from a larger number of ethnic groups, which allows for an increased resolution of the human genome. Moreover, the resulting 1000G map includes not only common variants, but also low-frequency and rare variants. In this thesis, the meta-GWAS used genotype data imputed to the HapMap Phase II as a reference panel. A meta-GWAS follow-up using a large-scale sequencing project reference panel such as the 1000G project is warranted as this would likely aid in fine-mapping of known loci and discovery of novel loci.

Other ethnicities

Due to the thousands of years of migration, mutations, new habitats, diverse climates and diets, exposure to different infectious diseases etc., natural selection has acted upon and created differences in (the frequency of) genetic variants between populations of different ethnicities. For example, African populations are characterized by a greater level of genetic diversity, extensive population substructure and less LD among loci compared to non-African populations [64].

In our GWAS we used both Hispanics/Latinos and African Americans in our replication stage to confirm whether SNPs associated to HRV identified in individuals of European ancestry also transfer to other ethnicities. However, for our GWAS results to be truly universal a discovery stage GWAS in other ethnicities, ideally using ethnicity specific arrays and reference panels such as available from the 1000G Project and/or the Human Genome Diversity Panel [65], would be needed.

Functional follow-up studies in model systems

At the time of writing of this thesis, we are expanding our post-GWAS analyses with experimental follow-up work of positional candidate genes in *Drosophila melanogaster* (i.e. fruit fly) and *Danio rerio* (i.e. zebrafish) as a powerful translational tool for our candidate genes that are likely to underlie the associations identified in our meta-GWAS and thereby hope to elucidate their function. The fruit fly has been an established model organism for over a century. This is due to practical reasons namely, they are small, have a simple diet, and therefore are inexpensively maintained. In addition, their life cycle is about two weeks which allows for large-scale crosses and follow-up through several generations within several months, and they can be reared under controlled environmental conditions. Also, mutations can be induced by exposing the fruit flies to mutagenic chemicals in their food which can then be easily recovered and investigated. More recently, small interfering RNA technology has allowed generation of fruit fly lines with specifically knocked down genes facilitating investigation of, e.g., genes from our HRV-associated loci for their downstream function. Fruit flies have four pairs of large polytene chromosomes (i.e. giant chromosomes of dipteran flies) of which the barcode patterns of light and dark bands permitted for an accurate gene map. In 2000, the fly genome was successfully sequenced (their genome encodes approximately 13,600 genes) [66] and now there are extensive public databases and genetic resources available such as FlyBase [67], which show that 60% of human disease [68] genes have homologs in the fruit fly. Importantly, it is possible to record cardiac function in fruit fly larvae [69], which has permitted the observation of various cardiac dysfunctions [70]. Furthermore, the fruit flies' CNS uses the same neurotransmitter system to mediate behaviors as conserved in mammals (including humans). This feature has been used to investigate the pathophysiology of neurodegenerative disease such as Alzheimer's disease [71-74], Parkinson's disease [75, 76], neuromuscular disease [77], seizures and epilepsy [78], cognitive [79] and psychiatric disorders [80].

Being vertebrates, zebrafish share even more homologs with humans than fruit flies where approximately 70% of human genes have at least one zebrafish orthologue [81, 82]). These have been widely used in vertebrate developmental biology studies due to their large transparent embryos that mature *ex vivo*, allowing for visual tracking. Similar to fruit flies, they are small and are able to produce large numbers of progeny per week per pair (200-300 new progeny), which take five days to reach full organ development

[83]. The zebrafish genome has been well characterized and sequenced [82] and has been used to in a variety of studies such as complex brain disorders [84], Parkinson's [85] and Alzheimer's disease [86, 87].

Similar to mammals, zebrafish actively regulate their cardiac output by using both PNS and SNS branches of their ANS [88]. Moreover, the zebrafish's two-chambered heart presents coronary vascularity with a heart rates (120-180 bpm) and QT intervals (400-500 ms) similar to those observed in humans, whereas mice have a higher HR (300-600 bpm) and shorter QT intervals (50ms) [89, 90]. Cardiac function can be measured by video-recording and analyzed using digital motion analysis and power spectral analysis [91].

