

Chromosome 2q31.1 Associates with ESRD in Women with Type 1 Diabetes

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ABSTRACT

Sex and genetic variation influence the risk of developing diabetic nephropathy and ESRD in patients with type 1 diabetes. We performed a genome-wide association study in a cohort of 3652 patients from the Finnish Diabetic Nephropathy (FinnDiane) Study with type 1 diabetes to determine whether sex-specific genetic risk factors for ESRD exist. A common variant, rs4972593 on chromosome 2q31.1, was associated with ESRD in women ($P < 5 \times 10^{-8}$) but not in men ($P = 0.77$). This association was replicated in the meta-analysis of three independent type 1 diabetes cohorts ($P = 0.02$) and remained significant for women ($P < 5 \times 10^{-8}$; odds ratio, 1.81 [95% confidence interval, 1.47 to 2.24]) upon combined meta-analysis of the discovery and replication cohorts. rs4972593 is located between the genes that code for the Sp3 transcription factor, which interacts directly with estrogen receptor α and regulates the expression of genes linked to glomerular function and the pathogenesis of nephropathy, and the CDCA7 transcription factor, which regulates cell proliferation. Further examination revealed potential transcription factor-binding sites within rs4972593 and predicted eight estrogen-responsive elements within 5 kb of this locus. Moreover, we found sex-specific differences in the glomerular expression levels of SP3 ($P = 0.004$). Overall, these results suggest that rs4972593 is a sex-specific genetic variant associated with ESRD in patients with type 1 diabetes and may underlie the sex-specific protection against ESRD.

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In the non-diabetic population, ESRD is more common in men than in women, and women seem protected from ESRD until menopause.¹ Similarly, diabetic nephropathy (DN) and ESRD are more common in diabetic men than in diabetic women.² However, the female “protection” from ESRD appears attenuated in diabetic women because women who were younger than age 15 years at onset of type 1 diabetes (T1D) do not differ from diabetic men regarding their incidence of ESRD.² The loss of female “protection” in diabetes remains controversial.^{3,4} Moreover, some risk factors for

DN differ between men and women,⁵ suggesting sex-specific mechanisms that may be related to differences in the hormonal profiles as estrogen exerts renoprotective effects in nondiabetic persons.⁴ Furthermore, women with T1D have lower estradiol concentrations and a hormonal profile that more closely resembles that of men.³ Nevertheless, the role of estrogen in the progression of DN still remains ambiguous.⁶

DN clusters in families, and the sibling risk ratio is conspicuously high for ESRD, suggesting that genetic variation influences the risk of ESRD.⁷ We previously

identified two genetic loci that are associated with ESRD in patients with T1D, namely *AFF3* and *RGMA-MCTP2*.⁸ However, no genetic variants have so far been robustly associated with ESRD in a sex-specific manner. Therefore, we studied common genetic variation affecting the risk of ESRD in men and women separately, using a genome-wide association study (GWAS) approach involving 3652 Finnish patients with T1D from the Finnish Diabetic Nephropathy (FinnDiane) Study.⁹

The FinnDiane discovery cohort included 258 women and 387 men with ESRD. These patients were compared with those without signs of DN despite long duration of diabetes. GWAS on ESRD was performed separately for men ($N_{cases} = 387$, $N_{controls} = 655$) and women ($N_{cases} = 258$, $N_{controls} = 935$). The main clinical characteristics of the patients are shown in Table 1.

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The Manhattan plots of the GWAS results for both sexes are shown in Supplemental Figure 1. GWAS on ESRD in women identified two highly correlated single-nucleotide polymorphisms (SNPs) ($r^2=1$ in HapMap2 CEU samples) on chromosome 2q31.1 that reached genome-wide significance: rs4972593, with an odds ratio (OR) of 2.39 (95% confidence interval [CI], 1.75 to 3.25; $P=3.02 \times 10^{-8}$), and rs530673, with an OR of 2.38 (95% CI, 1.75 to 3.23; $P=3.52 \times 10^{-8}$) (Figure 1A). No association was seen for this locus in men (OR, 0.97 [95% CI, 0.78 to 1.21]; $P=0.78$) (Figure 1B), even though we had 99% power to detect the same association with $\alpha=0.05$. Furthermore, no other loci reached genome-wide significance in men or women (Supplemental Table 1).

