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RESEARCH ARTICLE

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A meta-analysis of genome-wide data from five European isolates reveals an association of *COL22A1*, *SYT1*, and *GABRR2* with serum creatinine level

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Abstract

Background: Serum creatinine (S_{CR}) is the most important biomarker for a quick and non-invasive assessment of kidney function in population-based surveys. A substantial proportion of the inter-individual variability in S_{CR} level is explicable by genetic factors.

Methods: We performed a meta-analysis of genome-wide association studies of S_{CR} undertaken in five population isolates ('discovery cohorts'), all of which are part of the European Special Population Network (EUROSPAN) project. Genes showing the strongest evidence for an association with S_{CR} (candidate loci) were replicated in two additional population-based samples ('replication cohorts').

Results: After the discovery meta-analysis, 29 loci were selected for replication. Association between S_{CR} level and polymorphisms in the collagen type XXII alpha 1 (*COL22A1*) gene, on chromosome 8, and in the synaptotagmin-1 (*SYT1*) gene, on chromosome 12, were successfully replicated in the replication cohorts (p value = 1.0×10^{-6} and 1.7×10^{-4} , respectively). Evidence of association was also found for polymorphisms in a locus including the gamma-aminobutyric acid receptor rho-2 (*GABRR2*) gene and the ubiquitin-conjugating enzyme E2-J1 (*UBE2J1*) gene (replication p value = 3.6×10^{-3}). Previously reported findings, associating glomerular filtration rate with SNPs in the uromodulin (*UMOD*) gene and in the schroom family member 3 (*SCHROOM3*) gene were also replicated.

Conclusions: While confirming earlier results, our study provides new insights in the understanding of the genetic basis of serum creatinine regulatory processes. In particular, the association with the genes *SYT1* and *GABRR2* corroborate previous findings that highlighted a possible role of the neurotransmitters GABA_A receptors in the regulation of the glomerular basement membrane and a possible interaction between GABA_A receptors and synaptotagmin-I at the podocyte level.

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Background

In epidemiological population-based surveys, serum creatinine (S_{CR}) represents the most important biomarker for a quick and non-invasive assessment of kidney function, allowing estimation of the glomerular filtration rate (GFR) [1]. At the same time, an increased S_{CR} level has also been recognized as a risk factor for adverse outcomes in patients hospitalized for cardiac surgery or heart failure [2]. A substantial proportion of the inter-individual variability in S_{CR} level is explicable by genetic factors. Twin [3] and pedigree-based studies [4-7] yielded heritability estimates ranging from 0.19 to 0.53, with higher values observed in subjects not treated for hypertension [4]. A large number of genetic loci have emerged from genome-wide linkage analyses as being related to variation in S_{CR} level or in S_{CR} -based estimates of both GFR and creatinine clearance [4-15]. Focusing upon quantitative renal phenotypes, a recent GWA study [16] and a subsequent replication study [17] identified variants in the 5,10-methylenetetrahydrofolate synthetase (*MTHFS*) gene to be associated with chronic kidney disease (CKD; defined as $GFR < 60$ ml/min/1.73 m²). More recently, variants in the uromodulin (*UMOD*) gene have been shown to be associated with GFR and CKD independent of age, sex, hypertension, and diabetic status [18]. In the same study, SNPs in the schroom family member 3 (*SCHROOM3*) gene, the glycine amidinotransferase (*GATM*)-spermatogenesis associated 5-like 1 (*SPATA5L1*) locus, and in the jagged 1 (*JAG1*) gene were also found to be associated with GFR [18].

We have reported linkage between S_{CR} level and a region at 22q13 containing the myosin heavy chain 9 non-muscle (*MYH9*) gene [4], a locus that had been associated with non-diabetic end-stage renal disease (ESRD) [19,20] and glomerulosclerosis [21] before. This linkage was detected in families from three isolated European populations participating in the European Special Population Research Network (EUROSPAN). Here, we have performed a genome-wide association analysis of S_{CR} level combining data from all five EUROSPAN populations, with S_{CR} re-measured using an enzymatic method in one and the same central laboratory. We have performed population-specific GWA studies and subjected the results to an inverse-variance, fixed effects meta-analysis. Selected candidate regions were then tested in two additional, population-based samples from Europe.

Methods

Study samples

For all EUROSPAN and replication studies, written informed consent was obtained from all participants and

all protocols were approved by the institutional ethical review committees of the participating centres.

Eurospan

The EUROSPAN project <http://homepages.ed.ac.uk/s0565445/index.html> was initiated in 2006 and involves five population isolates from Italy, Croatia, Scotland, Sweden, and the Netherlands. The project aims at assessing the genetic structure of European isolates and at identifying genes underlying common traits, taking advantage of the genetic and environmental homogeneity that usually characterizes population isolates. In the current context, according to Neel [22], with population isolates we mean “secondary isolates”, i.e. groups that, for some reasons, detached or were detached from larger populations. In particular, EUROSPAN cohorts were derived from small population samples which have grown slowly, with little recruitment from outside the groups.

The *ERF* study is a family-based project including over 3000 participants that originated from 22 couples living in the Rucphen region of the Netherlands in the 19th century [23-25]. All descendants of these people were invited to visit a clinical research center in the region, where they were examined in person and where blood was taken after fasting. Height and weight were measured for each participant. All participants filled out a questionnaire on risk factors.

The *MICROS* study is part of the genomic health care program ‘GenNova’ and was carried out in three villages of the Val Venosta, South Tyrol (Italy), in 2001-2003. It comprised members of the populations of Stelvio, Valledlunga and Martello. A detailed description of the *MICROS* study can be found elsewhere [26]. Briefly, study participants were volunteers from three isolated villages located in the Italian Alps, in a German-speaking region bordering upon Austria and Switzerland. Owing to geographical, historical and political reasons, the entire region experienced a prolonged period of isolation from surrounding populations. Information on the participants’ health status was collected through a standardized questionnaire. Laboratory data were obtained from standard blood analyses. Genotyping was performed on >1400 participants, with 1334 of them suitable for analysis after data cleaning.

The Northern Swedish Population Health Study (*NSPHS*) is a family-based study including a comprehensive health assessment and the collection of data on family structure, lifestyle, diet, medical history and of samples for laboratory analyses [27,28]. Participants came from the northern part of the Swedish mountain region (County of Norrbotten, Parish of Karesuando). Historic population accounts show that little migration

or population changes have occurred in this area over the last 200 years.

The Orkney Complex Disease Study (*ORCADES*) is an ongoing, family-based and cross-sectional study in the isolated Scottish archipelago of Orkney [29]. Genetic diversity in this population is reduced in comparison to Mainland Scotland, consistent with high levels of historical endogamy. Participants were aged 18-100 years and came from a subgroup of ten islands. Fasting blood samples were collected and over 200 health-related phenotypes and environmental exposures were measured in each individual.

