Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Coronary Artery Calcification and Its Relationship to Validated Genetic Variants for Diabetes Mellitus Assessed in the Heinz Nixdorf Recall Cohort

Sonali Pechlivanis, André Scherag, Thomas W. Mühleisen, Stefan Möhlenkamp, Bernhard Horsthemke, Tanja Boes, Martina Bröcker-Preuss, Klaus Mann, Raimund Erbel, Karl-Heinz Jöckel, Markus M. Nöthen and Susanne Moebus

Arterioscler Thromb Vasc Biol. 2010;30:1867-1872; originally published online July 8, 2010;
doi: 10.1161/ATVBAHA.110.208496
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

Coronary Artery Calcification and Its Relationship to Validated Genetic Variants for Diabetes Mellitus Assessed in the Heinz Nixdorf Recall Cohort

Sonali Pechlivanis, André Scherag, Thomas W. Mühleisen, Stefan Möhlenkamp, Bernhard Horsthemke, Tanja Boes, Martina Bröcker-Preuss, Klaus Mann, Raimund Erbel, Karl-Heinz Jöckel, Markus M. Nöthen, Susanne Moebus, for the Heinz Nixdorf Recall Study Group

Objective—To examine the association between genomewide association study–based diabetes mellitus–related single-nucleotide polymorphisms (SNPs) and coronary artery calcification (CAC), a valid risk factor for coronary heart disease, in a large, unselected, population-based cohort.

Methods and Results—We genotyped 11 validated genomewide association study–based diabetes SNPs in 4459 participants of the Heinz Nixdorf Recall Study. We applied generalized linear regression models to explore the impact of the diabetes SNPs on CAC and to jointly model the effect of the SNPs and CAC on diabetes status. We observed a significant association between cyclin-dependent kinase inhibitor 2A/2B (*CDKN2A/2B*) variant *rs564398* and quantitative CAC ($P=1.81\times10^{-5}$ and adjusted $P=4.02\times10^{-4}$; odds ratio for the presence of CAC, 1.12 [95% CI, 1.02 to 1.25]). Moreover, we observed no strong impact of CAC on diabetes risk in the presence of the other genetic variants.

Conclusion—We show that a genetic variant near *CDKN2A/2B* that has been reported to be strongly associated with diabetes is strongly associated with CAC. In contrast, variants near insulin-like growth factor-binding protein 2 (*IGFBP2*), CDK5 regulatory subunit associated protein 1-like 1 (*CDKAL1*), solute carreir family 30 (zinc transporter), member 8 (*SLC30A8*), hematopoietically-expressed homeobox (*HHEX*), and transcription factor 7-like2 (*TCF7L2*) were clearly associated with diabetes; no evidence for an association to CAC was observable. This differential association pattern underlines the potential of endophenotypes, such as CAC, to extend the scope of disease outcome associations.

(*Arterioscler Thromb Vasc Biol*. 2010;30:1867-1872.)

Key Words: diabetes mellitus ■ coronary heart disease ■ coronary artery calcification ■ cohort study ■ polymorphism

The development of both diabetes mellitus and subclinical atherosclerosis is characterized by a presumed early onset.[1] Prediabetic subjects have an atherogenic pattern of risk factors, possibly caused by obesity, hyperglycemia, and hyperinsulinemia, which may be present for many years before diabetes.[2] Moreover, elevated coronary artery calcification (CAC) has also been repeatedly observed in diabetes cases in both case-control and cohort studies[1,3–5] from the general population.

Because individuals with diabetes are at high risk for coronary heart disease, we were primarily interested in examining whether there is a genetic association between diabetes-related single-nucleotide polymorphisms (SNPs) derived for genomewide association studies (GWAS)[6–11] and the extent of CAC in our population-based cohort study with 4459 unselected participants. As a secondary research question, we explored the known genetic association of the GWAS-based SNPs to diabetes as a clinical outcome in the presence or absence of CAC.

Methods

Study Population

We conducted a cross-sectional analysis among 4814 participants, aged 45 to 75 years, from the Heinz Nixdorf Recall (Risk Factors, Evaluation of Coronary Calcium, and Lifestyle) cohort. The participants were randomly selected from registration lists of the densely populated Ruhr metropolitan area in Germany (residents of Essen, Bochum, and Mülheim) between December 2000 and

Received on: February 10, 2010; final version accepted on: June 18, 2010.

From the Institute for Medical Informatics, Biometry and Epidemiology (S.P., A.S., T.B., K.-H.J., and S.M.), University Hospital of Essen, University Duisburg-Essen, Essen, Germany; the Department of Genomics (T.W.M. and M.M.N.), Life and Brain Center, University of Bonn, Bonn, Germany; the Clinic of Cardiology (S.M. and R.E.), West German Heart Centre, University Hospital of Essen, University Duisburg-Essen, Essen, Germany; the Institute of Human Genetics (B.H.), University Hospital of Essen, University Duisburg-Essen, Essen, Germany; the Department of Endocrinology and the Division of Laboratory Research (M.B.-P. and K.M.), University Hospital of Essen, University Duisburg-Essen, Essen, Germany.

Drs Pechlivanis and Scherag contributed equally.

