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Calpain-10 variants and haplotypes are associated with polycystic ovary syndrome in Caucasians

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Vollmert C, Hahn S, Lamina C, Huth C, Kolz M, Schöpfer-Wendels A, Mann K, Bongardt F, Mueller JC, Kronenberg F, Wichmann HE, Herder C, Holle R, Löwel H, Illig T, Janssen OE, the KORA group. Calpain-10 variants and haplotypes are associated with polycystic ovary syndrome in Caucasians. *Am J Physiol Endocrinol Metab* 292: E836–E844, 2007. First published November 14, 2006; doi:10.1152/ajpendo.00584.2005.—PCOS is known to be associated with an increased risk of T2DM and has been proposed to share a common genetic background with T2DM. Recent studies suggest that the Calpain-10 gene *(CAPN10)* is an interesting candidate gene for PCOS susceptibility. However, contradictory results were reported concerning the contribution of certain *CAPN10* variants, especially of UCSNP-44, to genetic predisposition to T2DM, hirsutism, and PCOS. By means of MALDI-TOF MS technique, we genotyped an expanded single nucleotide polymorphism panel, including the *CAPN10* UCSNP-44, -43, -56, ins/del-19, -110, -58, -63, and -22 in a sample of 146 German PCOS women and 606 population-based controls. Statistical analysis revealed an association between UCSNP-56 and susceptibility to PCOS with an odds ratio (OR) of 2.91 (95% CI = 1.51–5.61) for women carrying an AA genotype compared with GG. As expected, the 22-genotype of the ins/del-19 variant, which is in high linkage disequilibrium ($r^2 = 0.98$) with UCSNP-56, was also significantly associated (OR = 2.98, 95% CI = 1.55–5.73). None of the additionally tested variants alone showed any significant association with PCOS. A meta-analysis including our study (altogether 623 PCOS cases and 1,224 controls) also showed significant association only with ins/del-19. The most common haplotype TGG3AGCA was significantly associated with a lower risk for PCOS (OR = 0.487, $P = 0.0057$). In contrast, the TGA2AGCA haplotype was associated with an increased risk for PCOS (OR = 3.557, $P = 0.0011$). By investigating a broad panel of *CAPN10* variants, our results pointed to an allele dose-dependent association of UCSNP-56 and ins/del-19 with PCOS.

single nucleotide polymorphism; genetic association study; metabolic syndrome; type 2 diabetes mellitus

POLYCYSTIC OVARY SYNDROME (PCOS) is a common endocrinopathy characterized by chronic anovulation, hyperandrogenism, polycystic ovaries (PCO), and, frequently, insulin resistance (26, 20). It is prevalent in ~5–10% of women of reproductive age (13, 47). Previous studies (4, 9, 12) indicate that PCOS is a complex disease with both multiple genetic components and environmental factors contributing to its etiology. Since PCOS is associated with a two- to sevenfold increased risk to develop impaired glucose tolerance (IGT) or type 2 diabetes mellitus (T2DM), genes related to T2DM may also play a role in PCOS pathogenesis (41, 44). Several T2DM candidate genes, including those for insulin, insulin receptor, and insulin receptor substrates, have been investigated in this context and partly indicated significant evidence for a genetic contribution to PCOS susceptibility (12, 44, 53, 55, 56, 58).

Calpain-10 (*CAPN10)* was the first positionally cloned susceptibility gene for T2DM (24, 31). It encodes the ubiquitously expressed cysteine protease Calpain-10, which is essential for calcium-regulated intracellular signaling (35, 52). *CAPN10* plays a crucial role in proinsulin processing, insulin secretion, action, and sensitivity (3, 48). The single nucleotide polymorphism (SNP) of the *CAPN10* gene UCSNP-43 and the haplotype combination 112/121, defined by the three UCSNPs UCSNP-43, ins/del-19, and -63, were found to be associated with an up to threefold increased risk to develop T2DM in selected populations (31). The rare UCSNP-43 A allele was also found to be associated with idiopathic hirsutism (15) and with an approximately twofold increased risk for PCOS in both African-Americans and Europids in the case of the haplotype combination (14). Moreover, UCSNP-44 located in a transcription enhancer element next to UCSNP-43 was found to be associated with T2DM (17, 59) and with PCOS in a Spanish population (20, 21). It is in perfect linkage disequilibrium (LD) with the missense mutation $Thr^{504}Ala$ (UCSNP-110) and two additional polymorphisms in the 5′UTR (UCSNP-134 and UCSNP-135). These results suggest that either the UCSNP-44 or an SNP in high LD may contribute to T2DM and PCOS susceptibility. Nevertheless, the relationship between *CAPN10* and both T2DM and PCOS is highly controversial across

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studies and populations, because discordant or lacking associations of polymorphisms in the *CAPN10* region with T2DM (17, 39, 36) and PCOS (23) have been reported.