The *Vis* study includes 986 unselected Croatians, aged 18-93 years, who were recruited during 2003 and 2004 from the villages of Vis and Komiza on the Dalmatian island of Vis [30,31]. The settlements on Vis island have a unique history and remained isolated from other villages and the outside world for centuries. Participants were phenotyped for 450 disease-related quantitative traits. Biochemical and physiological measurements were performed, detailed genealogies reconstructed, questionnaires on lifestyle and environmental exposures collected, and blood samples and lymphocytes extracted and stored for further analyses.

All DNA samples were genotyped on Illumina Infinium HumanHap300 v2 SNP bead microarrays according to the manufacturer's instructions, except for samples from Vis for which version 1 was used (the Vis samples had 311,398 SNPs genotyped in common with the other populations).

For all five studies, serum or plasma creatinine was measured at the Institute for Clinical Chemistry and

Laboratory Medicine, Regensburg University Medical Center, Germany, using an enzymatic photometric assay on an ADVIA1650 clinical chemistry analyzer (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany) [32]. The number of individuals with available creatinine, sex, and age information is reported for each study in Table 1.

Replication Cohorts

Data on German healthy control individuals were obtained from the popgen biobank [33]. Genotyping constituted an essential part of the GWAS initiative of the German National Genome Research Network (NGFN) and was performed at an Affymetrix service facility (South San Francisco, CA, USA) using the Affymetrix Genome-Wide Human SNP Array 6.0 (1000 k) (Santa Clara, CA, USA). Genotype calling was carried out using Affymetrix' Birdseed v2 algorithm with default quality thresholds. Samples with more than 5% missing genotypes, showing excess genetic dissimilarity to the remaining subjects, or with evidence for a cryptic relatedness to other study participants were removed. These quality control measures left 1213 control samples for inclusion in the replication cohort. All sex assignments could be verified by reference to the proportion of heterozygous SNPs on the X chromosome. Serum creatinine was available for 1140 individuals and was measured at the Institute for Clinical Chemistry in Kiel, Germany, using an enzymatic *in vitro* assay (CREAplus, Cobas®, Roche Diagnostics, Indianapolis, IN).

The *Korcula* study included 944 unselected 18-98 year old Croatians, recruited into the study during 2007 from

Table 1 Characteristics of studies and study participants.

| Study name | EUROSPAN | | | | | REPLICATION STUDIES | |
|--|-----------------|----------------|----------------|----------------|----------------|---------------------|----------------|
| | ERF | MICROS | NSPHS | ORCADES | VIS | popgen | Korcula |
| Nationality | The Netherlands | Italy | Sweden | UK | Croatia | Germany | Croatia |
| Population type | isolated | isolated | isolated | isolated | isolated | general | isolated |
| Genotyping platform | Illumina 318 K | Illumina 318 K | Illumina 318 K | Illumina 318 K | Illumina 318 K | Affymetrix 1000 K | Illumina 370 K |
| Sample size* | 775 | 1086 | 653 | 718 | 774 | 1140 | 895 |
| Females: n (%) | 472 (61%) | 615 (57%) | 345 (53%) | 385 (54%) | 454 (59%) | 534 (47%) | 572 (64%) |
| Age: mean (sd) | 53 (15) | 45 (16) | 47 (21) | 54 (16) | 57 (15) | 54 (15) | 56 (14) |
| Diabetes: n (%) | 36 (4.9%) | 39 (3.6%) | 44 (6.7%) | 21 (3.0%) | 72 (9.4%) | 18 (1.6%) | 93 (10.3%) |
| AHT [#] : n (%) | 190 (24.5%) | 85 (7.8%) | 124 (19.0%) | 152 (21.2%) | 192 (25.2%) | Not available | 197 (22.0%) |
| S _{CR} mg/dl: mean (sd) | | | | | | | |
| Males | 1.01 (0.20) | 0.96 (0.14) | 0.94 (0.20) | 0.97 (0.15) | 1.01 (0.31) | 0.93 (0.15) | 0.92 (0.15) |
| Females | 0.85 (0.21) | 0.78 (0.12) | 0.75 (0.14) | 0.77 (0.19) | 0.79 (0.27) | 0.74 (0.12) | 0.75 (0.12) |
| eGFR [†] ml/min/1.73 m ² | | | | | | | |
| Males | 81.1 (19.0) | 87.8 (15.1) | 92.2 (22.1) | 84.3 (16.0) | 82.0 (19.4) | 88.0 (17.5) | 77.1 (14.9) |
| Females | 75.8 (20.5) | 83.4 (16.8) | 88.7 (19.2) | 81.5 (18.5) | 81.0 (19.8) | 85.5 (17.2) | 72.4 (14.1) |

* Number of individuals with available creatinine, sex, and age information.

[#] AHT: Anti-hypertensive treatment

[†] Estimated using the updated abbreviated MDRD study equation [58]

the island of Korcula [34]. The settlements on Korcula included the Eastern region of the island, which has a unique population history and has maintained a high level of isolation from other mainland populations and from the Western part of the island. Participants were phenotyped for >400 disease-related quantitative traits. Biochemical and physiological measurements were performed, genealogies reconstructed, questionnaires on lifestyle and environmental exposures collected, and blood samples and lymphocytes extracted and stored for further analyses. DNA was genotyped using the Illumina Infinium HumanCNV370v1 SNP bead microarrays. Serum creatinine as measured by the Jaffé rate method was available for 895 individuals.

Statistical analysis

To ensure normality within centers and comparability of S_{CR} values across centers, we applied a quantile normalization in all discovery and replication studies, which involves ranking all S_{CR} values and converting them to z-scores according to a standard normal distribution.

Discovery stage

To account for inter-individual relatedness within the five EUROSPAN cohorts, genome-wide association (GWA) analysis was carried out following a two stage approach. In the first stage, a sex- and age-adjusted linear model was fitted to the normalized S_{CR} in order to estimate the residuals. A polygenic model was then fitted to estimate the inverse of the variance-covariance matrix, which accounts for the inter-individual relatedness and is based upon a genomic kinship matrix as described in Amin *et al.* [35]. The association between SNPs and residuals was assessed by means of an approximate score test statistic [36], assuming an additive model, as implemented in the GenABEL package [37]. The results from the five EUROSPAN cohorts were then combined into a fixed-effects meta-analysis with inverse-variance weighting, using MetABEL <http://mga.bionet.nsc.ru/~yurii/ABEL/>. Only SNPs that had a call rate ≥ 0.95 , a Hardy-Weinberg equilibrium (HWE) test p value $> 10^{-6}$ and a minor allele frequency (MAF) ≥ 0.01 were included in the analyses. In total, 322,498 SNPs were tested for association with S_{CR} . The threshold for genome-wide statistical significance, according to Bonferroni adjustment for multiple testing, was set to 1.55×10^{-7} . Between-study heterogeneity was quantified using the I^2 statistic, i.e. the percentage of total variation explained by heterogeneity rather than sampling error [38].

