Genetic insights into resting heart rate and its role in cardiovascular disease

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Abstract

Resting heart rate (RHR) is associated with cardiovascular diseases and mortality in observational and Mendelian randomization studies. The aims of this study are to extend the number of RHR associated genetic variants and to obtain further insights in RHR biology and its clinical consequences. A genome-wide meta-analysis of 100 studies in up to 835,465 individuals revealed 493 independent genetic variants in 352 loci. We prioritized 670 genes and *in silico* annotations point to their enrichment in cardiomyocytes and provided insights in their ECG signature. Two-sample Mendelian randomization analyses indicated that higher genetically predicted RHR increases risk of dilated cardiomyopathy, but decreases risk of developing atrial fibrillation, ischemic stroke, and cardio-embolic stroke. We did not find evidence for a linear or non-linear genetic association between RHR and all-cause mortality in contrast to our previous Mendelian randomization study. Systematic alteration of key differences between the current and previous Mendelian randomization study indicated that the most likely cause of the discrepancy between these studies arises from false positive findings in previous one-sample MR analyses caused by weak-instrument bias at lower *P*-value thresholds. The results extend our understanding of RHR biology and give additional insights in its role in cardiovascular disease development.

Keywords

Resting heart rate, all-cause mortality, atrial fibrillation, stroke, dilated cardiomyopathy, Genome-wide association study, two-sample Mendelian randomization, ECG.

Introduction

Higher resting heart rate (RHR) is associated with cardiovascular diseases and all-cause mortality in traditional epidemiological studies[1–5]. However, RHR is influenced by disease status and a plethora of potential confounders, which could affect these associations.

A Mendelian randomization (MR) approach, in which genetic variants associated with the RHR are used as a proxy for RHR, can also be used to study the association between RHR and cardiovascular diseases and all-cause mortality. Since genetic variants are fixed from conception and then randomly assigned from parents to offspring, they are more immune to reverse causation and confounders[6]. In our previous study, we identified 64 loci associated with RHR and found evidence for a positive association between genetically predicted RHR and all-cause mortaliy[7]. However, a higher genetically predicted RHR was not found to increase risk of cardiovascular diseases[7–9] and appeared to decrease risks of atrial fibrillation and cardio-embolic stroke[9]. Identification of novel RHR loci could give new insights in the relationship between genetically predicted RHR and cardiovascular disease and mortality risk and will broaden our knowledge of the biological mechanisms underlying interindividual differences in RHR.

To increase our knowledge on the genetic make-up of RHR and the association with mortality and cardiovascular disease, we conducted a meta-analysis of 100 genome-wide association studies (GWAS's) in 835,464 participants (**Figure 1A**), performed multiple analyses to gain insights in the underlying biology of the identified variants (**Figure 1B**) and explored the relationship of genetically predicted RHR with mortality and cardiovascular diseases using a two-sample MR approach (**Figure 1C**).

Results

Genome-wide meta-analysis of resting heart rate

We performed a meta-analysis of RHR GWAS's using 99 cohorts consisting of up to 351,158 individuals, which from here on will be referred to as the International Consortium of Resting Heart Rate (IC-RHR). Second, we performed a GWAS in 484,307 individuals from the UK Biobank. These large cohorts were meta-analyzed to include up to 835,464 individuals, in whom 30,458,884 directly genotyped and imputed autosomal variants were analyzed (**Supplementary Data 1**, **Figure 1A**). The meta-analysis revealed 493 independent genetic variants in 352 loci. Out of these 493 independent genetic variants, 68 were outside previously identified RHR associated loci and 67 of those were internally replicated (**Figure 2A, Supplementary Data 2**)[7,10,11]. Out of the 425 genetic variants inside previously identified RHR associated loci, a total of 376 were internally replicated. In addition, 332 out of the 332 loci were considered internally replicated as they showed concordant direction of effects and nominal associations (P<0.01) in the UK Biobank GWAS and IC-RHR meta-analysis (**Figure 2B**). The RHR associated genetic variants identified previous studies from Eppinga *et al.* and Den hoed *et al.* were all replicated in the current study (**Supplementary Data 3)**. A total of 74 loci identified in the study from Guo *et al.* were not replicated in the current study, of which 40 would not have been identified as locus using the current GWAS clumping criteria. The remaining 34 loci did not reach genome-wide significance in the current meta-analysis with generally high *P*-values in the IC-RHR consortium, probably therefore failing replication (**Supplementary Data 3)**. The linkage disequilibrium (LD) score regression intercept of the meta-analysis was 1.051 ± 0.002, suggesting little evidence of genomic inflation (**Figure 2C**). The QQ plots for the UK Biobank GWAS and IC-RHR meta-analysis are shown in **Supplementary Figure 1**. The genomic control lambda's, LD-Score intercepts and the attenuation ratio statistics suggested no inflation due to non-polygenic signals[12,13]. Single nucleotide polymorphism (SNP) heritability of RHR as calculated by LD score regression was estimated to be 10%. A polygenic score weighted by the effect sizes of the IC-RHR explained 5.33% of the variation in RHR in the UK Biobank. A Chow-test indicated absence of strong differences between participants with a history of any cardiovascular disease or use of RHR-altering medication versus participants without such a medical history (**Supplementary Data 4**). Genetic correlation analyses

were performed and we observed significant correlations with anthropometric measurements, pulse wave reflection index and physical activity measurements (**Supplementary Data 5**). A query of the GWAS Catalog showed that the 493 genetic variants associated with RHR were most commonly in high LD (LD>o.8) with anthropometric measurements and blood pressure traits (**Supplementary Data 6**).

Candidate Genes and Insights into Biology

We explored the potential biology of the 352 RHR loci by prioritizing candidate genes in these loci (**Supplementary Data 2**). A total of 407 unique genes were in close proximity to the lead variant, defined as the nearest gene and any additional gene within 10kb (**Supplementary Data 2**). There were 52 genes that contained coding genetic variants in LD (R^2>0.80) with RHR lead variants. Functional annotation of these coding variants is provided in **Supplementary Data 7**. Using summary data based Mendelian randomization analysis (SMR) and heterogeneity in dependent instruments (HEIDI) tests, we found that the RHR associated loci and eQTLs colocalized at 88 genes (**Supplementary Data 8**)[14]. Lastly, 381 unique genes were taken forward by Data-driven Expression-Prioritized Integration for Complex Traits (DEPICT) analyses (**Supplementary Data 9**). Of the 670 unique candidate causal genes identified, 33 genes were prioritized by at least three out of four established methods, which may be used to prioritize candidate genes (**Figure 2D**). Of these genes, *PHACTR4*, *ENO3* and *SENP2* were prioritized by all four methods. Full annotations of all identified genes are in **Supplementary Data 10**.

Pathway analyses and tissue enrichment

Pathway analysis performed by DEPICT showed that RHR revolved around mainly cardiac biology, including cardiac tissue development, muscle cell differentiation and pro-arrhythmogenic pathways. A total of 1,471 reconstituted gene sets within 155 gene clusters were significantly associated with RHR (FDR<0.05). The newly discovered gene clusters consisted of mostly protein-protein interaction pathways and were commonly located in the periphery of the network (**Supplementary Data 11, Supplementary Figure 2**). The tissue enrichment analysis by DEPICT showed 28 tissues at FDR < 0.05 and implicated the cardiovascular system as the most important tissue type, with 8 of the 10 most

significantly enriched tissues located within the cardiovascular system (**Figure 3, Supplementary Data 12**). Non-cardiovascular tissues with enrichment included muscle and fat tissues, the adrenal glands, the esophagus and urogenital structures. Conditional analyses showed that associations with non-cardiovascular tissues were rather due to co-expression of RHR genes in cardiovascular tissue than independent enrichment of RHR associated genes in non-cardiovascular tissues (**Figure 3**).

ECG morphology

The ECGenetics browser, which contains genome-wide summary statistics of every time-point of the complete cardiac cycle at a resolution of 500 Hz, was used to gain insights into the electrophysiological effect of the RHR genetic variants[15]. A total of 86 genetic variants were strongly associated with at least one ECG time point on the non-normalized and normalized association patterns across the full RR-interval at a stringent Bonferroni-corrected *P*-value$<1 \times 10^{-7}$. The associations represented a plethora of ECG morphologies (**Supplementary Data 13, Supplementary Figure 3**). The *ACHE*, *ANKRD1*, and *SCN5A* genes exhibited their largest electrical effects on atrial depolarization, *BAG3* and *TTN* on ventricular depolarization and *RGS6* and *SYT10* on ventricular repolarization. The ECG-wide MR highlighted several loci that had not been associated with resting heart rate or cardiac rhythm and structure previously. The *CCLN1* gene exhibited strong effects on atrial depolarization, *RAP1A* and *ZBTB38* exerted strong effects on early and late ventricular repolarization respectively. The ECG-wide MR showed that RHR variants exert the largest effect on ventricular repolarization on both the non-normalized and normalized association pattern (**Supplementary Figure 4**).