However, there are some limitations to using fruit flies and zebrafish as model systems. Despite the conservation of basic cell processes between fruit flies/zebrafish and mammals, important differences are found. For example, it is known that the fruit fly larvae heart is innervated and neuronal inputs regulate their rate and cardiac contractions rhythm [92]. However, it is not known whether the heart innervation of fruit fly larvae is comparative to the human ANS and more specifically to the PNS. With regard to zebrafish, although they have a CNS morphology similar to mammalian models and their brain neurochemistry is highly conserved across vertebrates [84], the genetic tools in the study of zebrafish are currently not as advanced as those for fruit flies [93].

An alternative model system for functional work could be the *Mus musculus* (i.e. laboratory mouse) due to the well-defined human/mouse homology and already well-characterize functions [94, 95]. Functional elucidation of candidate genes could be achieved by using available mapped mouse quantitative trait loci (QTLs) or knockout experiments.

Does HRV cause HR?

Five of the HRV loci identified overlapped with previous HR loci, which is to be expected because of their known inverse relationship. However, it is not clear which of the two traits, HR or HRV, is the cause, the effect, or whether both are common effects of a third unobserved variable. For future studies Mendelian randomization could be used to determine whether HRV or HR is causal [96, 97]. The design mimics that of a randomized clinical trial, because it is based on Mendel's Law of Independent Assortment, i.e. the segregation of risk markers for a specific trait or outcome is inherited in a randomized fashion. The basic principle of Mendelian randomization is that if a biomarker (or intermediate or endophenotype such as HRV) is a causal factor for the clinical outcome (e.g., HR), the genetic variation underlying this biomarker should also be directly related to this outcome. If this is not the case, it may indicate that the relation between biomarker and outcome may be explained by reverse causation. The genetic markers individually or combined in a genetic risk score may be used as so called instrumental variables to estimate causal relationships in this approach.

Testing the causal role of HRV in psychopathology

Our meta-GWAS findings could be used as a stepping stone for future research attempting to illuminate possible pathways via which psychophysiological and psychosocial factors can influence mental health. A link between chronic HRV reduction and a compromised cholinergic anti-inflammatory reflex and downstream changes in allostatic systems, which increase the risk for cardiovascular disease and mortality, has been reviewed by Kemp and colleagues [98] in psychiatric populations. In addition, interventions that increase HRV and alleviate psychiatric symptoms also seem to lower the risk of morbidity and mortality [98]. Furthermore, there is direct evidence that HRV plays an important role in social communication via emotion recognition [99], which plays a major role in autism spectrum disorder. However, a major question plaguing research on the link between HRV and psychopathology is whether HRV is a true causal factor or simply an innocent bystander. For example, Licht and colleagues [100] showed that major depression was associated with lower HRV, but the association seemed to be driven by the effect of antidepressants. Based on the results of our meta-GWAS for HRV, researchers interested in these questions can now for the first time use (poly)genetic risk scores for HRV as instrumental variables in a Mendelian randomization approach and test the causal role of HRV in psychopathology.

RGS6 as potential therapeutic target

One of the most interesting discoveries of our meta-GWAS is the locus on Chromosome 14 harboring three independent genome-wide significant SNPs all mapping to the *RSG6* gene. This gene encodes the RGS6 protein, which is an essential regulator of parasympathetic signaling in the heart by acting on M2R signaling (Figure 6.1). Due to its key role in regulating M2R signaling in the heart and its effect on sinus rate, RGS6 may be a promising therapeutic target for patients with failing cardiovascular health such as those with atrial fibrillation or ventricular tachycardia. Applications may not be limited to cardiovascular disease as [45, 46] RGS6 may also constitute a novel therapeutic target for the treatment of mood disorders [101], ataxias [102], breast tumors [103], to overcome doxorubicin cardiotoxicity in cancer patients [104], and disorders of the cardiac conduction system [45, 46] by, perhaps, manipulating RGS6 activity through its regulatory mechanisms.

Ultimately with the studies reported in this thesis, I hope to have improved insight into the biology underlying cardiac vagal control and to stimulate future research into the causal role of HRV (as a proxy of PNS function) and its potential as a therapeutic target for clinical outcomes.

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