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Chapter 6
Systematic testing of literature reported genetic variation associated with coronary restenosis
Results of the GENDER study

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ABSTRACT

Background: Coronary restenosis after percutaneous coronary intervention still remains a significant problem, despite all medical advances. Unraveling the mechanisms leading to restenosis development remains challenging. Many studies have identified genetic markers associated with restenosis, but consistent replication of the reported markers is scarce. The aim of the current study was to analyze the joined effect of previously in literature reported candidate genes for restenosis in the GENetic DEterminants of Restenosis (GENDER) databank.

Methods and results: Candidate genes were selected using a MEDLINE search including the terms ‘genetic polymorphism’ and ‘coronary restenosis’. The final set included 36 genes. Subsequently, all single nucleotide polymorphisms (SNPs) in the genomic region of these genes were analyzed in GENDER using set-based analysis in PLINK. The GENDER databank contains genotypic data of 2,571,586 SNPs of 295 cases with restenosis and 571 matched controls. The set, including all 36 literature reported genes, was indeed significantly associated with restenosis, $p = 0.024$ in the GENDER study. Subsequent analyses of the individual genes demonstrated that the observed association of the complete set was determined by 6 of the 36 genes.

Conclusion: Despite overt inconsistencies in literature, with regard to individual candidate gene studies, this is the first study demonstrating that the joint effect of all these genes together, indeed is associated with restenosis.
INTRODUCTION

Restenosis is a complex disease for which the causative mechanisms have not yet been fully identified. Despite medical advances, restenosis still remains a significant complication after percutaneous coronary intervention (PCI).\textsuperscript{1} Identification of risk factors and underlying mechanisms could not only be useful in risk stratification of patients, they also contribute to our understanding of this condition. In addition, these factors could provide evidence on which to base individually tailored treatment and aid in the development of novel therapeutic modalities.\textsuperscript{2} Unraveling the mechanisms leading to restenosis development remains challenging. Genetic susceptibility is known to play a role in the individuals risk of developing this complication.\textsuperscript{1} Many studies have focused on identification of genetic markers associated with restenosis. Over the last decades genetic research has developed from candidate gene approaches\textsuperscript{3-5} to multiplex arrays\textsuperscript{6} and finally to genome wide association studies (GWAS).\textsuperscript{7} Genetic variation in large array of plausible candidate genes have been associated with restenosis, however, consistent replication of the reported markers is scarce.\textsuperscript{1} Possible explanations for this lack of consistency are the small sample size of many (especially relative more dated) studies, phenotype heterogeneity and lack of proper replication cohorts.

Currently more and more GWAS are being performed, investigating many diseases, including cardiovascular diseases.\textsuperscript{8,9} An advantage of GWAS is the hypothesis-free approach of this method, enabling identification of new genetic loci associated with the disease of interest. With respect to restenosis, a disadvantage of the GWAS approach is that due to the complexity of the disease the effect size of individual genetic markers is likely to be small and therefore hard to detect. Moreover, the availability of (large) replication cohorts is very limited. In 2011, the first GWAS on restenosis in the GENetic DEterminants of Restenosis (GENDER) study identified a new susceptibility locus on chromosome 12.\textsuperscript{7} The fact that this GWAS only identified this previously unknown locus does not mean that genetic variation in the previously proposed candidate genes does not affect restenosis development. It merely indicates that the influence of other individual markers is probably too small to detect in the GWAS setting. Especially for the complex traits, a more appropriate approach to interpret GWAS data is to analyze the combined effect of a single nucleotide polymorphism (SNP) set, grouped per pathway or gene region.\textsuperscript{10} To date, investigation into a possible joined effect of multiple genetic markers for restenosis has not been performed.