Single-nucleus RNA expression

Single-nucleus RNA sequencing data obtained from the healthy human heart revealed that RHR gene expression is highest in ventricular cardiomyocytes, followed by atrial cardiomyocytes (**Supplementary Data 14**)[16]. The candidate genes of genetic variants involved in non-isoelectric parts of the ECG showed stronger expression patterns than the isoelectric parts, for example those involved in left atrial depolarization (*ANKRD1*), ventricular depolarization (*FOHD3*, *RBM20*, *MYO18B*, *TTN*) and ventricular repolarization (*CACNA1C*) (**Supplementary Figure 3**).

Mendelian randomization analyses

A series of two-sample MR analyses was performed to test whether genetically predicted RHR is associated with all-cause mortality and cardiovascular diseases (definitions provided in **Supplementary Data 15-16**). We initially used the inverse variance weighted multiplicative random-effects (IVW-MRE) model, which provides a consistent estimate under the assumption of balanced pleiotropy. If we found evidence for a genetic association using the IVW-MRE model, we further interrogated this findings using several sensitivity analyses that are more robust to different sources of bias in MR analyses.

First, we assessed the association between genetically predicted RHR and all cause-mortality in the UK Biobank participants over a median follow-up of 8.9 years (interquartile range 8.2-9.5) (**Supplementary Data 17-19**). Genetically-predicted RHR was not associated with the risk of all-cause mortality (HR 1.024, 95% CI 0.993-1.057, *P*=0.13) as shown in **Figure 4**. We did not find evidence for an association between genetically predicted RHR and parental longevity. Neither did we find evidence for an association between RHR and the 35 leading causes of mortality in the UK Biobank. Systematic alteration of key differences between the current and previous Mendelian randomization study indicated that the most likely cause of the discrepancy between these studies arises from false positive findings in previous one-sample MR analyses caused by weak-instrument bias at lower *P*-value thresholds (**Supplementary Figure 5, Supplementary Data 20**)[7]. Non-linear MR analyses showed an always-increasing dose-response relation between genetically predicted RHR and all-cause mortality that was compatible with a null effect (**Figure 4, Supplementary Data 21-22**), providing no evidence for an U-shaped pattern that has been previously described[3].

We then explored the association between genetically predicted RHR and several prevalent cardiovascular diseases. We did not find evidence for an association between genetically predicted RHR and coronary artery disease in the UK Biobank (OR 0.977, 95% CI 0.946 – 1.009, *P*=0.16) or in the CARDIoGRAMplusC4D cohort (OR 0.976, 95% CI 0.944 – 1.010, *P*=0.17), in line with previous analyses[8,9]. Similarly, there was no evidence for an association between genetically predicted RHR and myocardial infarction in the UK Biobank or in the CARDIoGRAMplusC4D cohort (**Figure 5,**

Supplementary Data 23-26). We found no evidence for non-linear dose-response relations of genetically predicted RHR with coronary artery disease or myocardial infarction (**Supplementary Data 21-22**).

Higher genetically predicted RHR was suggestively associated with a lower risk of atrial fibrillation development in the UK Biobank (OR 0.946, 95% CI 0.897 – 0.998, *P*=0.04) and in the AFgen consortium (OR 0.942, 95% CI 0.897 – 0.989, *P*=0.02), but these results were not significant after correction for multiple testing ($P<4.17 \times 10^{-3}$). MR-Lasso, which can provide evidence for potential causal associations when there is a small number of genetic variants with heterogeneous ratio estimates, indicated that genetically predicted RHR was significantly inversely associated with atrial fibrillation (**Figure 5, Supplementary Data 23**). The contamination mixture model, which provides evidence for potential causal associations if the plurality of the genetic instruments is valid, provided evidence for a negative association between genetically predicted RHR and atrial fibrillation in the UK Biobank cohort, but this was not replicated in the AFgen consortium (**Figure 5**). Non-linear MR analyses showed a significant negative exponential growth pattern in the dose-response relation between genetically predicted RHR and atrial fibrillation (**Supplementary Data 21-22, Supplementary Figure 8**). Specifically, individuals at the extreme right tail of the distribution of genetically predicted RHR had a lower risk of atrial fibrillation. For example, compared with the population mean RHR of approximately 70 bpm, individuals with a genetically predicted RHR of 89 bpm and 98 bpm had a significantly lower risk of atrial fibrillation (OR 0.969, 95% CI 0.941 – 0.998, *P*=0.04; OR 0.922, 95% CI 0.897 – 0.948, $P=6.36 \times 10^{-9}$), while this was not true for a genetically predicted RHR of 80 bpm (OR 1.000, 95% CI 0.968 – 1.034, *P*=0.99).

We found that higher genetically predicted RHR is associated with risk of any stroke (OR 0.951, 95% CI 0.926 – 0.976, $P=1.59 \times 10^{-4}$), ischemic stroke (OR 0.940, 95% CI 0.915 – 0.967, $P=1.08 \times 10^{-5}$) and cardio-embolic stroke (OR 0.875, 95% CI 0.828 – 0.925, $P=2.11 \times 10^{-6}$), suggestively associated with large artery stroke (OR 0.939, 95% CI 0.884 – 0.998, *P*=0.04) and not with small vessel stroke (OR 1.001, 95% CI 0.950 – 1.055, *P*=0.97) in the MEGASTROKE consortium. The results were consistent across MR methods for any, ischemic and cardioembolic stroke (**Figure 5**, **Supplementary Data 23**). The

associations between genetically determined RHR and any stroke or ischemic stroke could not be replicated in the UK Biobank using an univariable MR IVW-MRE approach (OR 0.987, 95% CI 0.953 – 1.023, *P*=0.49; OR 0.970, 95% CI 0.928 – 1.015, *P*=0.19). We found no evidence for a non-linear association between genetically determined RHR and any or ischemic stroke (**Supplementary Data 21-22**). We used a multivariable MR approach to gain insights in potential mediating factors or pleiotropic pathways in the association between genetically predicted RHR and stroke. First, we found that the direct effects of RHR on cardio-embolic stroke to be attenuated by the effects of atrial fibrillation and estimate the attenuation through atrial fibrillation to be 18.4% (**Figure 6, Supplementary Data 27-28**). The direct effects of genetically predicted RHR on any stroke and ischemic stroke were most strongly attenuated by pulse pressure, with estimated attenuation of 28.1% and 31.5% respectively (**Figure 6, Supplementary Data 27-28**). There was a strong association between genetically predicted RHR and pulse pressure (β -0.192, SE 0.019, $P=1.81 \times 10^{-24}$), but MR-Steiger sensitivity analysis filtered a large part of the genetic variants and repeating the MR on the remaining subset did not show a significant association between RHR and pulse pressure (β -0.005, SE 0.008, *P*=0.57, **Supplementary Data 29-30**).

Lastly, we found that genetically predicted RHR is associated with an increased risk of dilated cardiomyopathy in the UK Biobank (OR 1.391, 95% CI 1.205 – 1.605, $P=6.27 \times 10^{-6}$). The results were robust to MR-Lasso (OR 1.411, 95% CI 1.228 – 1.622, $P=1.20 \times 10^{-6}$) and MR-contamination mixture models (OR 1.697, 95% CI 1.318 – 2.184, $P=4.03 \times 10^{-5}$). We excluded 96 variants associated with the Q-R upslope at −18 ms of the R peak (*P*<0.05), which has been established as a biomarker for dilated cardiomyopathy[15], to investigate whether reversed causation contributed to the association with dilated cardiomyopathy. The results were similar to the main analyses (**Supplementary Table 1**). We did not find evidence for an association between genetically predicted RHR and heart failure, heart failure excluding cardiomyopathies, and hypertrophic cardiomyopathy (**Figure 5, Supplementary Data 23**). We did not find evidence for a non-linear association between genetically determined RHR and any type of heart failure (**Supplementary Data 21-22**). Scatterplots and dose-response curves of the association between RHR and all assessed outcomes can be found in **Supplementary Figures 6-10**.

We assessed whether the Wald estimates between the RHR associated genetic variants and the cardiovascular diseases could identify risk loci not anticipated to be associated with these outcomes in the outcome GWASs. The locus *FOXC1* for coronary artery disease, *USP39* for myocardial infarction, and *SLC35F1* and *SSPN* for atrial fibrillation were significantly ($P<1.01 \times 10^{-4}$) and concordantly associated with their respective outcomes in both cohorts while not reaching genome-wide significance in either one of the outcome cohorts (**Supplementary Data 31**).