Testing for heterogeneity indicated a sex-specific difference in the association of rs4972593 with ESRD (heterogeneity $P=5.8 \times 10^{-5}$). rs4972593 was nominally associated with body mass index (BMI) in both men and women and with hemoglobin A_{1c} in women (Supplemental Table 2). Association with ESRD was enhanced in women after adjustment for BMI (OR, 2.64 [95% CI, 1.92 to 3.63]; $P=2.7 \times 10^{-9}$) and remained even after adjustment for both BMI and hemoglobin A_{1c}, although slightly attenuated (OR, 2.31 [95% CI, 1.65 to 3.24]; $P=1.3 \times 10^{-6}$). Thus, it is unlikely that the observed sex difference would be driven by a sex-related confounder.

Replication of the association at rs4972593 was then sought in three additional T1D cohorts within the GENIE collaboration, with substantial numbers of women with ESRD available (Ireland-Warren 3-Genetics of Kidneys in Diabetes UK [UK-ROI], $n=113$; Genetics of Kidneys in Diabetes US Study [GoKinD US], $n=252$; Italian study, $n=68$).⁸ The association between rs4972593 and ESRD in women was replicated with a combined P value of 0.02 for the three studies (OR, 1.41 [95% CI, 1.05 to 1.90]). Even though the results did not reach statistical significance in the UK-ROI and Italian studies, the effect was in the same direction also in these studies. Moreover, all the comparisons with high power ($>90\%$: GoKinD US and the replication studies combined) showed evidence of association. Of note, the OR of 2.07 in GoKinD US was similar to that in the FinnDiane discovery cohort. The association remained genome-wide significant after combined meta-analysis of the FinnDiane and replication cohorts (OR, 1.81 [95% CI, 1.47 to 2.24]; $P=3.85 \times 10^{-8}$) (Table 2 and Figure 2A). As seen in FinnDiane, there was no association between rs4972593 and ESRD in men in the replication cohorts ($P=0.90$), and the results remained nonsignificant after combined meta-analysis (OR, 0.97 [95% CI, 0.78 to 1.21]; $P=0.78$) (Table 2 and Figure 2B). Consequently, the meta-analysis indicated

heterogeneity between men and women ($P=5.7 \times 10^{-5}$).

To explore whether additional associated loci could be detected by including more patients, we performed a genome-wide meta-analysis of the FinnDiane, UK-ROI, and GoKinD US GWAS data. However, no association other than that observed between rs4972593 and ESRD in women reached genome-wide significance. Furthermore, no evidence of sex difference was found for the SNPs that have been previously reported to be associated ($P<5 \times 10^{-8}$) with ESRD in T1D (*AFF3*,⁸ *RGMA-MCTP2*,⁸ and *EPO10*) (Supplemental Table 3).

To ensure that the reported association with ESRD is due to diabetes, we excluded all patients with T1D known to have ESRD due to any nondiabetic cause. Supporting a diabetic background, most patients with ESRD in the FinnDiane had diabetic retinopathy, and only six patients with ESRD had no retinopathy. In both UK-ROI and GoKinD US, all patients had retinopathy as an inclusion criterion for the studies. Therefore, the genetic variation at rs4972593 appears to be associated with susceptibility to ESRD in women with T1D.

