

University of Groningen

The genetics of heart rate variability

Muñoz Venegas, Maria Loreto

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2016

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Muñoz Venegas, M. L. (2016). *The genetics of heart rate variability*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Identifying genetic variant for heart rate variability in the acetylcholine pathway

PLoS ONE 2014; 9(11):e112476

Harriëtte Riese^{1,2}, Loretto M. Muñoz², Catharina A. Hartman¹, Xiuhua Ding³, Shaoyong Su⁴, Albertine J. Oldehinkel¹, Arie M. van Roon⁵, Ilja M. Nolte², Peter J. van der Most², Joop Lefrandt⁵, Ron T. Gansevoort⁶, Pim van der Harst⁷, Niek Verweij⁷, Carmilla M.M. Licht⁸, Dorret I. Boomsma⁹, Jouke-Jan Hottenga⁹, Gonneke Willemsen⁹, Brenda W.J.H. Penninx⁸, Eco J.C. de Geus⁹, Xiaoling Wang^{*4}, Harold Snieder^{*2}
*shared last authors

1. Interdisciplinary Centre Psychopathology and Emotion regulation (ICPE), Department of Psychiatry, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands.
2. Department of Epidemiology, University of Groningen, University Medical Centre Groningen, Groningen, Netherlands.
3. Departments of Biostatistics & Epidemiology, College of Public Health, University of Kentucky, Lexington, KY, USA.
4. Georgia Prevention Institute, Department of Pediatrics, Georgia Regents University, Augusta, GA, USA.
5. Department of Vascular Medicine, University of Groningen, University Medical Centre Groningen, Groningen, Netherlands.
6. Division of Nephrology, Department of Internal Medicine, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands.
7. University of Groningen, University Medical Centre Groningen, Department of Cardiology, Groningen, the Netherlands.
8. VU University Medical Centre, Amsterdam, Netherlands.
9. Department of Biological Psychology, EMGO institute for Health and Care research, VU University & VU Medical Centre, Amsterdam, The Netherlands

ABSTRACT

Heart rate variability is an important risk factor for cardiovascular disease and all-cause mortality. The acetylcholine pathway plays a key role in explaining heart rate variability in humans. We assessed whether 443 genotyped and imputed common genetic variants in eight key genes (*CHAT*, *SLC18A3*, *SLC5A7*, *CHRNA4*, *CHRNA3*, *CHRNA*, *CHRM2* and *ACHE*) of the acetylcholine pathway were associated with variation in an established measure of heart rate variability reflecting parasympathetic control of the heart rhythm, the root mean square of successive differences (RMSSD) of normal RR intervals.

The association was studied in a two stage design in individuals of European descent. First, analyses were performed in a discovery sample of four cohorts (n = 3,429, discovery stage). Second, findings were replicated in three independent cohorts (n = 3,311, replication stage), and finally the two stages were combined in a meta-analysis (n = 6,740). RMSSD data were obtained under resting conditions. After correction for multiple testing, none of the SNPs showed an association with RMSSD.

In conclusion, no common genetic variants for heart rate variability were identified in the largest and most comprehensive candidate gene study on the acetylcholine pathway to date. Future gene finding efforts for RMSSD may want to focus on hypothesis free approaches such as the genome-wide association study.

INTRODUCTION

Heart rate variability (HRV) is the variation over time of the interval between consecutive heart beats. HRV in the respiratory frequency range is a specific marker of parasympathetic control of the heart rhythm [1], and can be reliably assessed by the root mean square of successive differences (RMSSD) of normal RR intervals [2]. Prior research has produced an extensive list of HRV correlates including a broad range of somatic and mental health problems [3]. High HRV is a sign of good adaptability, often used as a marker of well-functioning cardiac autonomic control mechanisms. Reduced HRV is a predictor of hypertension [4], all-cause mortality, arrhythmic events, and sudden death after acute myocardial infarction as well as in the general population [4–7].

The parasympathetic (vagal) branch of the autonomic nervous system has an inhibitory influence on the pace-making activity of the sinoatrial node of the heart. Parasympathetic regulation of the heart is mediated by acetylcholine neurotransmission. Acetylcholine activates mainly two types of receptors, the muscarinic and nicotinic receptors. Muscarinic receptors are found on all effector cells that are stimulated by the postganglionic cholinergic neurons of the parasympathetic nervous system. Nicotinic receptors are found on the postganglionic neurons of the autonomic ganglia. The two types of receptors have different functions, and specific drugs can be used to stimulate or block one or the other type [8].

Although there is consistent evidence for the influence of genetic factors on HRV [9–11], very few studies have tried to identify the genetic polymorphisms responsible for this heritability [12]. Genes involved in the regulatory pathways of acetylcholine, the neurotransmitter of the parasympathetic nervous system, seem plausible candidates to harbor such polymorphisms. In the present study we examined eight key genes (listed in Table 4.1) involved in biosynthesis, transport, breakdown, and receptor binding of acetylcholine. With the exception of the choline transporter [13], these genes have not been investigated in genetic studies of HRV before. Neumann et al [13] genotyped one single nucleotide polymorphism (SNP, rs3731683) in *SLC5A7*, and observed that this polymorphism was significantly associated with HRV in 413 white adults. The *ACHE*, *CHAT*, and *SLC18A13* genes have been investigated as functional candidates for Alzheimer's disease [14, 15]. The fact that patients with Alzheimer's disease have reduced HRV [16, 17] and this autonomic dysfunction can be normalized by acetylcholinesterase inhibitor treatment [16] suggests that these three genes are also candidate genes for HRV. *SLC18A3* lies within the first intron of *CHAT* [18].