Discussion

We report 493 genetic variants in 352 loci associated with RHR, discovered in the largest GWAS meta-analysis of RHR to date in up to 835,465 individuals[7,8,17,18]. This increase of samplesize allowed us to report 68 novel RHR associated genetic variants and, importantly, provide internal replication for 376 genetic variants previously associated with RHR. A total of 670 candidate genes were prioritized, providing a comprehensive data catalog for future studies on RHR and offering potential new insights into its biology. Four strategies were employed to prioritize candidate genes and *PHACTR4*, *ENO3* and *SENP2* were highlighted by all four strategies. The *PHACTR4* gene regulates protein phosphatase 1 which interacts with actin and is involved in processes ranging from angiogenesis to cell cycle regulation[19]. It has been associated with pulse pressure and systolic blood pressure in a previous GWAS analysis[20]. *ENO3* encodes beta-enolase, which plays an important role in glycolysis and striated muscle development[21]. It has been implicated in cardiac myocyte development through its function in energy metabolism in both humans and rats[22,23]. *SENP2* encodes sentrin-specific protease 2, which deconjugates small ubiquitin-related modifiers 1 and 2 that are involved in regulating posttranslational modification of a wide variety of proteins that affect a multitude different cellular processes[24,25]. Several of these affected proteins are critical in cardiac development and mouse models have shown that alterations in *SENP2* activity lead to congenital heart defects[26,27]. Involvement of *SENP2* in a multitude of cellular processes is reflected by its implication in GWAS of various conditions, including systolic and diastolic blood pressure[28], type 2 diabetes[29], the conduction system of the heart[30,31] and estimated glomerular filtration rate[32]. The loci we associated with coronary artery disease (*FOXC1*), myocardial infarction (*USP39)* and atrial fibrillation (*SLC35F1* and *SSPN*) through their effects on RHR have been associated with these cardiovascular diseases in recent studies[33–36].

To obtain further biological insights into RHR, we performed pathway analyses using DEPICT and found numerous newly associated pathways. The strongest associated clusters were identified previously and their importance to RHR biology was therefore validated in the current study[7]. Conditional analyses on the tissue enrichment demonstrates that genes influencing RHR are more likely co-expressed than primarily or solely located within non-cardiovascular tissues. However, it

should be noted that conditional analyses inherently attenuate tissue-enrichment considering DEPICT is based on co-regulation of gene expression[37]. Using cardiac single-nucleus RNA data, we demonstrate that RHR genes are mostly expressed in cardiomyocytes. We provide electrophysiological insights in the biology of the RHR associated variants and show that they exert diverse effects on ECG morphology with the largest effect on ventricular repolarization.

In-depth analyses were performed to assess genetic associations of RHR with clinical outcomes. In contrast to previous observational[1,2] and MR studies[7], we do not find evidence for an association between genetically predicted RHR and all-cause mortality. Moreover, genetically predicted RHR was not associated with parental longevity nor with any of the 35 leading causes of mortality. Lack of such associations suggest that follow-up length or large heterogenetic effects of RHR on different causes of mortality are unlikely causes for the absence of an association between genetically predicted RHR and all-cause mortality[38]. We demonstrate that the most likely cause of the discrepancy between current and previous results arises from false positive findings in previous one-sample MR analyses that were caused by weak-instrument bias at lower *P*-value thresholds[39]. We hypothesize that RHR is not on the causal pathway to mortality itself and that previous observational studies are more likely to reflect confounders, such as stress and socio-economic status, or reversed causation, in which an individual's disease status increases both RHR and mortality risk[40,41].

The linear MR between RHR and atrial fibrillation provided suggestive evidence for an inverse relationship between RHR and atrial fibrillation, in line with a previous linear MR study[9]. We do find a significant negative exponential dose-response curve between RHR and atrial fibrillation in support of an inverse relationship, and take the non-linear MR forward as the main result considering the fractional polynomial test indicated that a non-linear model fitted the localized average causal effect estimates better than the linear model. Previous observational studies on the relationship between RHR and atrial fibrillation have shown conflicting results and have described various relationships including inverse linear[42–44], U-shaped[44] and J-shaped[45] associations. All these association patterns support the hypothesis that individuals with a low RHR might exhibit a higher risk of atrial fibrillation development compared to those with an average RHR. A recent stratified Mendelian randomization showed an inverse genetic relationship between RHR and atrial fibrillation in individuals with a RHR

below 65 bpm as well[46]. Possible mechanisms that could underly an increased risk of atrial fibrillation in individuals with a low RHR include increased left atrial stroke volume and consequent atrial remodeling due to myocyte stretching[47], or an increased vagal tone promoting global disorganization in the left atrium due to increased heterogeneity of the refractory period[48]. In contrast to the often hypothesized U-shaped or J-shaped association[44,45], we find a decreasing risk of atrial fibrillation development in those with a high RHR. One potential explanation is that previous observational studies were affected by collider bias through confounding factors which increase atrial fibrillation risk and typically occur in tandem with a high rather than a low RHR, such as hypertension[49] and obesity[50]. We advocate for cautious interpretation of current result due to the diverse biological mechanisms through which the RHR associated genetic variants alter the risk of atrial fibrillation development[51].

We found that genetically predicted RHR was inversely associated with risk of any, ischemic and cardio-embolic stroke. The results were not replicated in the UK Biobank, possibly due to the substantially lower amount of cases. The inverse association is in contrast to many observational studies and we therefore performed multivariable MR analyses to pinpoint biological mechanisms that could underly the discrepancy[4,5]. We showed that atrial fibrillation attenuates the protective effect of higher genetically predicted RHR on developing cardio-embolic stroke. This indicates either biological or mediated pleiotropic effects of atrial fibrillation in the association between genetically determined RHR and cardio-embolic stroke, which cannot be distinguished based on the current results. Correction for atrial fibrillation only minimally affected the association between RHR and any or ischemic stroke, despite cardio-embolic stroke accounting for a substantial amount of ischemic stroke cases[52]. Although hypertension is another important risk factor for stroke, it commonly occurs in tandem with a higher and not a lower RHR[4,53,54] and we found that neither systolic nor diastolic blood pressure to affect the association between RHR and stroke. We did find that pulse pressure attenuates the association between RHR and any, ischemic and large-artery stroke. Lower RHR has previously been demonstrated to increase pulse pressure due to a higher likelihood of pressure wave reflections during prolonged systole[55] and increased pulse pressure has been established as a risk factor of stroke[55–57]. Moreover, the Conduit Artery Functional Endpoint Study (CAFE) study postulated that pulse pressure underlies the inferiority

of β-blocker based treatment (which lowers RHR) to amlodipine based treatment in prevention of stroke despite equal effects on peripheral blood pressure[55,58]. Our results could be considered as support for this mechanism in the scenario that the RHR associated genetic variants only affect pulse pressure through RHR and the association with stroke is primarily driven by RHR. However, we consider this unlikely as the MR-Steiger sensitivity analysis indicated that the association between the RHR associated genetic variants and pulse pressure is unlikely mediated through RHR entirely. Biological pleiotropic effects are therefore more likely to cause the attenuation of the association between RHR and stroke when correcting for pulse pressure.

Finally, our study provides evidence that higher genetically predicted RHR increases risks of developing dilated cardiomyopathy. The importance of decreasing RHR in the treatment of heart failure with reduced ejection fraction, the clinical phenotype of dilated cardiomyopathy, has been thoroughly studied. Beta-blockers have been shown to reduce mortality in individuals with heart failure with reduced ejection fraction and form the corner stone of pharmacological treatment[59–61]. There is also evidence that ivabradine lowers cardiovascular mortality in heart failure with a reduced ejection fraction[62]. This protective effect is more likely due to its effect on RHR than heart rate variability as it has a larger effect on RHR[63]. The fact that the MR results were robust to exclusion of SNPs associated with the -18 ms point of the R-peak, an established biomarker of dilated cardiomyopathy, supports the interpretation that current findings are driven by RHR differences which mimic pharmacological rate control[15]. Our MR on the compound definition of heart failure could be hampered by its phenotypical heterogeneity, as we were unable to differentiate between heart failure with reduced and preserved ejection fraction. It would be interesting to repeat current Mendelian randomization analysis if more in-depth phenotyping on left ventricular ejection fraction and function becomes available, especially considering the different effects of RHR on familial dilated versus hypertrophic cardiomyopathy in the current study.