Taken together, these studies suggest *CAPN10* to be a strong candidate for both T2DM and PCOS. However, it remains unclear whether and which DNA variation at *CAPN10* really affects the disease risk or is in LD with a causative variation nearby. Moreover, contradictory results of previous linkage and association studies (33, 50) can be attributed to study populations that have been far too small to yield any definite results. To further analyze the role of *CAPN10* in PCOS susceptibility, we selected an extended sample of eight variants (UCSNP-44, -43, -56, ins/del-19, -110, -58, -63, and -22) in the ~31-kb spanning region of the *CAPN10* gene on human chromosome band 2q37.3. Subsequently, we genotyped 146 PCOS subjects and 606 matched female controls to determine whether these variations in the *CAPN10* gene predispose to PCOS. Finally, to clarify putative associations between *CAPN10* and PCOS, we performed a meta-analysis on the basis of published data of all genetic association studies to date on *CAPN10* and PCOS.

MATERIALS AND METHODS

Study population. We recruited 146 PCOS patients (27 ± 6 yr) from the outpatient clinics of the Division of Endocrinology, Department of Medicine, and the Department of Gynecology at the University of Duisburg-Essen. On the basis of the criteria derived from the 1990 National Institutes of Health conference, diagnosis of PCOS was established when either oligomenorrhoea (menstrual cycles lasting >35 days) or amenorrhoea (absence of menstruation in the past 6 mo) and clinical signs of hyperandrogenism [either hirsutism with a Ferriman/Gallwey score of ≥6 or obvious acne or alopecia and/or an elevated total testosterone (normal range <2.0 nmol/l)] were found and when other pituitary, adrenal, or ovarian diseases could be excluded. All patients also fulfilled the 2003 Rotterdam Criteria of PCOS (44a). None of the PCOS subjects was taking any medication known to affect carbohydrate metabolism or endocrine parameters for ≥3 mo before entering the study. All PCOS patients were of Caucasian origin and German descent. The study protocol was approved by the Ethics Committee of the University of Essen. Written, informed consent was obtained from all participants.

Female controls ($n = 606$, matching rate 1:4) were taken from the population-based German study "Cooperative Health Research in the Region of Augsburg, Survey 4" (KORA S4) (19, 30, 60) that studied a representative sample of the adult general population of German nationality comprising 4,261 subjects aged 25–74 yr recruited from 1999 to 2001. All study methods were approved by the ethics committee of the "Bayerische Landesärztekammer," Munich, Germany. All participants signed a consent form to participate in genetic studies. Following frequency matching for age (best possible matching: tolerance maximum + 5 yr at ≥20 yr of age) and sex (perfect matching), 606 female individuals that in particular were not suffering from diabetes (according to self-report, medical diagnosis, and medication) were randomly selected. Body mass index (BMI) was calculated as measured weight (kg) divided by measured height squared (m^2).

DNA preparation. Genomic DNA of PCOS patients was isolated from whole blood with the QIAmp DNA Blood Midi Kit (Qiagen, Hilden, Germany). Genomic DNA of KORA participants was extracted from blood leukocytes by using the Puregene DNA Isolation Kit (MN 55441; Gentra Systems) according to the manufacturer's recommendation.

SNP selection criteria. Human *CAPN10* consists of 15 exons and extends over a region of ~31 kb on chromosome 2q37.3. For genotyping, we selected a gene-spanning panel of eight SNPs that had partly shown evidence for association with T2DM, PCOS, or related traits (14, 15, 17, 20, 21, 31, 51). Two of these, namely UCSNP-110 and UCSNP-58, result in amino acid substitutions and are located at the exonic splicing enhancers SC35 and SRp55, respectively, which are important *cis* elements required for exon inclusion. The other selected variants are located in the 3′UTR or at intronic sites and are thus suspected to influence stability of mRNA, *CAPN10* transcription, and expression (3, 31). There is a distinct overlap between HapMap II tagging SNP panel ($r^2 > 0.8$) and the SNP panel, which has been investigated in our study.

SNP genotyping. Genotyping of SNPs was performed using matrix-assisted laser desorption ionization time-of-flight-based mass spectrometry (MALDI-TOF MS) of allele-specific primer extension products (Mass Array; Sequenom, San Diego, CA), as described previously (57). Genomic DNA of each subject was tested individually. Primer information is given in Tables 1 and 2. A modified Bruker Biflex MALDI-TOF MS and a Bruker Autoflex HT MALDI-TOF MS were used for data acquisition from the SpectroCHIP. Genotype calling was performed in real time with Mass Array RT software version 3.0.0.4 (Sequenom). For quality control, 2% of negative controls were included in all assays. To control for reproducibility of genotyping data, 10% of randomly selected samples were genotyped in duplicate with an error rate <1%.

A fragment containing the *CAPN10* UCSNP-19 (rs3842570), which is an insertion/deletion polymorphism consisting of two or three repeats of a 32-bp sequence, was amplified (primer information is given in Table 1). PCR conditions were the same as for MALDI-TOF MS analysis. The PCR product was subjected to electrophoresis on a 3% agarose gel and stained with ethidium bromide. Allele 1 (2 repeats) comprised 155 bp, and allele 2 (3 repeats) consisted of 187 bp.