We further investigated whether the association is specific for women with T1D or is also observed in women with type 2 diabetes by exploring this association in the Family Investigation of Nephropathy and Diabetes (FIND) study.¹¹ No association was found in

Table 1. Clinical characteristics of the FinnDiane patients

Characteristic	Women		Men	
	ESRD ($n=258$)	Controls ($n=935$)	ESRD ($n=387$)	Controls ($n=655$)
Age at T1D onset (yr)	11.3±6.7	14.7±7.8	13.7±7.6	15.8±8.9
Age (yr)	44.4±8.9	42.9±11.1	47.7±8.6	43±11.9
T1D duration (yr)	33.1±8.8	28.3±9.6	34±8.3	27.2±9.2
Transplantation, n (%)	123 (47.7)	0 (0)	193 (49.9)	0 (0)
Antihypertensive medication, n (%)	229 (88.8)	204 (21.9)	361 (93.3)	179 (27.3)
Lipid-lowering medication, n (%)	99 (38.4)	114 (12.2)	162 (41.9)	99 (15.1)
BMI (kg/m^2)	24±3.9	25.1±3.8	25.1±4.3	25.4±3.1
Systolic BP (mmHg)	151±24	133±18	153±25	136±16
Diastolic BP (mmHg)	82±13	78±9	84±12	79±10
Hemoglobin A _{1c} (%)	8.8±1.8	8.1±1.2	8.7±1.7	8.0±1.2
Total cholesterol (mmol/L)	5.4±1.3	4.9±0.8	5.1±1.1	4.7±0.9
Triglycerides (mmol/L)	1.5 (1.0–1.8)	1.0 (0.7–1.1)	1.9 (1.1–2.3)	1.1 (0.7–1.3)
HDL cholesterol (mmol/L)	1.4±0.5	1.6±0.4	1.2±0.4	1.4±0.4

Data are mean ± SD, n (%), or mean (interquartile range).

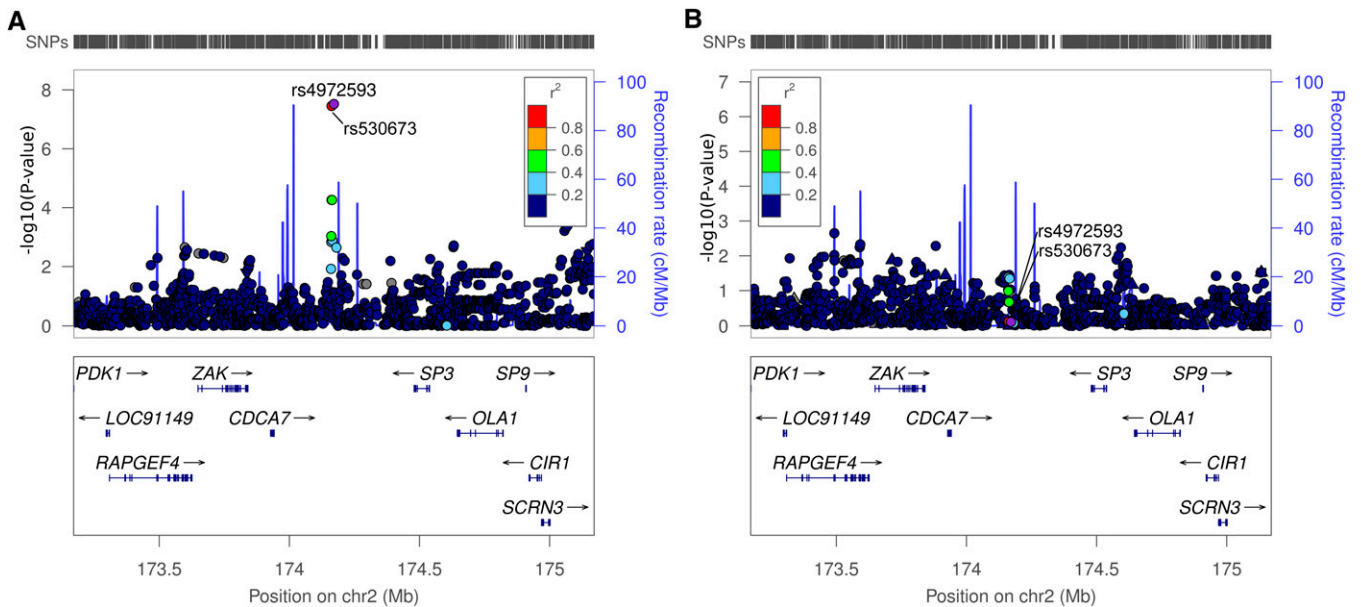


Figure 1. Regional Manhattan association plot of the associated region at rs4972593 showing association in women (A) but not in men (B). The color of the SNP symbol indicates the linkage disequilibrium (r^2) with the index SNP (purple) in the 1000 Genomes CEU population.