Several limitations should be considered. Although the current 493 RHR associated genetic variants explained more than double the RHR variance compared to the 64 loci from our previous study[7], there is still a large gap with heritability estimates from twin-studies that range between the 23% and 70%[64–

[66]. Future studies could include whole exome sequencing data to further increase our insights in the genetic architecture of RHR[67]. Second, individuals with cardiovascular diseases were included in the GWAS, which could potentially affect exposure-outcome associations. However, post-hoc analysis showed that UK Biobank participants with a history of cardiovascular disease or who used RHR-altering medication can be jointly analyzed with participants without such a medical profile. In addition, a two-sample MR strategy was adopted, reducing the risk that potential weak-instrument bias increases type 1 error rates through reintroduction of confounding, population stratification or correlated pleiotropy[39]. We note the broad biological nature of RHR genetic variants as illustrated by the diverse ECG patterns the genetic variants elicit on the full cardiac cycle. These broad effects should be taken into consideration for correct interpretation of the MR results, as pleiotropy and reversed causation might be introduced in the MR. For example, some genetic variants were included in the MR analyses which could be more specific for another trait (i.g. rs2234962 near *BAG3* for dilated cardiomyopathy). We believe that the influence of reversed causation on current results to be minimal, because we excluded variants more strongly associated with the outcome. The MR results were generally consistent across a multitude of sensitivity analyses, strengthening the interpretation of a true relationship. However, our study is not interventional in design and conservative interpretation of the results as generally unconfounded rather than causal estimates should be preferred. We stress that any causal claims can only be made if interventions or drugs alter RHR equal to the biological mechanisms in which RHR associated genetic variants affect RHR.

In conclusion, our GWAS meta-analysis discovered 493 RHR variants within 352 RHR loci, to which we prioritized 670 candidate causal genes. We demonstrated cardiovascular tissues as the primary enrichment sites for RHR gene effects and showed that their gene-expression is highest in cardiomyocytes. ECG signatures showed that RHR associated genetic variants exert the largest effect on RHR through ventricular repolarization. We found no evidence for linear and non-linear associations between genetically predicted RHR and all-cause mortality across several analyses, suggesting that the well-known link between higher RHR and all-cause mortality reflects confounding factors and reversed causation. The results point towards an inverse association between genetically predicted RHR and development of atrial fibrillation and any stroke, ischemic stroke and cardio-

embolic stroke, whereas it is positively associated with dilated cardiomyopathy development. Multivariable MR analysis showed that atrial fibrillation attenuates the protective effect of higher RHR on the development of cardio-embolic stroke. Pulse pressure attenuates the protective effects on any stroke, ischemic and large artery stroke, but this likely reflects biological pleiotropy rather than true mediation.

Methods

Method details

Populations

The full RHR meta-analysis included 100 studies with data on RHR in up to 835,465 individuals. RHR was obtained from ECG in 54 studies, from pulse rate in 31 studies (of which seven were self-measured by the participants), from blood pressure monitor in nine studies, from electronic medical records in three studies, from manual measurement in one study and through a combination of multiple of the before mentioned methods in two studies. Further information on cohort characteristics is provided in **Supplementary Data 1** and statistical details are provided in the "*Genome-wide association studies*" section.

Imputation and quality control

Genotyping and quality control before imputation were performed using different genome-wide genotyping arrays and methods, as further detailed in the **Supplementary Data 1**.

The UK Biobank was imputed to the Haplotype Reference v1.1 panel (HRC) by the Wellcome Trust Centre for Human Genetics. Analysis has been restricted to variants that are in the HRC v1.1. Quality control of samples and variants, and imputation was performed by the Wellcome Trust Centre for Human Genetics, as described in more detail elsewhere[68].

The 99 cohorts of the IC-RHR were imputed to 1000 Genomes Phase 1 and 3. For further information, please see **Supplementary Data 1**.

On cohort level, we performed quality control by: 1) re-formatting and SNP-name harmonization; 2) checking the used reference panel by plotting effect allele frequency plots using 1000G as a reference; 3) checking for genomic inflation by plotting QQ plots; 4) checking the betas by plotting histograms of the beta, frequency and info; 5) comparison of the expected *P*-value based on beta and standard error versus reported *P*-values.

Association with other traits

Genetic correlation analyses with GWAS of previously investigated traits were performed using LD Hub platform[69]. Genetic correlations were considered significant if they achieved a Bonferroni-

corrected significance threshold of $P<0.05/855=5.85 \times 10^{-5}$. The GWAS Catalog was queried to find previously established genetic variants ($P<1 \times 10^{-5}$) in LD ($R^2>0.8$) with all 493 RHR variants[70]. Summary statistics were downloaded from the NHGRI-EBI GWAS Catalog on 27/04/2020.

Functional annotation of genes

For all independent genetic variants that were genome-wide significantly associated in the final meta-analysis, candidate causal genes were prioritized as followed: 1) by proximity, the nearest gene and any other gene within 10 kb; 2) protein coding genes containing variants in LD with RHR associated variants at $R^2>0.8$; 3) eQTL genes in LD with RHR associated variants at $R^2>0.8$; and 4) DEPICT gene mapping using variants that achieved $P<1 \times 10^{-8}$ (further information described below). Annotation of all identified genes was performed by querying GeneALacart[71].

Query of dbNSFP

The dbNSFP database (version 3a) was queried to obtain functional prediction and annotation of all potential non-synonymous genetic variants[72]. The dbNSFP database contains information on multiple prediction algorithms and conservation scores further detailed elsewhere[72].

eQTL analyses

Colocalization of multiple expression quantitative trait loci (eQTL) was performed using SMR and HEIDI analyses (version 0.710)[14] in data repositories from GTEx V7[73], GTEx brain[73], Brain-eMeta eQTL[74] and blood eQTL from Westra[75] and CAGE[76]. Colocalization analyses were performed to test whether the effect size of the RHR associated variants on the phenotype are most likely mediated by gene expression[14]. eQTL genes were considered as a candidate causal gene if they achieved a significance after Bonferroni correction for the amount of eQTL's tested ($P<0.05/188{,}737=2.65 \times 10^{-7}$), passed the HEIDI test at $P>0.05$ and if the lead variants of the eQTL genes were in LD ($R^2 > 0.8$) with the RHR associated genetic variants.

DEPICT analyses

DEPICT was used to find genes associated with identified variants, enriched gene sets and tissues in which these genes are highly expressed. DEPICT.v1.beta version rel137 (obtained from

https://data.broadinstitute.org/mpg/depict/) was used to perform integrated gene function analyses as stated above[37]. DEPICT was run using all genetic variants that achieved $P<1 \times 10^{-8}$.

Pathway analyses

DEPICT was used to find enriched gene sets using the settings as described above[37]. Enriched genesets were further clustered on the basis of the correlation between scores for all genes using an Affinity Propagation method as provided by DEPICT[37]. Each cluster was named according to the name of the most central gene set as identified using the Affinity Propagation method. Identified meta-clusters were compared to the clusters found in the study of Eppinga *et al.* and were determined to be new if not a single cluster within the meta-cluster had been identified before. Clustering was performed using python 2.7[77] and visualization using Cytoscape 3.8.0[78].

Tissue enrichment

DEPICT was used to find enriched tissues using the settings as described above[37]. Enriched tissues were further investigated by performing conditional analyses to provide evidence for an independent association with RHR. The following formula was used:

$$\mathrm{T}cond = \frac{\mathrm{Z_t} - \rho_{ts}\,\mathrm{Z_s}}{\sqrt{1 - \rho_{\mathrm{ts}}{}^2}}$$

Here, Z_t is the maximum Z value of all tissue Z values, Z_s a vector of all tissue Z values and ρ_{ts} the correlation between the tissue of Z_t and the tissue of Z_s[79]. The maximum Z value (Z_t) was determined for every new iteration. Conditional analyses were performed up until the highest Z value reached ~2.58, which corresponds to the lowest Z value with an FDR < 0.05.

ECG morphology

The ECGenetics browser was used to gain insights in the electrophysiological effect of the RHR associated genetic variants[15]. Detailed information on the methodology can be found in the study of Verweij *et al.* and is briefly discussed below. The ECGenitics browser contains genome-wide summary statistics of the complete cardiac cycle. The complete cardiac cycle was defined using two methods, including a) the signal averaged electrocardiographic beat surrounding the R wave at a resolution of

500hz resulting in 500 averaged data points and b) R-R intervals corrected signal (made of equal length of 500 data points).

All RHR associated genetic variants were tested for their association with both the non-normalized and normalized association patterns. A heatmap was constructed containing all associated genetic variants associated with at least one point on the ECG at a Bonferonni-corrected *P*-value of $0.05/493/(500 \times 2)=1 \times 10^{-7}$. Effects were aligned to the most positively associated allele across all time points. The heatmap shows a hue ranging from red (positive effect) to a blue color (negative effect) color scale, with yellow indicating no effect. Secondly, the total effect of the 493 RHR associated genetic variants on ECG morphology was assessed using a ECG-wide MR approach (inverse variance weighted fixed-effects model) on the non-normalized and normalized association pattern.