Correspondence to Sonali Pechlivanis, PhD, Institute for Medical Informatics, Biometry and Epidemiology, University Hospital of Essen, University Duisburg-Essen, Essen, Germany. E-mail sonali.pechlivanis@uk-essen.de

Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.110.208496**

Table 1. Characteristics of the Study Population*

Variable	Males (n=2225)	Females (n=2234)
Age, y†	59.64±7.81	59.62±7.81
BMI†	28.01±3.96	27.64±5.19
BMI category		
<25	428 (19.30)	774 (34.60)
25–29.99	1215 (54.60)	830 (37.20)
≥30	582 (26.20)	630 (28.20)
Diabetes mellitus	389 (17.48)	221 (9.89)
CAC score‡	75.60 (6.70–352.00)	2.10 (0.00–43.65)
Log_e(CAC score+1)‡	4.33 (2.04–5.87)	1.13 (0.00–3.80)
CAC		
0	349 (15.69)	980 (43.87)
>0	1876 (84.31)	1254 (56.13)

BMI indicates body mass index (calculated as weight in kilograms divided by height in meters squared).

*Data are given as number (percentage) unless otherwise indicated.

†Data are given as mean±SD.

‡Data are given as median (quartile 1-quartile 3).

August 2003. The rationale and design of the study were previously described in detail.[12] The baseline response of the study was 56%,[13] which is comparable to rates of other population-based studies. Information on genotypes, sex, age, CAC, and diabetes was available for up to 4459 of the 4814 participants (Table 1). The study was approved by the local ethics committees and was conducted in accordance with the German *Gute Epidemiologische Praxis*, including extended quality management procedures and recertifications according to Deutsches Institut für Normung e.V. International Organization for Standardization (DIN ISO) 9001:2000. Informed consent was obtained from all participants.

The clinical outcome, diabetes (n=610), was defined as either of 4 criteria: (1) participants reported a history of diabetes, (2) participants took glucose-lowering drugs, (3) participants had fasting glucose levels of greater than 125 mg/dL, or (4) participants had nonfasting glucose levels of 200 mg/dL or greater. CAC was assessed by nonenhanced electron-beam computed tomographic scans (C-150 scanner; GE Imatron, San Francisco, Calif), as previously described.[3] Body mass index was calculated from standardized measurements of height and weight (weight in kilograms divided by height in meters squared), with participants in light underwear and without shoes. Table 1 presents the clinical characteristics of the study participants.

SNP Selection and Genetic Analyses

PubMed was searched for the keywords diabetes, SNP, CAC, polymorphism, GWAS, and replication between June 2008 and January 2009. Within this interval, 4 GWAS and several replication studies related to diabetes were published.[6–11] The following SNPs (with the related genes and the minimum reported pairwise r^2 in parentheses) were selected using a threshold of $P<10^{-6}$ from the studies of Zeggini et al[10] and Steinthorsdottir et al[11]: *rs4402960* (*IGF2BP2*), *rs1801282* (peroxisome proliferator-activated receptor gamma [*PPARG*]), *rs7754840*, *rs7756992*, and *rs10946398* (*CDKAL1*, $r^2>0.93$), *rs13266634* (*SLC30A8*), *rs10811661* and *rs564398* (*CDKN2A/2B*, $r^2<0.001$), *rs7903146* in *TCF7L2*[7] ($r^2=0.94$ with *rs7901695*), *rs1111875* (*HHEX*, $r^2=1$ with *rs5015480*), and *rs8050136* (fat mass and obesity associated [*FTO*]). In addition, we also selected *rs5219* potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*), but this SNP could not be included in the same genotyping assay and had to be omitted from this report.

Genotyping of all other SNPs in the population-based Heinz Nixdorf Recall Study was performed by matrix-assisted laser desorption ionization–time of flight mass spectrometry–based iPLEX Gold assay at the Department of Genomics, Life and Brain Center, Bonn, Germany.

Statistical Analyses

The genotype distributions of all 11 SNPs were tested for deviations from the Hardy-Weinberg equilibrium (exact 2-sided $P>0.05$). All analyses were performed under a (log-) additive genetic model for each SNP, as suggested in the previous studies.[8,9]

First, we investigated the primary outcome, CAC, for an association to the GWAS-based SNPs (1). Second, we checked the association of the validated GWAS-based SNPs to the clinical outcome, diabetes, in the presence or absence of CAC (2). For the primary outcome, CAC, we performed 3 analyses (1a–1c). As the first analysis for the primary research question (1a), we used linear regression to analyze the untransformed quantitative CAC outcome in individuals for whom CAC was present (adjusted for sex, age, and diabetes status). This restriction was done to meet the normality assumption on which the modeling and the power consideration is based (as explained later). Next (1b), we used logistic regression for the binary CAC outcome (CAC >0 as presence of CAC and CAC=0 as absence of CAC, adjusted for sex, age, and diabetes status). Finally, we used all available CAC data and addressed the marked right-skewed distribution of CAC by a log_e transformation of CAC score plus 1, as previously suggested.[3] We applied linear regression to the transformed quantitative CAC outcome (adjusted for sex, age, and diabetes status; 1c). Because the conclusions for all these analyses were similar, we decided to limit the presentation to the analyses (1c) (Table 2).

To address the secondary research question (ie, the association of the validated GWAS-based SNPs to the clinical outcome, diabetes, in the presence or absence of CAC), we used logistic regression analyses adjusted for sex and age with and without log_e-transformed CAC (Table 3). In a last step, we also explored the impact of the dependency between the GWAS-based SNPs (related to 8 genes) and the other covariates on each of the 2 outcomes (CAC and diabetes) using backward selection for all predictors.