Statistical analysis. Each SNP was tested for deviation from Hardy-Weinberg equilibrium by means of a chi-square test or Fisher's exact test depending on allele frequency. To evaluate the relationship between SNPs, pairwise LD statistics D′ and correlation coefficients r^2 were calculated using Haploview version 3.2 (http://www.broad.

Table 1. *Primer information: SNP genotyping and PCR reaction*

SNP ID	1st PCR Primer	2nd PCR Primer	Product length, bp
rs2975760	5′-AGAGAGTTTCTGTGTGTGGG-3′	5′-CTTAGCCTCACCTTCAAACG-3′	100
rs3792267	5′-TTTCTGTGTGTGGGCAGAGG-3′	5′-AAGTCAAGGCTTAGCCTCAC-3′	103
rs2975762	5′-TTGTTCTCAAGGGTGGTGTC-3′	5′-TAGTGGAAAGGACTGGTCAG-3′	118
rs3842570	5′-CATGAACCCTGGCAGGGTCTAAG-3′	5′-GTTTGGTTCTCTTCAGCGTGGAG-3′	187/155
rs7607759	5′-TGTTTGTCTTCTTGGCAGCG-3′	5′-ACTCTCCAAGAACCCCGTAG-3′	131
rs2975766	5′-GGCCACCCTGTTAGGTTTTC-3′	5′-GCCCCCCATCTGTCTTTGCA-3′	81
rs5030952	5′-TTAGGAAGCTTCTGAGCCTG-3′	5′-TTCACTCGGTCAGAGCCCTA-3′	118
rs2953152	5′-GCTCCGAACAGGATTCCTTC-3′	5′-AGTGTTGGACAAGCTTGCTG-3′	133

SNP, single nucleotide polymorphism.

Table 2. *Primer information: SNP genotyping and extension reaction*

SNP ID	Extension Primer	ddNTPs	Extension 1	Ext1_Mass, Da	Extension 2	Ext2_Mass, Da
rs2975760	5′-GGCGCTCACGCTTGCTG-3′	ACG	C	5451.5	T	5795.8
rs3792267	5′-TTGCTGTGAAGTAAGGC-3′	ACT	A	5562.6	G	5882.8
rs2975762	5′-TCAGTTTGTGACCTTCCCCT-3′	ACT	A	6307.1	G	6612.3
rs7607759	5′-CAGTGGCCAAGAACACC-3′	ACT	A	5470.6	G	5775.8
rs2975766	5′-CTGTCTTTGCAGGTCTCC-3′	ACT	A	5729.7	G	6049.9
rs5030952	5′-GACGCGGCCCACCCCTC-3′	ACT	T	5374.5	C	5679.7
rs2953152	5′-AGGATTCCTTCCTGCCC-3′	ACG	G	5370.5	A	5978.9

mit.edu/mpg/haploview/index.php; Whitehead Institute for Biomedical Research).

To estimate the genetic association of each SNP with PCOS, differences in genotype distributions between case and control groups were tested using a chi-square test. Subsequently, logistic regression analyses were performed and adjusted for age and BMI. To assess independent genotypic effects, heterozygotes and homozygotes for the rare allele were each tested against homozygotes for the common allele as reference group. Only in the meta-analysis for polymorphism ins/del-19 were the homozygotes for the rare allele used as reference group, according to the nomenclature of UCSNP-19 by Horikawa et al. (31). In a second step, we tested whether a dose effect per copy of the rare allele was available.

The impact of the different age and BMI structures in the case and control groups was explored by sensitivity analyses. Logistic regression analyses were constrained to participants aged 25–35 yr or to individuals above or below BMI of 25 kg/m^2, respectively, each adjusted for the other covariate, to ensure a balanced proportion of cases and controls.

The expected numbers of copies of each haplotype were estimated for all subjects using the expectation-maximization algorithm (18). A global test on all haplotypes (frequency >1%) was performed via logistic regression with stepwise selection procedure using the expected number of copies. Haplotype estimation and logistic regression analyses were adjusted for age and BMI and calculated using SAS software version 8.0 (Cary, NC).

A meta-analysis including our own and all available published data has been performed for all UCSNPs, on which at least three studies were available (UCSNP-44, -43, -19, -63). Between-study heterogeneity was tested by the χ^2-based Q-statistic, and its impact was quantified by I^2, the proportion of total variation in the estimates of genotype associations that is due to heterogeneity between studies (27). To derive a combined odds ratio (OR) estimate, study-specific ORs were pooled by using the inverse-variance fixed effect and the DerSimonian and Laird (10) random effects models. The random effects model incorporates an estimate of the between-study variance and tends to provide wider confidence intervals in the case of heterogeneity. In the absence of heterogeneity, the two models provide identical results.

Forest plots displaying study-specific ORs with 95% confidence intervals (95% CIs) were prepared using Review Manager software version 4.2 (Cochrane Collaboration, Copenhagen, Denmark).

