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Epistatic interaction between haplotypes of the ghrelin ligand and receptor genes influence susceptibility to myocardial infarction and coronary artery disease

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Data from both experimental models and humans provide evidence that ghrelin and its receptor, the growth hormone secretagogue receptor (ghrelin receptor, GHSR), possess a variety of cardiovascular effects. Thus, we hypothesized that genetic variants within the ghrelin system (ligand ghrelin and its receptor GHSR) are associated with susceptibility to myocardial infarction (MI) and coronary artery disease (CAD). Seven single nucleotide polymorphisms (SNPs) covering the *GHSR* region as well as eight SNPs across the ghrelin gene (*GHRL*) region were genotyped in index MI patients (864 Caucasians, 'index MI cases') from the German MI family study and in matched controls without evidence of CAD (864 Caucasians, 'controls', MONICA Augsburg). In addition, siblings of these MI patients with documented severe CAD (826 'affected sibs') were matched likewise with controls ($n = 826$ Caucasian 'controls') and used for verification. The effect of interactions between genetic variants of both genes of the ghrelin system was explored by conditional classification tree models. We found association of several *GHSR* SNPs with MI [best SNP odds ratio (OR) 1.7 (1.2–2.5); $P = 0.002$] using a recessive model. Moreover, we identified a common *GHSR* haplotype which significantly increases the risk for MI [multivariate adjusted OR for homozygous carriers 1.6 (1.1–2.5) and CAD OR 1.6 (1.1–2.5)]. In contrast, no relationship between genetic variants and the disease could be revealed for *GHRL*. However, the increase in MI/CAD frequency related to the susceptible *GHSR* haplotype was abolished when it coincided with a common *GHRL* haplotype. Multivariate adjustments as well as permutation-based methods conveyed the same results. These data are the first to demonstrate an association of SNPs and haplotypes within important genes of the ghrelin system and the susceptibility to MI, whereas association with MI/CAD could be identified for genetic variants across *GHSR*, no relationship could be revealed for *GHRL* itself. However, we found an effect of *GHRL* dependent upon the presence of a common, MI and CAD susceptible haplotype of *GHSR*. Thus, our data suggest that specific haplotypes of the ghrelin ligand and its receptor act epistatically to affect susceptibility or tolerance to MI and/or CAD.

INTRODUCTION

Coronary artery disease (CAD) and its most adverse complication, acute myocardial infarction (AMI), are the leading causes of morbidity and mortality in western societies with an increasing prevalence due to the aging of the population and the obesity epidemic (1).

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The etiology of these multifactorial diseases is complex, determined by the interplay of genetic and environmental factors (2). Indeed, family history of cardiovascular disease is a risk factor independent of other traditional risk factors and is largely driven by genetic variation (3,4). Genetic predisposition for MI and CAD probably results from additive or synergistic effects of many genes, which have only a small role by themselves. Moreover, epistatic gene interactions are believed to be a major factor in the genetic architecture of complex disease and molecular geneticists have begun to identify genetic pathways involved in the expression of phenotypic features of complex diseases (5,6).

Dissecting the genetic architecture of the biggest causes of morbidity and mortality in the western world is of critical importance in developing better therapeutics to reduce adverse clinical outcomes and in their ultimate prevention. Towards this goal, several large-scale genome-wide scans using hundreds of families or affected sib pairs have been performed and chromosomal regions linked to CAD or MI traits have been revealed (7). Case–control association studies have led to the identification of genes encoding cytokine precursors, factors influencing vessel wall integrity and lipoproteins that have been associated with MI or CAD, but most of them have not been replicated or confer only modest risk increase. In essence, the genetic basis of the disease is not fully understood (8) suggesting that variations in genes from alternative biological pathways presumably also contribute to the overall MI/CAD susceptibility. Given the marked risk increase for MI and CAD by the presence of obesity and the metabolic syndrome, neuroendocrine and metabolic candidate pathways might be involved in the pathogenesis of MI/CAD.

Recently, a meta-analysis revealed a quantitative trait locus (QTL) on chromosome 3q26 that has been linked to CAD (9). Interestingly, a significant linkage signal for metabolic syndrome-associated phenotypes has been found independently in the same region (10–14). This QTL harbors the growth hormone secretagogue or ghrelin receptor gene, *GHSR*, representing the target of the endogenous ligand ghrelin, a gut–brain regulatory orexigenic hormone, which is secreted from the stomach. GHSR along with ghrelin provide the only hormonal appetite-stimulatory input in regulating food intake and energy homeostasis, thereby playing a critical role in the pathogenesis of obesity and related metabolic traits (15–21). In addition, both components of this 'ghrelin system', ligand ghrelin and its receptor GHSR, have been reported to exercise additional central and peripheral biological functions such as growth hormone secretion, exocrine functions, cell proliferation and cardiovascular actions (22–24). Accordingly, GHSR and ghrelin are widely distributed in central and peripheral tissues, including hypothalamus, pituitary and peripheral tissues, and most importantly for our study, in cardiovascular tissues (22,25–28). Moreover, the density of ghrelin receptors is up-regulated in atherosclerotic coronary arteries (29). These findings suggest that ghrelin could have direct effects on cardiovascular regulation, cardiac structure and function as well as atherosclerotic lesions, mediated by GHSR. In fact, recent studies in both experimental models and humans provide evidence that the receptor GHSR and its ligand ghrelin, play a crucial role in the tolerance or susceptibility of ischemic injury, possessing a variety of cardioprotective effects against myocardial ischemia, vasoactive and cardiotropic actions (25,30–41).

In this context, we hypothesized that genetic variants within the ghrelin system (preproghrelin gene, *GHRL*, plus its receptor gene, *GHSR*) are associated with susceptibility to MI and CAD and carried out a comprehensive genetic association analysis to address this question.

We systematically explored the linkage disequilibrium (LD) and haplotype structures of the *GHSR* and *GHRL* genomic regions with single-nucleotide polymorphisms (SNPs) and assessed the role of common sequence variants and haplotypes in MI/CAD. In addition, we describe epistatic interactions between both genes playing a role in the same biological pathway and illustrate how the identified genetic predictors interact. Since recent data suggest that ascertainment of familial cases are more advantageous than are randomly selected cases (42–44), we compared siblings with a strong family history for premature MI with randomly selected controls from the general population in the present study.