African American ($N_{cases}=570$), European American ($N_{cases}=165$), or Mexican American ($N_{cases}=413$) women with diabetes (Supplemental Table 4). Furthermore, we sought to investigate whether rs4972593 affects the risk for ESRD in women without diabetes by studying female patients of the Wellcome Trust Case-Control Consortium (WTCCC) study on Renal Transplant Dysfunction. However, no adequate proxies were available for rs4972593 in the WTCCC GWAS, and, thus, the role of rs4972593 in the nondiabetic women remains unclear.

The SNP rs4972593 is located in an intergenic region between the *SP3* and *CDCA7* genes. *In silico* analysis of the associated SNP for transcription factor-binding sites (TFBS) indicates that the presence of the minor A allele results in loss of several TFBSs, including binding sites for E-box and hypoxia inducible factors (Supplemental Table 5). Moreover, eight estrogen-responsive elements (EREs) were predicted within 5k base pairs (bp) up- or downstream of rs4972593 (Supplemental Table 6). rs530673, a SNP in high linkage disequilibrium with rs4972593 ($D'=1$; $r^2=1$ in HapMap II CEU), indicated potential regulatory activity (GATA2 binding,

SMAD4 binding motif, DNase hypersensitivity peak) in the ENCODE RegulomeDB (Supplemental Table 7).¹² However, no significant expression quantitative trait loci (eQTL) association was observed between rs4972593 or any other SNP in linkage disequilibrium with rs4972593 and the expression level of the genes within a 1-Mbp region up- or downstream of rs4972593 in the human HapMap3 lymphoblastoid cell line (Supplemental Table 8).¹³

We then assessed DN- and sex-related differential gene expression of the flanking genes *SP3* and *CDCA7*.^{14,15} Interestingly, *SP3* was among the top 3% for the sex-specific gene expression in glomeruli (fold change, -1.45 [*i.e.*, higher expression in women]; $P=0.004$) Supplemental Table 9).¹⁵ No renal expression was reported for *CDCA7* in studies of DN.^{14,15}

CDCA7 encodes a transcription factor that participates in the regulation of cell proliferation, targeted by Myc, and is frequently overexpressed in human cancers.¹⁶ *SP3* encodes the transcription factor Sp3, which binds to consensus GC-box and GT-box regulatory elements in target genes. Interestingly, Sp3 directly interacts with the estrogen receptor- α (ER α), and 17 β -estradiol has been shown to activate GC-rich promoter

constructs through the ER α /Sp3 pathway, independent of the classic EREs. Among the ER α /Sp3 pathway target genes, 17 β -estradiol differentially regulates *VEGFA* expression in various cell models.^{17,18} Vascular endothelial growth factor plays a key role in the angiogenesis, and it has been suggested as one of the common pathogenic factors behind retinopathy and nephropathy.¹⁹

We recently reported an induction of the Sp1/Sp3 transcriptional network in human renal tubule epithelial cells stimulated with the profibrotic cytokine TGF- β and identified the Sp3 target gene *TGFBI* as the top-ranked upregulated gene.²⁰ Other noteworthy Sp3 target genes include *COL1A1*,²¹ *NOTCH1*,²² and *CD2AP*, encoding an adapter protein essential for the glomerular filtration barrier interacting with the slit diaphragm proteins nephrin and podocin.^{23,24} In addition to the sex-specific glomerular expression reported for *SP3*, these data highlight this gene as a plausible positional and biologic candidate for future fine-mapping studies in women with T1D.