The genes for the $\beta 4$, $\alpha 3$, and $\alpha 5$ subunits of nicotinic receptors (*CHRNB4*, *CHRNA3*, and *CHRNA5*) are clustered on Chromosome 15q24 and physically linked [19]. Interestingly, the only genome-wide linkage scan conducted so far for HRV [20] yielded a multipoint LOD score of 1.84 on Chromosome 15 close to this gene cluster. Among the five subtypes

Table 4.1: Eight key genes involved in synthesis, metabolism and receptor binding of acetylcholine

Protein	Gene	Chr	Physical position (bp)	Genotyped tSNPs*	Imputed SNPs
Acetylcholinesterase	<i>ACHE</i>	7	100,487,615 - 100,494,594	4/4 /1	3/3 /6
Choline transporter	<i>SLC5A7</i>	2	108,602,979 -108,630,450	12/12 /16	43/43 /39
Choline acetyltransferase	<i>CHAT</i>	10	50,817,141-50,901,925	26/29 /19	63/60 /70
Vesicular acetylcholine transporter	<i>SLC18A3</i>	10	50,818,347 – 50,820,765		
Nicotinic receptors α3 subunit	<i>CHRNA3</i>	15	78,885,394-78,913,637	10/9 /10	66/67 /66
Nicotinic receptors α5 subunit	<i>CHRNA5</i>	15	78,857,862 – 78,887,611		
Nicotinic receptors β4 subunit	<i>CHRNB4</i>	15	78,916,461 – 79,020,096		
Muscarinic receptor 2	<i>CHRM2</i>	7	136,553,416-136,705,002	35/36** /45	181/180 /171

Note: Chr, chromosome. bp, base pair; *GA/ (TRAILS-P & TWINS)/NESDA, as TRAILS-P and TWINS were jointly genotyped the middle number represents genotyped SNPs of both cohorts; **One tSNP was replaced with two substitutes during the assay design stage

of muscarinic receptors (M1-M5), the mammalian heart predominantly expresses M2 receptors [21]. In *CHRM2* knockout mice, bradycardia caused by vagal stimulation was completely abolished [22].

In the current study, we assessed whether common genetic variants in these eight key genes of the acetylcholine pathway were associated with RMSSD in a total of 3429 individuals of European descent from four cohorts. Findings were replicated in 3155 subjects from three further cohorts of European descent. The key objective of this study was to identify genetic variants in the acetylcholine pathway contributing to variation in RMSSD.

METHODS

Subjects

For the discovery stage, we included subjects from four cohorts: The Georgia cohort (GA) [23], the TRacking Adolescents’ Individual Lives Survey population cohort (TRAILS-P) [24], the Twin Interdisciplinary Neuroticism Study (TWINS) [25], and the Netherlands Study of Depression and Anxiety (NESDA) cohort [26]. As replication samples The Prevention of Renal and Vascular End-stage Disease study (PREVEND) [27], the TRacking Adolescents’ Individual Lives Survey clinical cohort (TRAILS-CC) [24], and the Netherlands Twin Registry (NTR) [28] were used. A short description of the different cohorts is given below; the general characteristics are listed in Table 4.2. The study protocol was approved by the Research Ethics Board at The Institutional Review Board at the Medical College of Georgia, USA (GA), Central Committee on Research Involving Human Subjects (CCMO; TRAILS-P,

Table 4.2: General characteristics of the subjects included in the study of the four discovery cohorts and the three replication cohorts. Means and SD are given unless indicated otherwise.

	Discovery Cohorts				Replication Cohorts		
	GA	TRAILS-P	TWINS	NESDA	PREVEND	TRAILS-CC	NTR
N	682	1029	216	1502	2793	307	211
Men, %	50.6	47.6	0	31.4	50.5	69.3	31.2
Age (years)	19.9±4.4	11.5±0.53	23.2±3.5	41.6±12.4	53.21±11.67	11.11±0.48	30.83±14.42
RMSSD (ms)	73.8±38.6	75.5±49.9	39.0±26.3	39.9±25.8	29.35±19.70	88.77±54.26	61.02±34.41
log RMSSD	4.16±0.55	4.10±0.69	3.49±0.58	3.51±0.60	3.23±0.51	4.30±0.63	4.11±3.54

Note: details on the different cohorts are given in the method section.

TRAILS-CC), Medical Ethics Committee of the University Medical Centre Groningen, Groningen (TWINS, PREVEND), and the Ethical Review Board of the VU University Medical Centre (NTR, NESDA). Participants were treated in accordance with the guidelines of the Declaration of Helsinki, and all measurements were carried out with their adequate understanding and written consent provided by all subjects and by parents if subjects were < 18 years.

The GA cohort included subjects from two studies: the BP Stress Study and the Georgia Cardiovascular Twin Study. Both are ongoing longitudinal studies, which have followed the subjects more than 10 years including roughly equal numbers of Caucasians and African Americans and men and women. The BP Stress Study was established in 1989 with subjects aged 7-16 years at baseline [29], while the Georgia Cardiovascular Twin Study was established in 1996 with subjects aged 7-25 years at baseline [30]. For the twin study, the twin pairs were reared together and zygosity was determined using five standard microsatellite markers. Subjects in both studies were recruited from the south-eastern United States and were overtly healthy and free of any acute or chronic illness based on parental report. Study design, selection criteria and the criteria to classify subjects as Caucasians or African Americans for these two studies have been described before [31, 32]. A total of 682 Caucasians were available for this study of which i) 261 subjects (mean ± SD age, 23.1 ± 2.88 years; range, 15.6 to 31.8 years) from the BP stress cohort who had RMSSD measured on visit 13 and, ii) 421 twins (mean ± SD age, 17.9 ± 3.95 years; range, 11.9 to 32.9 years; 45 singletons and 188 twin pairs) from the Georgia Cardiovascular Twin Study who had RMSSD data available from the third visit.

The TRAILS-P cohort included subjects from the ongoing longitudinal population cohort, which started in 2001. Sample selection procedures and methods of study have been described before [24, 33, 34]. For the current study, data of 1029 10-to-13-year-old Dutch preadolescents (47.6% boys, mean ± SD age, 11.5 ± 0.53 years) were used for whom RMSSD data were available from the first measurement wave. A more detailed descrip-

tion of the group that participated in the cardiovascular measurements has been given elsewhere [35].

The TWINS cohort was drawn from the Groningen Twin Register, a 3-wave study including approximately 800 twin pairs from the Northern part of the Netherlands [25]. At the second wave a selection of 125 female twin pairs between 18 and 30 years old were invited to visit a psychophysiological laboratory to perform various rest and mental stress tasks. For the current study, valid RMSSD data of 216 subjects were available [11]. Zygosity of this target group was determined using 10 microsatellite markers.

The NESDA cohort was drawn from a larger 8-year longitudinal study conducted among 652 persons without depression or anxiety disorder and 2,329 with a remitted or current diagnosis of depression or an anxiety disorder [26]. For the current study, 1,502 NESDA subjects were included from which RMSSD data were collected on the baseline measurement session of the study and who had been genotyped on a genome-wide platform.