RESULTS

Anthropometric and clinical characteristics of study subjects

The clinical characteristics of the study populations are described in Table 1. As expected, cardiovascular risk factors were more prevalent in both case samples [index MI cases from the German MI family study (cases Study A), single affected CAD siblings of index patients (cases Study B)] when compared with control subjects [age- and sex-matched healthy individuals from the WHO MONICA general population (controls Study A), MONICA controls plus healthy unrelated spouses, brothers- and sisters in-law from the MI family study (controls Study B)], despite matching for age and gender. Risk factor distribution was similar in index MI cases and affected siblings. However, affected CAD siblings were older and more often male.

Hardy–Weinberg equilibrium and LD mapping

In MI cases, affected siblings and controls, none of the genotyped *GHSR* and *GHRL* SNPs deviated from Hardy–Weinberg proportions.

The LD block structures defined by the seven SNPs within the *GHSR* region and the eight SNPs within the *GHRL* region are depicted in Figure 1. Strong LD was detected between five SNPs (rs509035, rs572169, rs519384, rs512692, rs863441) across *GHSR* spanning a 11.6 kb region and encompassing most of the intron, exon 1 and the 5′ adjacent region of *GHSR*. Less LD with recombination patterns was observed within *GHRL*, dividing this gene region into two correlated two-marker LD-blocks of 1.5 kb and 2.0 kb size.

Association of *GHSR* and *GHRL* SNPs with MI and CAD

In a case–control association study, we tested each of the seven *GHSR* SNPs and each of the eight *GHRL* SNPs individually for association with the disease. Table 2 summarizes

Table 1. Anthropometric and clinical characteristics of the study populations

	Study A		Study B	
	Index MI cases ($N = 864$)	Control group A ($N = 864$)	Affected CAD siblings ($N = 826$)	Control group B ($N = 826$)
Age (of onset, years)[a,b]	49.4 ± 0.4*	54.9 ± 0.4	54.2 ± 0.3*	58.3 ± 0.3
Gender (%) male[a]	71.4	71.4	86.2	86.2
Obesity (%)	23.8	20.5	19.7	18.8
Hypertension (%)	59.7*	47.9	59.2*	50.9
Hypercholesterolemia (%)	83.2*	67.4	82.6*	65.4
Diabetes (%)	23.8*	4.9	22.9*	7.9
Former and current smoking (%)	70.6*	34.1	73.6*	45.4

[a]Matched for age and gender.
[b]Age of onset (cases) or age at inclusion (controls).
*$P < 0.01$ versus controls.

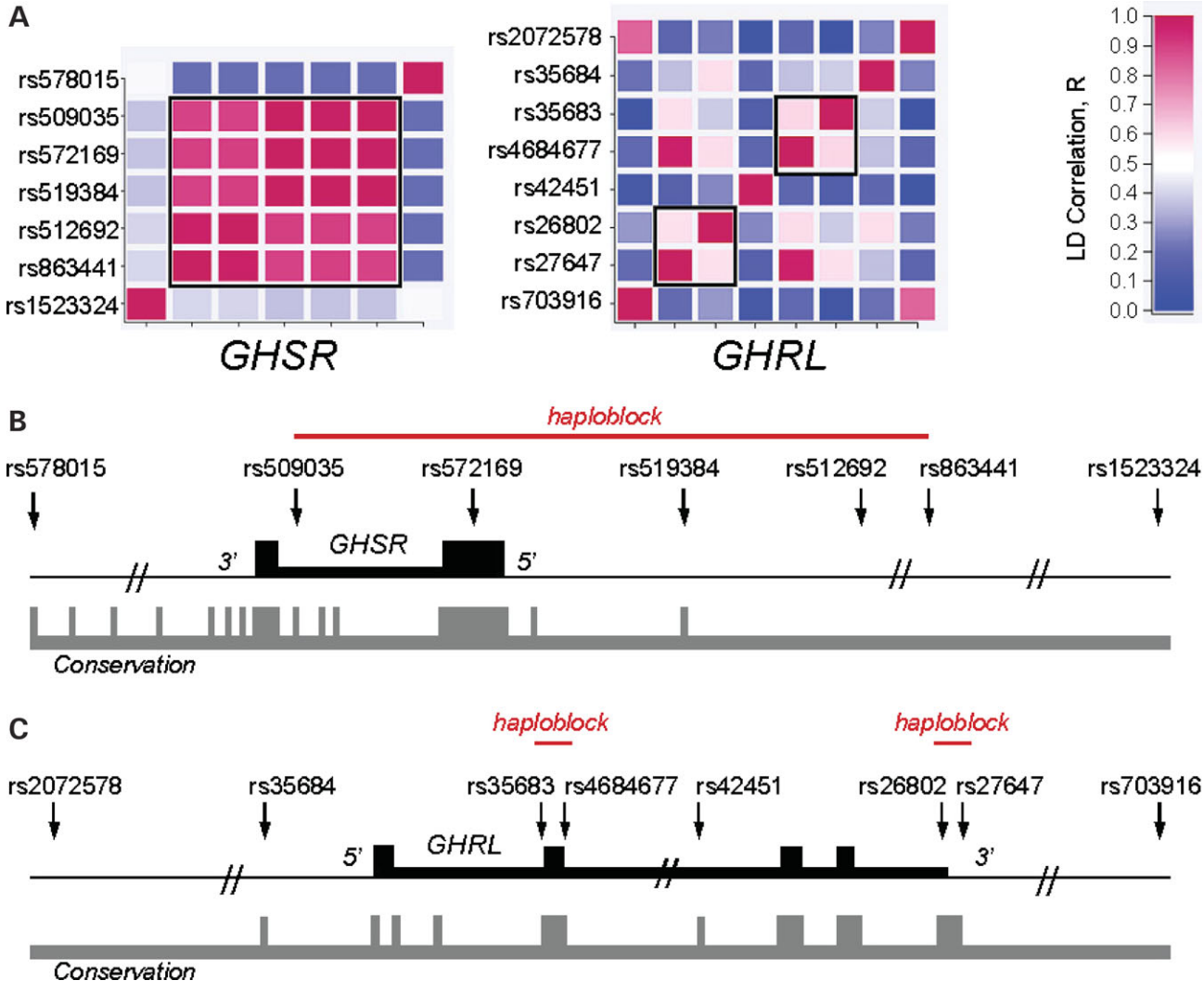

Figure 1. Genomic structures of the *GHSR* and *GHRL* genes. (**A**) Pair-wise LD analysis of seven SNPs across the *GHSR* and eight SNPs across the *GHRL* gene. Using the standard LD measure r^2, a five-marker haploblock within the *GHSR* and two two-marker LD-blocks within the *GHRL* gene were identified (LD blocks are inframed). A scale for r^2 of the LD strength is provided on the right. (**B**) Schematic representation of physical locations of SNPs and haplotypes across *GHSR* with corresponding evolutionary conservation track. The SNPs rs 509035, rs 572169 and rs519385 are located within highly conserved regions. (**C**) Physical locations of SNPs and haplotypes across *GHRL*. The SNPs rs 35684, rs 35683, rs42451, rs26802 and rs 27647 are located within highly conserved regions.