The goal of the current study is to investigate whether the last decade of research on genetics of restenosis has led to a set of genes that is associated with restenosis in a set-based analysis using the available genotypic data of the GENDER databank.
METHODS

Gene Selection
Candidate genes previously associated with restenosis were selected after a search of literature of papers published up to November 2011. Genes were identified searching MEDLINE using keywords as ‘genetic polymorphism’, ‘candidate gene’, ‘restenosis’ and ‘percutaneous coronary intervention’. Selection criteria included a sample size of > 250 patients and the observation of a significant association of a SNP with restenosis. The final set included 36 genes (Table 2). All available SNPs from the GENDER GWAS databank within a 10-Kb window around these genes were analyzed.

Study Population
The design of GENDER and the genome-wide association study (GWAS), which has been performed in a subset of this study population, have both been described previously. In brief, GENDER included 3,104 consecutive unrelated symptomatic patients treated successfully by PCI for angina. The study protocol conforms to the Declaration of Helsinki and was approved by the ethics committees of each participating institution. Written informed consent was obtained from each participant before the PCI procedure. During a follow-up period of 9 months, the endpoint clinical restenosis, defined as renewed symptoms requiring target vessel revascularization (TVR) either by repeated PCI or CABG, by death from cardiac causes or myocardial infarction not attributable to another coronary event than the target vessel, was recorded. During follow-up, 346 patients developed clinical restenosis. Blood samples were collected at the index procedure for DNA isolation. The GWAS was performed in 325 cases of restenosis and 630 controls matched by gender, age, and some possible confounding clinical variables for restenosis in the GENDER study such as total occlusion, diabetes, current smoking and residual stenosis. Genotyping was performed using the Illumina Human 610-Quad Beadchips following the manufacturer’s instructions. After genotyping, samples and genetic markers were subjected to a stringent quality control protocol. The final dataset consisted of 866 individuals (295 cases, 571 controls) and 556,099 SNPs that passed all quality control criteria, together covering 89% of the common genetic variation in the European population. Imputation was performed with MACH software based on the HapMap II release 22 CEU build 36 using the default settings. This program infers missing genotypes based on the known genotypic data of the samples together with haplotypes from a reference population provided by HapMap taken into account the degree of linkage disequilibrium (LD). After subsequent quality control, we excluded SNPs for further analyses with a call rate lower than 95% (n = 3335) or with a significant deviation from Hardy–Weinberg equilibrium (HWE) in controls (P < 0.00001) (n = 79). The final GENDER Biobank dataset consisted of 866 (295 cases, 571 controls) individuals and 2,571,586 SNPs.
Statistical Analysis

The statistical analyses were performed using the set-based test of PLINK v1.07. During this test, first a single SNP analysis of all SNPs within the set is performed. Subsequently a mean SNP statistic is calculated from the single SNP statistics of a maximum amount of independent SNPs below a certain p-value threshold. If SNPs are not independent and the LD (expressed in $R^2$) is above a certain threshold, the SNP with the lowest p-value in the single SNP analysis is selected. This analysis is repeated in a certain amount of permutations of the phenotype. An empirical p-value for the SNP set is computed by calculating the number of times the test statistic of the simulated SNP sets exceeds that of the original SNP set. For the analysis of this study, the parameters were set to p-value threshold $< 0.05$, $R^2$ threshold $< 0.1$, maximum number of SNPs = 5 and 10,000 permutations.

Initially, the set including all 36 genes is tested as a whole for the association with restenosis. Subsequent analysis of the individual genes will be justified only when the complete set is significantly associated with the endpoint.

RESULTS

Patient characteristics are presented in Table 1. No significant differences were found between cases and controls regarding the known risk factors for restenosis (age, diabetes, smoking, stenting and previous restenosis). Hypertension and multivessel disease were more common in the cases compared to the controls.