To our knowledge, because an association of the SNP to CAC has not been demonstrated previously, we decided to control the familywise error rate of the primary research question relating the SNPs to CAC at $\alpha=5\%$. Consequently, we corrected for 11 SNPs/statistical tests that translate into $\alpha_{BF}\approx0.005$ using the Bonferroni procedure. For the primary analysis, we also performed power calculations using QUANTO Version 1.2.3 (http://hydra.usc.edu/gxe) for common variants, assuming a minor allele frequency of 7.5%, which is the smallest minor allele frequency of all explored SNPs, as based on the HapMap-Utah residents with Northern and Western European ancestry (CEU) samples (http://www.hapmap.org) and $\alpha=0.005$ (2-sided). For a sample of 3130 individuals (those with CAC >0 mean, 336.20; SD, 715.30), the comparisonwise power estimate was 91% (or 24%) assuming a standard normally distributed quantitative trait locus and standardized effect sizes of 0.2 (or 0.1) in SDs for each risk allele under an additive mode of inheritance without dominance effects. Thus, our study was well powered to detect relatively strong effect sizes of CAC-predisposing variants when controlling for multiple testing.

In addition, we determined 95% CIs for all estimates and report nominal 2-sided probability values that are not adjusted for multiple testing (unless otherwise stated).

Results

Genetic Associations to CAC

Table 2 shows the effect size estimators (βs) for the linear regression models with quantitative transformed CAC (log_e[CAC score +1]) for 11 genetic markers related to 8 GWAS-based confirmed diabetes candidate genes. The marker *rs564398* (*CDKN2A/2B*) was the only marker that showed an association to quantitative CAC with a significant

Table 2. Results of the Genetic Association Analyses for Log_e(CAC Score+1) Using SNPs (Additive Genetic Model), Sex, Age, and Diabetes Mellitus Status as Predictors in a Linear Regression Model*

				Minor Allele		Log_e(CAC Score+1)		All-Marker Model for Log_e (CAC Score+1)	
CHR	Gene	SNP	Physical Position	Designator	Frequency in the Cohort	β (95% CI)	*P* Value	β (95% CI)	*P* Value
3	*PPARG*	*rs1801282*	12368125	G	0.14	.04 (−.08 to .17)	0.50	NA	NA
	IGF2BP2	*rs4402960*	186994381	T	0.31	.04 (−.06 to .13)	0.50	NA	NA
6	*CDKAL1*	*rs10946398*	20769013	C	0.33	.01 (−.08 to .10)	0.80	NA	NA
		rs7754840	20769229	C	0.33	.02 (−.07 to .12)	0.63	NA	NA
		rs7756992	20787688	G	0.29	.01 (−.09 to .11)	0.82	NA	NA
8	*SLC30A8*	*rs13266634*	118253964	T	0.31	.02 (−.08 to .11)	0.74	NA	NA
9	*CDKN2A/2B*	*rs564398*	22019547	G	0.42	−.20 (−.30 to −.11)	1.81×10^{-5}	−.20 (−.29 to −.10)	$<10^{-4}$
		rs10811661	22124094	C	0.17	.05 (−.06 to .18)	0.35	NA	NA
10	*HHEX*	*rs1111875*	94452862	A	0.40	−.03 (−.12 to .06)	0.53	NA	NA
	TCF7L2	*rs7903146*	114748339	T	0.27	−.01 (−.11 to .09)	0.90	NA	NA
16	*FTO*	*rs8050136*	52373776	A	0.41	.05 (−.04 to .14)	0.25	NA	NA

NA indicates not applicable; OR, odds ratio; CHR, chromosome.

*The columns to the right display the impact of all predictors on the outcome log_e(CAC score+1), applying backward selection on all predictors (sex, age, diabetes status, and 9 SNPs related to 8 genes); only the results for the finally included SNPs are displayed. The Statistical Analyses subsection of the Methods section provides additional information.

probability value even after adjustment for multiple testing in the primary model (model 1a; data not shown; $P=0.003$). The findings for the transformed quantitative CAC in Table 2 ($\beta_{rs564398}=-.20$ [95% CI, −.30 to −.11]; $P=1.81\times10^{-5}$) and for the dichotomized CAC outcome (CAC=0 versus CAC >0; data not shown) were consistent with this observation (for dichotomized CAC: odds ratio of *rs564398*=0.89 [95% CI, 0.80–0.98]; $P=0.03$). In addition, Table 2 also shows that the impact of *CDKN2A/2B rs564398*, in the presence of all other covariates (age, sex, diabetes status, and 9 SNPs related to 8 genes), is based on backward model selection for transformed CAC. To explore the observed effect of *rs564398* beyond the models described in the Statistical Analyses subsection of the Methods section, we performed sex-stratified analyses and allowed for an interaction of genotype and sex in the analyses of the outcome-transformed CAC. We observed an effect for the interaction with sex ($\beta_{rs564398\times sex}=.21$ [95% CI, .03-.40]; $P=0.03$) and when stratified according to sex; the effect was stronger in or limited to males ($\beta_{rs564398}=-.30$ [95% CI, −.43 to −.15]; $P=3.27\times10^{-5}$) versus females ($\beta_{rs564398}=-.13$ [95% CI, −.26 to −.0007]; $P=0.05$) (supplemental Table I; available

Table 3. Results of the Genetic Association Analyses for Diabetes Mellitus Using SNPs (Additive Genetic Model), Sex, and Age, With and Without Log_e(CAC+1) as Predictors in a Logistic Regression Model*