Table 2. Association of *GHSR* SNPs in MI index patients versus controls (Study A) and in CAD siblings versus controls (Study B)

SNP[a]	Genotype													
	Cases (HIFAM)				Controls (S3)				Allele 2 versus Allele 1		Genotype 22 versus 11		Genotype 22 versus 11 + 12	
	11	12	22	MAF[b]	11	12	22	MAF[b]	OR (95% CI)[c]	*P*	OR (95% CI)[c]	*P*	OR (95% CI)[c]	*P*
rs509035														
Study A	378	372	109	0.34	352	357	66	0.32	1.11 (0.96–1.29)	0.148	0.92 (0.75–1.13)	0.446	1.60 (0.16–2.22)	**0.004**
Study B	346	383	90	0.34	361	352	63	0.31	1.18 (1.01–1.37)	**0.032**	1.49 (1.05–2.12)	**0.027**	1.40 (1.00–1.97)	0.053
rs572169														
Study A	378	364	107	0.34	379	385	70	0.31	1.12 (0.97–1.30)	0.113	1.53 (1.10–2.14)	**0.012**	1.57 (0.15–2.17	**0.005**
Study B	350	382	87	0.34	382	358	66	0.30	1.18 (1.02–1.36)	**0.030**	1.44 (1.01–2.04)	**0.042**	1.33 (0.95–1.87)	0.094
rs519384														
Study A	411	332	88	0.31	422	368	57	0.28	1.11 (0.95–1.28)	0.180	1.59 (1.11–2.27)	**0.012**	1.64 (1.16–2.23)	**0.005**
Study B	388	356	76	0.31	422	336	53	0.28	1.20 (1.03–1.39)	**0.019**	1.56 (1.07–2.27)	**0.020**	1.46 (1.02–2.11)	**0.042**
rs512692														
Study A	420	344	94	0.31	406	359	54	0.29	1.13 (0.97–1.31)	0.115	1.68 (1.17–2.41)	**0.004**	1.74 (1.23–2.49)	**0.002**
Study B	389	356	76	0.31	412	325	52	0.27	1.20 (1.03–1.40)	**0.019**	1.55 (1.06–2.26)	**0.023**	1.45 (1.00–2.10)	**0.049**
rs863441														
Study A	420	345	90	0.31	411	360	56	0.29	1.11 (0.96–1.29)	0.170	1.57 (1.10–2.26)	**0.013**	1.62 (1.15–2.31)	**0.007**
Study B	387	355	76	0.31	413	332	54	0.28	1.18 (1.02–1.38)	**0.031**	1.50 (1.03–2.19)	**0.033**	1.41 (0.98–2.04)	0.062

Significant *P*-values are depicted in bold.
[a]SNPs are shown as 'rs' numbers from the dbSNP database.
[b]Minor allele frequency.
[c]ORs reported with their 95% CI and are based on the Armitage test for trend.

the association results between the five individual SNPs contributing to the *GHSR* LD block in index MI cases and controls (Study A) and in affected CAD siblings of the second control set (Study B). There was a significant relationship between the SNPs within the *GHSR* haplotype block and MI (Study A) when applying a homozygous trait comparison and when applying a recessive genetic model. In the latter, crude odds ratios (OR) for MI ranged between 1.57 and 1.74 for homozygous carriers of the minor alleles. Association could be confirmed between the same markers and the trait 'CAD' in a second case–control study of comparable size (Study B). To ensure that the SNPs outside the high LD block were not associated with MI/CAD, we performed a separate association analysis for these SNPs. None of the SNPs showed any evidence for association with the MI/CAD affection status (data not shown). Contrary to the GHSR results, no significant relationship could be found for SNPs within the *GHRL* region and MI or CAD when applying the same models (Table 3).

Haplotype association with MI and CAD

In addition to the SNP analysis, we searched for haplotypes in both gene regions associated with the disease using the same cohorts in order to assess LD structure and haplotype association. Two common haplotypes spanning the five-marker LD block across the *GHSR* region were significantly associated with the disease. Specifically, we observed an association between a common (frequency 66.1%) protective haplotype ('11111'), comprising the five major alleles of individual SNPs, with a *P*-value <0.01 (Table 4). We found that this association persists even after adjusting for age and gender, or additionally for cardiovascular risk factors. The association results were even more pronounced for a second haplotype, comprising all minor alleles ('22222') of rs509035 to rs863441 (frequency 28.9%). Homozygous carriers of this haplotype had a multivariably adjusted 64% risk increase for suffering from MI in Study A. These results were highly comparable in Study B when examining the association of the second haplotype in affected CAD siblings versus controls (Table 5). After performing a permutation-based procedure to test for empirical significance, the *P*-value for the susceptible haplotype ('22222') reached the level of significance ($P = 0.01$), while the significance level only marginally failed for the protective haplotype ('11111', $P = 0.058$).

Notably, carriers and non-carriers of the protective haplotype '11111' as well as carriers and non-carriers of the susceptible haplotype '22222' did not significantly differ with respect to age, gender, blood pressure and lipid levels, the prevalence of hypertension, hypercholesterolemia, diabetes and smoking (data not shown).

Similar to our approach to address association of *GHSR* to MI and CAD, both two-marker haploblocks across *GHRL* were likewise tested for association with the disease. In essence, none of the seven inferred haplotypes with allele frequencies of more than 3% showed significant association with MI/CAD or cardiovascular risk factor distribution, similar to the results of the single-SNP association study in Table 3 (data not shown).