Despite the evidence that sex influences the risk of ESRD in patients with T1D, no large-scale sex-specific genetic studies have thus far been reported, and none of

Table 2. Association analysis results for rs4972593

Variable	Women					Men				
	Allele Frequency in Cases/Controls	Cases/Controls (n/n)	Power	OR (95% CI)	P Value	Allele Frequency in Cases/Controls	Cases/Controls (n/n)	Power	OR (95% CI)	P Value
FinnDiane	0.17/0.09	258/935	0.97	2.39 (1.75 to 3.25)	3.02×10^{-8}	0.11/0.11	387/655	0.99	0.95 (0.69 to 1.32)	0.77
Replication										
UK-ROI	0.18/0.16	113/508	0.86	1.16 (0.75 to 1.80)	0.50	0.16/0.14	146/395	0.87	1.10 (0.70 to 1.71)	0.68
GoKinD US	0.17/0.13	252/479	0.97	2.07 (1.24 to 3.46)	0.0055	0.14/0.13	256/342	0.92	1.04 (0.58 to 1.86)	0.89
Italy	0.18/0.15	65/86	0.48	1.20 (0.64 to 2.24)	0.63	0.14/0.17	105/99	0.53	0.78 (0.44 to 1.36)	0.40
Meta	0.16	430/1073	1.00	1.41 (1.05 to 1.90)	0.021	0.14	507/836	1.00	0.98 (0.73 to 1.32)	0.90
replication										
Meta all	0.14	688/2009	1.00	1.81 (1.47 to 2.24)	3.85×10^{-8}	0.13	894/1491	1.00	0.97 (0.78 to 1.21)	0.78

Association analyses are calculated with the minor A allele as the effect allele. Meta replication is the meta-analysis of the three replication cohorts, and meta all is the meta-analysis of all four cohorts; for both, a weighted estimate of the minor allele frequency over all patients is given. Allele frequency refers to the frequency of the minor A allele. Power refers to the statistical power to detect association with $\alpha=0.05$ and OR of 1.75 (i.e., the lower 95% CI for women in FinnDiane). Power to detect the association with $\alpha=5 \times 10^{-8}$ was 0.08 in women and 0.12 in men in the discovery cohort and 0.93 in women and 0.92 in men in the combined meta-analysis.

the earlier reported genetic susceptibility loci for ESRD in T1D showed sex-specific association. Here we were able to detect a novel locus near *SP3* associated with ESRD in women. Because no association was seen in men for rs4972593, it is not surprising that this locus was not detected in our previous GWAS on ESRD, achieving a modest *P* value of 6.7×10^{-5} when men and women were analyzed together. This emphasizes the importance of analyzing well characterized, homogeneous subgroups in a GWAS setting in parallel to combining all available patients. Other sex-specific susceptibility loci for ESRD may be discovered when larger GWASs become available. In conclusion, our data point to a biologically plausible pathway and suggest that the association on 2q31.1 may be one explanation for why some women with T1D lose their protection against ESRD.

CONCISE METHODS

Patients

Study participants have previously been described in detail.⁸ The discovery cohort consisted of 3652 patients with T1D from FinnDiane, a Finnish nationwide multicenter study.⁹ The main clinical characteristics of the patients are shown in Table 1. Replication was performed on three independent cohorts participating in the GENIE collaboration: the UK-ROI collection, with 1830 genotyped patients

with T1D; the GoKinD US cohort, with 1792 patients; and an Italian cohort from the Milan region comprising of 397 patients with T1D.

We compared patients who had T1D and ESRD with T1D controls who had no evidence of diabetic kidney disease despite long duration of diabetes. ESRD was defined as the need for dialysis treatment or having received a kidney transplant, and the minimum duration of T1D was 10 years. We excluded all patients with T1D who were known to have ESRD due to any nondiabetic cause. In FinnDiane, 85% of the patients with ESRD had laser treatment (9% had missing data) and only six had no retinopathy. In UK-ROI and GoKinD US, all ESRD patients had retinopathy as an inclusion criterion. Controls were defined as patients with T1D who had stable normal urinary albumin excretion and diabetes duration of at least 15 years, as described earlier.⁸