Replication stage

In the absence of clear methodological guidance on what may be the best strategy for passing SNPs to a replication stage, we selected SNPs for replication based on a tradeoff that enabled us to include our best

findings from the discovery analysis, whilst avoiding the risk of an excessively long list. This was achieved by including all SNPs with a p value $\leq 10^{-5}$, but also allowing for SNPs with higher p values to get into the replication list in the presence of additional evidence for association provided by other SNPs within 100 kb. The following criteria were applied: (i) at least one p value $\leq 10^{-5}$; (ii) at least one p value $\leq 10^{-4}$ and at least one additional SNP with p value $\leq 10^{-3}$ within 100 kb; (iii) at least three SNPs with p value $\leq 10^{-3}$ within 100 kb. For each group of candidate SNPs, we selected a candidate gene (all SNPs included in the gene were considered for replication) or region (all SNPs included within ± 100 kb of the candidate SNPs were considered for replication) to test in the independent cohorts of Korcula and popgen. The complete workflow of the test procedure is depicted in Figure 1.

In popgen, the association between S_{CR} and each SNP within a candidate region was assessed by means of a sex- and age-adjusted linear regression model assuming additive genetic effects, using PLINK version 1.05 <http://pngu.mgh.harvard.edu/purcell/plink/> [39]. For Korcula, given that it is a family-based study, we used the same approach as described above for the EUROSPAN cohorts.

To assess the significance in the replication cohorts of associations with different SNPs from those genotyped in the discovery cohorts, we defined region-specific p values as follows: for a genomic region containing N SNPs, we counted the number n of SNPs that achieved p value < 0.05 . Assuming that p values are uniformly distributed between 0 and 1 under the null hypothesis of no association, the distribution function of n , $F(\cdot)$, is that of a binomial with parameters N and 0.05. The region-specific p value then equals $1 - F_{N,0.05}(n-1)$. Given that in small genomic regions the assumption of independency is rarely met, the estimated p value should be considered as conservative since the effective number of independent tests can only be lower than the total number of SNPs tested. Meta-analysis of region-specific p values from popgen and Korcula was finally performed using the Fisher's combined probability test [40], which is suitable for combining tests performed in independent samples. We further evaluated rejection of the null hypothesis of no association based on a false discovery rate (FDR) of 0.05 [41], where p values are sorted in ascending order and the first k tests with p value $\leq i/m \times \alpha$ are considered significant (in our case, $i = 1..29$, $m = 29$, $\alpha = 0.05$).

Replication of previous findings

We finally assessed whether any of the four loci reported to be associated with eGFR by Köttgen *et al.* [18] showed evidence for an association with S_{CR} in our discovery meta-analysis. Köttgen *et al.* reported four

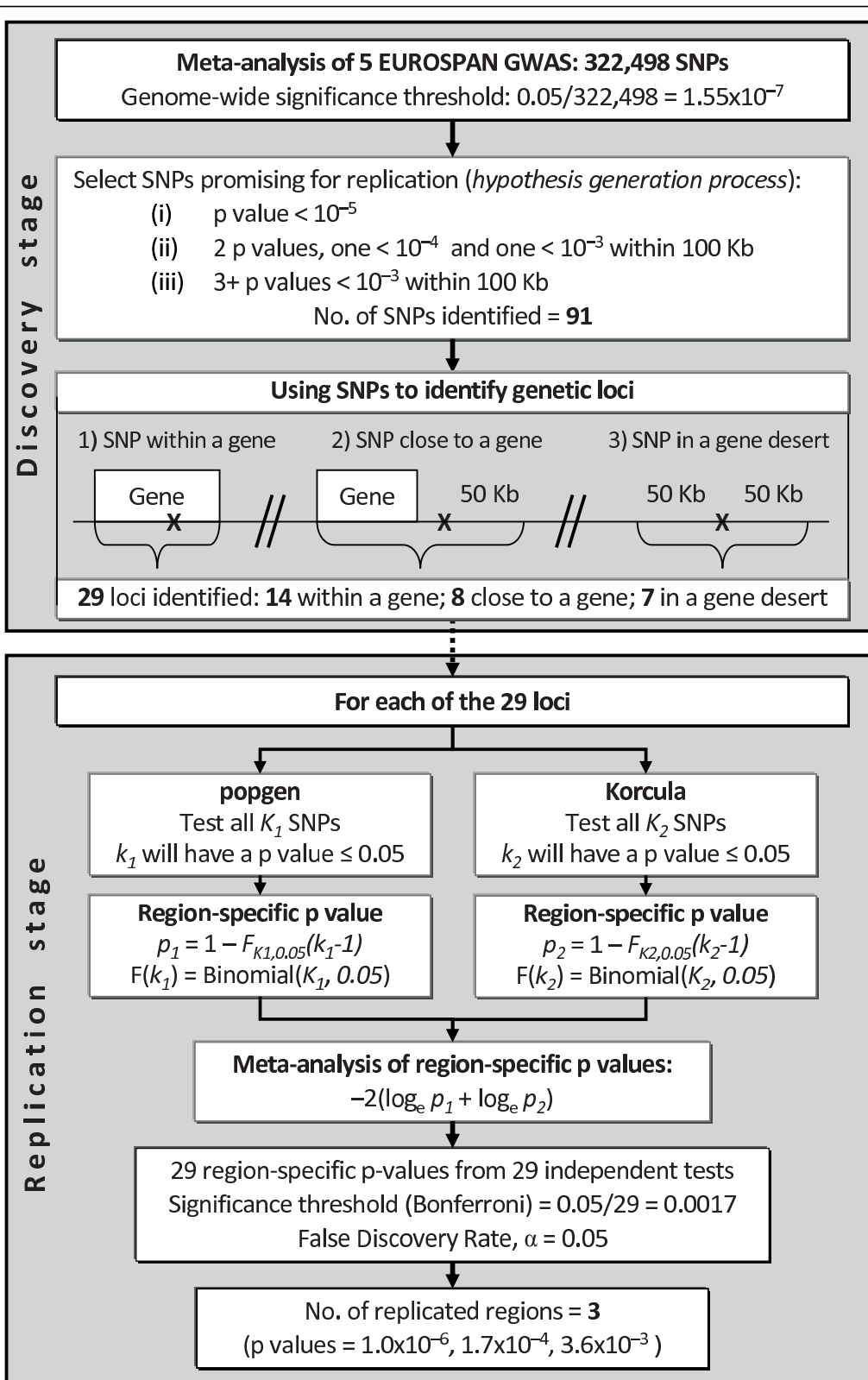


Figure 1 Workflow of the discovery and replication stages of the study (for details, see the *Statistical Analysis* section).