In Figure 1 the QQ-plot of the GENDER GWAS after imputation is shown, demonstrating that no genomic inflation has occurred in this analysis ($\lambda$ = 1.027). The complete set of 36 genes, previously associated with restenosis in literature, contained 2,581 SNPs. A detailed description of the individual studies and candidate genes can be found in Table 2. The largest gene was chemokine (C-X3-C motif) receptor 1 (CX3CR1) of 316.54kb, contributing 384 SNPs (14.8%), and glutathione peroxidase 1 (GPX1) was with 1.18kb the smallest gene, only contributing 8 SNPs (0.3%). Analysis of the complete set using the set-based test demonstrated a significant association with clinical restenosis, with an empirical p-value of 0.024.

To determine which genes are mainly responsible for this association we subsequently investigated the association of the individual gene based sets. Six of the 36 genes were demonstrated to have an empirical p-value below 0.05 (Table 3). In order of descending p-values the associated genes are; angiotensin II receptor type 1 (AGTR, $p = 0.028$), glutathione peroxidase 1 (GPX1, $p = 0.025$), K(lysine) acetyltransferase 2B (KAT2B, also known as PCAF, $p = 0.023$), matrix metallopeptidase 12 (MMP12, $p = 0.019$), fibrinogen beta chain (FGB, $p = 0.013$) and vitamin D receptor (VDR, $p = 0.012$). Detailed
Table 1, Demographic, clinical and lesion characteristics of the study population.

|                         | Cases (n=295) | Controls (n=571) | p-value |
|-------------------------|---------------|------------------|---------|
| Age (years)             | 62.8 ± 10.6   | 62.4 ± 10.9      | 0.59    |
| BMI (kg.m⁻²)            | 26.7 ± 3.6    | 27.1 ± 3.7       | 0.20    |
| Male sex                | 213 (72)      | 421 (74)         | 0.63    |
| Diabetes                | 58 (20)       | 119 (21)         | 0.68    |
| Hypercholesterolemia    | 179 (61)      | 341 (60)         | 0.79    |
| Hypertension            | 138 (47)      | 211 (37)         | 0.005   |
| Current smoker          | 68 (23)       | 148 (26)         | 0.36    |
| Family history of MI    | 117 (40)      | 210 (37)         | 0.41    |
| Previous MI             | 119 (40)      | 246 (43)         | 0.44    |
| Stable angina           | 188 (64)      | 400 (68)         | 0.06    |
| Multivessel disease     | 155 (53)      | 248 (43)         | 0.01    |
| Restenotic lesion       | 23 (8)        | 48 (8)           | 0.76    |
| Total occlusion         | 57 (19)       | 97 (17)          | 0.40    |
| Type C lesion           | 95 (38)       | 154 (27)         | 0.11    |
| Stenting                | 199 (68)      | 385 (67)         | 0.99    |

Values were given as n (%) or mean ± SD. Patients using anti-diabetic medication or insulin at study entry were considered to be diabetics. Hypertension was defined as a blood pressure of either above 160 mmHg systolic or 90 mmHg diastolic. Hypercholesterolaemia was defined as total cholesterol concentrations of above 5 mmol/L. BMI: body mass index, MI: myocardial infarction. P-values are determined by Pearson's Chi-Square (discrete variables) or unpaired 2-sided t-test (continuous variables).

Table 2 Candidate genes and the studies that reported their association with restenosis.