Genotyping

Genotyping of the FinnDiane patients was performed using the Illumina 610Quad chip; genotype calling, quality control, and imputation procedures have been described earlier.⁸ In brief, genotypes with low genotyping quality or low minor allele frequency (<0.01) were discarded, as were samples with low genotyping quality or cryptic relatedness and geographic outliers based on principal component analysis. A total of 3546 samples and 549,530 SNPs remained after quality control. Imputation was based on the HapMap II CEU population and resulted in

approximately 2.4 million SNPs across the autosomal genome. For the replication in UK-ROI and GoKinD US, rs4972593 was selected from the UK-ROI and GoKinD US GWAS data, where the quality control and imputation procedures were the same as described above. Quality control resulted in 1726 UK-ROI samples and 1595 GoKinD US samples. The DNA samples from Italy were genotyped using Sequenom IPLEX assays (Sequenom Inc., San Diego, CA). Imputation and genotyping quality metrics are given in Supplemental Table 10.

Patients and Genotyping in the FIND Study

The FIND cohort, consisting of 885 samples from European Americans, 1460 samples from African Americans, 889 samples from American Indians, and 1535 samples from Mexican Americans, underwent genotyping using the Affymetrix SNP 6.0 GeneChip at the Genotyping Core Facility at Affymetrix (Santa Clara, CA). Samples were plated by ethnic group, randomly assigned by case/control status; 217 pairs of duplicate samples were included for quality control. Genotype calls used the Birdsuite algorithm as implemented in GCOS software (Affymetrix). Samples with call rates $>95\%$ were subject to additional quality control procedures for sample and SNP heterozygosity, sample and SNP missingness, sex verification, expected and unexpected relatedness, and population substructure analysis *via* principal components analysis. After trimming, 342 cases