RESULTS

SNP genotyping, allelic frequencies, and LD. In the present investigation, 752 individuals, including 146 females suffering from PCOS and 606 unrelated nondiabetic female controls from the population-based KORA study, were analyzed. The PCOS participants were 14–41 yr of age (mean = 27). Because the KORA study does not include individuals <25 yr of age, we performed a best possible frequency matching for age (tolerance maximum ± 5 yr at ≥20 yr of age). Thus the KORA controls were 25–38 yr old (mean = 32). The range of BMI was 17–58 (PCOS, mean = 30.2 kg/m^2) and 16–46 (controls, mean = 24.7 kg/m^2) kg/m^2.

Eight *CAPN10* variants were genotyped. Genetic locations of the polymorphisms, genotyping success rates, and allelic frequencies are given in Table 3. The observed genotype frequencies agreed with the expected frequencies according to the Hardy-Weinberg equilibrium. Pairwise comparison showed extensive LD between two pairs of variations: UCSNP-44 and UCSNP-110 ($D' = 0.994$, $r^2 = 0.975$); UCSNP-56 and UCSNP-19 ($D' = 1.000$, $r^2 = 0.976$) (Fig. 1).

Association results of single SNPs. Chi-square statistics showed significant associations between PCOS and the genotype distribution of UCSNP-56 ($P = 0.047$) and the highly correlated ins/del-19 polymorphism ($P = 0.027$), respectively (data not shown). Adjustment for age and BMI yielded an even stronger association with $P = 0.0045$ for UCSNP-56 and $P = 0.0035$ for ins/del-19 (Table 3). In adjusted logistic regression analyses, these two *CAPN10* polymorphisms showed an allele dose effect, as described in detail in Table 4. Homozygote carriers of the rare alleles UCSNP-56_AA and ins/del-19_22 bear the highest risk of predisposition to PCOS with an age-

Table 3. *SNP information, genotyping, and association results*

SNP ID	UCSNP	Chromosome Position 2q37.3	Gene Location	GSR, %	c/r Allele	MAF PCOS	MAF Controls	Logistic Regression* WS, *P* Value
rs2975760	44	241251153	intron 3	89.4	T/C	14.3	14.7	0.802
rs3792267	43	241251164	intron 3	86.4	G/A	32.2	30.5	0.950
rs2975762	56	241251727	intron 4	95.2	G/A	44.7	37.5	0.0045
rs3842570	19	241254283^241254284	intron 6	98.5	3/2	44.4	36.7	0.0035
rs7607759	110	241256116	exon 10 T = >V	94.7	A/G	14.6	15.3	0.941
rs2975766	58	241258064	exon 13 I = >V	96.7	G/A	6.3	4.5	0.095
rs5030952	63	241262693	3′UTR	96.9	C/T	7.6	6.2	0.807
rs2953152	22	241282108	3′UTR	92.0	G/A	42.8	43.3	0.797

UCSNP, SNP of the Calpain-10 (*CAPN10*) gene; GSR, genotyping success rate; c/r allele, common/rare allele; MAF, minor allele frequency; PCOS, polycystic ovary syndrome; WS, Wald statistics. *Adjusted for age and body mass index (BMI).

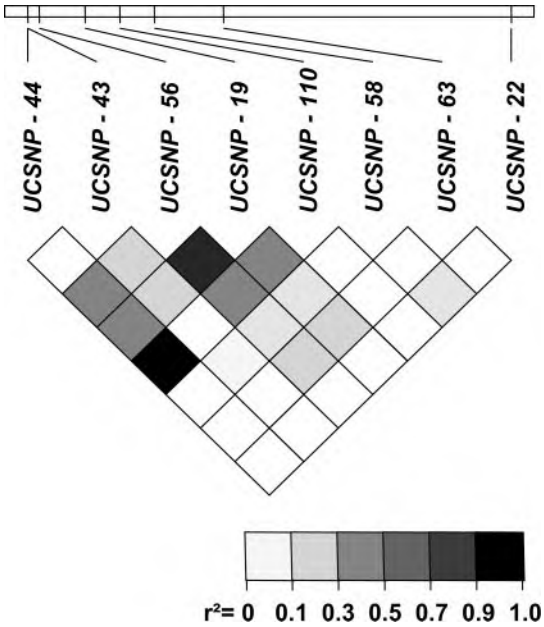

Fig. 1. Pairwise linkage disequilibrium between genotyped variants in Calpain-10 (*CAPN10*). *Top*: physical single nucleotide polymorphism (SNP) locations. The shaded diamonds represent the pairwise r^2 between the 2 SNPs defined by the *top left* and the *top right* sides of the diamond. Shading represents magnitude and significance of the pairwise r^2, with black reflecting high r^2 ($r^2 > 0.9$) and white reflecting low r^2 ($r^2 < 0.1$).

and BMI-adjusted OR of 2.913, $P = 0.0014$, and 2.976, $P = 0.0011$, respectively. This is an approximately threefold increased risk compared with the carriers of the homozygote common allele, whereas heterozygotes showed only a nonsignificantly higher risk (Table 4). Bonferroni correction for the number of analyzed SNPs still yielded comparable stable and statistically significant results for age and BMI-adjusted regression analyses.