The PREVEND cohort was drawn from a larger community-based prospective cohort which was originally initiated to investigate the natural course of increased albuminuria levels and its association to renal and cardiovascular disease [27, 36]. PREVEND subjects completed the baseline survey from 1997 to 1998. At the second screening (2001 to 2003) RMSSD data were obtained from 2,793 subjects.

The TRAILS-CC cohort included subjects from the ongoing longitudinal cohort of clinically referred subjects following the same protocol as the TRAILS-P cohort described above. Sample selection procedures and methods of study have been described before [24, 33, 37]. For the current study data of 307 TRAILS-CC 10-to-12-year-old Dutch preadolescents (69.3% boys, mean \pm SD age, 11.11 \pm 0.48 years) were used for whom RMSSD data were obtained at the first measurement wave.

The NTR cohort that was established in the late 1980s, consisted of Dutch adult twins (mean age \pm SD, 30.83 \pm 14.42 years) that had participated in one of various studies of cardiovascular regulation [38, 39] and had been part of the GAIN genotyping effort [40]. RMSSD data obtained during a resting condition were used.

In all cohorts subjects with heart disease and those taking antidepressant or anticholinergic medications were excluded from the analyses.

Heart rate variability measurement and processing

For the GA cohorts, the continuous RR intervals were recorded with BioZ (CardioDynamics, San Diego, CA) impedance monitors. Four dual sensors connected to the BioZ were placed on the subject's neck and thorax, which formed four electrocardiogram (ECG) vectors. These ECG vectors can be detected by the BioZ. The BioZ then converted the RR interval into beat-to-beat heart rate with a precision of 2 decimal places, including a record of the real time of each beat. At the 10th, 12th, and 14th minute of the 15-minute resting period, a 30-second continuous period of RR intervals was recorded. Prior to calculation of HRV

parameters, the raw RR interval data were firstly processed for artificial readings using the following two criteria: (1) RR intervals were between 300 and 2000 ms; (2) the successive RR interval ratios were between 0.8 and 1.2. Dedicated HRV software (Kubios HRV analysis) [41] was used to compute the RMSSD from the RR intervals. RMSSD was calculated as the average of the three 30-second segments of continuous RR intervals.

In the TRAILS-P and TRAILS-CC cohorts, RMSSD was computed on the ECG-derived RR interval time series. ECG measurements took place in a quiet room at school (TRAILS-P) or at the hospital research center (TRAILS-CC). First, subjects lied down and were encouraged to relax and not to move or speak. Next, the procedure was explained to them and data acquisition did not commence until the signal was considerably stable (i.e., as visually observed through the levelling out of the signals after initial fluctuations), usually within five minutes. RR intervals were registered for four minutes in the supine position. To obtain RR intervals with sufficient resolution for HRV determination, a special interpolation algorithm was used, increasing the time resolution for R-peak detection by a factor of 2.5. After initial visual inspection and exclusion of unusable signals, stationarity of the time series was checked and artifacts were corrected using the CARSPAN program (IECProgramma, Rijks Universiteit Groningen, Groningen, The Netherlands) [42]. A more detailed description of cardiovascular data assessment, analysis, attrition, and internal reliability has been given before [35].

In the TWINS cohort RMSSD was computed on the ECG-derived RR interval time series, which were assessed in a quiet laboratory with a three-lead ECG during four standardized conditions: a rest condition, two stress conditions, and a second rest condition. Cardiovascular measurements started after the subjects had relaxed in sitting position for at least 10 minutes. Each condition lasted for about five minutes. The average of the two rest conditions was used in the current study. The same data cleaning procedure as described above for the TRAILS-P and TRAILS-CC studies was used. Further details have been described before [43, 44].

In the NESDA cohort RMSSD was computed on the ECG-derived RR interval time series measured with the Vrije Universiteit Ambulatory Monitoring System (VU-AMS) [45, 46]. Subjects wore the VU-AMS device during a large part of the baseline assessment while participating in other assessments (medical examination, an interview and a computer task). Further details on the assessment have been described before [47].

In the PREVEND cohort RMSSD was computed on the RR interval time series based on the beat-to-beat blood pressure signals measured with the Portapres® device (FMS Finapres Medical systems BV, Amsterdam, The Netherlands [48]). Subjects were measured in the supine resting condition, after resting for five minutes, during 15 minutes, while holding their hand with the Portapres cuff at heart level. They were not allowed to talk or to move during the measurement. In short, from the 15 minute recording an artifact free period of at least 100 seconds but no longer than five minutes was selected for RMSSD calculations.

The same data cleaning procedure as described above for the TRAILS-P, TRAILS-CC, and TWINS studies was used. Further details have been described before [49].

In the NTR cohort RMSSD was computed on the ECG-derived RR interval time series measured with the VU-AMS device during a 24-h ambulatory monitoring procedure which has been described before [38, 39]. Prompted by the VU-AMS device, the subjects filled out a diary every 30 (\pm 10) minutes in which they logged their physical activities and bodily postures since the previous prompt. This diary information was combined with simultaneously recorded vertical accelerometer data to divide the entire 24-h recording into periods of homogeneous activities/postures (median duration 26.2 minutes). From the total set of ambulatory RMSSD measurements only the evening measurements were selected where subjects were seated calmly for at least five minutes but no longer than 60 minutes. The average of these measurements was taken as their RMSSD resting value.

SNP selection

For the GA cohorts, tagging SNPs were selected from HapMap phase II data (July, 2007) for seven genes including *CHAT*, *SLC18A3*, *SLC5A7*, *CHRNB4*, *CHRNA3*, *CHRNA5* and *CHRM2* using Haploview pairwise tagging with a $r^2 \geq 0.8$. All the common SNPs with a minor allele frequency (MAF) $\geq 5\%$ within the gene as well as -10kb upstream and +10kb downstream of the gene were covered. Since the HapMap had a limited number of SNPs available in the *ACHE* gene at the time when tagging SNPs were selected (July, 2007), the SNP data from the SeattleSNPs Variation Discovery Resource (www.pga.gs.washington.edu), which had completely re-sequenced *ACHE* in 23 white Americans, was used for tagging SNPs selection. Table 4.1 lists the physical position, number of the tagging SNPs selected, the genotyped and imputed SNPs for the eight key genes. Since *SLC18A3* is within the first intron of the *CHAT* gene and *CHRNB4*, *CHRNA3*, and *CHRNA5* are within one gene cluster, these genes were listed together as two loci in Table 4.1.