SNPs to be associated with GFR: rs17319721 (*SHROOM3*), rs2467853 (*SPATA5L1-GATM*), rs12917707 (*UMOD*), and rs6040055 (*JAG1*). For each of the four SNPs, we defined LD blocks around them, following the method by Gabriel *et al.* [42] and using SNPs with MAF $\geq 1\%$ from the HapMap-CEU database, Phase III/Release 2. Given that the genes were in four independent loci, the threshold for claiming significant replication, according to Bonferroni, was set to $0.05/4 = 0.0125$. To compare our results with the commonly used method of testing replication at the same SNP or at a proxy SNP, we repeated the test procedure at one SNP per locus, using the same threshold for statistical significance.

If not specified otherwise, all data management, data analysis, programming, and the creation of graphs were performed using R 2.8.0 [43].

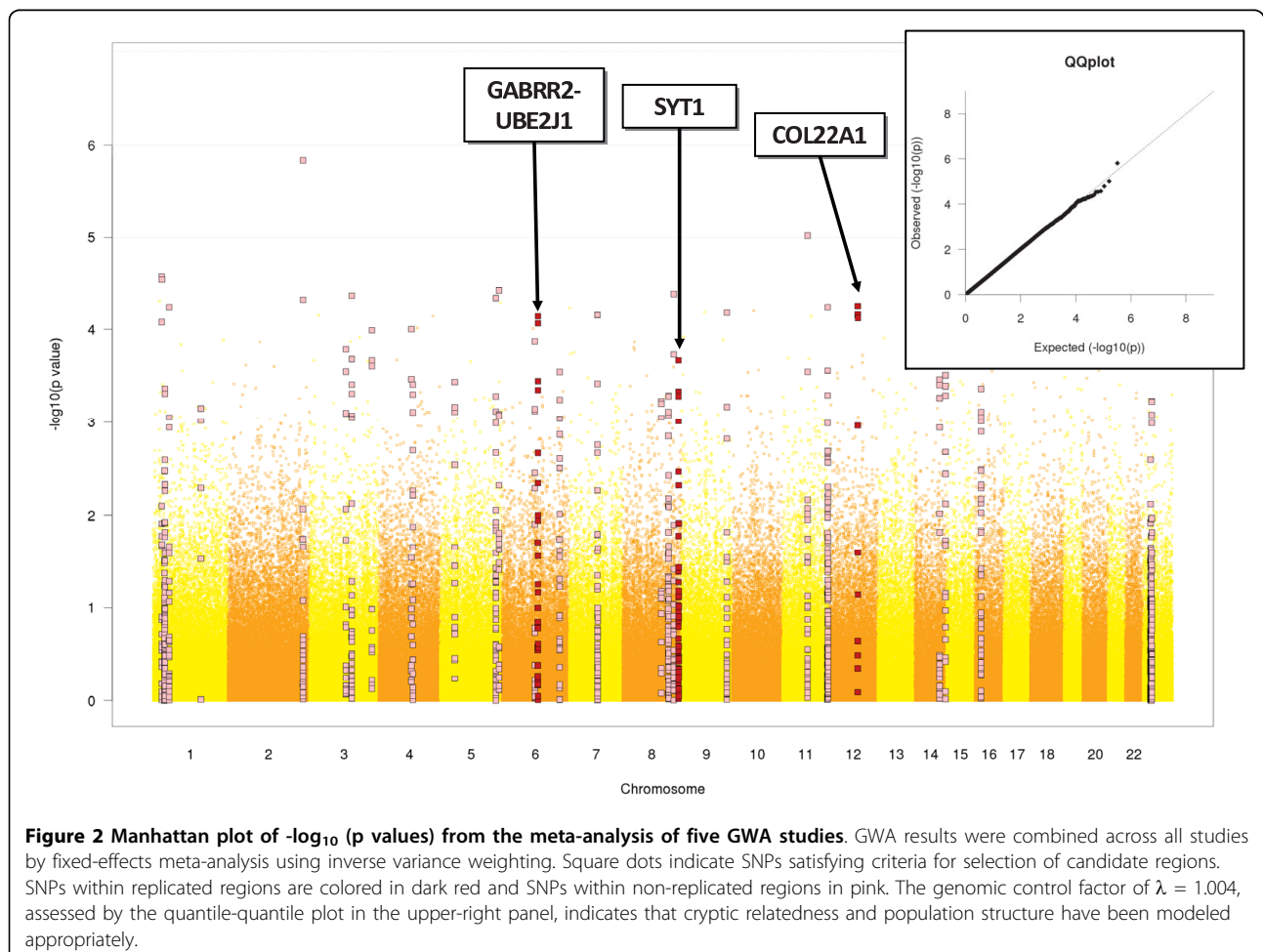
Results

Study-specific characteristics of the participants are reported in Table 1. A total of 4006 individuals were

included in the meta-analysis whilst the replication cohorts comprised 2035 individuals. All EUROSPAN cohorts comprised a higher percentage of females (between 53% and 61%) than males. The mean age ranged from 45 years in MICROS to 57 years in VIS. The replication samples had a similar age range, but a smaller percentage of females than males included in popgen.

The results of the GWA meta-analysis are depicted in Figure 2. The genomic control factor (λ), as assessed by the quantile-quantile plot included in Figure 2, was 1.004 (SE $< 10^{-5}$), indicating that no cryptic relatedness or gross population structure affected our results [44].

The smallest p value in the meta-analysis was 1.5×10^{-6} and was obtained for SNP rs2396463 in the collagen type IV alpha-3 (*COL4A3*) gene on chromosome 2q. In the absence of hits of genome-wide significance, we selected a set of promising regions for follow-up in two independent replication samples, namely Korcula (an island population from Croatia) and popgen (a random population sample from the most northern part of



Germany). Twenty-nine genomic regions were considered for replication. The full list of regions and the results of the discovery meta-analysis are provided in the Additional file 1 - Table S1. Since the SNP panels genotyped in the replication samples differed from one another and from that used in the discovery GWA studies, all markers present in the candidate regions were included in the replication phase. A total of 2224 SNPs were analyzed in the popgen samples and 1136 SNPs were analyzed in the Korcula samples. Results of the replication analysis are fully reported in the Additional file 1 - Table S2, with loci sorted by significance.

Two candidate regions from the discovery GWA studies also showed significant evidence for an association with S_{CR} level in the popgen and Korcula samples after Bonferroni correction for multiple testing of the combined p value (significance threshold for 29 independent tests: $\alpha = 0.0017$). When controlling for the FDR, i.e. for the probability of reporting a false positive result, a

third region could be added to the list of replicated findings (Figure 3).