					Diabetes Without Log_e(CAC+1)		Diabetes With Log_e(CAC+1)		All Marker-Model for Diabetes	
CHR	Gene	SNP	Physical Position	Minor Allele	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
3	*PPARG*	*rs1801282*	12368125	G	0.90 (0.75–1.08)	0.27	0.91 (0.76–1.10)	0.32	NA	NA
	IGF2BP2	*rs4402960*	186994381	T	1.27 (1.12–1.44)	3.04×10^{-5}	1.26 (1.10–1.44)	7.09×10^{-4}	1.23 (1.06–1.41)	0.004
6	*CDKAL1*	*rs10946398*	20769013	C	1.07 (0.94–1.22)	0.28	1.08 (0.95–1.24)	0.23	NA	NA
		rs7754840	20769229	C	1.06 (0.93–1.20)	0.41	1.06 (0.93–1.21)	0.38	NA	NA
		rs7756992	20787688	G	1.16 (1.01–1.33)	0.04	1.17 (1.02–1.35)	0.03	1.20 (1.04–1.40)	0.01
8	*SLC30A8*	*rs13266634*	118253964	T	0.87 (0.76–0.99)	0.04	0.86 (0.75–0.99)	0.03	0.85 (0.73–0.98)	0.03
9	*CDKN2A/2B*	*rs564398*	22019547	G	0.92 (0.81–1.05)	0.21	0.96 (0.84–1.10)	0.58	NA	NA
		rs10811661	22124094	C	0.99 (0.84–1.18)	0.96	0.99 (0.83–1.17)	0.90	NA	NA
10	*HHEX*	*rs1111875*	94452862	A	0.88 (0.78–0.99)	0.04	0.89 (0.79–1.01)	0.09	NA	NA
	TCF7L2	*rs7903146*	114748339	T	1.28 (1.18–1.46)	1.67×10^{-5}	1.30 (1.14–1.49)	1.08×10^{-4}	1.30 (1.13–1.49)	3×10^{-4}
16	*FTO*	*rs8050136*	52373776	A	1.08 (0.95–1.22)	0.23	1.08 (0.95–1.22)	0.26	NA	NA

NA indicates not applicable; OR, odds ratio; CHR, chromosome.

*The columns to the right display the impact of all predictors on the outcome diabetes, applying backward selection on all predictors (sex, age, log_e[CAC+1], and 9 SNPs related to 8 genes); only the results for the finally included SNPs are displayed. The Statistical Analyses subsection of the Methods section provides additional information.

online at http://atvb.ahajournals.org). We also stratified the sample by diabetes status and found similar effect size estimators for *rs564398* and transformed CAC ($\beta_{\text{with diabetes}}=-.18$ and $\beta_{\text{without diabetes}}=-.20$). Supplemental Table II summarizes the genotype distribution of the *CDKN2A/2B rs564398* by dichotomized CAC and diabetes status. Clearly, even in the absence of diabetes, the effect of *rs564398* on CAC is still present. Finally, we explored the association of this particular marker (*rs564398*) with coronary heart disease as the result of reports on nearby coronary heart disease markers[14] (eg, *rs4977574*, which is 69 kb downstream of *rs564398* (database SNP 36.3), with an r^2 of 0.27 between the markers; HapMap CEU release 22). We observed a crude odds ratio of *rs564398* of 0.90 (95% CI, 0.76–1.08; $P=0.27$) for each *G* allele, which changed to an odds ratio of *rs564398* of 0.88 (95% CI, 0.74–1.06; $P=0.18$) after adjustment for sex and age using logistic regression.

Genetic Associations to Diabetes in the Presence or Absence of CAC as a Risk Factor for Diabetes

Table 3 shows the association of the 11 GWAS-based diabetes SNPs with diabetes in the presence or absence of CAC. We found the strongest effect sizes for markers related to *IGF2BP2*, *CDKAL1*, *SLC30A8, HHEX*, and *TCF7L2*. When CAC was included in this model, the point estimators were not altered by more than 0.04. Finally, exploring the impact of sex, age, CAC, and the 9 SNPs related to 8 genes, again the same 4 of 5 diabetes-associated SNPs contributed independently to diabetes risk in addition to age, sex, and CAC (Table 3).

Discussion

The results of our study show the impact of the GWAS-based diabetes SNPs on the outcome of CAC and the joint relationship of these SNPs and CAC on the outcome of diabetes. We observed a significant association between *rs564398* (*CDKN2A/2B*) and CAC. This association was significant after conservatively controlling for multiple testing by the Bonferroni method. Moreover, *rs564398* (*CDKN2A/2B*) remained an independent predictor of CAC even after adjusting for sex, age, and diabetes status or all other diabetes markers in the multiple regression model. Moreover, we observed that the association of *rs564398* (*CDKN2A/2B*) with CAC was largely because of stronger effects in males. In contrast to the problems that have been reported when testing for sex-specific effects,[15] we expected to see such sex differences given the larger load of CAC in males.[16,17] However, follow-up studies are necessary to confirm our CAC findings.

With regard to the secondary research question (ie, the impact of the genetic markers on diabetes risk in the presence or absence of CAC), we replicated the diabetes association of risk alleles reported in the literature for 5 SNPs, *rs4402960* (*IGF2BP2*), *rs7756992* (*CDKAL1*), *rs13266634* (*SLC30A8*), *rs1111875* (*HHEX*), and *rs7903146* (*TCF7L2*), in an unselected population-based cohort at a nominal α level of 5%.[6–10] All other SNPs showed directionally consistent effects when compared with those reported in the literature[6–10,18–20] ($P<0.001$ for a binomial test). Except for *rs564398*, the point estimates for diabetes risk of all investigated SNPs did not vary much after including CAC in the model.