For the TRAILS-P, TRAILS-CC and TWINS cohorts, the same tagging SNPs as selected for the Caucasian GA cohorts described above were selected. For the NESDA cohort, which had genome wide genotyping data, SNPs within the same physical regions were selected for this study.

DNA extraction and genotyping

In the GA cohorts, genotyping of blood derived DNA was performed by BioServe Biotechnologies (Laurel, MD) using Sequenom's high-throughput matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. All genotyping results were reviewed manually for quality control. Two 'no template' controls and two positive controls were included on each plate. In addition, 107 sets of blinded controls were distributed throughout the plates for quality control purposes. There was excellent

observer agreement in the randomly selected duplicates that were included for quality control purposes. The kappa statistic ranged from 0.89 to 1, with an average of 0.974.

In a subsample of the TRAILS-P, TRAILS-CC and TWINS cohorts, DNA was extracted from blood samples or (in a few cases) buccal swabs (Cytobrush[®]) using a manual salting out procedure [50]. Genotyping was performed on the Golden Gate Illumina BeadStation 500 platform (Illumina Inc., San Diego, CA, USA), following the manufacturers protocol. Genotyping data and clustering was performed in BeadStudio 3.0 (Illumina Inc., San Diego, CA, USA). Concordance between DNA replicates showed a genotyping accuracy of 100%. Data cleaning was in line with standard procedures [51]. The call rate in the combined set of TRAILS-P, TRAILS-CC, and TWINS after data cleaning was 99.8%. In addition to this candidate gene genotyping, genome-wide genotyping of TRAILS-CC was performed using the Illumina HumanCytoSNP12 v2 beadchip assay (Illumina, Inc.; San Diego, CA, USA). Genotypes were called with the Illumina GenomeStudio software package (Illumina, Inc.). Details on genotyping and quality control have been given elsewhere [52].

In the NESDA and NTR cohorts, genome-wide genotyping of a subsample of the subjects was performed as part of the GAIN initiative to detect genetic variation associated with major depression [40]. Genotyping was performed on Affymetrix 500K GeneChip arrays. A detailed description of genotyping and quality control criteria has been given previously [53].

For PREVEND genome-wide genotyping of a subsample of the cohort was performed using the Illumina HumanCytoSNP12 v2 beadchip assay (Illumina, Inc.; San Diego, CA, USA). Genotypes were called with the Illumina GenomeStudio software package (Illumina, Inc.). Details on genotyping and quality control have been described before [54].

Imputation

Non-genotyped SNPs within the eight genes, as well as -10kb upstream and +10kb downstream of the genes, were imputed. For the GA, TRAILS-P and TWINS cohorts, for seven genes (*CHAT*, *SLC18A3*, *SLC5A7*, *CHRNA4*, *CHRNA3*, *CHRNA5* and *CHRM2*), imputation was performed using Phase II CEU HapMap data (release 24, build 36) as the reference database using IMPUTE version 0.3.2 [55], while for the *ACHE* gene, imputation was performed using Phase III CEU HapMap data (release 2, build 36) because coverage of this gene in HapMap Phase II (release 24) was minimal. For the NESDA cohort imputation was done with IMPUTE version 2.1.2 [56] using the ALL panel of the 1000 Genomes phase I integrated variant set of March 2012. For the NTR and TRAILS-CC cohorts imputation was done with IMPUTE version 0.3.2 [55] using Phase II CEU HapMap data (release 22, build 36) reference population. For the PREVEND cohort imputation was done with Beagle version 3.1.0 [57] using the Phase II CEU HapMap (release 23a, build 36) reference population.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was tested in each cohort by a χ^2 test with 1 df. To prevent inflated significance, these tests were performed in data including only one of the twins, chosen at random, in the GA and TWINS cohorts. For the GA cohort, SNPs were excluded if they violated HWE ($p < 0.01$) and at the same time the kappa statistic for the randomly selected duplicates on the genotype of these SNPs was less than 0.90. For the TRAILS-P and TWINS cohorts, SNPs were excluded if they violated HWE ($p < 0.01$). For the NESDA, NTR, PREVEND, and TRAILS-CC cohorts SNPs with a HWE p-value below 10^{-5} were excluded.

The genotyped and imputed SNPs were examined for their associations with RMSSD under an additive model of inheritance with adjustment for age and sex. RMSSD was log-transformed in the analyses. For the GA and TWINS cohorts, generalized estimating equations were used with exchangeable correlation structure to account for familial correlations. A fixed effects meta-analysis on these four cohorts was conducted using the inverse variance weighted method. The Q and I^2 statistics were used to estimate between study heterogeneity. To help interpret I^2 's magnitude we used guidelines from Higgins and colleagues [58] in which $I^2 \leq 25\%$ marks low, $I^2 = 50\%$ medium, and $I^2 \geq 75\%$ high heterogeneity.

To optimize the chances of discovery we used a relatively lenient $p < 0.05$ cut-off in the discovery stage. That is, all SNPs with $p < 0.05$ in the discovery stage were taken forward for replication in three independent samples. For the PREVEND and NTR cohorts results for these SNPs were extracted from genome-wide analyses of log-transformed RMSSD, who analyzed it by means of SNPTTEST [55] using an additive model with age and sex as covariates. The results for the TRAILS-CC cohort were obtained from SNPTTEST [55] using an additive model with age and sex as covariates. Results from discovery and replication phase were subsequently combined in a meta-analysis using METAL [59]. When not mentioned analyses were performed in StataSE software (version 12, StataCorp, College Station, Texas, USA).

RESULTS

The characteristics of the subjects included in the analysis in each of the cohorts are given in Table 4.2. Of the 95 SNPs selected in the design stage, 87 were successfully genotyped in the GA cohort and 90 in the TRAILS-P and TWINS cohorts (Table 4.1). No further SNPs were excluded as all met our criteria for inclusion (see methods). In total, 443 genotyped and imputed SNPs were included in the association analysis with RMSSD.

Table 4.3: Results of discovery stage, replication stage and the overall meta-analysis of the 25 SNPs associated with RMSSD ($p < 0.05$) in the discovery stage. SNPs are sorted according to p-value in the discovery cohort.