Association results using an 'affected sib pair–unrelated control' design

In order to potentially increase our power to detect disease association with the genetic variants, we took advantage of our family-based ascertainment strategy by constructing an efficient 'affected sib pair–unrelated controls' design, using a novel unified framework that allows joint analysis of all

Table 3. Association of *GHRL* SNPs in MI index patients versus controls (Study A) and in CAD siblings versus controls (Study B)

SNP[a]	Genotype													
	Cases (MI or CAD)				Controls				Allele 2 versus Allele 1		Genotype 22 versus 11		Genotype 22 versus 11 + 12	
	11	12	22	MAF[b]	11	12	22	MAF[b]	OR (95% CI)[c]	*P*	OR (95% CI)[c]	*P*	OR (95% CI)[‡]	*P*
rs2072578														
Study A	717	136	7	0.09	723	123	4	0.08	1.14 (0.90–1.46)	0.280	1.76 (0.51–6.05)	0.360	1.74 (0.52–6.64)	0.381
Study B	700	118	2	0.07	665	147	4	0.09	0.77 (0.60–0.98)	0.034	0.48 (0.09–2.60)	0.380	0.50 (0.07–2.55)	0.419
rs35684														
Study A	452	330	81	0.29	419	343	66	0.29	0.99 (0.85–1.15)	0.909	1.14 (0.80–1.62)	0.471	1.20 (0.85–1.68)	0.302
Study B	440	316	64	0.27	414	324	56	0.27	0.98 (0.84–1.15)	0.807	1.08 (0.73–1.58)	0.710	1.12 (0.77–1.62)	0.565
rs35683														
Study A	241	409	202	0.48	234	422	182	0.47	1.03 (0.90–1.18)	0.636	1.08 (0.82–1.41)	0.586	1.12 (0.89–1.41)	0.329
Study B	216	413	192	0.49	234	401	168	0.46	1.11 0.97–1.28)	0.1310	1.24 (0.94–1.63)	0.131	1.15 (0.91–1.46)	0.232
rs4684677														
Study A	747	109	4	0.07	734	105	3	0.07	1.03 (0.79–1.35)	0.806	1.31 (0.29–5.87)	0.723	1.31 (0.29–6.65)	0.727
Study B	700	114	5	0.08	701	102	3	0.07	1.14 (0.87–1.49)	0.335	1.67 (0.40–7.01)	0.480	1.64 (0.40–8.03)	0.497
rs42451														
Study A	457	323	76	0.28	418	347	62	0.28	0.96 (0.83–1.12)	0.637	1.12 (0.78–1.61)	0.534	1.20 (0.85–1.71)	0.302
Study B	441	317	58	0.27	459	297	45	0.24	1.13 (0.97–1.33)	0.121	1.34 (0.89–2.02)	0.160	1.29 (0.86–1.93)	0.221
rs26802														
Study A	415	340	97	0.31	398	361	91	0.32	0.97 (0.84–1.12)	0.705	1.02 (0.74–1.40)	0.892	1.07 (0.79–1.45)	0.655
Study B	381	358	79	0.32	383	353	77	0.31	1.02 (0.88–1.18)	0.825	1.03 (0.73–1.46)	0.861	1.02 (0.73–1.42)	0.898
rs27647														
Study A	295	399	148	0.41	297	413	128	0.40	1.06 (0.92–1.21)	0.424	1.16 (0.87–1.55)	0.298	1.18 (0.91–1.53)	0.203
Study B	293	384	137	0.40	286	404	120	0.40	1.03 (0.89–1.18)	0.700	1.11 (0.83–1.50)	0.471	1.16 (0.89–1.52)	0.266
rs7039167														
Study A	689	164	5	0.10	684	150	6	0.10	1.06 (0.84–1.33)	0.628	0.83 (0.25–2.72)	0.757	0.81 (0.23–2.71)	0.736
Study B	665	148	3	0.09	623	177	5	0.12	0.79 (0.63–0.99)	0.043	0.56 (0.13–2.36)	0.425	0.59 (0.12–2.41)	0.472

[a]SNPs are shown as 'rs' numbers from the dbSNP database.
[b]Minor allele frequency.
[c]ORs reported with their 95% CI and are based on the Armitage trend test.

Table 4. ORs for MI according to *GHSR* haplotypes in index MI cases versus controls (Study A)

Number of copies	Frequencies (%) cases/controls	OR (95% CI)		
		Model 1: crude model[b]	Model 2: adjusted for age and gender	Model 3: multivariable adjusted[a]
Haplotype 1-1-1-1-1	64.8/66.6			
0		1.00 (reference)	1.00 (reference)	1.00 (reference)
1		0.62 (0.45–0.85)*	0.63 (0.45–0.87)	0.62 (0.43–0.90)
2		0.69 (0.50–0.95)*	0.70 (0.50–0.98)	0.70 (0.48–1.02)
Haplotype 2-2-2-2-2	30.2/27.4			
0		1.00 (reference)	1.00 (reference)	1.00 (reference)
1		0.97 (0.79–1.18)†	0.97 (0.79–1.19)	0.95 (0.76–1.19)
2		1.74 (1.21–2.52)†	1.72 (1.18–2.51)	1.64 (1.07–2.52)

[a]Adjusted for age, gender, diabetes, hypertension, smoking, hyperlipidemia, obesity.
[b]In model 1, 10 000 Monte Carlo permutations have been performed to test for empirical significance.
The corresponding permutated *P*-values were $^{*}P = 0.058$ and $^{\dagger}P = 0.010$.

available data (42). In Table 6, *P*-values are shown for the additive, multiplicative and recessive genetic models, respectively, for the *GHSR* markers in LD, as well as for all *GHRL* SNP markers. Again, statistically significant association results could be demonstrated for the *GHSR* SNPs with CAD but not for any of the *GHRL* SNPs.

In summary, whereas association with MI and CAD could be clearly identified for genetic variants across *GHSR*, no relationship could be revealed for the *GHRL* itself.

Testing for interaction between *GHSR* and *GHRL* genetic variants

As both genes are playing a role in the same biological pathway, we explored whether there is interaction between *GHSR* and *GHRL* SNPs and/or haplotypes using a recursive partitioning method (RP) (45–50). All *GHSR* and *GHRL* genetic variants (SNPs and haplotypes) were considered for this analysis. RP identifies a parsimonious set of the most relevant genetic

Table 5. ORs for CAD according to *GHSR* haplotypes in affected CAD siblings of MI cases versus controls (Study B)

Number of copies	Frequencies (%) cases/controls	OR (95% CI)		
		Model 1: crude model[b]	Model 2: adjusted for age and gender	Model 3: multivariable adjusted[a]
Haplotype 1-1-1-1-1	65.3/68.2			
0		1.00 (reference)	1.00 (reference)	1.00 (reference)
1		0.77 (0.55–1.08)*	0.63 (0.45–0.87)	0.62 (0.43–0.90)
2		0.71 (0.51–1.00)*	0.70 (0.50–0.98)	0.70 (0.48–1.02)
Haplotype 2-2-2-2-2	30.5/26.5			
0		1.00 (reference)	1.00 (reference)	1.00 (reference)
1		1.19 (0.97–1.45)†	1.18 (0.96–1.44)	1.14 (0.91–1.42)
2		1.58 (1.08–2.33)†	1.52 (1.02–2.25)	1.61 (1.05–2.48)

[a]Adjusted for age, gender, diabetes, hypertension, smoking, hyperlipidemia, obesity.
[b]In model 1, 10 000 Monte Carlo permutations have been performed to test for empirical significance.
The corresponding permutated *P*-values were $^{*}P = 0.058$ and $^{\dagger}P = 0.046$.