The first replicated region was defined by four SNPs (rs4075073, rs4588898, rs9324496, and rs2873682) in the collagen type XXII alpha 1 (*COL22A1*) gene, all of which yielded p values $\leq 9.79 \times 10^{-4}$ in the meta-analysis of the discovery stage, with homogeneous effects observed across populations: $I^2 = 0\%$ for all the four SNPs. A forest plot of the marker with the strongest evidence for association, SNP rs4588898, is provided in Figure 4A. Six other SNPs in the gene (out of 72) had p values between 3.42×10^{-3} and 0.0414 (Table 2). As it can be inferred from Figure 5, some 19 of 140 *COL22A1* SNPs genotyped in popgen were found to be significantly associated with S_{CR} (red squares), as were 11 out of 71 SNPs in Korcula (green squares). Fisher's combined probability test p value was 1.0×10^{-6} . Nevertheless, the gene regions with the strongest S_{CR} association in EUROSPAN and either popgen or Korcula failed

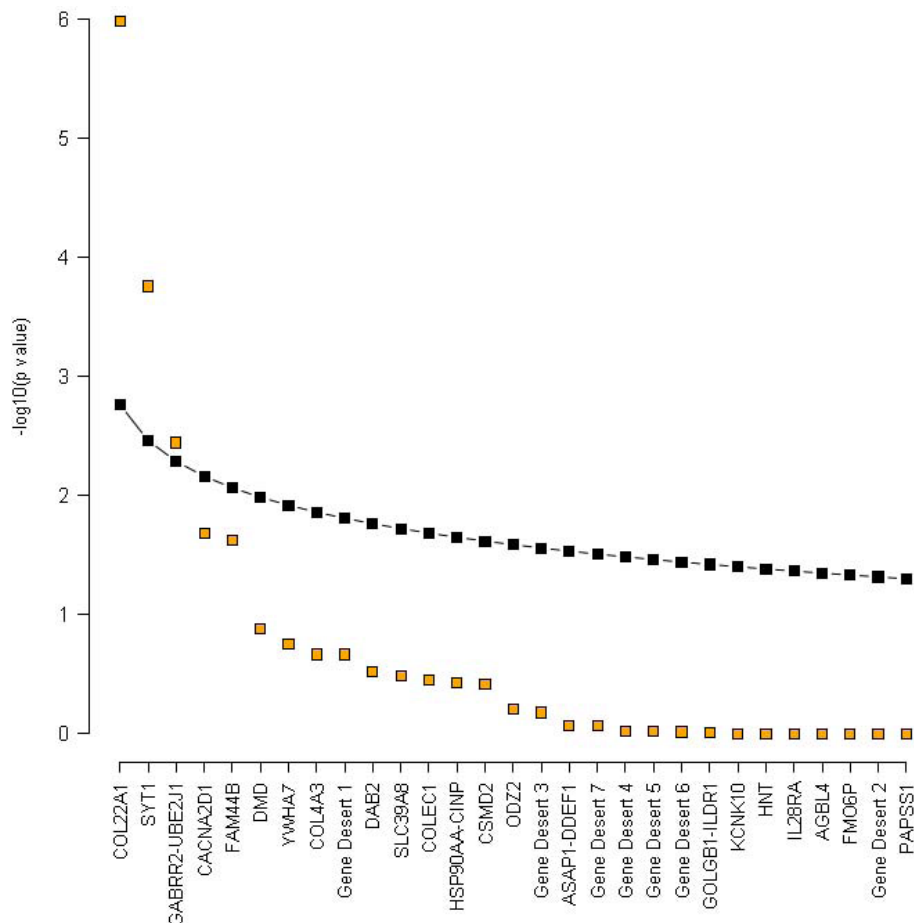
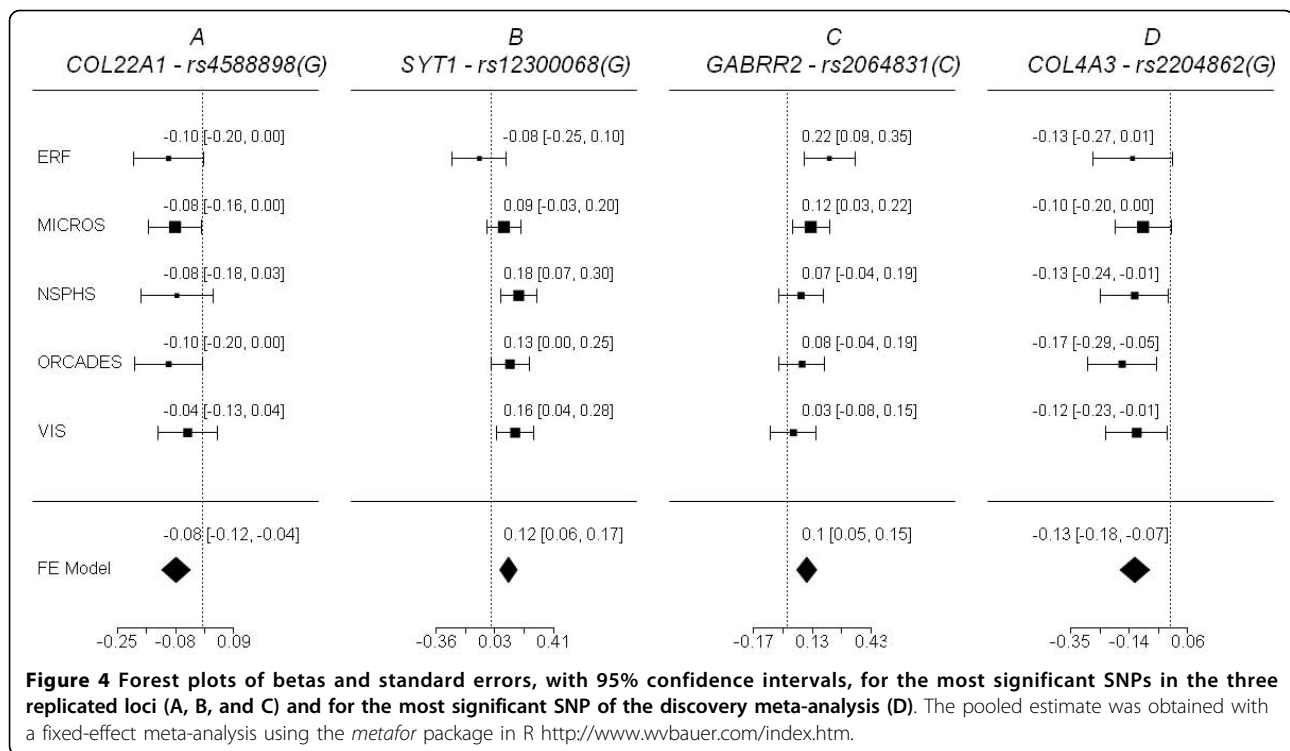


Figure 3 Results of the false discovery rate analysis. $-\log_{10}$ (Fisher p value) of the replication analysis (orange) and $-\log_{10}$ (significance thresholds) for FDR (black) are plotted for each candidate locus. According to [41], FDR thresholds have been calculated as $i/m \times \alpha$, with $\alpha = 0.05$, $m =$ no. of test (i.e.: no. of regions, 29), and i ranging between 1 and 29. After sorting the Fisher p values in ascending order, the first k tests for which the p value is \leq threshold are significant.



to overlap and were found to be located in distinct LD blocks. Interestingly, however, the smallest p values for popgen and Korcula fell into the same LD block.