Gene	SNP	C/NC	Discovery							Replication							Overall meta-analysis						
			CAF	Beta	SE	P value	I ² (%)	Q statistics	CAF	Beta	SE	P value	I ² (%)	Q statistics	Beta	SE	P value	I ² (%)	Q statistics				
1	SLCSA7	rs3731683	G/A	0.66	-0.052	0.016	0.002	14.9	0.318	0.67	0.048	0.024	0.044	0	0.484	-0.020	0.013	0.144	91.6	0.001			
2	SLCSA7	rs333214	A/G	0.88	-0.061	0.022	0.005	18.4	0.298	0.87	0.031	0.057	0.583	37.9	0.204	-0.049	0.020	0.016	56.6	0.129			
3	CHRM2	rs17506824	T/C	0.47	-0.035	0.014	0.014	0	0.965	0.47	-0.008	0.012	0.511	0	0.482	-0.020	0.010	0.034	50.1	0.157			
4	CHRM2	rs6967953	T/C	0.43	-0.035	0.015	0.017	0	0.755	0.41	-0.007	0.014	0.591	0	0.399	-0.020	0.010	0.044	47.7	0.167			
5	SLCSA7	rs17269293	C/G	0.82	-0.043	0.018	0.021	75.9	0.006	0.79	0.018	0.023	0.436	0	0.825	-0.019	0.015	0.185	76	0.041			
6	CHRM2	rs324654	T/C	0.07	-0.066	0.029	0.022	0	0.660	0.08	0.018	0.023	0.426	0	0.941	-0.015	0.018	0.417	80.9	0.022			
7	CHRM2	rs6962027	T/A	0.49	-0.032	0.014	0.024	0	0.893	0.47	-0.009	0.013	0.473	0	0.376	-0.019	0.010	0.042	31.9	0.226			
8	CHRM2	rs10246819	T/C	0.49	-0.031	0.014	0.029	0	0.920	0.47	-0.009	0.012	0.461	0	0.422	-0.019	0.010	0.047	27.7	0.240			
9	CHRM2	rs324576	T/C	0.08	-0.058	0.027	0.032	0	0.621	0.08	0.012	0.021	0.600	0	0.827	-0.016	0.017	0.352	74.9	0.046			
10	CHRM2	rs324578	A/T	0.08	-0.058	0.027	0.033	0	0.622	0.07	0.098	0.056	0.077	0	0.471	-0.028	0.025	0.255	84.3	0.012			
11	CHRM2	rs324579	G/A	0.08	-0.058	0.027	0.034	0	0.616	0.08	0.012	0.022	0.582	0	0.756	-0.015	0.017	0.367	75.1	0.045			
12	CHRM2	rs324580	T/C	0.08	-0.058	0.027	0.034	0	0.614	0.08	0.012	0.022	0.582	0	0.756	-0.015	0.017	0.368	75	0.045			
13	CHRM2	rs324587	T/A	0.08	-0.057	0.027	0.034	0	0.617	0.08	0.012	0.022	0.591	0	0.696	-0.016	0.017	0.350	74.4	0.048			
14	CHAT	rs10508915	A/G	0.85	-0.044	0.021	0.035	0	0.915	0.85	-0.003	0.017	0.848	0	0.519	-0.020	0.013	0.137	56.2	0.131			
15	CHRM2	rs324582	G/A	0.08	-0.056	0.027	0.038	0	0.608	0.08	0.012	0.022	0.582	0	0.754	-0.015	0.017	0.382	74	0.050			
16	CHRM2	rs324584	G/A	0.08	-0.056	0.027	0.039	0	0.651	0.08	0.012	0.022	0.581	0	0.753	-0.015	0.017	0.389	73.8	0.051			
17	CHRM2	rs324632	C/T	0.08	-0.056	0.027	0.041	0	0.568	0.06	0.040	0.044	0.357	0	0.970	-0.029	0.023	0.214	71.3	0.062			
18	SLCSA7	rs333227	G/A	0.15	-0.041	0.020	0.041	0	0.556	0.16	-0.003	0.017	0.858	49.8	0.137	-0.019	0.013	0.148	53	0.145			
19	SLCSA7	rs333230	G/T	0.15	-0.041	0.020	0.042	0	0.557	0.15	0.001	0.019	0.981	50.9	0.130	-0.019	0.014	0.172	56.2	0.131			
20	CHRM2	rs324612	T/C	0.08	-0.055	0.027	0.043	0	0.651	0.08	0.027	0.026	0.313	0	0.861	-0.130	0.019	0.490	78.5	0.031			

Table 4.3: Results of discovery stage, replication stage and the overall meta-analysis of the 25 SNPs associated with RMSSD ($p < 0.05$) in the discovery stage. SNPs are sorted according to p-value in the discovery cohort. (continued)

Gene	SNP	C/NC	Discovery						Replication						Overall meta-analysis					
			CAF	Beta	SE	P value	i^2 (%)	Q statistics	CAF	Beta	SE	P value	i^2 (%)	Q statistics	Beta	SE	P value	i^2 (%)	Q statistics	
21	SLC5A7	G/A	0.15	-0.041	0.020	0.044	0	0.480	0.16	-0.005	0.017	0.768	11.7	0.322	-0.020	0.013	0.131	45.9	0.174	
22	CHRM2	A/G	0.08	-0.054	0.027	0.046	0	0.688	0.06	0.049	0.043	0.253	0	0.973	-0.025	0.023	0.280	75.7	0.043	
23	CHRM2	G/A	0.08	-0.054	0.027	0.047	0	0.694	0.07	0.021	0.026	0.424	0	0.826	-0.015	0.019	0.426	74.8	0.047	
24	CHAT	A/G	0.85	-0.041	0.021	0.048	0	0.908	0.85	-0.003	0.017	0.852	0	0.569	-0.019	0.013	0.159	49	0.162	
25	CHAT	G/C	0.85	-0.041	0.021	0.048	0	0.911	0.84	0.005	0.031	0.886	0	0.591	-0.027	0.017	0.117	32.4	0.224	

Note: RMSSD was log-transformed prior data analysis. Analyses are age & sex adjusted. Discovery stage sample size (N) = 3429; Replication stage N = 3311 (except for one SNP [rs333214] in the SLC5A7 gene due to low imputation quality where N=5116); and overall meta-analysis N = 6740. C/NC, coded/non-coded allele; CAF, coded allele frequency

Discovery stage

In the discovery phase the association between the candidate SNPs and RMSSD were tested in four cohorts ($n = 3,429$; GA, TRAILS-P, TWINS and NESDA). For 25 SNPs an association with RMSSD ($p < 0.05$) was found (see Table 4.3).