Table 6. Test of genetic association of affected sibship data with unrelated controls

	P-values		
	Additive model	Multiplicative model	Recessive model
GHSR			
rs509035	0.011	0.020	0.050
rs572169	0.010	0.020	0.050
rs519384	0.020	0.030	0.040
rs512692	0.009	0.020	0.020
rs863441	0.020	0.040	0.060
GHRL			
rs2072578	0.900	0.900	0.500
rs35684	0.500	0.600	0.120
rs35683	0.300	0.300	0.200
rs4684677	0.300	0.300	0.300
rs42451	0.400	0.500	0.120
rs26802	0.600	0.600	0.200
rs27647	0.400	0.400	0.200
rs7039167	0.600	0.500	0.800

All *P*-values are derived by the LAMP statistical genetic software, which allows to combine data from different family structures (here, affected sibpair–controls have been used for analysis).

variants defining best the risk of having the disease. In the present study, the highest risk confers to subjects presenting the susceptible *GHSR* haplotype '22222' and lacking the protective *GHSR* ('11111') and *GHRL* ('11') haplotypes (Node 6, Fig. 2): as illustrated in Figure 2A, the best single split separated carriers from non-carriers of the protective *GHSR* haplotype '11111' (Node 2). The MI frequency increased from 50 to 60% in individuals lacking this protective haplotype (Node 2). The subsequent analysis was conditional on the absence of this haplotype: almost all individuals lacking the protective *GHSR* haplotype '11111' were carriers of the risk *GHSR* haplotype '22222' (Node 4). In the following step, an additional split was observed for a two-marker *GHRL* haplotype '11' (population frequency 24.3%): in subjects carrying the risk *GHSR* haplotype '22222', lacking the two-marker *GHRL* haplotype '11' further increased the MI frequency to 69% (Node 6). In contrast, carrying the *GHRL* haplotype '11' (Node 7) abolished the increase in MI frequency due to the susceptible *GHSR* haplotype '22222'.

The same relationship could be observed in Study B relating genetic variants to CAD in affected siblings of MI patients, with the exception that the effect of the risk *GHSR* haplotype '22222' was stronger than the effect of the protective *GHSR* haplotype '11111'. However, the subjects representing the highest risk group were identical to Study A (Node 6 in Study A equals Node 6 in Study B).

Multivariable logistic regression analyses were used to compare risks between predicted haplotype constellations (Fig. 3). OR of having MI (left panel of Fig. 3, Study A) or CAD (right panel of Fig. 3, Study B) was significantly increased in homozygous carriers of the risk *GHSR* haplotype '22222' compared with non-carriers of the protective *GHSR* haplotype '11111' [MI: OR = 1.7 (1.2–2.5), $P = 0.003$; CAD: OR = 1.6 (1.1–2.3), $P = 0.020$]. Whereas the OR further increased in subjects also lacking the two-marker *GHRL* haplotype '11' (Fig. 3, 'C') in index MI cases and affected sibs to 2.2 (1.4–3.7), $P = 0.0015$ and 1.9 (1.2–3.2), $P = 0.020$. Respectively, they decreased markedly in subjects presenting with such haplotype [MI: OR = 1.3 (0.8–2.1), $P = 0.390$ and CAD: OR = 1.2 (0.7–2.2), $P = 0.519$]. Thus, whereas this *GHRL* haplotype had no effect on the disease by itself, it modified the risk of MI and CAD when coinciding with particular *GHSR* haplotypes.

DISCUSSION

Our study presents a comprehensive analysis of polymorphisms and haplotypes of relevant genes within the biological pathway of the ghrelin system, i.e. *GHSR* encoding the ghrelin receptor and *GHRL* encoding the endogenous ligand ghrelin in families with MI and individuals of the general population.

Ghrelin, an endogenous gut–brain regulatory hormone, is produced by the stomach, has been recently shown to stimulate eating and plays a role in regulating energy balance and growth hormone secretion. These effects are exerted through interaction with a specific G-protein-coupled receptor, GHSR, which is expressed mainly in the hypothalamus–pituitary complex, but also in cardiovascular tissues (22,25–28,31,51). In fact, GHSR receptor density has been shown to be up-regulated in atherosclerotic arteries (29). Exploration of the cardiovascular functions of ghrelin revealed cardiopro-