We performed sensitivity analyses with regard to the age structure of the study as well as to BMI to exclude possible bias by study design-driven age and BMI structures. These analyses showed that the different age and BMI structures in cases and controls did not modify the observed associations between the genotypes and PCOS (data not shown). Additionally performed analyses with continuous BMI or other definitions of BMI as outcome also revealed a lack of direct association between genotypes and BMI both investigating all study participants and PCOS patients alone (data not shown).

Haplotype association results. Eleven *CAPN10* haplotypes with frequencies >1% were identified in the entire group of 752 individuals. Two of these haplotypes emerged from the stepwise selection procedure of the global test on all haplotypes. The most common haplotype TGG3AGCA (frequency 22%) comprised common alleles of all SNPs, except for the 3′ UCSNP-22, and it only differed in UCSNP-56 and -19 from haplotype TGA2AGCA (4.9%). TGG3AGCA showed a protective association with PCOS (OR = 0.487, $P = 0.0057$), whereas TGA2AGCA increased the risk (OR = 3.557, $P = 0.0011$), being consistent with the results of the single SNP analyses. Frequencies of these two haplotypes clearly differed as follows: 14.64 vs. 23.56% for TGG3AGCA ($p^2_{chi} = 0.0198$) and 10.18 vs. 3.55% for TGA2AGCA ($p^2_{chi} = 2.5 \times 10^{-8}$) in cases and controls, respectively.

Meta-analysis results. To clarify the putative associations between *CAPN10* and PCOS, we performed a meta-analysis that was based on our own data and the published data of all genetic association studies to date that have investigated *CAPN10* and PCOS (Table 5). Unfortunately, in the publication by Ehrmann et al. (14), essential data from 177 Europid PCOS cases were missing and were therefore not included in the meta-analysis. Since the data published in Gonzalez et al. 2002 (20) are part of the data published in Gonzalez et al. 2003 (21), only the latter were analyzed in the meta-analysis.

Including our own data, the meta-analysis comprised 761 cases vs. 1,828 controls for UCSNP-43 and UCSNP-44, as well as 623 cases vs. 1,224 controls for UCSNP-19 and UCSNP-63, respectively. In addition to these case-control studies, one family study comprising 146 parent-offspring trios has been included in the meta-analysis of UCSNP-43, -44, -19, and -63 (Table 5).

Results of the meta-analysis presenting fixed and random effects ORs on the basis of allele frequencies are given in Table 6. In the presence of heterogeneity between studies ($I^2 > 0$), the results of the fixed and random effects models differ. In this case we assessed the results of the random effects model, which usually are more conservative than the results of the fixed effects model, to be more qualified and valid. Analyzing the pooled data of five case-control studies, we revealed a significant association between UCSNP-19 and PCOS, with the insertion being protective (OR = 0.83, $P = 0.02$) and the deletion being a risk factor for PCOS (OR = 1.21, $P = 0.02$), respectively. Study-specific as well as the pooled ORs with 95% CI are illustrated in the forest plot in Fig. 2. There was no association between PCOS and UCSNP-44, -43, and -63; that is, the association findings of our single study were well supported by the meta-analysis.

When further including the family study in the meta-analysis, heterogeneity between studies was considerably higher for UCSNP-19 and -63, and no significant association with PCOS was found (Table 6); only UCSNP-19 showed a borderline significant association.

Table 4. *Association results of logistic regression analysis (adjusted for age and BMI) of selected SNPs*

SNP ID	UCSNP	Genotypes Compared	OR	95% CI	*P* Value	
					Logistic regression	Logistic regression WS
rs2975762	56	AA vs. GG	2.913	1.512–5.613	0.0014	0.0045
		AG vs. GG	1.266	0.751–2.133	0.3759	
rs3842570	19	22 vs. 33	2.976	1.545–5.733	0.0011	0.0035
		23 vs. 33	1.247	0.742–2.097	0.4046	

OR, odds ratio; CI, confidence interval.

Table 5. *Genetic association studies investigating CAPN10 and PCOS that are published to date*

Study References	Analyzed *CAPN10*_UCSNP	Cases (*n*)	Controls (*n*)
Ref. 15	**43 44** 45	76 Caucasian, hyperandrogenic hirsutism 5 Caucasian, PCOS	37 Caucasian
Ref. 14	43 19 63	117 White European, PCOS 33 African-American, PCOS	
Ref. 23	**43 44 19 63**	185 Europid, PCOS 146 Europid (>80% UK) parent-offspring-trios, PCOS	525 White Europid (UK)
Ref. 20	43 44 19 63	55 Europid (Spain), PCOS	93 White Europid
Ref. 21	**43 44 19 63**	146 Europid (Spain), PCOS	93 White Europid
Ref. 22	**43 44** 45	57 Caucasian, PCOS	567 Caucasian
Present study	**43 44** 58 **19** 56 **63** 22	146 White Europid (German), PCOS	606 White Europid (German), population-based KORA study

Europid, European White population; KORA, Cooperative Health Research in the Region of Augsburg. Data of *CAPN10* UCSNPs that have been implemented to the meta-analysis are highlighted in bold.