Single-nucleus RNA expression

All genes prioritized in the current study were queried in the single cell data from the study of Tucker *et al.* through the Broad Institute's Single Cell Portal (available at: https://singlecell.broadinstitute.org/single_cell/study/SCP498/transcriptional-and-cellular-diversity-of-the-human-heart under study ID SCP49) to gain insights in their transcriptional and cellular diversity[16]. We selected the 86 genetic variants strongly associated with at least one ECG time point. We took forward the most likely candidate gene per genetic variant, based on the amount gene identification strategies by which the gene was identified. When a genetic variant highlighted multiple genes identified by the same amount of gene identification strategies, we took forward the gene with the highest biological plausibility of involvement in RHR biology. A dotted heatmap was constructed for this subset of genes.

UK Biobank definitions

In the UK Biobank, we captured prevalence and incidence of functional outcomes through data collected at the Assessment Centre in-patient Health Episode Statistics (HES), and data on cause of death from the National Health Service (NHS) Information Centre. Prevalent disease was also based on an interview with a trained nurse at the baseline visit (self-reported). HES data was available up to 31-03-2017 for English participants, 29-02-2016 for Welsh participants and 31-10-2016 for Scottish.

Information on cause of death was available for participants from England and Wales until 31-01-2018, and from the NHS Central Register Scotland for participants from Scotland until 30-11-2016. Definitions of all-cause mortality, 35 leading causes of mortality (defined as any cause of mortality with a prevalence > 0.1%), coronary artery disease, myocardial infarction, atrial fibrillation, stroke (any stroke, any ischemic stroke), heart failure and subtypes (hypertrophic cardiomyopathy, dilated cardiomyopathy) are provided in **Supplementary Data 15**. Longevity was obtained through questionnaires in which participants were asked to provide the age of death of both parents. Individuals were excluded in case the answer was older than 115 years, if they reported themselves as adopted, if their parent was still alive but not yet long-lived or their parent died prematurely (fathers <46 years or mothers <57 years), in line with previously established methods[38]. Combined parental longevity was assessed by summing Z scores of age of death from both parents if information on both parents was provided[38]. Systolic and diastolic blood pressure values were obtained through two automated and/or two manual blood pressure measurements. The average value of all available blood pressure measurements was used per phenotype. Automated measurements were corrected according to previously described methodology[80]. In addition, we corrected systolic and diastolic blood pressure for medication use, by adding respectively 15 and 10 mmHg to the blood pressure trait[81]. Pulse pressure was calculated by subtracting diastolic from systolic blood pressure.

Statistical details of the analyses on functional outcomes are provided in the "*Genetics and Regression Analyses on functional outcomes in the UK Biobank*" and "*Mendelian randomization*" sections.

External cohort definitions

External cohorts included the CARDIoGRAMplusC4D[82], AFGen[83], MEGASTROKE[52] and ICBP-plus[20] consortia and descriptions have been detailed previously. Effect sizes from the ICBP-plus consortium were obtained from the meta-analysis of the UK Biobank and ICBP-plus, after subtracting the effects from the UK Biobank (for further details, see "*Meta-subtract of blood pressure traits*"). An overview of these studies is provided in **Supplementary Data 16**. We searched for proxies (LD>0.8) in case variants could not be found within the outcome datasets.

Quantification and Statistical Analysis

Genome-wide association studies

All included cohorts performed genetic variant association analyses on RHR using linear regression analyses assuming an additive genetic model (**Supplementary Data 1**). No transformation of heart rate was performed and extreme (>4SD) phenotypic outliers were excluded analogous to previous GWAS on RHR and as per predefined criteria[7]. The GWAS model was adjusted for age, age^2, body mass index (BMI), sex and study specific covariates (e.g. principal components, genotyping array and RHR measuring method in case multiple RHR methods were used within a study).

The UK Biobank GWAS was performed using BOLT-LMM v2.3beta2, employing a mixed linear model that corrects for population structure and cryptic relatedness[84]. A total of 484,307 participants from the UK Biobank remained available for the GWAS after exclusion of 14,242 individuals for whom no genetic data was available, 1,341 individuals who failed genetic quality control, 1,058 individuals who were outside the 4 SD range for RHR and 1,587 individuals due to missing covariates (**Supplementary Data 1**).

Study-specific details and methodology of the 99 cohorts of the IC-RHR were are provided in **Supplementary Data 1**. A fixed effect meta-analysis using the inverse variance method in METAL was performed on all 99 cohorts, including up to a total of 351,158 individuals[85]. Genomic control was applied at study-level by correcting for the study-specific lambda.

All genetic variants were excluded if they had poor imputation quality score (Info<0.3) and effective sample size (N_{eff}) < 25 for the genetic variants computed as sample size x Info x 2 x minor allele frequency (MAF) x (1 - MAF). After these exclusions, a total of 19,400,415 and 27,082,649 variants remained available for the UK Biobank and IC-RHR GWAS respectively.

Again, a fixed effects meta-analysis using the inverse variance method in METAL was performed to pool the data from the UK Biobank and IC-RHR up to 835,465 participants using ~30 M genetic variants[85]. LD score regression software (v1.0.0) was used to calculate linkage disequilibrium score regression intercepts[12,13]. We corrected for genomic inflation prior to the meta-analysis by multiplying the standard-errors with the square root of linkage disequilibrium score regression intercepts in the UK Biobank (1.132 ± 0.017) and the IC-RHR (1.020 ± 0.010)[12,13].

PLINK (version 1.9) was used to prune genetic variants in a set of independently associated variants[86]. An independent genetic variant was defined as a genome-wide significant genetic variant in low LD (R^2<0.005) with another genome-wide significant variant within a five megabase window. A genetic locus was defined as the most significant variant in an one megabase region at either side of the independent genetic variant.

An one-stage replication analysis was performed next. The following criteria had to be satisfied for a signal to be reported as a replicated signal for RHR:

1. the sentinel genetic variant has $P<1 \times 10^{-8}$ in the discovery (UKB+ IC-RHR) meta-analysis;
2. the sentinel genetic variant shows support (P<0.01) in the UKB GWAS alone;
3. the sentinel genetic variant shows support (P<0.01) in the IC-RHR meta-analysis alone;
4. the sentinel genetic variant has concordant direction of effect between UKB and IC-RHR datasets;

The sentinel genetic variants were compared with previous loci from previous GWAS of RHR and were determined novel if located outside a 1 megabyte distance of previously RHR associated loci[7,10,11]. We selected the *P*-value thresholds to be an order of magnitude more stringent than a genome-wide significance *P*-value to ensure robust results and to minimize false positive findings.

Post-hoc quality control

We performed additional analyses to investigate whether individuals with a history of cardiovascular disease or those who took RHR-altering medication could influence the results of the GWAS. The UK Biobank population was stratified by a medical history of any cardiovascular disease or those who reported taking RHR altering medication. A history of any cardiovascular disease was defined according to the definition in **Supplementary Data 15**. RHR altering medication was defined as intake of beta-blockers, calcium antagonists, sotalol, amiodarone, flecainide, anti-depressants, atropine, other anti-cholinergic medication, cardiac glycosides, diuretics, ACE-inhibitors or angiotensin II receptor blockers, analogous to previous methods[87]. Linear regressions on RHR were performed in both populations, using cluster-robust standard errors with genetic family IDs as clusters to account for relatedness among participants. Exclusions and covariates were similar as to those used

for the GWAS. Individuals belonging together based on 3rd-degree or closer as indicated by the kinship matrix (kinship coefficient > 0.0442) provided by UK Biobank received a family ID. A Chow-test was used to investigate whether there were significant differences in beta estimates in participants with and without cardiovascular disease or RHR-altering medication[88]. The post-hoc quality control was performed using statistical software STATA 15 (StataCorp LP).

Genetics and regression analyses

All of the outcomes assessed in the UK Biobank that are reported in this manuscript have been adjusted for age, sex, the first 30 principal components (PCs) to account for population stratification, and genotyping array (Affymetrix UK Biobank Axiom® array or Affymetrix UK BiLEVE Axiom array). The exclusions were performed according to the above mentioned methods for the GWAS of RHR in the UK Biobank. In addition, we excluded 74,471 individuals based on familial relatedness, after which 412,481 individuals remained available for further analyses.

SNP-outcome associations for all outcomes (see section "*UK Biobank outcome definitions*") were obtained for all 493 variants with a $P<1 \times 10^{-8}$ in the RHR GWAS (see section "*Mendelian randomization*"). The associations with all-cause mortality and 35 leading causes of mortality within the UK Biobank (defined as a prevalence higher than 0.1%) were obtained using a Cox proportional hazard model during a median (interquartile range) follow-up of 8.9 (8.2-9.5) years. The association with parental longevity was assessed using linear regression analysis. The associations with both prevalent and incidence of cardiovascular diseases were assessed using logistic regression analyses. Cox and linear regressions were corrected for age at baseline, while the logistic regression analysis were corrected for age until the last date of follow-up to correctly account for both prevalent and incident disease.