Three closely linked SNPs (rs10506807, rs12300068, and rs11112829) on chromosome 12q21, yielded p values $\leq 7.55 \times 10^{-5}$ in the EUROSPAN discovery meta-analysis. I^2 for heterogeneity was 21.88%, 24.11%, and 19.94%, respectively, none of which was statistically significant. A forest plot for SNP rs12300068 is provided in Figure 4B. Depending upon the annotation of the 5'-UTR sequence, all SNPs are located either >100 kb upstream (NCBI) or directly inside (Ensembl and UCSC) the synaptotagmin-1 (*SYT1*) gene. In the popgen sample, nine SNPs located less than 50 kb upstream of the three discovery SNPs were significantly associated with S_{CR} , with p values between 2.0×10^{-5} and 8.5×10^{-3} (Table 3). The Fisher's combined probability test p value for the whole region was as low as 1.7×10^{-4} .

The third locus was defined by the four SNPs rs3777514 (p value = 4.5×10^{-4}), rs2064831 (p value = 7.2×10^{-5}), rs1998576 (p value = 8.6×10^{-5}), and rs7744005 (p value = 3.6×10^{-4}). The effect of the three SNPs upon S_{CR} level was homogeneous across populations, with an I^2 of 2.20% (p value = 0.38), 6.73% (p value = 0.29), 9.70% (p value = 0.38), and 0.00% (p value = 0.54), respectively. A forest plot of the SNP with the strongest evidence for association (rs2064831), is reported in Figure 4C. The SNPs in question are located in a region on chromosome 6q15 containing the

gamma-aminobutyric acid receptor rho-2 (*GABRR2*) and the ubiquitin-conjugating enzyme E2-J1 (*UBE2J1*) genes (Figure 6). Five additional SNPs (out of a total of 28 SNPs tested) in this region had p values between 2.2×10^{-3} and 0.0280 (Table 4). Of the 37 SNPs genotyped in the popgen sample, eight were associated with S_{CR} with a p value ≤ 0.05 ; seven of them overlapped with the *GABRR2* and *UBE2J1* genes (see the blue squares in Figure 6). The most consistent association signals in EUROSPAN and popgen were found near the *GABRR2* gene and its promoter, and the respective SNPs were in linkage disequilibrium ($r^2 > 0.59$, Figure 6, middle panel). SNP rs12195070 was genotyped in both EUROSPAN and popgen: effect direction was discordant but in both cases association was very significant with p values of 4.5×10^{-3} in EUROSPAN and p value = 7.2×10^{-3} in popgen, respectively. In the Korcula samples, the smallest p value in the region was 0.0616 so that we cannot formally claim replication in this population. However, Fisher's combined probability test p value equaled 3.6×10^{-3} . This value does not meet the Bonferroni threshold for multiple testing, but it does meet the FDR threshold of 0.0052, which controls the probability of reporting false positives at a 0.05 level (see Additional file 1 - Table S2 and Figure 3). Detailed results for this locus are reported in Table 4.

Although consistently observed in all EUROSPAN populations (Figure 4D), the association noted between S_{CR} and the *COL4A3* gene could not be verified in the

Table 2 SNPs showing the highest evidence of association in the COL22A1 gene (chromosome 8).#

| study | name | position | Genotype (Ref. All.) | Ref. All. Freq. | n | Beta* | SE | p value** |
|----------|------------|-----------|----------------------|-----------------|------|---------|--------|----------------------|
| EUROSPAN | rs4075073 | 139808143 | A/C (A) | 0.37 | 3996 | 0.0671 | 0.0203 | 9.8×10^{-4} |
| EUROSPAN | rs4588898 | 139815390 | A/G (A) | 0.30 | 3999 | 0.0786 | 0.0212 | 2.1×10^{-4} |
| EUROSPAN | rs13255079 | 139828786 | C/T (T) | 0.16 | 4001 | 0.0769 | 0.0263 | 3.4×10^{-3} |
| EUROSPAN | rs9324496 | 139833578 | C/T (T) | 0.34 | 3995 | 0.0716 | 0.0205 | 4.6×10^{-4} |
| EUROSPAN | rs7824025 | 139839898 | A/C (A) | 0.07 | 3998 | 0.0762 | 0.0374 | 0.0414 |
| EUROSPAN | rs2873682 | 139840693 | A/G (A) | 0.30 | 2610 | 0.0915 | 0.0264 | 5.2×10^{-4} |
| EUROSPAN | rs11166844 | 139848062 | C/T (C) | 0.39 | 3993 | 0.0485 | 0.0202 | 0.0166 |
| EUROSPAN | rs1574172 | 139941802 | A/G (A) | 0.14 | 3998 | -0.0713 | 0.0284 | 0.0122 |
| EUROSPAN | rs4736078 | 139944298 | C/T (C) | 0.22 | 3996 | -0.0503 | 0.0242 | 0.0373 |
| EUROSPAN | rs6986041 | 139988449 | C/T (C) | 0.35 | 3936 | -0.0583 | 0.0207 | 4.8×10^{-3} |
| POPGEN | rs4282617 | 139795530 | C/G (C) | 0.25 | 1139 | -0.0822 | 0.0398 | 0.0391 |
| POPGEN | rs10092896 | 139856976 | C/T (C) | 0.27 | 1139 | -0.1123 | 0.0380 | 3.1×10^{-3} |
| POPGEN | rs2318334 | 139859320 | C/T (C) | 0.25 | 1140 | -0.1115 | 0.0390 | 4.2×10^{-3} |
| POPGEN | rs1574370 | 139866164 | A/G (A) | 0.24 | 1126 | -0.0992 | 0.0402 | 0.0137 |
| POPGEN | rs6991720 | 139871695 | C/G (G) | 0.25 | 1139 | -0.1074 | 0.0391 | 6.1×10^{-3} |
| POPGEN | rs7837787 | 139875897 | A/G (G) | 0.25 | 1140 | -0.1106 | 0.0391 | 4.7×10^{-3} |
| POPGEN | rs12155960 | 139886087 | A/G (G) | 0.26 | 1134 | -0.1152 | 0.0388 | 3.0×10^{-3} |
| POPGEN | rs11779129 | 139888085 | A/C (A) | 0.25 | 1140 | -0.0992 | 0.0388 | 0.0105 |
| POPGEN | rs16893541 | 139890024 | C/G (C) | 0.27 | 1124 | -0.1089 | 0.0390 | 5.2×10^{-3} |
| POPGEN | rs12549194 | 139896019 | A/C (C) | 0.26 | 1137 | -0.0830 | 0.0388 | 0.0327 |
| POPGEN | rs10093925 | 139904787 | C/T (T) | 0.36 | 1124 | -0.0687 | 0.0347 | 0.0478 |
| POPGEN | rs6577953 | 139914021 | C/G (C) | 0.40 | 1139 | -0.1032 | 0.0336 | 2.1×10^{-3} |
| POPGEN | rs6993839 | 139914702 | A/G (G) | 0.39 | 1131 | -0.1029 | 0.0342 | 2.6×10^{-3} |
| POPGEN | rs9650564 | 139918011 | C/T (C) | 0.41 | 1139 | -0.1017 | 0.0338 | 2.6×10^{-3} |
| POPGEN | rs9650565 | 139918089 | C/T (C) | 0.41 | 1133 | -0.0944 | 0.0339 | 5.3×10^{-3} |
| POPGEN | rs7014497 | 139923457 | C/T (C) | 0.39 | 1133 | -0.0892 | 0.0341 | 8.9×10^{-3} |
| POPGEN | rs9650566 | 139923872 | A/G (G) | 0.46 | 1139 | -0.1054 | 0.0332 | 1.5×10^{-3} |
| POPGEN | rs17740495 | 139941879 | C/T (C) | 0.08 | 1136 | 0.1393 | 0.0603 | 0.0209 |
| POPGEN | rs16893545 | 139948143 | A/C (C) | 0.18 | 1140 | 0.0953 | 0.0440 | 0.0304 |
| KORCULA | rs4076439 | 139690550 | A/G (G) | 0.25 | 895 | 0.0907 | 0.0393 | 0.0209 |
| KORCULA | rs4341165 | 139692067 | C/T (C) | 0.25 | 895 | 0.0907 | 0.0393 | 0.0209 |
| KORCULA | rs4909439 | 139727929 | C/T (C) | 0.17 | 895 | 0.1080 | 0.0518 | 0.0371 |
| KORCULA | rs3923549 | 139732799 | C/T (T) | 0.10 | 895 | 0.1513 | 0.0704 | 0.0316 |
| KORCULA | rs4073446 | 139735056 | C/T (T) | 0.11 | 895 | 0.1555 | 0.0658 | 0.0182 |
| KORCULA | rs7839680 | 139762863 | A/G (A) | 0.18 | 895 | 0.0950 | 0.0472 | 0.0440 |
| KORCULA | rs4909444 | 139770391 | G/T (T) | 0.38 | 895 | 0.0623 | 0.0289 | 0.0309 |
| KORCULA | rs4074052 | 139785008 | C/T (T) | 0.46 | 894 | -0.0504 | 0.0248 | 0.0419 |
| KORCULA | rs10112806 | 139806115 | A/C (C) | 0.41 | 895 | -0.0589 | 0.0280 | 0.0357 |
| KORCULA | rs1320279 | 139899978 | C/T (C) | 0.11 | 895 | -0.1823 | 0.0662 | 5.9×10^{-3} |
| KORCULA | rs2318345 | 139970360 | C/T (C) | 0.09 | 894 | 0.1755 | 0.0720 | 0.0147 |