DISCUSSION

CAPN10: a suitable candidate gene for PCOS. There is clear evidence for a genetic component in the heterogeneous disorder PCOS, based on familial clustering of cases supporting an autosomal dominant form of inheritance. Although a number of candidate genes have been proposed, the putative PCOS genes have yet to be identified (1, 12). Existing hypotheses for a possible biological role of the ubiquitously expressed cysteine protease calpain-10 in the etiology of T2DM, obesity, and the metabolic syndrome include effects on proinsulin processing and on glucose-induced insulin secretion, action, and sensitivity (3, 28, 29, 40, 46, 48) and thus suggest *CAPN10* as a suitable candidate gene for PCOS. Previous association studies with PCOS (14, 15, 20, 21, 23) produced promising, but also conflicting, data. This may be due to small effects and severely underpowered studies that are unable to significantly detect modest odds ratios (43). Furthermore, the heterogeneity of phenotype features of the PCO syndrome, including the fact that the disorder is only expressed clinically in women during reproductive age, and in consequence the lack of uniform selection criteria of PCOS study samples, might have contributed to these contradictory results (12).

Possible modification of the transcription process. The genetic association between UCSNP-19 and UCSNP-56 observed in our study may originate from a possible modification of the transcription process. Whereas UCSNP-56 does not modify any interacting or binding site domains, the UCSNP-19 32-bp repeat sequence includes a possible binding site of the Pdx1 (IDX1/IPF-1) pancreatic and intestinal homeodomain transcription factor (MatInspector, Genomatix). In the case of UCSNP-19 deletion there are two possible binding sites of this transcription factor, and in the case of insertion there are three. Pdx1 was originally described as insulin promoter factor-1 (IPF-1), a homeodomain protein that is expressed in β-cells of mouse pancreas and binds to and transactivates the insulin promoter. The pattern of IPF-1 expression and its ability to stimulate insulin gene transcription suggest that IPF-1 functions in the maturation of the pancreatic β-cell (38). Given the known association between PCOS and diabetes and considering the positive impact of insulin sensitizers like metformin on

Table 6. *Results of meta-analysis of both the 5 CC studies and the 5 CC studies and 1 trio study (FAM)*

UCSNP	*n* Studies	*n* Cases/*n* Controls	I^2, %	Q-Test for Heterogeneity *P* Value	FE/RE Model	OR	95% CI	*P* Value
44	5 CC	761/1,828	32.4	0.21	FE	1.06	0.87–1.30	0.55
					RE	1.08	0.84–1.41	0.54
	5 CC + 1 FAM	761/1,828 + 146 Trios	28.9	0.22	FE	1.02	0.85–1.22	0.87
					RE	1.03	0.82–1.29	0.81
43	5 CC	761/1,828	0.0	0.57	FE	1.05	0.89–1.23	0.58
					RE	1.05	0.89–1.23	0.58
	5 CC + 1 FAM	761/1,828 + 146 Trios	0.0	0.67	FE	1.03	0.89–1.19	0.69
					RE	1.03	0.89–1.19	0.69
19	3 CC	623/1,224	0.0	0.40	FE	0.83	0.70–0.97	0.02
					RE	0.83	0.70–0.97	0.02
	3 CC + 1 FAM	623/1,224 + 146 Trios	38.1	0.18	FE	0.88	0.76–1.01	0.08
					RE	0.88	0.73–1.07	0.19
63	3 CC	623/1,224	0.0	0.51	FE	1.09	0.81–1.46	0.58
					RE	1.09	0.81–1.46	0.58
	3 CC + 1 FAM	623/1,224 + 146 Trios	49.2	0.12	FE	0.95	0.73–1.25	0.73
					RE	0.90	0.61–1.34	0.61

FE, fixed effect; RE random effect; CC, case controls; FAM, family study. I^2 measures the impact of inconsistency across studies and can range between 0 and 100%. The pooled OR of the FE and RE models are given with 95% CIs for the association between *CAPN10* UCSNPs, allele 2 vs. allele 1 according to Horikawa et al. (31), and PCOS.