The marker *rs564398* is located approximately 100 kb 5′ of gene *CDKN2A/2B*. *CDKN2A* and *CDKN2B* encode the prototypic tumor suppressor protein (INK)-4 proteins p16INK4a and p15INK4b, respectively, which play an important role in the regulation of the cell cycle, β-cell proliferation, and transforming growth factor β–induced growth inhibition.[21–23] Through transforming growth factor β/Sma- and Mad-related protein signaling, this region may be implicated in the pathogenesis of atherosclerosis.[21] Recently, it was shown that *ANRIL* (another predicted gene within the 9p21 region) expression is associated with atherosclerosis and 9p21 region SNPs.[24] To summarize, the 9p21 region has been strongly associated with susceptibility to coronary artery disease, myocardial infarction (to other largely independent markers, such as *rs4977574*), and diabetes (in particular *rs564398*), suggesting the possibility of a shared mechanism causing subclinical atherosclerosis, coronary artery disease, and diabetes.[7,8,10,25–27] In the light of our results, however, functional or animal studies should focus on atherosclerotic phenotypes first and might benefit from including sex information as well.

However, this study also has some limitations. In light of emerging GWAS meta-analyses, a sample size of approximately 4400 individuals is relatively limited with regard to statistical power to confirm the reported diabetes associations. As a consequence, this study focused on CAC and a particular set of GWAS-based diabetes markers attenuating the multiplicity problem. Furthermore, our population-based cohort has not been included in any of the GWAS detection meta-analyses. Thus, we were able to take an independent look at the available evidence. Another limitation is the selection of GWAS-based diabetes markers, given that the recent review already described 19 genomic loci related to diabetes status.[28] Thus, our investigation of CAC and its relationship to diabetes is not comprehensive. On the other hand, the effects on diabetes risk are even smaller for the new markers, most likely leading to even more pronounced power problems for our analyses. Some of these issues may become addressable once genotype data for the Metabochip (designed for fine mapping to follow up on findings from several large-scale consortia, such as CARDIoGRAM (Coronary ARtery DIsease Genome-wide Replication And Meta-Analysis), DIAGRAM (Diabetes Genetics Replication And Meta-analysis Consortium), GIANT (Genomewide Investigation of ANThropometric measures), MAGIC (Meta-Analysis of Glucose and Insulin-related traits Consortium), Lipids (Genome-wide association study of Lipids), ICBP-GWAS (Genome-wide association study of blood pressure), and QT-IGC (Genome-wide association study for QT interval) become available for the Heinz Nixdorf Recall Study cohort.

In conclusion, we provide evidence for an association of *rs564398* near *CDKN2A/2B* to CAC. Thus, this genetic locus is more likely related to subclinical atherosclerotic processes and to diabetes as a secondary phenomenon. We further substantiate that genetic variation in *IGF2BP2, CDKAL1, SLC30A8, HHEX*, and *TCF7L2* is associated with diabetes and largely independent of subclinical atherosclerotic pro-

cesses. By investigating the effects of GWAS-based diabetes SNPs of likely functional relevance and the burden of CAC in an unselected central European population, we underline the potential of using (endo)phenotypes, such as CAC, to address yet unknown properties of the genetic markers from a genetic-epidemiological point of view.

Acknowledgments

We are indebted to the all study participants and to the dedicated personnel of both the study centre of the Heinz Nixdorf Recall Study and the electron beam tomography-scanner facilities as well as to the investigative group, in particular to U Roggenbuck, S Slomiany, EM Beck, A Öffner, S Münkel, M Bauer, S Schrader, R Peter, and H Hirche. Advisory Board: Meinertz T, Hamburg, Germany (Chair); Bode C, Freiburg, Germany; de Feyter PJ, Rotterdam, Netherlands; Güntert B, Hall i. T. (Austria); Gutzwiller F, Bern, Switzerland; Heinen H, Bonn, Germany; Hess O, Bern, Switzerland; Klein B, Essen, Germany; Löwel H, Neuherberg, Germany; Reiser M, Munich, Germany; Schmidt G (†), Essen, Germany; Schwaiger M, Munich, Germany; Steinmüller C, Bonn, Germany; Theorell T, Stockholm, Sweden; Willich SN, Berlin, Germany. Criteria and endpoint committee: C. Bode, Freiburg, Germany (Chair); K. Berger, Münster, Germany; HR. Figulla, Jena, Germany; C. Hamm, Bad Nauheim, Germany; P. Hanrath, Aachen, Germany; W. Köpcke, Münster, Germany; Ringelstein, Münster, Germany; C. Weimar, Essen, Germany; A. Zeiher, Frankfurt, Germany.

Sources of Funding

This study was supported by the Heinz Nixdorf Foundation; grants Nationales Genomforschungsnetz and 01GS0820 from the German Ministry of Education and Science; projects SI 236/8-1 and SI 236/9-1 from the German Research Council; and Sarstedt AG & Co (laboratory equipment).

Disclosures

None.