We performed in depth assessment of the association between RHR and all-cause mortality in the UK Biobank by systematically altering the differences between the current study and the previous study from Eppinga *et al.*, which included a) the set of SNPs, b) the *P*-value threshold for SNP inclusion, c) the assessment of the outcome in an independent cohort and d) the follow-up length[7]. Genetic risk scores for RHR were created following an additive model by summing the number of alleles (0, 1 or 2)

for each individual after multiplication with the effect size for RHR. Genetic risk scores were constructed using the 493 discovered variants within the full meta-analyses using the effect sizes of the IC-RHR, the effect sizes of the UK Biobank and using the 73 previously discovered variants at five *P*-value thresholds (1×10^{-8}, 5×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5}). These were transformed to translate to a change of 5 bpm. The association with all-cause mortality was tested using Cox regression analyses in different populations of the UK Biobank. One population included all individuals (ncases = 396,183, ncontrols = 16,289). Another population included a subset of individuals which were genotyped for the UK Biobank interim release from May 2015, which included in the GWAS by Eppinga *et al*. (ncases = 113,102, ncontrols = 4,953). The final population consisted of a subset of individuals without genetic information at the time of the UK Biobank interim release, which was therefore not included in the GWAS by Eppinga *et al*., ncases = 28,3081, ncontrols = 11,336). Please note that sample sizes might slightly differ from those in the previous GWAS due to updated exclusions. Lastly, point d) was taken into account by re-performing above mentioned steps using mortality data up until the previously available follow-up (All individuals, ncases = 405,373, ncontrols = 7,099; individuals not included in the GWAS by Eppinga *et al*., ncases = 289,417, ncontrols = 5,000; and those included in the GWAS by Eppinga *et al*., ncases = 115,956, ncontrols = 2,099). All regression analyses were performed using statistical software STATA 15 (StataCorp LP).

Mendelian randomization analyses

All 493 independent genetic variants at $P<1 \times 10^{-8}$ in the final meta-analysis were taken forward in the MR. To minimize overlap between exposure and outcome cohorts, effect sizes were taken from the IC-RHR data to test the associations with outcomes within the UK Biobank, whereas effects sizes were taken from the UK Biobank to test the association within other independent cohorts. Proxies (LD>0.8) were searched in case genetic variants could not be found within the UK Biobank or IC-RHR. All effect sizes were transformed to translate to a change in RHR of 5 bpm.

Potential weak instrument bias was assessed by calculating the F statistic using the following equation:

$$F = \frac{R^2\ (n-2)}{1-R^2}$$

In this formula, *n* is the sample size of the exposure and R^2 is the amount of variance of the exposure explained by the SNP[89]. R^2 was calculated based on summary statistics using a previously established formula[90]. Genetic variants were not excluded from further analyses if the F-statistic was < 10 as this can exacerbate bias by increasing the chance of winner's curse[39]. Exposure and outcome summary statistics were then harmonized using the TwoSample MR package[91]. Forward strand alleles were inferred using allele frequency information and palindromic SNPs were removed if the MAF was above the recommended setting of 0.42[91].MR-Steiger filtering was applied to explore pleiotropic effects through assessment of potential reversed causation. R^2 for both the exposure and outcome were calculated and variants were removed from further analyses if the R^2 of the exposure is significantly lower (*P*-value < 0.05) than the R^2 of the tested outcome[92]. R^2 for linear traits was calculated as described above[90], R^2 for binary outcomes was calculated on the liability scale[93]. A true causal direction was assumed if the R^2 for binary outcomes was too small to be correctly estimated. Variants were excluded from further analyses in case a false causal direction was indicated.

The linear association between genetically determined RHR on all outcomes was initially assessed using the IVW multiplicative random-effects method, which provides a consistent estimate under the assumption of balanced pleiotropy. The Rücker framework was applied to assess heterogeneity and thus potential pleiotropy within the MR effect estimates[94]. A Cochran's Q *P*-value of <0.05 was considered as prove of heterogeneity within the IVW estimate and, as a consequence, balanced horizontal pleiotropy. An I^2 index > 25% supports this conclusion[95]. The MR-Egger test was performed to allow SNPs to exert unbalanced horizontal pleiotropy[96]. The Rücker framework assesses heterogeneity within the MR-Egger regression (Rucker's Q) and calculates the difference between heterogeneity within the IVW effect estimate (Q-Q')[94]. A significant Q-Q' (*P*<0.05) in combination with a significant non-zero intercept of the MR-Egger regression (*P*<0.05) was considered as indication for unbalanced horizontal pleiotropy. We then moved from an IVW-model to the MR-Egger model as initial analysis, as the MR-Egger can provide causal estimates if SNPs exert unbalanced horizontal pleiotropy under the assumption that Instrument Strength Independent of Direct Effect (InSIDE) assumption holds. Weak instrument bias in the MR-Egger regression analysis was assessed by I^2_{GX} and was considered to indicate low risk of measurement error if larger than 95%[97]. The MR-

Lasso method was used to find consistent estimates under the same assumptions as the IVW method, but only for the set of genetic variants not identified as outlier[98]. This method is most valuable in the scenario that a small proportion of the genetic variants is invalid and show heterogeneous ratio estimates[98]. The weighed median approach was used to provide a consistent estimate if up to half of the variants are invalid. Finally, we performed the MR contamination mixture method to provide a consistent estimate if no larger subset of invalid genetic variants estimate the same causal association than the subset of valid genetic variants[99].

The non-linear associations between genetically determined RHR, all-cause mortality and cardiovascular diseases were assessed using a fractional polynomial method[100,101]. This association was assessed using the UK Biobank as outcome cohort, considering this was the largest cohort with individual level data available to us. Consequently, we used the independent weights of the IC-RHR meta-analysis to construct a weighted polygenetic risk score of RHR by summing the number of alleles (0, 1 or 2) for each individual after multiplication with the effect size between the genetic variant and RHR. We first calculate residual RHR by subtracting the results of the regression of RHR to the polygenetic score of RHR from RHR itself. Covariates of the regression included age, age^2, sex, BMI, genotyping array and PC1-PC30, analogous the GWAS. Residual RHR, which characterizes the predicted RHR for an individual if their polygenetic score took the value zero, was then divided in 30 quantiles. Stratifying on residual RHR rather than total RHR avoids overadjustment and collider bias as residual RHR is not downstream of the effect of the genetic variants on the outcome in a causal diagram. We then calculated the genetic associations with the exposure in each stratum of residual RHR using linear regression analyses, correcting for the same covariates as described above. Two tests for non-linearity in the genetic association with the exposure (trend and Cochran's Q tests) were performed to investigate heterogeneity in the polygenetic score of RHR on residual RHR in different strata. We then calculated the genetic associations with the outcome in each stratum. The same methodology was used as described in "*Genetics and regression analyses*", including the same covariate model (age, sex, genotyping array and PC1-PC30) and regression type (Cox regression for all-cause mortality and logistic regression for cardiovascular diseases). The outcome regression coefficient was then divided by the exposure regression coefficient as a ratio of coefficients to obtain

local average causal effects (LACE) in each stratum. These localized average causal effect were meta-regressed against the mean of the exposure in each stratum in a flexible semiparametric framework, using the derivative of fractional polynomial models of degrees 1 and 2. All possible fractional polynomials of degree 1 and 2 were fitted using the powers -2, -1, 0, 0.5, 1, 2, and 3 as described previously[101]. The fractional polynomial of degree 1 is fit to the data if the fractional polynomial of degree 1 was as good of a fit (P>0.05) as the degree 2 as indicated by the likelihood ratio test. Three tests for non-linearity of the association between genetically predicted RHR and the outcomes are reported: a trend test, which assesses for a linear trend among the localized average causal effect estimates, a Cochran's Q test, and a fractional polynomial test, which assesses whether a non-linear model fits the localized average causal effect estimates better than a linear model. Please note that before fitting the fractional polynomials, we subtracted 45 from values of RHR as the most flexible fit is achieved when the exposure is close to 0 but still positive. A reference of RHR of 70 bpm was taken as this was close to the mean RHR of 69.3 bpm. An additional 1,390 individuals were dropped compared to the linear MR estimates obtained from the UK Biobank cohort due to missing BMI values necessary for the correction of the exposure regression coefficients.