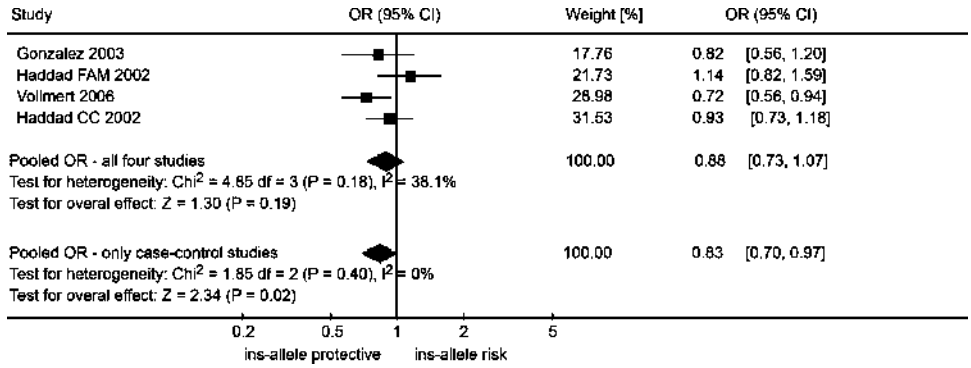

Fig. 2. Forest plot illustrating the study-specific odds ratios (ORs) with 95% confidence intervals (95% CIs) for the association between SNP of the *CAPN10* UCSNP-19 (ins vs. del allele) and polycystic ovary syndrome (PCOS). Additionally, pooled random effects ORs, with and without inclusion of the family study, are shown. The addenda CC and FAM to the Haddad study denote the case-control and the family study, respectively. The studies are sorted according to the weight with which they contribute to the pooled OR estimate.

PCOS (7, 11, 25, 32, 37, 54), a consequence of the UCSNP-19 ins/del polymorphism on PCOS risk driven by the transcription factor Pdx1 may be assumed.

Significant association of UCSNP-56 and ins/del-19 but not of other UCSNPs, including UCSNP-44. Our data provide support for the involvement of the *CAPN10* UCSNP-19 ins/del polymorphism that is, as shown previously (31), in strong LD with UCSNP-56 as a determinant of PCOS susceptibility. In addition, we found clear evidence for a risk increase by one allelic change in single SNP analyses, which was consistent with a copy number effect of haplotypes.

Our positive association findings are in accordance with previous single study results. Gonzalez et al. (20) described a statistically significant ($P = 0.045$) positive correlation between the UCSNP-19 deletion variant with PCOS in 55 Caucasian PCOS cases and 93 controls that was based on comparable minor allele frequencies of 47.2 and 34.4%, respectively. Additionally, they described an association between PCOS and the C allele of UCSNP-44 ($P = 0.023$), which is in LD with the ins/del polymorphism and has been also associated with T2DM in European populations (17). Additional studies revealed PCOS or associated phenotypes like IGT and hirsutism score to be associated with the heterozygous T2DM risk haplotype combination 112/121 of the polymorphisms UCSNP-43 (allele 1 = G), UCSNP-19 (allele 1 = deletion), and UCSNP-63 (allele 1 = C) in 124 white and 45 black PCOS cases (14) and with UCSNP-43 and -44 in 97 idiopathic hirsutism or PCOS cases and 37 controls (15). Although given a higher power, in our study, we did not confirm these findings. Moreover, other large studies did not reveal any association between PCOS and either the haplotype combination in family-based and case-control studies (23) or the single *CAPN10* UCSNP-43, -44, and -45 in 57 nondiabetic PCOS and 567 nondiabetic controls (22). Taken together, these data suggest that the UCSNP-44 may not be involved in the PCOS pathogenesis, although, in contrast, there is strong evidence for a contribution to T2DM predisposition for two reasons. First, a trio study conducted in the UK found the UCSNP-44 C allele to be more often transmitted to affected T2DM offspring (17); and second, two recent meta-analyses, including 3,303 and 7,667 subjects, confirmed that the rare C allele is associated with increased risk for T2DM (8, 59).

Finally, stating a significant association between UCSNP-19 and PCOS as well as a lack of association between other variants of the *CAPN10*, including UCSNP-43, -44, and -63, the association findings described in our manuscript were well supported by the meta-analysis including our data. In addition, we also performed meta-analysis for UCSNP-19 in only the two other published case-control studies to avoid overlap with our own data. With an OR of 0.89, the result of this association analysis points in the same direction as our study, but lacking power due to sample size, the small effect size does not reach significance (95% CI = 0.73–1.10, $P = 0.28$), which is not surprising. In contrast, the meta-analysis of parent-offspring trios, together with case-control studies, revealed no significant association of any of the *CAPN10* polymorphisms tested but borderline association between UCSNP-19 and PCOS. It is noteworthy that, for UCSNP-19 and -63, heterogeneity between studies was remarkably reduced when eliminating the trio study from the meta-analysis. Haddad et al. (23) already described a difference between their case-control and trio studies, with only the latter showing nominal evidence of excess transmission of the more common allele of UCSNP-63. Due to a typically lower age of onset in cases of trio studies, since both parents must still be alive, a different genetic background may prevail in study participants of trio studies compared with case-control studies, which might explain the heterogeneity between study results.

Relationship between CAPN10 and BMI. The only distinctive feature with respect to UCSNP-44 we observed in our study was one single PCOS homozygote carrier of the very rare UCSNP-44CC/-110GG genotype that showed an extreme BMI >40 kg/m^2, whereas the BMI of heterozygote and wild-type carriers was inconspicuous and the BMI of KORA controls was independent of genotype. Although it lacks significance, this is an interesting observation given that we found a significant interaction between *CAPN10* and BMI in a large T2DM case-control study (Huth and Kolz, personal communication).