| Candidate gene | Literature based study characteristics and results |
|----------------|---------------------------------------------------|
| adrenergic beta-2-receptor (ADRB2) | HR 1.33 (1.06-1.68) [6] |
| advanced glycosylation end product-specific receptor (AGER) | ↓ [15] |
| angiotensin II receptor, type 1 (AGTR1) | OR 1.85 (1.28-2.66) [18] |
| Butyrylcholinesterase (BCHE) | OR 5.5 (1.6-21.4) [19] |
| chemokine (C-C motif) ligand 11 (CCL11) | HR 0.73 (0.58-0.93) [6] |
| CD14 | RR 3.8 (1.2-11.6) [20] |
| Candidate gene | Location | Study size | % of cases | FU (mo) | Top SNP | Effect size (95% CI)* | Ref |
|----------------|----------|------------|------------|--------|---------|----------------------|-----|
| cyclin-dependent kinase inhibitor 1B (p27, Kip1) (CDKN1B) | 12p13.1-p12 | 433 | 11.3 | 12 | rs34330 | NS | [21] |
| collagen, type III, alpha 1 (Col3A1) | 2q31 | 527 | 9.1 | 6 | rs1800255 | OR 4.2 (1.4-11.2) | [23] |
| colony stimulating factor 2 (CSF2) | 5q31.1 | 3104 | 9.8 | 9 | rs25882 | HR 0.76 (0.61-0.94) | [6] |
| chemokine (C-X3-C motif) receptor 1 (CX3CR1) | 3p21.3 | 365 | 25.5 | 6 | rs3732379 | OR 2.4 (1.3-4.2) | [24] |
| cytochrome b-245, alpha polypeptide (CYBA) | 16q24 | 730 | 35.8 | 6 | rs4673 | OR 0.5 (0.3-0.8) | [25] |
| cytochrome P450, family 2, subfamily C, polypeptide 19 (CYP2C19) | 10q24 | 928 | 19.1 | 12 | rs12248560 | ↓ | [26] |
| fibrinogen beta chain (FGB) | 4q28 | 527 | 9.1 | 6 | rs1800790 | OR 2.7 (1.2-6.2) | [23] |
| coagulation factor V (F5) | 1q23 | 3104 | 9.8 | 9 | rs6025 | HR 0.40 (0.19-0.85) | [28] |
| glutathione peroxidase 1 (GPX1) | 3p21.3 | 461 | 23.2 | 6 | rs1050450 | OR 2.1 (1.2-3.8) | [19] |
| interleukin 10 (IL10) | 1q31-q32 | 162 | 39.5 | UK | rs1800871 | HR 0.39 (0.16-0.94) | [29] |
| interleukin 1 receptor antagonist (IL1RN) | 2q14.2 | 183 | 46.4 | 12 | VNTR | HR 3.24 (1.63-16.81) | [32] |
| insulin receptor (INSR) | 19p13.3-p13.2 | 461 | 23.2 | 6 | rs419598 | OR 0.73 (0.58-0.92) | [3] |
| integrin, beta 2 (ITGB2) | 21q22.3 | 1207 | 21.2 | 12 | rs235326 | OR 1.9 (1.2-3.1) | [19] |
| lipoprotein lipase (LPL) | 8p22 | 3104 | 9.8 | 9 | rs328 | OR 0.62 (0.44-0.86) | [34] |
| matrix metallopeptidase 12 (MMP12) | 11q22.3 | 527 | 9.1 | 6 | rs2276109 | OR 3.9 (1.0-12.4) | [23] |
| matrix metallopeptidase 9 (MMP9) | 20q11.2-q13.1 | 461 | 23.2 | 6 | rs2664538 | OR 2.0 (1.0-3.9) | [19] |
### Table 2 Candidate genes and the studies that reported their association with restenosis. (continued)