A multivariable MR approach was used to gain additional insights in the relationship between RHR (effect sizes of the UK Biobank) and (subtypes of) stroke from the MEGASTROKE consortium. We used either atrial fibrillation (AFgen consortium[83]), systolic blood pressure, diastolic blood pressure or pulse pressure (ICBP consortium, please see "*Meta-substract of blood pressure traits*" for further details[20]) as secondary exposures to obtain insights in the direct effect of RHR on (subtypes of) stroke that are independent of these secondary exposures[102]. First, a multivariable MR-IVW method was used, in which for each exposure the instruments are selected and regressed together against the outcome, weighting for the inverse variance of the outcome[102]. Weak instrument bias for any of the exposures was assessed using Q_{x1} and Q_{x2}[102]. When both are larger than the critical value on the χ^2 distribution, there is little evidence of weak instrument bias. The critical value on the χ^2 distribution was calculated by subtracting one degree of freedom from the amount of SNPs at a P-value of 0.05. Q_a was considered to indicate potential pleiotropy when larger than the critical value on the $\chi 2$ distribution as calculated by the amount of SNPs minus two degrees of freedom at a P-value of 0.05[102].

Multivariable MR-Egger was performed to allow for unbalanced horizontal pleiotropy[103]. An MR-Egger intercept with a *P*-value < 0.05 in combination with a significant Q_a was considered proof of unbalanced horizontal pleiotropy and the MR-Egger regression to provide a robust causal estimate[103]. Multivariable MR-Lasso analysiswas performed as this method provides consistent estimates even when half of the genetic variants are invalid instruments and display unbalanced pleiotropy[104]. We also performed multivariable weighted median analysis as this type of analysis has been shown to perform well under higher levels of pleiotropy[104]. We did not search for proxies in the multivariable MR-setting as this could introduce uncertainty through different LD-patterns between the secondary exposure and outcome and we therefore re-estimate the effect of RHR on the outcome with the eligible SNPs to allow for a better comparison of the results.

We assessed whether the Wald estimates between the RHR associated genetic variants and the cardiovascular disease outcomes could identify risk loci not previously associated with these outcomes in their respective GWASs. The genetic variants were considered associated with the outcome if a) the Wald estimates had concordant effects within the UK Biobank as well as either the CARDIoGRAMplusC4D[82], AFGen[83] or MEGASTROKE[52] cohorts, b) when the Wald estimates were significant at a Bonferonni corrected threshold of $P<1.01 \times 10^{-4}$, that is, $\alpha=0.05$ with Bonferroni correction for a maximum of 493 independent tests, and c) the genetic variant did not reach a genome-wide significant threshold of *P*-value < 5×10^{-8} in either one of the outcome cohorts used in the current study.

MR analyses were performed using R (version 3.6.3), the TwoSampleMR package (version 0.5.3)[91] and the MR-Lasso source code[98]. The multivariable MR analyses were performed using the MVMR (version 0.3)[102] and MendelianRandomization (version0.5.1) packages[105]. Non-linear MR analyses were performed based on previously described methods[100]. For the MR on (subtypes of) mortality, we considered a liberal *P*-value of $P < 0.05$ significant for any of the outcomes using the IVW-MR random effects model. For the MR on cardiovascular diseases, we considered a Bonferroni corrected *P*-value for the amount of unique outcomes ($P = 0.05/12 = 4.17 \times 10^{-3}$) to be significant for the main IVW-MR random effects analyses, and a *P*-value between 4.17×10^{-3} and 0.05 to indicate suggestive evidence for an assocation. A *P*-value tresthold of $P < 0.05$ was adopted for the sensitivity analyses.

Meta-subtract of blood pressure traits

We used the MetaSubtract (version 1.60) package in R to remove the effects sizes of the UK Biobank from the largest blood pressure GWAS's to date in order to obtain the independent effect sizes of the ICBP consortium[20,106]. Effect sizes for the UK Biobank were obtained through linear regression analyses using every RHR SNP available in the UK Biobank as exposure, and systolic, diastolic and pulse pressure as outcomes. Covariates included age, age^2, sex, BMI, genchip and PC1-PC30. Cluster-robust standard errors with genetic family IDs as clusters were used to account for relatedness among participants. Individuals belonging together based on 3rd-degree or closer as indicated by the kinship matrix (kinship coefficient > 0.0442) provided by UK Biobank received a family ID. To keep the cohort similar to the one used in the study from Evangelou *et al.,* we excluded those who self-reported as of non-European ancestry (n=18,405) and pregnant women (n=306) from the 484,307 included in the GWAS, leaving 465,659 individuals for the analysis[20]. We note that we did not correct the standard errors for the genomic inflation reported for the GWASs of blood pressure traits in the UK Biobank as our linear regression estimates, while resembling the GWAS data, will not be exactly equal to BOLT-LMM estimates due to different methodologies[84].

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Competing interests

N.V. is currently employed at Regeneron plc. The 23andMe Research team members are current or former employees of 23andMe, Inc. and hold stock or stock options in 23andMe. P. Sever has received research awards from Pfizer Inc. I.N. is a now a full time employee at Gilead. B.M.P. serves on the DSMB of a clinical trial funded by Zoll LifeCor and on the Steering Committee of the Yale Open Data Access Project funded by Johnson & Johnson. K.S., H.H., D.F.G., D.O.A., G.S. are employees of deCODE genetics / Amgen Inc. W.M. is employed with Synlab Holding Deutschland GmbH. M.E.K. is employed with Synlab Holding Deutschland GmbH. The remaining authors have no disclosures.

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Data availability

Individual level data are available to researchers on successful application to the UK Biobank. Genome-wide summary statistics, excluding the 23andMe data, can be downloaded from <https://data.mendeley.com/datasets/9b725x7mvb/draft?a=f8619d91-5c4d-4e4f-8f44-a73692a332a5>. The top 10.000 genetic variants, including the 23andMe data, can be downloaded from the same repository. Any additional information required to reproduce this work is available from the Lead Contact upon reasonable request.

Code availability

Information on scripts and coding required to reproduce this work is available from the Lead Contact upon reasonable request.

Figures

Figure 1. Study Flowchart showing the study design, in silico annotations and function analyses.
A) Schematic overview of the study design for the discovery and replication of genetic loci associated with resting heart rate (RHR). The black bordered boxes show the methodology, the red bordered boxes show the most important results. B) Analyses performed to evaluate RHR variants and to gain further insights in the underlying biology. C) Schematic presentation of the MR analyses of RHR on mortality and cardiovascular diseases. Effect sizes were taken from the IC-RHR data to test the associations with mortality and cardiovascular diseases in the UK Biobank. Effect sizes were taken from the UK Biobank to test the association with coronary artery disease and myocardial infarction in the CARDIoGRAMplusC4D cohort, atrial fibrillation in the AFGen cohort and any, ischemic, cardio-embolic, large artery and small vessel stroke within the MEGASTROKE consortium. BMI = body mass index, GWAS = genome-wide association study, HRC = Haplotype Reference Panel, IC-RHR = International Consortium for Resting Heart Rate, MB = megabase, N = sample size, Neff = Effective sample size, PC = principal components, RHR = resting heart rate, SNPs = single nucleotide polymorphisms, QC = quality control, 1000G = 1000 Genomes.

Figure 2. Overview of the findings in the genome-wide association study and *in silico* search of candidate causal genes.
A) Manhattan plot showing the -$\log_{10}$(*P*-value) for the association of all genotyped or imputed genetics variants with resting heart rate (RHR). Red indicates novel and internally replicated RHR associated loci and black indicates novel but unreplicated RHR associated loci. Dark grey indicates RHR associated genetic variants within 1 MB of previously identified RHR associated loci, which were internally replicated in the current study. Light grey indicates RHR associated genetic variants within 1 MB of previously identified RHR associated loci, which were not internally replicated in the current study. B) Venn diagram of the 352 identified loci. Of the 352 loci, 332 were internally replicated. C) Quantile-quantile (QQ) plot of the final meta-analysis. The black dots represent the observed statistic for the genotyped genetic variants against the corresponding expected statistic. The linkage disequilibrium score regression intercept after the final meta-analysis was 1.051, suggesting little evidence of genomic inflation due to non-polygenic signal. D) Venn diagram of the prioritization of the 670 unique candidate causal genes as identified by one or multiple strategies. Venn plot shows overlap of genes tagged by one or multiple strategies, including 1) by proximity, the nearest gene or any gene within 10 kb; 2) genes containing coding variants in LD with RHR associated variants at R^2>0.8; 3) eQTL genes in LD (R^2 >0.8) with RHR associated variants; and 4) DEPICT gene mapping using variants that achieved $P<1 \times 10^{-8}$. DEPICT = Data-driven Expression Prioritized Integration for Complex Traits, eQTL = expression quantitative trait loci.

Figure 3. Conditional analyses of tissue enrichment by DEPICT emphasizes cardiac tissue for RHR biology.
A) Shows the results of the depict tissue enrichment analysis. The Y-axis shows the tissues clustered by first MeSH term, ordered on Z value per cluster. The X-axis shows the Z-value. An FDR <0.05, corresponding to a *P*-value < 9.75×10^{-3} and Z-value of 2.585 was considered to be statistically significant. Significant tissues are plotted in red and annotated, other tissues are plotted in grey. Conditional analyses were performed by correcting for the tissue with the highest Z value to investigate whether significant tissues were independently associated with RHR. Not a single tissue remained significant at a FDR < 0.05 after three consecutive corrections (for heart, heart valve and arteries). Panel B), C) and D) show Z values of all tissues after consecutive correction for respectively heart and heart valves, heart and arteries and heart valve and arteries and jointly provide information on which the other tissues co-dependent.