When testing the genetic heterogeneity of the SNPs in this phase, the point estimate for I^2 was significant for only one SNP (rs17269293; $I^2 = 75.8\%$). Heterogeneity for all other SNPs was either completely absent or low and non-significant, confirming that performing a fixed effects meta-analysis on these data is justified.

Replication stage and the overall meta-analysis

To validate the association found in the discovery phase, the 25 lead SNPs (Table 4.3), were taken forward to the replication phase in three independent cohorts ($n = 3,311$ PREVEND, TRAILS-CC and NTR). The replication sample size was lower ($n = 516$) for one of the SNPs (rs333214 in the *SLC5A7* gene), because data of this SNP had to be excluded from the PREVEND cohort as the imputation quality was too low (0.09). None of the SNPs were replicated with a nominal $p < 0.05$, except for one SNP. However, this SNP (rs3731683) showed an effect in the opposite direction as observed in the discovery stage (see the opposite beta coefficients for this SNP in Table 4.3). Thus, none of the SNPs were replicated.

The overall meta-analysis combining discovery and replication stage ($n = 6,740$) gave no significant Bonferroni corrected ($p < 0.0005$, conservatively assuming 100 tests for the almost 100 independent tagging SNPs from Table 4.1) findings for any of the 25 SNPs (Table 4.3).

DISCUSSION

The current study comprehensively tested the association between all common ($MAF \geq 5\%$) variants in eight key genes of the acetylcholine pathway and an established measure of HRV, the RMSSD, in subjects of European descent. The eight key genes were carefully selected in this two-stage candidate gene study on the basis of their involvement in biosynthesis, transport, breakdown, and receptor binding of acetylcholine. However, none of the common genetic variants in these eight genes of the acetylcholine pathway were significantly associated with RMSSD.

Neumann and colleagues [13] genotyped a single SNP (rs333229) in the choline transporter gene *SLC5A7* in their study of 413 individuals of European ancestry (aged 30 to 54 years). They found an association with the power in the high frequency (HF) band (HF power of Fourier transformed inter-beat-interval time series of RR intervals of the ECG, is another measure of HRV comparable to RMSSD) obtained during five minutes rest: compared with GG homozygotes, T allele carriers had *higher* HF power. In a more recent study

on air pollution in 61 glucose intolerant subjects, T allele carriers of this SNP (rs333229, which was one of the 139 tested SNPs) had *lower* SDNN in response to air pollution [60]. Interestingly, the exact same SNP in *SLC5A7* (rs333229) was genotyped in the current study but did not survive our discovery stage (i.e., was not significantly associated with RMSSD). As such, the significant results in the above two underpowered studies should probably be interpreted as chance findings, because our discovery stage alone ($n = 3,429$) already had more than eight times the sample size of the largest of those two previous studies ($n = 413$).

Our study has a number of strengths compared to previous candidate gene studies that investigated only a single SNP in a single gene and/or had (very) small sample sizes. We comprehensively tested 443 genotyped and imputed common SNPs in eight key genes of the acetylcholine pathway in which our selected tagging SNPs covered all SNPs with $MAF \geq 5\%$ and $r^2 \geq 0.8$ ranging from -10kb upstream to +10kb downstream of those genes. Another strong point of our study is that we aimed to replicate the initially discovered associations in independent samples. As replication failed, our initial findings should be labeled as false-positive findings, reinforcing the idea that independent replication is essential in candidate gene studies. Our two stage association study with large combined sample size of $n = 6,740$ had more than 80% power with a Bonferroni corrected alpha of 0.0005 for 100 tests to detect SNPs explaining as little as 0.32% of the variance in RMSSD. As such it is highly unlikely that common SNPs in the investigated genes explain a considerable part of individual differences in RMSSD in rest.

A potential limitation of our study is that the RMSSD measures in the different cohorts were assessed in both sitting and supine positions, which might have introduced heterogeneity in our RMSSD data. Using these heterogeneous phenotypes might have decreased our power to detect associations somewhat. Furthermore, the findings and conclusions of the current study are not generalizable to individuals of non-European descent.

Another, perhaps more compelling, reason for our null-finding is that our approach was based on currently existing knowledge about the acetylcholine pathway. Using a hypothesis-driven design is a strength, but also has clear downsides. First of all, not all known elements of the acetylcholinergic signaling cascade were considered. Secondly, current knowledge about the acetylcholine pathways may be vastly incomplete. Thirdly, RMSSD is known to be a complex phenotype influenced by multiple genes [61], physiological and psychological systems, and their interactions, many of which may be outside of the acetylcholine pathways.

In conclusion, no common genetic variants for HRV were identified in the largest and most comprehensive candidate gene study on the acetylcholine pathway to date. Future gene finding efforts for RMSSD may want to focus on hypothesis free approaches such as the genome-wide association study.