References

1. Meigs JB, Larson MG, D'Agostino RB, Levy D, Clouse ME, Nathan DM, Wilson PW, O'Donnell CJ. Coronary artery calcification in type 2 diabetes and insulin resistance: the Framingham offspring study. *Diabetes Care*. 2002;25:1313–1319.
2. Haffner SM, Stern MP, Hazuda HP, Mitchell BD, Patterson JK. Cardiovascular risk factors in confirmed prediabetic individuals: does the clock for coronary heart disease start ticking before the onset of clinical diabetes? *JAMA*. 1990;263:2893–2898.
3. Moebus S, Stang A, Mohlenkamp S, Dragano N, Schmermund A, Slomiany U, Hoffmann B, Bauer M, Broecker-Preuss M, Mann K, Siegrist J, Erbel R, Jockel KH. Association of impaired fasting glucose and coronary artery calcification as a marker of subclinical atherosclerosis in a population-based cohort: results of the Heinz Nixdorf Recall Study. *Diabetologia*. 2009;52:81–89.
4. Schurgin S, Rich S, Mazzone T. Increased prevalence of significant coronary artery calcification in patients with diabetes. *Diabetes Care*. 2001;24:335–338.
5. Wolfe ML, Iqbal N, Gefter W, Mohler ER III, Rader DJ, Reilly MP. Coronary artery calcification at electron beam computed tomography is increased in asymptomatic type 2 diabetics independent of traditional risk factors. *J Cardiovasc Risk*. 2002;9:369–376.
6. Cauchi S, Meyre D, Durand E, Proenca C, Marre M, Hadjadj S, Choquet H, De GF, Gaget S, Allegaert F, Delplanque J, Permutt MA, Wasson J, Blech I, Charpentier G, Balkau B, Vergnaud AC, Czernichow S, Patsch W, Chikri M, Glaser B, Sladek R, Froguel P. Post genome-wide association studies of novel genes associated with type 2 diabetes show gene-gene interaction and high predictive value. *PLoS ONE*. 2008;3:e2031.
7. Saxena R, Voight BF, Lyssenko V, Burtt NP, de Bakker PI, Chen H, Roix JJ, Kathiresan S, Hirschhorn JN, Daly MJ, Hughes TE, Groop L, Altshuler D, Almgren P, Florez JC, Meyer J, Ardlie K, Bengtsson BK, Isomaa B, Lettre G, Lindblad U, Lyon HN, Melander O, Newton-Cheh C, Nilsson P, Orho-Melander M, Rastam L, Speliotes EK, Taskinen MR, Tuomi T, Guiducci C, Berglund A, Carlson J, Gianniny L, Hackett R, Hall L, Holmkvist J, Laurila E, Sjogren M, Sterner M, Surti A, Svensson M, Svensson M, Tewhey R, Blumenstiel B, Parkin M, Defelice M, Barry R, Brodeur W, Camarata J, Chia N, Fava M, Gibbons J, Handsaker B, Healy C, Nguyen K, Gates C, Sougnez C, Gage D, Nizzari M, Gabriel SB, Chirn GW, Ma Q, Parikh H, Richardson D, Ricke D, Purcell S. Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science*. 2007;316:1331–1336.
8. Scott LJ, Mohlke KL, Bonnycastle LL, Willer CJ, Li Y, Duren WL, Erdos MR, Stringham HM, Chines PS, Jackson AU, Prokunina-Olsson L, Ding CJ, Swift AJ, Narisu N, Hu T, Pruim R, Xiao R, Li XY, Conneely KN, Riebow NL, Sprau AG, Tong M, White PP, Hetrick KN, Barnhart MW, Bark CW, Goldstein JL, Watkins L, Xiang F, Saramies J, Buchanan TA, Watanabe RM, Valle TT, Kinnunen L, Abecasis GR, Pugh EW, Doheny KF, Bergman RN, Tuomilehto J, Collins FS, Boehnke M. A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants. *Science*. 2007;316:1341–1345.
9. Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D, Boutin P, Vincent D, Belisle A, Hadjadj S, Balkau B, Heude B, Charpentier G, Hudson TJ, Montpetit A, Pshezhetsky AV, Prentki M, Posner BI, Balding DJ, Meyre D, Polychronakos C, Froguel P. A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature*. 2007;445:881–885.
10. Zeggini E, Weedon MN, Lindgren CM, Frayling TM, Elliott KS, Lango H, Timpson NJ, Perry JR, Rayner NW, Freathy RM, Barrett JC, Shields B, Morris AP, Ellard S, Groves CJ, Harries LW, Marchini JL, Owen KR, Knight B, Cardon LR, Walker M, Hitman GA, Morris AD, Doney AS, McCarthy MI, Hattersley AT. Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. *Science*. 2007;316:1336–1341.
11. Steinthorsdottir V, Thorleifsson G, Reynisdottir I, Benediktsson R, Jonsdottir T, Walters GB, Styrkarsdottir U, Gretarsdottir S, Emilsson V, Ghosh S, Baker A, Snorradottir S, Bjarnason H, Ng MC, Hansen T, Bagger Y, Wilensky RL, Reilly MP, Adeyemo A, Chen Y, Zhou J, Gudnason V, Chen G, Huang H, Lashley K, Doumatey A, So WY, Ma RC, Andersen G, Borch-Johnsen K, Jorgensen T, van Vliet–Ostaptchouk JV, Hofker MH, Wijmenga C, Christiansen C, Rader DJ, Rotimi C, Gurney M, Chan JC, Pedersen O, Sigurdsson G, Gulcher JR, Thorsteinsdottir U, Kong A, Stefansson K. A variant in CDKAL1 influences insulin response and risk of type 2 diabetes. *Nat Genet*. 2007;39:770–775.
12. Schmermund A, Mohlenkamp S, Stang A, Gronemeyer D, Seibel R, Hirche H, Mann K, Siffert W, Lauterbach K, Siegrist J, Jockel KH, Erbel R. Assessment of clinically silent atherosclerotic disease and established and novel risk factors for predicting myocardial infarction and cardiac death in healthy middle-aged subjects: rationale and design of the Heinz Nixdorf RECALL Study; Risk Factors, Evaluation of Coronary Calcium and Lifestyle. *Am Heart J*. 2002;144:212–218.
13. Stang A, Moebus S, Dragano N, Beck EM, Mohlenkamp S, Schmermund A, Siegrist J, Erbel R, Jockel KH. Baseline recruitment and analyses of nonresponse of the Heinz Nixdorf Recall Study: identifiability of phone numbers as the major determinant of response. *Eur J Epidemiol*. 2005;20:489–496.
14. Arking DE, Chakravarti A. Understanding cardiovascular disease through the lens of genome-wide association studies. *Trends Genet*. 2009;25:387–394.
15. Patsopoulos NA, Tatsioni A, Ioannidis JP. Claims of sex differences: an empirical assessment in genetic associations. *JAMA*. 2007;298:880–893.
16. McClelland RL, Chung H, Detrano R, Post W, Kronmal RA. Distribution of coronary artery calcium by race, gender, and age: results from the Multi-Ethnic Study of Atherosclerosis (MESA). *Circulation*. 2006;113:30–37.
17. Newman AB, Naydeck BL, Sutton-Tyrrell K, Feldman A, Edmundowicz D, Kuller LH. Coronary artery calcification in older adults to age 99: prevalence and risk factors. *Circulation*. 2001;104:2679–2684.
18. Cauchi S, El AY, Choquet H, Dina C, Krempler F, Weitgasser R, Nejjari C, Patsch W, Chikri M, Meyre D, Froguel P. TCF7L2 is reproducibly associated with type 2 diabetes in various ethnic groups: a global meta-analysis. *J Mol Med*. 2007;85:777–782.
19. Lango H, Palmer CN, Morris AD, Zeggini E, Hattersley AT, McCarthy MI, Frayling TM, Weedon MN. Assessing the combined impact of 18 common genetic variants of modest effect sizes on type 2 diabetes risk. *Diabetes*. 2008;57:3129–3135.
20. Zeggini E, Scott LJ, Saxena R, Voight BF, Marchini JL, Hu T, de Bakker PI, Abecasis GR, Almgren P, Andersen G, Ardlie K, Bostrom KB,