Although Shima et al. (45) found an association of *CAPN10* UCSNP-19 genotype 33 with higher BMI and increased hemoglobin A_{1c} levels, which was consistent with results of haplotype analysis, suggesting the contribution of UCSNP-19 to mild obesity and glucose intolerance in Japanese, we did not confirm these findings in our Caucasian sample. Our study did not reveal any significant association between *CAPN10* variants and BMI in PCOS patients and controls. By sensitivity analyses for BMI subgroups we could also exclude any significant interaction of the BMI on associations between genotypes of the investigated *CAPN10* polymorphisms and PCOS.

Associations between CAPN10 and T2DM might be influenced by associations between CAPN10 and PCOS. Our data suggest that *CAPN10* UCSNP-19 could be related to both pathologies, PCOS and T2DM. Depending on the population studied, $\leq$90% of PCOS women suffer from impaired insulin

secretion, IGT, insulin resistance, and T2DM (5, 13, 34). In addition, the prevalence of metabolic syndrome is nearly twofold higher in PCOS patients than in age-matched women in the general population (2). Only a few studies have assessed the prevalence of PCOS among women with T2DM (6). Given the high prevalence of 5–10% of PCOS in the population and especially in T2DM patients (~27%), these PCOS patients may be severely underdiagnosed in samples of T2DM association studies (42). In this case, results of *CAPN10* and T2DM association studies are likely to depend on the male-female ratio and on the degree of hyperandrogenism of the study participants.

Limitations and strengths of the study. Regarding limitations of our study, it needs to be noted that the control individuals were on average 5 yr older than the PCOS patients. Because genetic structure as our main outcome is independent of age (at least in the investigated age groups), we accepted this best possible matching for age for the advantages of investigating population-based controls. In addition, because the control individuals were older and not suffering from diabetes yet, they should be suitable controls and are probably not PCOS patients. Due to the high prevalence of 5% of PCOS in the normal population, it is nevertheless possible that PCOS patients are included in the control group. In this case, however, the true association would have been rather underestimated. The BMI was the only age-dependent phenotype we tested. Although the controls were of older age, they showed on average 5 kg/m^2 lower BMI than the PCOS cases. Thus there seemed to be no bias by study design-driven age structure. This was also confirmed by sensitivity analyses.

In an independent genomic control approach, we tested for population stratification by genotyping 212 genome-wide selected markers in 2,159 individuals from KORA and two Northern German populations (49). No major population substructuring either between or within these populations was found, indicating that the Northwestern, Northeastern, and Southern German populations are genetically homogeneous and that population stratification plays only a minor role in association studies for complex diseases in the German population. In a second independent genomic control study, restless leg patients that had been recruited all over the country were compared with KORA controls with a subset of 70 of the same 212 genome-wide markers (Juliane Winkelmann, personal communication). Again, there was no major population stratification. In this second genomic control study, one recruiting center was quite close to our PCOS recruiting center in the central western part of Germany. However, because we did not genotype the PCOS cases for the same genome-wide markers and did not test whether there are genetic differences between them and the KORA population, we cannot entirely exclude a possible, although not very likely, population stratification.

Compared with the literature, the present study has the great advantage of comprising one of the largest PCOS case-control studies. All patients were recruited in a single-center study using the same standardized diagnosis criteria. The controls are population-based and were matched at the high ratio of 1:4. One-hundred forty-six PCOS patients may still be a rather small number of cases regarding the expected small effects and low allele frequencies that are typical for complex diseases. However, given the small *P* values and rather high minor allele frequencies between 36.7 and 48.6%, the stated associations seemed to overcome the risk of being false positive. Even if corrected for multiple testing, the results remained statistically significant. In addition, the study results based on single SNP and haplotype association analyses were highly consistent. Finally, the association findings that we described here have been well supported by meta-analysis of all available data of association studies that have investigated the relationship of PCOS and *CAPN10* to date.

Our data provide support for the contribution of the *CAPN10* UCSNP-19 ins/del and the UCSNP-56 polymorphism to PCOS susceptibility. We found clear evidence for an allele-dose relationship in single SNP analyses, which was consistent with haplotype analyses. Thus, despite the fact that a broad panel of *CAPN10* variants did not show significant associations, our study suggests that *CAPN10* is an interesting candidate gene for PCOS. Although there is possible evidence for the UCSNP-19 ins/del polymorphism to influence the transcription factor Pdx1, it is not yet clear whether this variation at *CAPN10* causally affects the risk for PCOS or is in LD with a nearby variation that does. Therefore, although our study is population based and is one of the larger case-control association studies in PCOS up to now, and is supported by meta-analysis of published data, we insistently recommend extended case-control studies and meta-analysis on the basis of individual participants' data that additionally permit to compose homogenous subgroups by higher consensus criteria in PCOS sample definitions to confirm our observations.

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