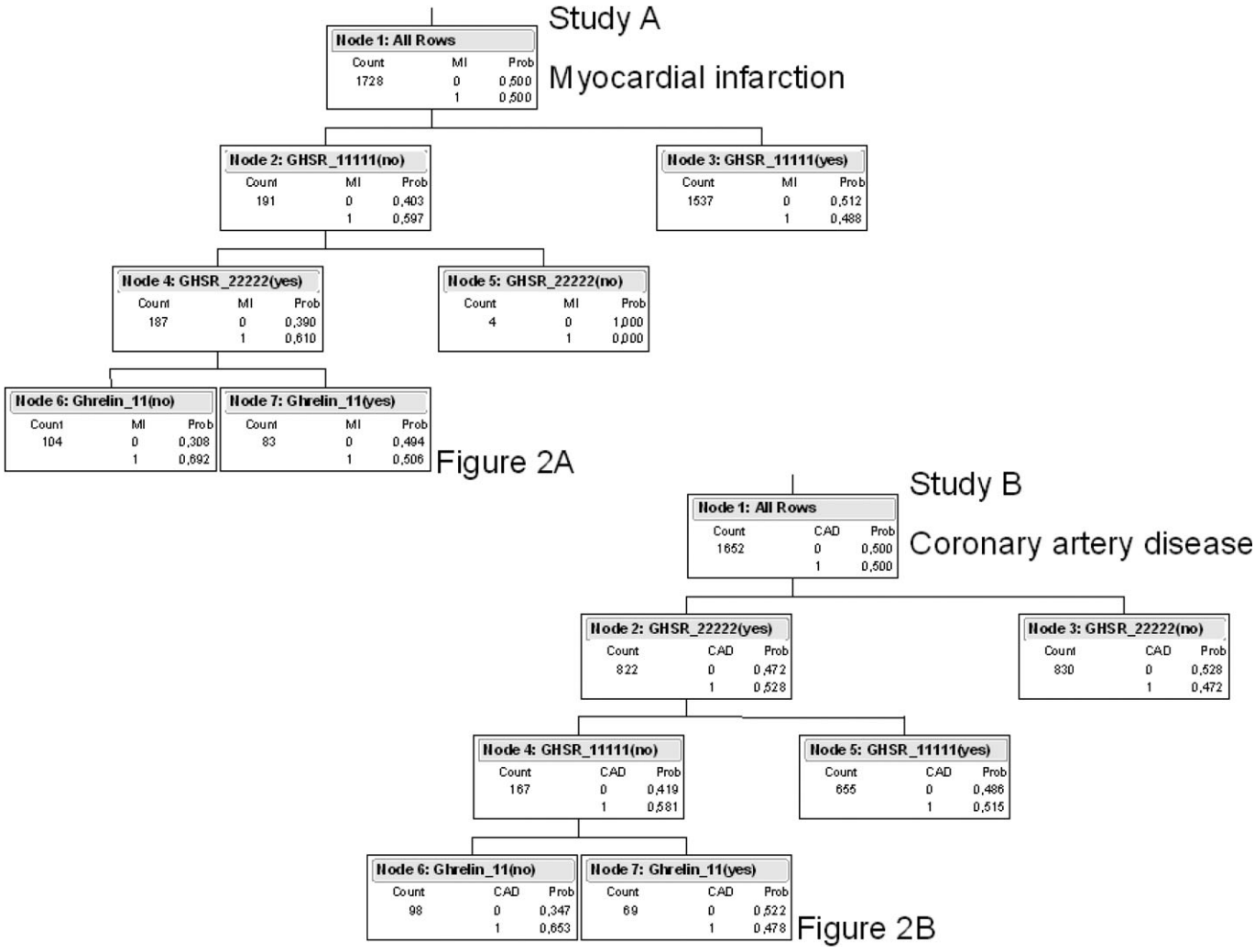

Figure 2. Recursively partitioned tree model for MI (Study A) and CAD (Study B). The *GHSR* haplotypes operate in interaction with a *GHRL* haplotype. The effects of the *GHSR* risk haplotypes operate differently depending upon the presence or absence of the *GHRL* haplotype.

tective effects against myocardial ischemia, and vasoactive and cardiotropic actions in both experimental models and human (30,32–41). These effects are mediated by the interaction of the ligand with receptor binding sites, including that of GHSR, for which the signaling pathways are not yet fully documented. Based on these data it was tempting to speculate that genetic markers across both genes encoding ghrelin and its receptor GHSR are related to susceptibility of MI and/or CAD.

While we found a significant association of both individual SNPs and haplotypes across *GHSR* with MI/CAD, no significant association could be detected between genetic variations across *GHRL* and MI/CAD. However, we found an effect of *GHRL* dependent upon the presence of a common, MI and CAD susceptible haplotype of *GHSR*. Specifically, a common haplotype within *GHRL* significantly affects the relationship between the *GHSR* risk haplotype and MI/CAD, while this *GHRL* haplotype alone does not show any association with the disease or its associated cardiovascular risk factors. Thus, our data suggest that specific haplotypes of the ghrelin ligand and its receptor act epistatically to affect susceptibility or tolerance to MI and/or CAD.

Factors regulating the expression of *GHSR* and its physiological ligand ghrelin as well as the impact of genetic variations on transcriptional regulation and receptor and ligand properties have not been clarified so far. Several hormones, e.g. leptin, insulin and glucagon may present potential regulators of ghrelin production and GHSR activation (52). On the other hand, receptor properties, such as the constitutive activity of *GHSR*, receptor signaling or response to the ligand ghrelin might be relevant in regulating receptor–ligand expression and interaction, thereby influencing susceptibility to disease (15,20,53). Recently, a *GHSR* missense mutation has been identified that segregates with short stature within two unrelated families (54). This mutation results in decreased cell-surface expression of *GHSR* and, moreover, impairs its constitutive activity while preserving its ability to respond to ghrelin (54). As speculated in a previous investigation, genetic *GHSR* variations, i.e. the risk haplotype '22222', could result in an increase of constitutive

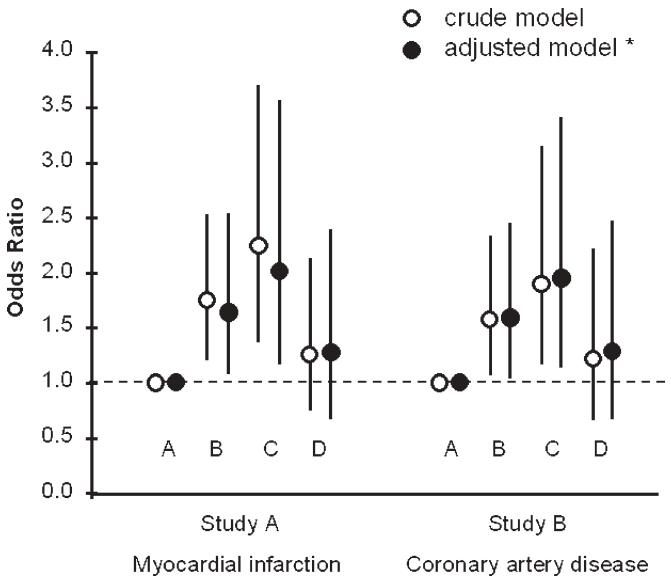

Figure 3. Crude and adjusted logistic regression analysis for MI (Study A) and CAD (Study B) in subjects with different constellations of *GHSR* and *GHRL* haplotypes: (**A**) *GHSR* risk haplotype not present (reference); (**B**) homozygous carriers of *GHSR* risk haplotype ('22222'); (**C**) homozygous carriers of *GHSR* risk haplotype, *GHRL* haplotype ('11') not present; (**D**) homozygous carriers of *GHSR* risk haplotype, *GHRL* haplotype present. Asterisk denotes adjusted for age, sex, hypertension, diabetes, hyperlipidemia, smoking.

activity and a decreased sensitivity to multiple inhibitory signals and consequently promote disease susceptibility (15).