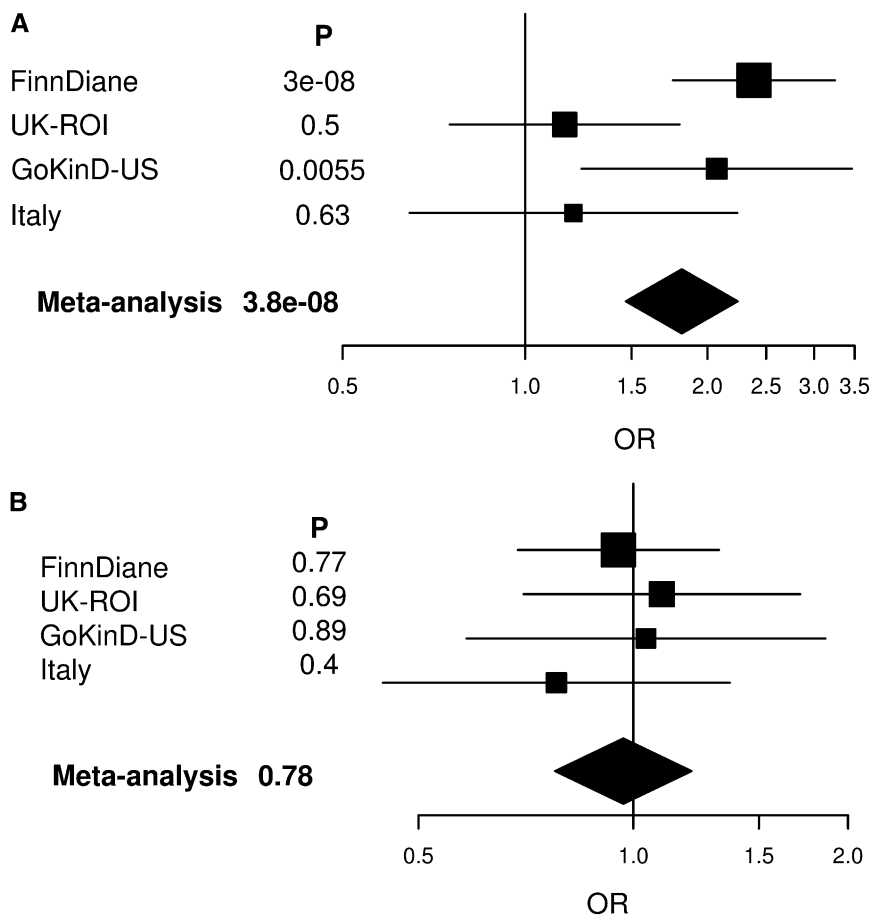


Figure 2. Forest plot of the meta-analysis of ESRD association at rs4972593 showing association in women (A) but not in men (B). Plots show the study-specific association estimates (ORs and 95% CIs) for the discovery and replication cohorts. The OR and 95% CI for the meta-analysis of the discovery and replication cohorts are denoted by the diamond.

and 404 controls of European American ancestry, 979 cases and 304 controls of African American ancestry, 538 cases and 319 controls of American Indian ancestry, and 779 cases and 594 controls of Mexican American ancestry were included in a final meta-analysis.

Statistical Analyses

The association analysis that compared ESRD cases with T1D controls with normal albumin excretion despite long duration of T1D was performed with PLINK 1.07²⁵ using logistic regression and adjustment for age, T1D duration, and the 10 first principal components that were obtained with the EIGENSTRAT software.²⁶ Estimated allele dosages were used rather than the most likely genotypes in order to account for the uncertainty arising from the imputation process. Women and men were analyzed separately. The quantile-

quantile plots of both analyses showed good adherence to the diagonal line of expected *P* values, and very little excess genomic inflation was observed ($\lambda=1.034$ for women, $\lambda=1.045$ for men). The association results were adjusted for the genomic inflation. The same methods were used for the statistical analysis of UK-ROI and GoKinD US. Because of the relatively small number of women with ESRD in the Italian cohort, for those samples we used the Fisher exact test of association, which is deemed more robust for small sample numbers. Consequently, the Italian replication cohort was not adjusted for any covariates. Meta-analysis of the four cohorts was performed using a fixed-effect model based on the standard errors and *P* values, implemented with METAL software.²⁷ Power calculations were performed with Genetic Power Calculator.²⁸

TFBS and Regulatory Function

We looked for the TFBSs directly created or deleted due to rs4972593 using MatInspector (release professional 8.06; Matrix Family Library, version 8.4,) from the Genomatix software suite (Genomatix Software, GmbH, Munich, Germany). The flanking region 5 kbp up- and downstream of rs4972593 was downloaded from National Center for Biotechnology Information SNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP/>), and MatInspector was used to detect EREs (V\$EREF family) within this region. Furthermore, we sought evidence of the regulatory function of the SNPs using the RegulomeDB database, which annotates SNPs with known and predicted regulatory elements in the intergenic regions. The annotation is based on regions of DNAse hypersensitivity, TFBS, and promoter regions that have been biochemically characterized to regulate transcription.¹²

Renal Gene Expression

Disease- and sex-associated gene expression in published human DN microarray data sets was determined using Nephromine (www.nephromine.org). Selected data sets comprised microdissected renal biopsy specimens from patients with DN versus living donors or patients who had minimal-change disease.^{14,15} We performed a total of 10 various sex- and disease-specific association look-ups (Supplemental Table 9). Therefore, the *P* values were adjusted for multiple testing according to 10 performed tests; a *P* value of 0.005 was required for significance after adjustment.

eQTL Gene Expression

We studied whether rs4972593 was associated with the gene expression level of any of the genes within a 1-Mbp region up- and downstream in the HapMap3 lymphoblastoid cell lines¹³ using the Genevar user interface (<http://www.sanger.ac.uk/resources/software/genevar/>). The analysis included all the SNPs in full linkage disequilibrium ($r^2=1$) with rs4972593 in the HapMap2 CEU samples and with data on HapMap3 eQTL in Genevar; three SNPs filled the criteria (rs530673, rs4972590, rs4972591). The *P* value threshold for statistical significance after multiple testing was $P<0.00063$ based on $\alpha=0.05$ significance level, 10 studied

genes, and 8 included HapMap populations. Because the selected SNPs were in strong linkage disequilibrium, we did not adjust for the number of SNPs.

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