REFERENCES

1. Task Force of the European Society of Cardiology and the North American Society of pacing and Electrophysiology (1996) Heart rate variability: standards of measurement, physiological interpretation and clinical use. *Circulation* 93: 1043-1065.
2. Goedhart AD, Van der Sluis S, Houtveen JH, Willemsen G, De Geus EJC (2007) Comparison of time and frequency domain measures of RSA in ambulatory recordings. *Psychophysiology* 44: 203-215.
3. Thayer JF, Yamamoto SS, Brosschot JF (2010) The relationship of autonomic imbalance, heart rate variability and cardiovascular disease risk factors. *Int J Cardiol* 141: 122-131.
4. Singh JP, Larson MG, Tsuij H, Evans JC, O'Donnell CJ, et al. (1998) Reduced heart rate variability and new-onset hypertension: insights into pathogenesis of hypertension: the Framingham Heart Study. *Hypertension* 32: 293-297.
5. Thayer JF, Lane RD (2007) The role of vagal function in the risk for cardiovascular disease and mortality. *Biol Psychol* 74: 224-242.
6. Buccelletti F, Gilardi E, Scaini E, Galiuto L, Persiani R, et al. (2009) Heart rate variability and myocardial infarction: systematic literature review and meta-analysis. *Eur Rev Med Pharmacol Sci* 13: 299-307.
7. Dekker JM, Crow RS, Folsom AR, Hannan PJ, Liao D, et al. (2000) Low heart rate variability in a 2-minute rhythm strip predicts risk of coronary heart disease and mortality from several causes - The ARIC study. *Circulation* 102: 1239-1244.
8. Guyton, A. C. and Hall, J. E. (2006) *Textbook of medical physiology*. Philadelphia: Elsevier Saunders.
9. Snieder H, Boomsma DI, Van Doornen LJ, de Geus EJ (1997) Heritability of respiratory sinus arrhythmia: dependency on task and respiration rate. *Psychophysiology* 34: 317-328.
10. Wang X, Thayer JF, Treiber F, Snieder H (2005) Ethnic differences and heritability of heart rate variability in African- and European American youth. *Am J Cardiol* 96: 1166-1172.
11. Riese H, Rosmalen JGM, Ormel J, van Roon AM, Oldehinkel AJ, et al. (2007) The genetic relationship between neuroticism and autonomic function in female twins. *Psychol Med* 37: 257-267.
12. Imumorin IG, Dong Y, Zhu H, Poole JC, Harshfield GA, et al. (2005) A gene-environment interaction model of stress-induced hypertension. *Cardiovasc Toxicol* 5: 109-132.
13. Neumann SA, Lawrence EC, Jennings JR, Ferrell RE, Manuck SB (2005) Heart rate variability is associated with polymorphic variation in the choline transporter gene. *Psychosom Med* 67: 168-171.
14. Cook LJ, Ho LW, Wang L, Terrenoire E, Brayne C, et al. (2005) Candidate gene association studies of genes involved in neuronal cholinergic transmission in Alzheimer's disease suggests choline acetyltransferase as a candidate deserving further study. *Am J Med Gen Part B-Neuropsychiatr Gen* 132B: 5-8.
15. Mubumbila V, Sutter A, Ptok U, Heun R, Quirin-Stricker C (2002) Identification of a single nucleotide polymorphism in the choline acetyltransferase gene associated with Alzheimer's disease. *Neurosci Lett* 333: 9-12.
16. Giubilei F, Strano S, Imbimbo BP, Tisei P, Calcagnini G, et al. (1998) Cardiac autonomic dysfunction in patients with Alzheimer disease: Possible pathogenetic mechanisms. *Alzheimer Dis Assoc Disord* 12: 356-361.
17. Aharonperetz J, Harel T, Revach M, Benhaim SA (1992) Increased sympathetic and decreased parasympathetic cardiac innervation in patients with Alzheimers disease. *Arch Neurol* 49: 919-922.
18. Erickson JD, Varoqui H, Schafer MKH, Modi W, Diebler MF, et al. (1994) Functional identification of a vesicular acetylcholine transporter and its expression from a cholinergic gene locus. *J Biol Chem* 269: 21929-21932.

19. Duga S, Solda G, Asselta R, Bonati MT, Dalpra L, et al. (2001) Characterization of the genomic structure of the human neuronal nicotinic acetylcholine receptor CHRNA5/A3/B4 gene cluster and identification of novel intragenic polymorphisms. *J Hum Genet* 46: 640-648.
20. Singh JP, Larson MG, O'Donnell CJ, Tsuji H, Corey D, et al. (2002) Genome scan linkage results for heart rate variability (the Framingham Heart Study). *Am J Cardiol* 90: 1290-1293.
21. Brodde OE, Bruck H, Leineweber K, Seyfarth T (2001) Presence, distribution and physiological function of adrenergic and muscarinic receptor subtypes in the human heart. *Basic Res Cardiol* 96: 528-538.
22. Fisher JT, Vincent SG, Gomeza J, Yamada M, Wess J (2004) Loss of vagally mediated bradycardia and bronchoconstriction in mice lacking M-2 or M-3 muscarinic acetylcholine receptors. *Faseb Journal* 18: 711-739.
23. Ge DL, Dong YB, Wang XL, Treiber FA, Snieder H (2006) The Georgia Cardiovascular Twin Study: Influence of genetic predisposition and chronic stress on risk for cardiovascular disease and type 2 diabetes. *Twin Res Hum Genet* 9: 965-970.
24. Ormel J, Oldehinkel AJ, Sijtsema J, van Oort F, Raven D, et al. (2012) The TRacking Adolescents' Individual Lives Survey (TRAILS): Design, current status, and selected findings. *J Am Acad Child Adolesc Psychiatry* 51: 1020-1036.
25. Riese H, Rijdsdijk FV, Snieder H, Ormel J (2013) Twin Interdisciplinary Neuroticism Study (TWINS). *Twin Res Hum Genet* 16: 268-270.
26. Penninx BWJH, Beekman ATF, Smit JH, Zitman FG, Nolen WA, et al. (2008) The Netherlands Study of Depression and Anxiety (NESDA): rationale, objectives and methods. *Int J Methods Psychiatr Res* 17: 121-140.
27. Pinto-Sietsma SJ, Janssen WM, Hillege HL, Navis G, de Zeeuw D, et al. (2000) Urinary albumin excretion is associated with renal functional abnormalities in a nondiabetic population. *J Am Soc Nephrol* 11: 1882-1888.
28. Willemsen G, Vink JM, Abdellaoui A, den Braber A, van Beek JHDA, et al. (2013) The adult Netherlands Twin Register: Twenty-five years of survey and biological data collection. *Twin Res Hum Genet* 16: 271-281.
29. Li ZB, Snieder H, Su SY, Ding XH, Thayer JF, et al. (2009) A longitudinal study in youth of heart rate variability at rest and in response to stress. *Int J Psychophysiol* 73: 212-217.
30. Wang XL, Ding XH, Su SY, Li ZB, Riese H, et al. (2009) Genetic influences on heart rate variability at rest and during stress. *Psychophysiology* 46: 458-465.
31. Dekkers JC, Snieder H, van den Oord EJ, Treiber FA (2002) Moderators of blood pressure development from childhood to adulthood: a 10-year longitudinal study. *J Pediatr* 141: 770-779.
32. Snieder H, Dong Y, Barbeau P, Harshfield GA, Dalageogou C, et al. (2002) Beta2-adrenergic receptor gene and resting hemodynamics in European and African American youth. *Am J Hypertens* 15: 973-979.
33. Huisman M, Oldehinkel AJ, de WA, Minderaa RB, de Bildt A, et al. (2008) Cohort profile: the Dutch 'TRacking Adolescents' Individual Lives' Survey'; TRAILS. *Int J Epidemiol* 37: 1227-1235.
34. de Winter A, Oldehinkel AJ, Veenstra R, Brunnekreef JA, Verhulst FC, et al. (2005) Evaluation of non-response bias in mental health determinants and outcomes in a large sample of pre-adolescents. *Eur J Epidemiol* 20: 173-181.
35. Dietrich A, Riese H, van Roon AM, Van Engelen K, Ormel J, et al. (2006) Spontaneous baroreflex sensitivity in (pre)adolescents. *J Hypertens* 24: 345-352.
36. Joosten H, Izaks GJ, Slaets JPJ, de Jong PE, Visser ST, et al. (2011) Association of cognitive function with albuminuria and eGFR in the general population. *Clin J Am Soc Nephrol* 6: 1400-1409.
37. de Winter A, Oldehinkel AJ, Veenstra R, Brunnekreef JA, Verhulst FC, et al. (2005) Evaluation of non-response bias in mental health determinants and outcomes in a large sample of pre-adolescents. *Eur J Epidemiol* 20: 173-181.