Bergman RN, Bonnycastle LL, Borch-Johnsen K, Burtt NP, Chen H, Chines PS, Daly MJ, Deodhar P, Ding CJ, Doney AS, Duren WL, Elliott KS, Erdos MR, Frayling TM, Freathy RM, Gianniny L, Grallert H, Grarup N, Groves CJ, Guiducci C, Hansen T, Herder C, Hitman GA, Hughes TE, Isomaa B, Jackson AU, Jorgensen T, Kong A, Kubalanza K, Kuruvilla FG, Kuusisto J, Langenberg C, Lango H, Lauritzen T, Li Y, Lindgren CM, Lyssenko V, Marvelle AF, Meisinger C, Midthjell K, Mohlke KL, Morken MA, Morris AD, Narisu N, Nilsson P, Owen KR, Palmer CN, Payne F, Perry JR, Pettersen E, Platou C, Prokopenko I, Qi L, Qin L, Rayner NW, Rees M, Roix JJ, Sandbaek A, Shields B, Sjogren M, Steinthorsdottir V, Stringham HM, Swift AJ, Thorleifsson G, Thorsteinsdottir U, Timpson NJ, Tuomi T, Tuomilehto J, Walker M, Watanabe RM, Weedon MN, Willer CJ, Illig T, Hveem K, Hu FB, Laakso M, Stefansson K, Pedersen O, Wareham NJ, Barroso I, Hattersley AT, Collins FS, Groop L, McCarthy MI, Boehnke M, Altshuler D. Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes. *Nat Genet*. 2008; 40:638–645.

21. Kalinina N, Agrotis A, Antropova Y, Ilyinskaya O, Smirnov V, Tararak E, Bobik A. Smad expression in human atherosclerotic lesions: evidence for impaired TGF-beta/Smad signaling in smooth muscle cells of fibrofatty lesions. *Arterioscler Thromb Vasc Biol*. 2004;24:1391–1396.
22. Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: progress and puzzles. *Curr Opin Genet Dev*. 2003;13:77–83.
23. Mettus RV, Rane SG. Characterization of the abnormal pancreatic development, reduced growth and infertility in Cdk4 mutant mice. *Oncogene*. 2003;22:8413–8421.
24. Holdt LM, Beutner F, Scholz M, Gielen S, Gabel G, Bergert H, Schuler G, Thiery J, Teupser D. ANRIL expression is associated with atherosclerosis risk at chromosome 9p21. *Arterioscler Thromb Vasc Biol*. 2010;30: 620–627.
25. Helgadottir A, Thorleifsson G, Magnusson KP, Gretarsdottir S, Steinthorsdottir V, Manolescu A, Jones GT, Rinkel GJ, Blankensteijn JD, Ronkainen A, Jaaskelainen JE, Kyo Y, Lenk GM, Sakalihasan N, Kostulas K, Gottsater A, Flex A, Stefansson H, Hansen T, Andersen G, Weinsheimer S, Borch-Johnsen K, Jorgensen T, Shah SH, Quyyumi AA, Granger CB, Reilly MP, Austin H, Levey AI, Vaccarino V, Palsdottir E, Walters GB, Jonsdottir T, Snorradottir S, Magnusdottir D, Gudmundsson G, Ferrell RE, Sveinbjornsdottir S, Hernesniemi J, Niemela M, Limet R, Andersen K, Sigurdsson G, Benediktsson R, Verhoeven EL, Teijink JA, Grobbee DE, Rader DJ, Collier DA, Pedersen O, Pola R, Hillert J, Lindblad B, Valdimarsson EM, Magnadottir HB, Wijmenga C, Tromp G, Baas AF, Ruigrok YM, van Rij AM, Kuivaniemi H, Powell JT, Matthiasson SE, Gulcher JR, Thorgeirsson G, Kong A, Thorsteinsdottir U, Stefansson K. The same sequence variant on 9p21 associates with myocardial infarction, abdominal aortic aneurysm and intracranial aneurysm. *Nat Genet*. 2008;40:217–224.
26. McPherson R, Pertsemlidis A, Kavaslar N, Stewart A, Roberts R, Cox DR, Hinds DA, Pennacchio LA, Tybjaerg-Hansen A, Folsom AR, Boerwinkle E, Hobbs HH, Cohen JC. A common allele on chromosome 9 associated with coronary heart disease. *Science*. 2007;316: 1488–1491.
27. Samani NJ, Erdmann J, Hall AS, Hengstenberg C, Mangino M, Mayer B, Dixon RJ, Meitinger T, Braund P, Wichmann HE, Barrett JH, Konig IR, Stevens SE, Szymczak S, Tregouet DA, Iles MM, Pahlke F, Pollard H, Lieb W, Cambien F, Fischer M, Ouwehand W, Blankenberg S, Balmforth AJ, Baessler A, Ball SG, Strom TM, Braenne I, Gieger C, Deloukas P, Tobin MD, Ziegler A, Thompson JR, Schunkert H. Genomewide association analysis of coronary artery disease. *N Engl J Med*. 2007;357: 443–453.
28. Wolfs MG, Hofker MH, Wijmenga C, van Haeften TW. Type 2 diabetes mellitus: new genetic insights will lead to new therapeutics. *Curr Genomics*. 2009;10:110–118.