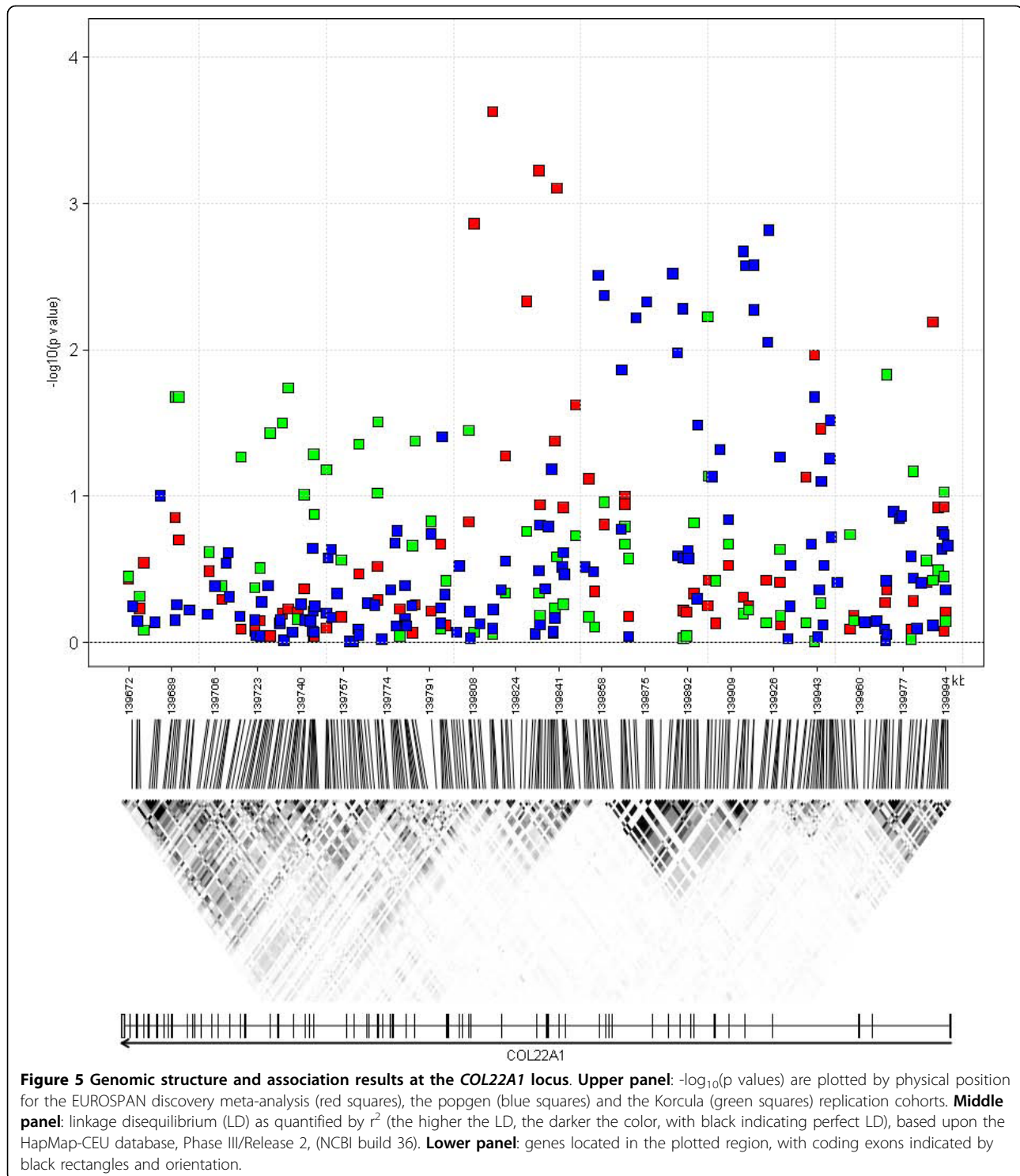
association models were adjusted for age and sex; *in standard deviations; **p value: for EUROSPAN, the p value refers to the meta-analysis of the five individual GWA studies, for popgen and Korcula, the p value is from the linear regression analysis (for details, see Methods).

replication cohorts (Fisher's combined probability test p value = 0.216).