Here, we show that the protective *GHRL* haplotype '11' reduces the risk of MI/CAD by influencing the relationship between its receptor *GHSR* and the disease, while it does not independently show any direct effect. Potentially, it might reduce the risk of disease by altering its binding properties to the receptor thereby influencing receptor–ligand interactions. This specific haplotype comprises the promotor region of *GHRL* and displays a high degree of sequence homology between species. Recently, a study of characterization and regulation of the rat and human *GHRL* promotor identified putative initiator sites and TATA box elements. Mutations in such functional elements markedly reduced or even abolished promotor activity indicating the importance of these sites for promotor activity and regulation of *GHRL* transcription in both species (55). Thus, it seems convincing that genetic variations in specific regions of the receptor or ligand genes may change transcriptional regulation, protein expression or ligand/receptor properties and thereby have an effect on the susceptibility to disease.

So far, the roles of ghrelin in the cardiovascular system have not yet been unambiguously established. Since ghrelin is an effective vasodilator (34), it has been suggested that the increase of receptor density in atherosclerotic vessels might reflect the beneficial role of ghrelin in human atherosclerosis (29). In addition, ghrelin appears to have beneficial features in relation to other essential processes of atherosclerotic activity, namely inflammation and apoptosis (24,56–58). Moreover, there is evidence that ghrelin modulates intracellular energy balance in a cell-specific manner by increasing the activity of the key enzyme 5′-AMP-activated protein kinase in heart cells resulting in protection from ischemia-reperfusion injury (59). A negative correlation between fasting plasma ghrelin concentrations and both systolic and diastolic blood pressure has been shown in a population-based cohort (60), in pregnant women (61), and in experimental settings (25), suggesting that low ghrelin plasma concentration might be hemodynamically harmful. This notion is supported by findings showing a lower frequency of hypertension among non-carriers of the Arg51Gln mutation of *GHRL* than among carriers (60). This is in accordance with an earlier report showing an association between the Arg51Gln mutation and low plasma ghrelin concentrations (62). However, the frequency of the Arg51Gln mutation is quite low and therefore does not explain the increased risk for hypertension at the population level (60). In addition, low ghrelin concentrations have been associated with metabolic disturbances such as insulin resistance, type-2 diabetes and low HDL cholesterol levels (63,64).

In contrast to the studies showing beneficial effects of ghrelin in cardiovascular system, there is another line of recent evidence showing vasoconstrictive effects of ghrelin in coronary arterioles (65). Plasma ghrelin concentrations were positively associated with the degree of subclinical atherosclerosis measured as intima media thickness of carotid arteries in men (66). This observation was surprising because it is opposite to the view that majority of the currently available data suggest. Thus, further studies are warranted to elucidate the role of ghrelin in atherosclerosis.

Our results suggest a new paradigm in the design and interpretation of genetic association studies. Many reported associations with common gene polymorphisms have not been replicated, presumably because of factors such as population stratification, inadequate statistical power and genotyping errors. Moreover, most complex traits show different etiologies in different subgroups or families, variability in age of onset or severity and are determined by interactions among multiple genes and environmental attributes. This heterogeneity can particularly confound attempts to detect the underlying genes. As shown by our conditional gene finding approach, our results indicate that epistatic effects, i.e. haplotype × haplotype genetic interactions, might indeed seriously account for the frustrating reproducibility of genetic association results. In fact, the positive association between the identified *GHSR* risk haplotype and MI/CAD appears to be completely masked in carriers of other favorable variants. It seems therefore straightforward that many genes within a pathway could be dependent on the presence or absence of other genes and genetic variants within these genes. Therefore, it is likely that the search for genetic variants generally should be 'conditional', particularly since in the genetic architecture of complex disease traits, epistatic interactions may well be the rule, not the exception. In this respect, recursive partitioning has been successful in analyzing single dependent variables which are determined by complex, interacting models and/or mixtures of models (46,47,67). Indeed, it has been shown that the statistical significance of two purported diabetes genes are greatly increased if the analysis is conditional (68).

With respect to our findings it should be noted, however, that our approach is possibly still too simplified, because we accounted only for common variation within the two genes

and did not include environmental factors or traditional risk factors in the classification tree analysis. In addition, the algorithm of recursive partitioning performs several statistical comparisons during the construction of the tree that makes the overall type I error rate difficult to interpret. Studies that test several genetic variants can lead to false-positive associations, whereas a conservative correction for multiple testing can lead to false-negative associations. We addressed this dilemma by performing permutation-based methods to test for empirical significance and were largely able to confirm the significance of the described associations. Furthermore, we tested for association in a second case–control group, consisting of affected siblings of our index MI cases and revealed essentially the same results.

Mindful of the fact that conventional risk factors were enriched in the case groups as compared with the control groups, we investigated confounding by traditional risk factors. However, with the use of multivariate analysis to adjust for age, gender, diabetes, hypertension, smoking, hyperlipidemia and obesity, the risk estimates were only incrementally changed and we did not observe any association between *GHSR* and *GHRL* alleles with these cardiovascular risk factors.

We used novel statistical methods allowing the joint association analysis of two or more affected family members versus randomly selected unrelated controls (42). This study design might be ideal for our ascertained study sample comprising affected sib pairs with MI and/or severe CAD. Indeed, it has been shown that the power can be substantially increased by including families with more affected siblings in association studies (43,44,69). However, our findings are restricted to cases with premature MI/CAD and a strong positive family history of MI, and might not be representative for incidental MI cases from the typical hospital setting. Thus, independent confirmation of our results is certainly warranted. Finally, the case–control studies conducted in this investigation are limited because this retrospective study did not include fatal cases of early-onset MI. Nevertheless, the variants we described are good candidates for genetic analysis in further population-based studies of cardiovascular disease. If the association of the *GHSR* gene and its interaction with the *GHRL* gene is further confirmed, these variants could advance our understanding of disease mechanism, suggest targets for therapeutic intervention, and be useful in assessing genetic risk of early-onset MI.

MATERIALS AND METHODS

Study populations

Cases from the German MI family study. The ascertainment strategy and study design have been described in detail elsewhere (70,71). Briefly, MI kindreds were ascertained through index patients, who were identified by screening 200 000 patient charts in 14 cardiac in-hospital rehabilitation centers distributed throughout Germany. Index patients had all suffered from MI before 60 years. If at least one sibling had suffered from MI or had severe CAD [percutaneous transluminal coronary angioplasty (PTCA) or bypass surgery (CABG) before 70 years], the nuclear family (index patient, available parents and all siblings) was contacted and invited to participate in the study. The study protocol was approved by Ethics Committee of the University of Regensburg, Germany, and all participants gave informed consent.