Figure 4. Mendelian randomization shows absence of linear and non-linear associations between genetically predicted RHR and all-cause mortality.
Linear and non-linear Mendelian randomization analyses were performed to test the association between genetically predicted RHR and all-cause mortality. Panel A) shows a forestplot of the linear

MR analyses between genetically predicted RHR and all-cause mortality. Hazard ratios and 95% confidence intervals are shown. Panel B) shows the dose-response curve of the non-linear MR analyses between genetically predicted RHR and all-cause mortality. The comparisons are conducted within strata and therefore the graph provides information on the expected average change in the outcome if a person with a RHR of (say) 70 bpm instead had a RHR value of 90 bpm. The gradient at each point of the curve is the localized average causal effect. Shaded areas represent 95% confidence intervals. RHR = resting heart rate; HR = hazard ratio; CI = Confidence interval; MR = Mendelian randomization; IVW = inverse variance weighted; FE = Fixed effects; MRE = multiplicative random effects.

Figure 5. Mendelian randomization of genetically predicted RHR on cardiovascular diseases.
Forestplots of the linear Mendelian randomization analyses of resting heart rate (RHR) on cardiovascular diseases. Effect sizes were taken from the IC-RHR data to test the associations with mortality and cardiovascular diseases in the UK Biobank (panel A). Effect sizes were taken from the UK Biobank to test the association with cardiovascular diseases in in the CARDIoGRAMplusC4D, AFGen and MEGASTROKE consortia (panel B). Results of the MR-IVW, outlier-robust MR-Lasso and plurality valid MR-Mix are provided. Odds ratios and 95% confidence intervals are shown. RHR = resting heart rate; MR = Mendelian randomization; IVW = inverse variance weighted multiplicative random effects; OR = odds ratio; CI = Confidence interval.

Figure 6. Multivariable Mendelian randomization reveals pulse pressure and atrial fibrillation as potential mediators of the association of genetically predicted RHR with ischemic and cardio-embolic stroke, respectively.
Forestplots of the results of the two-sample multivariable Mendelian randomization analyses of resting heart rate on any, ischemic and cardio-embolic stroke, when using atrial fibrillation, systolic, diastolic and pulse pressure as secondary exposures. Shown in red are the univariable Mendelian randomization estimates which represent the total estimates of resting heart rate on the outcome. In black are the multivariable Mendelian randomization estimates, which show the direct effect of RHR when corrected for the secondary exposure. These results indicate that atrial fibrillation attenuates the beneficial effect of higher resting heart rate on cardio-embolic stroke, while pulse pressure attenuates the beneficial effect on any and ischemic stroke. MR-Steiger sensitivity analysis indicated that the association between the RHR associated genetic variants and pulse pressure is unlikely mediated through RHR entirely and biological pleiotropic effects are therefore more likely to cause the attenuation of the association between RHR and stroke when correcting for pulse pressure. Odds ratios and 95% confidence intervals are shown. RHR = resting heart rate; MV = multivariable, Nsnp = number of SNPs.

Supplementary Information

Supplementary Figures

Supplementary Figure 1: quantile–quantile (QQ) plot for the GWAS of RHR in A) the UK Biobank and B) the IC-RHR.

Supplementary Figure 2: Network plot of DEPICT gene set enrichment analyses way.

Supplementary Figure 3: ECG-wide heatmap and single cell gene expression dotplot of RHR SNPs.

Supplementary Figure 4: ECG-wide Mendelian randomization analyses of RHR SNPs.

Supplementary Figure 5: Forestplot of the results of the association between the genetic risk score of RHR and all-cause mortality across different sets of SNPs, effect sizes, *P* value thresholds, populations and follow-up lengths.

Supplementary Figure 6: Scatterplots of the Mendelian randomization analyses between genetically predicted RHR and mortality and longevity within the UK Biobank.

Supplementary Figure 7: Scatterplots of the Mendelian randomization analyses between genetically predicted RHR and cardiovascular diseases within the UK Biobank.

Supplementary Figure 8: Dose-response curve of the non-linear Mendelian randomization analyses between genetically predicted RHR and cardiovascular diseases within the UK Biobank.

Supplementary Figure 9: Scatterplots of the Mendelian randomization analyses between genetically predicted RHR and cardiovascular diseases within the CARDIoGRAMplusC4D, AFGen or MEGASTROKE cohorts.

Supplementary Figure 10: Scatterplots of the Mendelian randomization analyses between genetically predicted RHR and blood pressure phenotypes within the ICBP consortium

Supplementary Tables

Supplementary Table 1: Sensitivity analysis for the two-sample Mendelian randomization analysis between RHR and dilated cardiomyopathy.

Supplementary Data

Supplementary Data 1: Study characteristics of the cohorts.

Supplementary Data 2: 493 Genome-wide significant RHR SNPs.

Supplementary Data 3: Comparison of previously RHR associated loci and/or genetic variants identified in the studies from Guo *et al*., Eppinga *et al.*, and Den hoed *et al*., with the genetic variants associated with RHR in the current study.

Supplementary Data 4: Chow-test for all genome-wide significant RHR SNPs to assess differences of effect estimates between participants taking RHR-altering medication or with a history of any cardiovascular disease versus those who did not.

Supplementary Data 5: Genetic correlation between RHR and previously performed GWAS's.

Supplementary Data 6: List of RHR variants associated with previously discovered variants.

Supplementary Data 7: List of coding variants.

Supplementary Data 8: List of functional eQTL genes.

Supplementary Data 9: List of DEPICT genes.

Supplementary Data 10: List of gene annotations for all identified genes.

Supplementary Data 11: Results of gene set enrichment analyses by DEPICT.

Supplementary Data 12: Results of tissue enrichment analysis by DEPICT.

Supplementary Data 13: Effect of RHR SNPs on the ECG.

Supplementary Data 14: Mean scaled expression per gene and tissue from the Single-nucleus RNA sequencing data obtained from the healthy human heart.

Supplementary Data 15: Definitions of mortality and cardiovascular disease phenotypes in the UK Biobank.

Supplementary Data 16: Definitions of cardiovascular disease phenotypes in the CARDIoGRAMplusC4D, AFGen and MEGASTROKE consortia.

Supplementary Data 17: Results of the two-sample Mendelian randomization analyses of RHR on mortality within the UK Biobank.

Supplementary Data 18: Additional sensitivity analyses of the two-sample Mendelian randomization analyses of RHR on mortality within the UK Biobank.

Supplementary Data 19: Single SNP exposure, outcome and exposure-outcome associations between RHR and mortality.

Supplementary Data 20: Association between genetic risk scores of RHR and all-cause mortality across different sets of SNPs, effect sizes, *P* value thresholds, populations and follow-up lengths.

Supplementary Data 21: Results of the non-linear Mendelian randomization estimates between genetically predicted RHR and all-cause mortality and cardiovascular diseases in the UK Biobank.

Supplementary Data 22: Localized average causal effects on all-cause mortality and cardiovascular diseases in the UK Biobank for 30 quantiles of RHR.

Supplementary Data 23: Results of the Mendelian randomization between RHR and cardiovascular diseases.

Supplementary Data 24: Additional sensitivity analyses of the two-sample Mendelian randomization analyses of RHR and cardiovascular diseases.

Supplementary Data 25: Single SNP exposure, outcome and exposure-outcome associations between RHR (effect sizes IC-RHR) and cardiovascular disease (UK Biobank).

Supplementary Data 26: Single SNP exposure, outcome and exposure-outcome associations between RHR (effect sizes UK Biobank) and cardiovascular diseases (CARDIoGRAMplusC4D, AFGen and MEGASTROKE consortia).

Supplementary Data 27: Results of the two-sample multivariable Mendelian randomization analyses between resting heart rate, atrial fibrillation, blood pressure traits and stroke.

Supplementary Data 29: Sensitivity analyses in the two-sample multivariable MR between resting heart rate, atrial fibrillation, blood pressure traits and stroke.

Supplementary Data 29: Results of the Mendelian randomization between RHR and blood pressure phenotypes within the ICBP consortium.

Supplementary Data 30: Additional sensitivity analyses of the Two-sample Mendelian randomization analyses of RHR and blood pressure phenotypes within the ICBP consortium.

Supplementary Data 31: List of Wald estimates with significant ($P < 1.01 \times 10^{-4}$) associations with the cardiovascular outcomes.