38. Kupper NH, Willemsen G, van den Berg M, de Boer D, Posthuma D, et al. (2004) Heritability of ambulatory heart rate variability. *Circulation* 110: 2792-2796.
39. Kupper N, Willemsen G, Posthuma D, de Boer D, Boomsma DI, et al. (2005) A genetic analysis of ambulatory cardiorespiratory coupling. *Psychophysiology* 42: 202-212.
40. Boomsma DI, Willemsen G, Sullivan PF, Heutink P, Meijer P, et al. (2008) Genome-wide association of major depression: description of samples for the GAIN Major Depressive Disorder Study: NTR and NESDA biobank projects. *Eur J Hum Genet* 16: 335-342.
41. Niskanen JP, Tarvainen MP, Ranta-Aho PO, Karjalainen PA (2004) Software for advanced HRV analysis. *Comput Methods Programs Biomed* 76: 73-81.
42. Mulder LJM, van Dellen HJ, van der Muelen P, Opheikens B (1988) CARSPAN: a spectral analysis program for cardiovascular time series. In: Maarse FJ, Mulder L, Akkerman A, editors. *Computers in psychology: methods, instrumentation and psychodiagnostics*. Amsterdam: Swets and Zeitlinger. pp. 39-47.
43. Riese H, Rijdsdijk FV, Ormel J, van Roon AM, Neeleman J, et al. (2006) Genetic influences on baroreflex sensitivity at rest and during mental stress. *J Hypertens* 24: 1779-1786.
44. Rijdsdijk FV, Riese H, Tops M, Snieder H, Brouwer WH, et al. (2009) Neuroticism, recall bias and attention bias for valenced probes: a twin study. *Psychol Med* 39: 45-54.
45. De Geus EJC, Willemsen GHM, Klaver CHAM, Van Doornen LJP (1995) Ambulatory measurement of respiratory sinus arrhythmia and respiration rate. *Biol Psychol* 41: 205-227.
46. Willemsen GHM, De Geus EJC, Klaver CHAM, Van Doornen LJP, Carroll D (1996) Ambulatory monitoring of the impedance cardiogram. *Psychophysiology* 33: 184-193.
47. Licht CM, de Geus EJ, Zitman FG, Hoogendijk WJ, van DR, et al. (2008) Association between major depressive disorder and heart rate variability in the Netherlands Study of Depression and Anxiety (NESDA). *Arch Gen Psychiatry* 65: 1358-1367.
48. Wesseling KH (1990) Finapres, continuous noninvasive finger arterial pressure based on the method of Peñáz. In: Meyer-Sabellek W, Gotzen R, Anlauf M, Steingard RJ, editors. *Blood Pressure Measurements*. Frankfurt am Main: Steunkopff. pp. 161-172.
49. Muñoz ML, van Roon AM, Riese H, Oostenbroek E, Westrik I, et al. (2014) Validity of (ultra-)short heart rate variability recordings . unpublished data .
50. Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* 16: 1215.
51. Nolte IM, McCaffery JM, Snieder H (2010) Candidate gene and genome-wide association studies in behavioral medicine. In: Steptoe A, editors. *Handbook of behavioral medicine: methods and applications*. New York: Springer.
52. Ganesh SK, Tragante V, Guo W, Guo YR, Lanktree MB, et al. (2013) Loci influencing blood pressure identified using a cardiovascular gene-centric array (vol 22, pg 1663, 2013). *Hum Molec Genet* 22: 3394-3395.
53. Sullivan PF, De Geus EJC, Willemsen G, James MR, Smit JH, et al. (2009) Genome-wide association for major depressive disorder: a possible role for the presynaptic protein piccolo. *Mol Psychiatry* 14: 359-375.
54. Verweij N, Mahmud H, Leach IM, de Boer RA, Brouwers FP, et al. (2013) Genome-wide association study on plasma levels of midregional-proadrenomedullin and C-terminal-pro-endothelin-1. *Hypertension* 61: 602-608.
55. Marchini J, Howie B, Myers S, McVean G, Donnelly P (2007) A new multipoint method for genome-wide association studies by imputation of genotypes. *Nat Genet* 39: 906-913.
56. Howie BN, Donnelly P, Marchini J (2009) A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. *Plos Genetics* 5.
57. Browning BL, Browning SR (2009) A unified approach to genotype imputation and haplotype-phase inference for large data sets of trios and unrelated individuals. *Am J Hum Genet* 84: 210-223.

58. Higgins JPT, Thompson SG, Deeks JJ, Altman DG (2003) Measuring inconsistency in meta-analyses. *BMJ* 327: 557-560.
59. Willer CJ, Li Y, Abecasis GR (2010) METAL: fast and efficient meta-analysis of genomewide association scans. *Bioinformatics* 26: 2190-2191.
60. Hampel R, Breitner S, Schneider A, Zareba W, Kraus U, et al. (2012) Acute air pollution effects on heart rate variability are modified by SNPs involved in cardiac rhythm in individuals with diabetes or impaired glucose tolerance. *Environ Res* 112: 177-185.
61. Neumann SA, Tingley WG, Conklin BR, Shrader CJ, Peet E, et al. (2009) AKAP10 (I646V) functional polymorphism predicts heart rate and heart rate variability in apparently healthy, middle-aged European-Americans. *Psychophysiology* 46: 466-472.