| Candidate gene | Gene entrez nr | Location | Study size | % of cases | FU (mo) | Top SNP | Effect size (95% CI) | Ref |
|----------------|----------------|----------|------------|------------|---------|---------|----------------------|-----|
| methylenetetrahydrofolate reductase (NAD(P)H) (MTHFR) | 4524 | 1p36.3 | 260 | 36.9 | 6 | rs1801133 | OR 3.58 (1.51-8.46) | [35] |
| | | | 800 | 18.9 | 12 | rs1801133 | NS | [36] |
| nitric oxide synthase 3 (NOS3) | 4846 | 7q36 | 205 | 29.3 | 6 | rs2070744 | OR 2.06 (1.08-3.94) | [37] |
| | | | 901 | 10.2 | 9 | rs1799983 | HR 1.67 (1.09-2.54) | [38] |
| purinergic receptor P2Y, G-protein coupled, 12 (P2RY12) | 64805 | 3q24-q25 | 2062 | 8.4 | 9 | Haplotype of 5 SNPs | HR 1.6 (1.2-2.0) | [40] |
| serpin peptidase inhibitor, clade E, member 1 (SERPINE1) | 5054 | 7q21.3-q22 | 1850 | 20.3 | 12 | rs1799899 | NS | [41] |
| (K)lysine acetyltransferase 2B (KAT2B, PCAF) | 8850 | 3p24 | 3104 | 9.8 | 9 | rs2948080 | HR 0.80 (0.67-0.97) | [42] |
| peroxisome proliferator-activated receptor gamma (PPARG) | 5468 | 3p25 | 565 | 28.7 | 6 | rs3856806 | ↓ | [43] |
| c-ros oncogene 1 , receptor tyrosine kinase (ROS1) | 6098 | 6q22 | 461 | 23.2 | 6 | rs529038 | HR 1.8 (1.1-2.8) | [19] |
| thrombomodulin (THBD) | 7056 | 20p11.2 | 730 | 35.8 | 6 | rs1042579 | OR 2.1 (1.3-3.53) | [25] |
| thrombospondin 4 (THBS4) | 7060 | 5q13 | 628 | 6-10 | UK | rs1866389 | OR 2.67 (1.04-6.80) | [45] |
| thrombopoietin (THPO) | 7066 | 3q27 | 527 | 9.1 | 6 | rs6141 | OR 2.4 (1.1-5.3) | [23] |
| tumor necrosis factor (TNF) | 7124 | 6p21.3 | 1850 | 17.6 | 12 | rs1800629 | NS | [30] |
| | | | 3104 | 9.8 | 9 | rs361525 | HR 0.60 (0.37-0.98) | [5] |
| tumor protein p53 (TP53) | 7157 | 17p13.1 | 132 | 0 | UK | rs1042522 | ↑ | [46] |
| | | | 433 | 11.3 | 12 | rs1042522 | NS | [21] |
| | | | 779 | 43.9 | 6 | Haplotype of 3 SNPs | OR 0.58 (0.40-0.83) | [47] |
| uncoupling protein 3 (UCP3) | 7352 | 11q13.4 | 527 | 9.1 | 6 | rs1800849 | OR 5.2 (1.9-13.0) | [23] |
| vitamin D receptor (VDR) | 7421 | 12q13.11 | 3104 | 9.8 | 9 | rs11568820/ rs4516035 haplotype | HR 0.72 (0.57-0.93) | [48] |

*The direction of the association between genetic variation and the risk of restenosis, when effect size is not available; ↓ protective effect, ↑ deleterious effect. Entrez nr; unique gene ID number used in NCBI database. Abbreviations: FU, follow-up; UK, unknown; NS, not significant; OR, odds ratio; HR, hazard ratio; RR, relative risk; Ref, reference.*
Candidate gene-set analysis of restenosis

The SNP with the lowest individual p-value was rs11574027 in the VDR gene, $p = 1.4 \times 10^{-4}$. In the complete GWAS analysis, which has been published in 2011, this SNP ranked 116th. The strongest association in that analysis was found with a SNP in an intergenic region on chromosome 12, $p = 1.0 \times 10^{-6}$.

Logistic regression models with and without the 11 SNPs described in Table 4 demonstrated that together these SNPs explained 9.0% (R Square improved from 0.008 to 0.098) of the occurrence of clinical restenosis in this cohort.

As a final analysis we removed the 6 significantly associated genes from the complete set. Subsequent analysis of the subset of the other 30 genes did not demonstrate a remaining joined effect, $p = 0.65$ after 10,000 permutations.