Supplementary Table I. Results of the genetic association analyses for $\log_e$(CAC score+1) in a linear regression model using SNPs (additive genetic model), age and diabetes as predictors for females and males.

CHR	Gene	SNP	physical position	Minor allele	Minor allele Frequency in females	Females		Males	
						Beta (95%CI)	P	Beta (95%CI)	P
3	*PPARG*	rs1801282	12368125	G	0.14	0.15 (-0,3;0.33)	0.10	-0.05 (-0.24;0.14)	0.60
	IGF2BP2	rs4402960	186994381	T	0.32	0.11 (-0.01;0.24)	0.08	-0.04 (-0.19;0.10)	0.56
6	*CDKAL1*	rs10946398	20769013	C	0.33	0.03 (-0.10;0.16)	0.68	-0.005 (-0.15;0.14)	0.94
		rs7754840	20769229	C	0.33	0.04 (-0.94;0.16)	0.60	0.008 (-0.13 ;0.15)	0.92
		rs7756992	20787688	G	0.29	0.01 (-0.13 ;0.15)	0.89	0.01 (-0.14 ;0.16)	0.89
8	*SLC30A8*	rs13266634	118253964	T	0.31	0.04 (-0.09;0.17)	0.58	-0.007 (-0.15;0.13)	0.92
9	*CDKN2A/2B*	rs564398	22019547	G	0.42	-0.13 (-0.26;-0.0007)	0.05	-0.30 (-0.43;-0.15)	$3.27x10^{-5}$
		rs10811661	22124094	C	0.18	0.03 (-0.13;0.20)	0.70	0.09 (-0.09;0.27)	0.33
10	*HHEX*	rs1111875	94452862	A	0.40	-0.06 (-0.17;0.07)	0.41	-0.01 (-0.14;0.12)	0.86
	TCF7L2	rs7903146	114748339	T	0.27	0.10 (-0.04;0.24)	0.15	-0.11 (-0.26;0.03)	0.13
16	*FTO*	rs8050136	52373776	A	0.40	0.10 (-0.02;0.22)	0.12	0.01 (-0.12;0.14)	0.88

CAC: coronary artery calcification; SNP: single nucleotide polymorphism; 95%CI: 95% confidence interval.

Supplementary Table II. Chromosome 9 (*CDKN2A/2B*) rs564398 SNP genotypes distribution for dichotomized CAC and diabetes mellitus.

Sample	Diabetes	Coronary artery calcification	Genotype distribution			Diabetes mellitus-stratified ORs and 95% CIs for dichotomized CAC (CAC >0) ‡		
			GG n (%)	GA n (%)	AA n (%)	GG	GA	AA
All	Yes	CAC > 0	82 (16.6)	235 (47.7)	176 (35.7)	1.03 (0.49;2.18)	1.01 (0.70;1.48)	1 (Ref)
		CAC = 0	11 (15.5)	35 (49.3)	25 (35.2)			
	No	CAC > 0	407 (16.8)	1,186 (49.1)	824 (34.1)	0.80 (0.65;0.98)	0.89 (0.81;0.99)	1 (Ref)
		CAC = 0	227 (19.5)	573 (49.2)	364 (31.3)			
Females	Yes	CAC > 0	31 (19.0)	78 (47.9)	54 (33.1)	1.12 (0.41;3.16)	1.06 (0.64;1.78)	1 (Ref)
		CAC = 0	8 (19.5)	18 (43.9)	15 (36.6)			
	No	CAC > 0	160 (15.9)	498 (49.4)	349 (34.7)	0.79 (0.61;1.04)	0.89 (0.78;1.02)	1 (Ref)
		CAC = 0	157 (18.3)	430 (50.1)	271 (31.6)			
Males	Yes	CAC > 0	51 (15.4)	157 (47.6)	122 (37.0)	1.08 (0.34;3.56)	1.04 (0.58;1.89)	1 (Ref)
		CAC = 0	3 (10.0)	17 (56.6)	10 (33.4)			
	No	CAC > 0	247 (17.5)	688 (48.8)	475 (33.7)	0.71 (0.49;1.01)	0.84 (0.70;1.01)	1 (Ref)
		CAC = 0	70 (22.9)	143 (46.7)	93 (30.4)			

CAC: coronary artery calcification; ORs: odds ratios; 95%CI: 95% confidence interval.
‡ based on the exact Cochran-Armitage trend test (assuming a linear trend)