An independent case population, referred to as 'index MI cases' (cases Study A) consisting of 890 male and 300 female index patients, was selected. A second case population (cases Study B) consisting of single siblings of index patients suffering from MI or severe CAD (documented CABG or PTCA; 750 men, 190 women) was used for verification (referred to as 'affected siblings').

Controls from the WHO MONICA general population. Subjects participated in the MONICA Augsburg LVH substudy, as part of the third WHO MONICA Augsburg survey. The study population of the LVH substudy was sampled from the general population of the city of Augsburg, Germany, in 1994–1995. The study design, sampling frame and data collection have been described in detail elsewhere (72,73). The LVH substudy represents individuals 25–74 years of age from a sex- and age-stratified cluster sample of all German residents (total $n = 1674$). Of these, 1418 DNA samples were available for genotyping in the present study (724 men and 694 women). The study was approved by the Local Ethics Committee and all participants gave written informed consent.

Controls from the German MI family study. To serve as controls, non-affected sisters ($n = 502$) and brothers in-law ($n = 136$) of MI patients were included in the study. These subjects were examined by the same protocol as the cases and had no evidence of CAD by history and physical examination.

Study design

Index MI cases and affected siblings were each carefully matched with controls by age and gender in order to avoid systematic differences in genetic composition between the groups. Using this strategy, appropriate controls from the MONICA general population could be found for 864 of MI index patients (Study A). The same matching strategy was used for siblings of index patients, except that the control subjects comprised also the unaffected brothers in-law and sisters in-law of the German MI family study in order to increase the pool of controls to be able to match the largest possible number of subjects (Study B). The allele frequencies of all marker alleles were well comparable in controls from the MONICA population and married-in spouses. In total, 826 siblings with CAD or MI were matched randomly with the same number of appropriate controls (596 from the MONICA population and 230 brother or sisters in-law).

SNPs and genotyping methods

Single nucleotide polymorphisms. To obtain complete coverage of both genes, seven SNPs covering the *GHSR* and eight SNPs covering the *GHRL* gene and their flanking regions were selected from public databases (dbSNP, http://www.ncbi.nlm.nih.gov/SNP/, Fig. 1). We preferred validated SNPs with a minor allele frequency of >5%. Priority was given to

SNPs submitted multiple times followed by SNPs discovered by the TSC (74,75). Regarding the intergenic regions we prioritized SNPs located in highly conserved non-coding regions. Of the seven selected *GHSR* SNPs, one SNP was located in exon 1, one was in the intron, one was within 41.5 kb past the 3′ end of the gene and four covered a region of 53.5 kb past the 5′ end of the gene. The coding SNP (rs572169) led to a synonymous amino acid substitution. Of the eight selected *GHRL* SNPs, three SNPs were in introns, and one SNP (rs4684677) was located in exon 4 leading to a non-synonymous amino acid substitution (glutamine at position 90 to leucine). Four SNPs were selected in the locus region. The SNPs located beyond the boundaries of the genes were picked to determine the extent of LD and to explore the impact of sequence variations in non-coding and intergenic regions on the disease.

Genotyping. SNPs were genotyped using the 5′-exonuclease activity (TaqMan) assay on a HT7900 (Applied Biosystems, Darmstadt, Germany). H_2O controls, as well as blind duplicate samples (10%) were included in each round of amplification to check for consistency and to ensure intra- and inter-plate genotype quality control.

In both case–control cohorts, no genotyping discrepancies were detected between the repeated samples. The overall mis-genotyping rate of $<0.5\%$ was due to insufficient PCR amplification.

Definitions

Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Obesity was defined by a BMI ≥ 30 kg/m^2. Arterial hypertension was a history of hypertension, a blood pressure of $\geq 140/90$ mmHg or intake of antihypertensive drugs. Hyperlipidemia was defined as a history of hypercholesterolemia, an LDL cholesterol ≥ 130 mg/dl or intake of lipid-lowering agents. Smoking was defined as former or current smoking of more than five cigarettes per day. Diabetes was a history of diabetes or the use of anti-diabetic medication.

Statistical analysis

For each of the SNPs, we tested whether the observed allele frequencies departed from Hardy–Weinberg proportion. Frequencies of alleles and genotypes in cases and controls were explored using an allele frequency comparison, a homozygous and a recessive trait comparison, and ORs with their 95% CIs were reported (76). We assessed LD between all pairs of SNPs, applying the standard definition of r^2 (77). In addition to single-locus analysis, statistically inferred haplotypes were tested for association with the traits MI and CAD. An $r^2 > 0.4$ was used to define haplotypes. Haplotype frequencies were estimated using the expectation-maximization algorithm. Simple and multiple logistic regressions were used to assess the relationship between a haplotype and two or more continuous or categorical explanatory variables and the categorical response variable (MI, CAD). Three models were analyzed: a crude model, an age- and sex-adjusted model and a multivariate adjusted model controlling for age, gender, diabetes, hypertension, smoking, hyperlipidemia and obesity. Permutation tests were used to test for empirical significance.

In addition, to potentially increase the power of the study and given our fixed genotyping resources we applied an extension of the likelihood-based method of Li *et al.* (42), which assesses whether there is LD between a disease locus and a SNP, to accommodate sib-ships of arbitrary size and disease-phenotype configuration. Here, we consider the scenario of using both affected individuals (MI index cases plus their affected siblings) from our affected sib pairs and one unrelated control to form an 'affected sib pair–unrelated control' design (in addition to the 'one sibling per affected sib pair–control' design). For this test statistic, a retrospective likelihood is used that avoids the problem of ascertainment bias and ensures that the test remains valid even if there are additional genetic or environmental factors that induce correlation between family members.

Examination of a biochemical pathway suggests that the effect of a gene will be dependent on the presence or absence of other genes. Thus, interactions between *GHSR* and *GHRL* variants were explored by the recursive partitioning method (RP) (46,47). RP is a classification methodology to fit tree-based models for predicting the value of an outcome from a potentially large pool of independent variables (i.e. genetic variants) to arrive at a parsimonious set of 'important' ones which define risk. RP identifies optimal cut-points (splits), partitioning the data set into two mutually exclusive subsets, and can be viewed as conditional gene finding: once a split is based upon one gene variant, then the subsequent analysis is conditional on the presence or absence of that variant. All *GHSR* and *GHRL* SNPs and haplotypes were considered for this analysis. Five-fold cross-validation was used to determine optimal tree size.

Multivariable logistic regression analyses were used for verification and to compare risks between predicted leaf groups. A two-sided P-value <0.05 was considered significant.

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Conflict of Interest statement. All authors declare that they have no competing financial interests.

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