**DISCUSSION**

With this study we aimed at clarifying the ambiguities regarding genetic predisposition for developing restenosis after PCI. We show that the joined effect of the complete spectrum of candidate genes, so far proposed to be involved in the restenotic process, results in a significant association with restenosis. This association is determined by six individual genes. Analyzing a subset containing the 30 genes not associated with the endpoint on an individual basis, did not show a remaining joined effect, making the involvement of genetic variation in these genes on restenosis development more unlikely.

The six associated genes span a wide range of different functions underlining the complexity of the disease. When examining the biological pathways with involvement...
| Gene     | Chr | Start (bp) | End (bp) | Size (kb) | SNPs | Sign. SNPs | Indep. SNPs | P-value |
|---------|-----|------------|----------|-----------|------|------------|-------------|---------|
| ADRB2   | 5   | 148 186 349| 148 188 381| 2.03      | 32   | 8          | 2           | 0.088   |
| AGER    | 6   | 32 256 724 | 32 260 001| 3.28      | 37   | 1          | 1           | 0.228   |
| AGTR1   | 3   | 149 898 348| 149 943 480| 45.13     | 100  | 5          | 1           | 0.028   |
| BCHE    | 3   | 166 973 387| 167 037 944| 64.56     | 101  | 8          | 2           | 0.314   |
| CCL11   | 17  | 29 636 800 | 29 639 312| 2.51      | 18   | 0          | 0           | 1.000   |
| CD14    | 5   | 139 991 501| 139 993 439| 1.94      | 22   | 4          | 2           | 1.000   |
| CDKN1B  | 12  | 12 761 576 | 12 766 569| 4.99      | 13   | 0          | 0           | 1.000   |
| Col3A1  | 2   | 189 547 344| 189 585 717| 38.37     | 97   | 2          | 2           | 0.649   |
| CSF2    | 5   | 131 437 384| 131 439 757| 2.37      | 28   | 0          | 0           | 0.965   |
| CX3CR1  | 3   | 39 279 990 | 39 596 531| 316.54    | 384  | 3          | 1           | 0.358   |
| CYBA    | 16  | 87 237 199 | 87 244 958| 7.76      | 14   | 1          | 1           | 0.182   |
| CYP2C19 | 10  | 96 512 453 | 96 602 660| 90.21     | 43   | 1          | 1           | 1.000   |
| F5      | 1   | 167 747 816| 167 822 393| 74.58     | 200  | 1          | 1           | 1.000   |
| GPX1    | 3   | 49 369 615 | 49 370 795| 1.18      | 8    | 1          | 1           | 0.024   |
| IL10    | 1   | 205 007 571| 205 012 462| 4.89      | 30   | 5          | 1           | 0.053   |
| IL1RN   | 2   | 113 601 609| 113 608 063| 6.45      | 62   | 0          | 0           | 0.991   |
| INSR    | 19  | 7 063 266  | 7 245 011 | 181.75    | 172  | 20         | 5           | 0.263   |
| ITGB2   | 21  | 45 130 299 | 45 165 303| 35.00     | 57   | 6          | 4           | 0.663   |
| LPL     | 8   | 19 841 058 | 19 869 049| 27.99     | 75   | 14         | 5           | 1.000   |
| MMP12   | 11  | 102 238 675| 102 250 922| 12.25     | 36   | 3          | 3           | 0.019   |
| MMP9    | 20  | 44 070 954 | 44 078 606| 7.65      | 23   | 10         | 3           | 0.067   |
| MTHFR   | 1   | 11 768 374 | 11 788 702| 20.33     | 61   | 1          | 1           | 1.000   |
| NOS3    | 7   | 150 319 080| 150 342 608| 23.53     | 20   | 0          | 0           | 0.987   |
| P2RY12  | 3   | 152 538 066| 152 585 234| 47.17     | 121  | 0          | 0           | 1.000   |
| SERPINE1| 7   | 100 556 303| 100 558 421| 2.12      | 27   | 0          | 0           | 0.863   |
| KAT2B   | 3   | 20 056 528 | 20 170 898| 114.37    | 144  | 19         | 4           | 0.023   |
| PPARG   | 3   | 12 304 349| 12 450 854| 146.51    | 144  | 14         | 5           | 1.000   |
| ROS1    | 6   | 117 716 223| 117 853 711| 137.49    | 206  | 1          | 1           | 0.631   |
| THBD    | 20  | 22 974 271 | 22 978 301| 4.03      | 22   | 0          | 0           | 1.000   |
| THBS4   | 5   | 79 366 747 | 79 414 861| 48.11     | 61   | 3          | 2           | 0.292   |
| THPO    | 3   | 185 572 467| 185 578 626| 6.16      | 16   | 1          | 1           | 0.165   |
| TNF     | 6   | 31 651 329 | 31 654 089| 2.76      | 41   | 2          | 2           | 0.370   |
| TP53    | 17  | 7 512 445 | 7 531 642| 19.20     | 17   | 1          | 1           | 0.120   |
| UCP3    | 11  | 73 388 958 | 73 397 778| 8.82      | 34   | 1          | 1           | 0.183   |
| VDR     | 12  | 46 521 589 | 46 585 081| 63.49     | 93   | 2          | 2           | 0.012   |

Chromosome and genomic region based on HapMap Rel 28 Phase II+III. P-value based on permutation (10,000). Abbreviations: SNPs, number of SNPs in genomic region including 10kb window; Sign.SNPs, number of SNPs with p < 0.05; Indep.SNPs, number of significant and independent SNPs, considering threshold of $R^2 < 0.1$. 

Table 3 Results of individual gene set-based analysis of genes previously associated with restenosis.
of these genes, only the VDR and KAT2B genes share a common pathway. The genes are both involved in the Vitamin D receptor pathway described by BioCarta.\textsuperscript{15} This pathway mainly involves the transcriptional regulating capacities of this receptor and is involved in controlling cellular growth, differentiation and apoptosis. Since these processes are all thought to be important contributors to the restenotic process, this indeed is a plausible pathway to be involved in restenosis development.\textsuperscript{1}

The rationale of set-based analysis is to overcome the marginally weak effect of single SNPs by analyzing a set of SNPs, since these SNPs could jointly have strong genetic effects. Most studies utilizing the candidate gene approach analyzed only one or at most a few SNPs within the gene of interest. The likelihood that exactly those SNPs are the causal or associated SNPs is of course small. A broader approach, like this set-based analysis, is therefore more likely to detect an associated gene by combining multiple SNPs with a possible marginal individual effect.\textsuperscript{16,17} For the current study we used the PLINK software\textsuperscript{14}, although multiple statistical programs are available for this type of analysis. Gui et al. compared 7 tests analyzing the WTCCC Crohn’s Disease dataset.\textsuperscript{18} One of their overall conclusions was that the set-based test in PLINK was the most powerful algorithm. Another study, applying PLINK set-based test, Global test, GRASS and SNP ratio test, for the analysis of three pathways regarding human longevity observed similar results with the different tests.\textsuperscript{19}

| Table 4 | Significantly associated SNPs of the 6 top genes. |
|---------|-----------------------------------------------|
| Gene    | SNP   | Chr | bp            | Function            | Alleles   | MAF  | OR  | p-value | Origin | Imputation quality |
|---------|-------|-----|---------------|---------------------|-----------|------|-----|---------|--------|-------------------|
|         | rs5182 | 3   | 149942085     | Exon, synonymous    | T/C       | 0.43 | 0.50 | 0.75    | 0.0040 | -                 |
| AGTR1   | rs1044291 | 4 | 155712802    | 3’UTR               | T/C       | 0.38 | 0.30 | 1.40    | 0.0028 | 0.970             |
| FGB     | rs8179164 | 3 | 49372288     | Promoter            | A/T       | 0.02 | 0.04 | 0.42    | 0.0077 | 0.993             |
| GPX1    | rs12808148 | 11 | 102238373    | Downstream         | C/T       | 0.16 | 0.09 | 1.82    | 0.00021| 0.953             |
| MMP12   | rs17099726 | 11 | 102257062    | Promoter            | G/T       | 0.03 | 0.06 | 0.54    | 0.032  | 0.957             |
|         | rs6776870 | 3 | 20126544     | Introns             | G/C       | 0.14 | 0.21 | 0.62    | 0.00064| 0.999             |
| KAT2B   | rs2929404 | 3 | 20069570     | Introns             | T/C       | 0.21 | 0.15 | 1.49    | 0.0026 | 0.981             |
|         | rs17796904 | 3 | 20096353     | Introns             | T/C       | 0.16 | 0.12 | 1.43    | 0.012  | -                 |
|         | rs4858767 | 3 | 20141941     | Introns             | G/C       | 0.29 | 0.34 | 0.79    | 0.037  | 0.994             |
| VDR     | rs11574027 | 12 | 46573640     | Introns             | T/G       | 0.03 | 0.007| 4.19    | 0.00014| -                 |
|         | rs11574077 | 12 | 46539194     | Introns             | G/A       | 0.07 | 0.04 | 1.60    | 0.029  | -                 |

SNP, single nucleotide polymorphism; Chr, chromosome; bp, base pair; MAF, minor allele frequency in control group; OR, odds ratio. The imputation quality indicates the average posterior probability for the most likely genotype generated by MACH, ranging from 0-1.
For the current study we analyzed the data using a threshold of linkage disequilibrium defined by $R^2 \geq 0.1$. The standard setting in PLINK is a $R^2$ of 0.5. In our opinion this threshold is too high for the intended analysis for this study. A higher threshold will include more SNPs in higher LD, which would be unfavorable, since we were interested in independent loci contributing to the risk of restenosis. By decreasing this threshold, only SNPs were selected that had a $R^2$ below 0.1, and thus independent of each other.

Although hypertension and multivessel disease were more frequent in cases compared to controls we decided not to correct for these variables. In the complete GENDER population these variables were not independent predictors for restenosis development\(^1\), so the differences in the current subpopulation likely resulted by chance during the selection process. Also, other studies provide no convincing data that hypertension is related to restenosis.\(^1\) It is therefore unlikely that previous associations of some of the current candidates genes (VDR, FGB, AGTR1 and GPX1) with hypertension\(^20-23\), have influenced our results, although this cannot be completely excluded.

A limitation of the current study could be that we analyzed imputed genotypic data, which introduces some amount of uncertainty. However, since we were interested in the combined effect of SNPs, an extensive genomic coverage was paramount for this analysis. Only analyzing the genotyped GWAS data would have resulted in the coverage of some of the smaller genes by only 1 or 2 SNPs. Therefore we decided that the more extensive genomic coverage of the imputed dataset outweighed the small introduction of possible error. A second limitation is that the analyses were only performed in the GENDER population. Availability of other populations with thorough genetic data on restenosis is however very limited. To our knowledge, the GWAS on restenosis in the GENDER population is the first, and only, examining this endpoint on a genome wide scale. Finally, the conclusions of this study are only based on genetic analyses. Functional studies should be performed to elucidate the biological consequences of these findings.

In conclusion, with these results we demonstrate that the efforts in unraveling the genetic factors influencing the risk of restenosis of the last years has resulted in a set of genes that joint together is indeed likely to be associated with restenosis, despite the overt inconsistencies of the individual studies. Confirmation of the association of these genes with the occurrence of restenosis after PCI helps our understanding of the genetic etiology of the disease. Future additional research strategies, like biological pathway analysis of GWAS data or even (exome) sequencing, might help us find the missing heritability of restenosis after PCI and increase our knowledge of the biological mechanistic background of restenosis development. This knowledge could subsequently result in identification of new treatment targets or development of novel preventive measure or risk stratification models.
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