Unfortunately, the number of people with diabetes included in the EUROSPAN populations was too small to allow any meaningful subgroup analysis, adjusting for disease state. However, when we repeated our GWA meta-analysis in non-diabetic subjects only, the p values

for the three replicated gene regions changed so little that any strong interaction of the respective loci with diabetes status appears rather unlikely.

Results of the replication analysis at the four loci reported to be associated with eGFR by Köttgen *et al.* [18] are reported in the Additional file 1 - Table S3. We identified three SNPs in the *UMOD* gene locus:



rs4293393 was significantly associated with S_{CR} level (p value = 3.9×10^{-4}) and weaker signals were observed for rs11647727 (p value = 0.011) and rs4506906 (p value = 0.028). The region-specific p value for this locus was 1.3×10^{-4} . Of the five SNPs identified in the *SPATA5L1-GATM* locus, rs1153860, rs1346268, and

rs1719247 were associated with S_{CR} at p values of 5.0×10^{-4} , 1.9×10^{-3} , and 4.6×10^{-3} , respectively (region-specific p value = 1.2×10^{-3}). At the *SCHROOM3* gene locus, 53 SNPs were tested: three of them resulted in a p value ≤ 0.05 (region-specific p value = 0.50). The smallest p value of 1.7×10^{-3} was observed for SNP

Table 3 SNPs with the highest evidence of association in the region upstream the *SYT1* gene.#

| Study | name | position | Genotype (Ref. All.) | Ref. All. Freq. | n | Beta* | SE | p value** |
|----------|------------|----------|----------------------|-----------------|------|---------|--------|----------------------|
| EUROSPAN | rs11838060 | 77997790 | G/T (T) | 0.14 | 3163 | -0.0696 | 0.0312 | 0.0258 |
| EUROSPAN | rs10506807 | 78005428 | C/T (C) | 0.13 | 4000 | -0.1141 | 0.0287 | 7.0×10^{-5} |
| EUROSPAN | rs12300068 | 78005502 | A/G (A) | 0.13 | 3997 | -0.1158 | 0.0288 | 5.7×10^{-5} |
| EUROSPAN | rs7972593 | 78013149 | C/T (T) | 0.12 | 3989 | -0.0978 | 0.0300 | 1.1×10^{-3} |
| EUROSPAN | rs11112829 | 78029267 | C/T (C) | 0.13 | 4000 | -0.1135 | 0.0287 | 7.5×10^{-5} |
| POPGEN | rs12312807 | 77957336 | A/G (A) | 0.11 | 1139 | 0.1480 | 0.0539 | 6.1×10^{-3} |
| POPGEN | rs1527119 | 77964145 | G/T (G) | 0.11 | 1139 | 0.1471 | 0.0539 | 6.4×10^{-3} |
| POPGEN | rs1033196 | 77966968 | C/T (T) | 0.14 | 1128 | 0.1320 | 0.0479 | 5.8×10^{-3} |
| POPGEN | rs2950383 | 77973538 | A/G (A) | 0.37 | 1103 | -0.1553 | 0.0359 | 2.0×10^{-5} |
| POPGEN | rs1356022 | 77974533 | C/T (C) | 0.14 | 1140 | 0.1300 | 0.0475 | 6.2×10^{-3} |
| POPGEN | rs12317960 | 77977824 | C/T (T) | 0.14 | 1140 | 0.1249 | 0.0474 | 8.5×10^{-3} |
| POPGEN | rs10506806 | 77979113 | C/T (T) | 0.31 | 1120 | 0.1012 | 0.0362 | 5.1×10^{-3} |
| POPGEN | rs11610381 | 77979918 | C/T (C) | 0.14 | 1134 | 0.1321 | 0.0476 | 5.5×10^{-3} |
| POPGEN | rs7971081 | 77980051 | C/T (C) | 0.14 | 1140 | 0.1296 | 0.0474 | 6.3×10^{-3} |

association models were adjusted for age and sex; *in standard deviations; **p value: for EUROSPAN, the p value refers to the meta-analysis of the five individual GWA studies, for popgen, the p value is from the linear regression analysis (for details, see Methods).

rs4859682 (this SNP was in strong LD with the rs17319721 reported in the paper, $r^2 = 0.94$). None of the 8 polymorphisms tested in the *JAG1* gene was significantly associated with S_{CR} level, including the rs6040055 which was included in our database as well. Our conclusions would have been slightly different had we attempted replication at the level of the individual variants rather than at the locus level. In this case, also the *SCHROOM3* locus would have been replicated.

Discussion

The main result of our study was the identification of three novel genetic associations with S_{CR} level. These were detected in a meta-analysis of five European isolated population samples and replicated in two independent population samples, one from the general Central-European population (popgen), and one from another isolate (Korcula). The replicated loci included the *COL22A1* gene on chromosome 8 and the *SYT1* gene on chromosome 12. The third locus, including the *GABRR2* and *UBE2J1* genes on chromosome 6, showed substantial evidence of association as well. In addition, the association of two loci (*UMOD* and *SPATA5L1-GATM*) and one SNP in the *SCHROOM3* locus, previously reported with GFR, was also found to apply to S_{CR} . This confirmation also supports our choice of using S_{CR} adjusted by age and sex which is equivalent to study GFR calculated from S_{CR} weighted by age and sex [1] (black or white ethnicity, which should also be accounted for, was not relevant in our study involving individuals of European origin).

The two SNPs with the strongest evidence for association in the discovery stage of our study were located in

the promoter region of the *COL4A3* gene, which is expressed in the glomerular basement membrane (GBM) of the kidney, and which has been associated with Alport syndrome, Goodpasture syndrome [45] and benign hematuria [46]. Despite making biological sense, however, the association between *COL4A3* and S_{CR} as observed in our meta-analysis could not be verified in the replication stage. On the one hand, this could be interpreted to indicate that no common variants of that gene are involved in the regulation of kidney function. On the other hand, the lack of replication could be due either to the involvement of the gene in regulatory pathways, implying a strong interaction with other genes, or to a particularly prominent interaction with population-specific environmental conditions [47].

In any case, a slightly less significant association between three other genes and S_{CR} from the discovery meta-analysis could be replicated in either the popgen or the Korcula data. Different SNPs in the *COL22A1* gene were found to be associated with S_{CR} in all discovery and replication cohorts. This association may reflect the biological relationship between muscle mass formation and creatinine levels. In fact, *in situ* hybridization of myotendinous junctions has revealed that muscle cells produce collagen XXII, and functional tests have shown that collagen XXII acts as a cell adhesion ligand for skin epithelial cells and fibroblasts [48].

The discovery stage association of S_{CR} with the *SYT1* gene was well replicated in the popgen sample. Even though the annotation of gene reference sequences is not uniform across different databases (NCBI, UCSC, Ensembl), the associated SNPs are located in the fourth and last intron of the 5' *SYT1* un-translated region

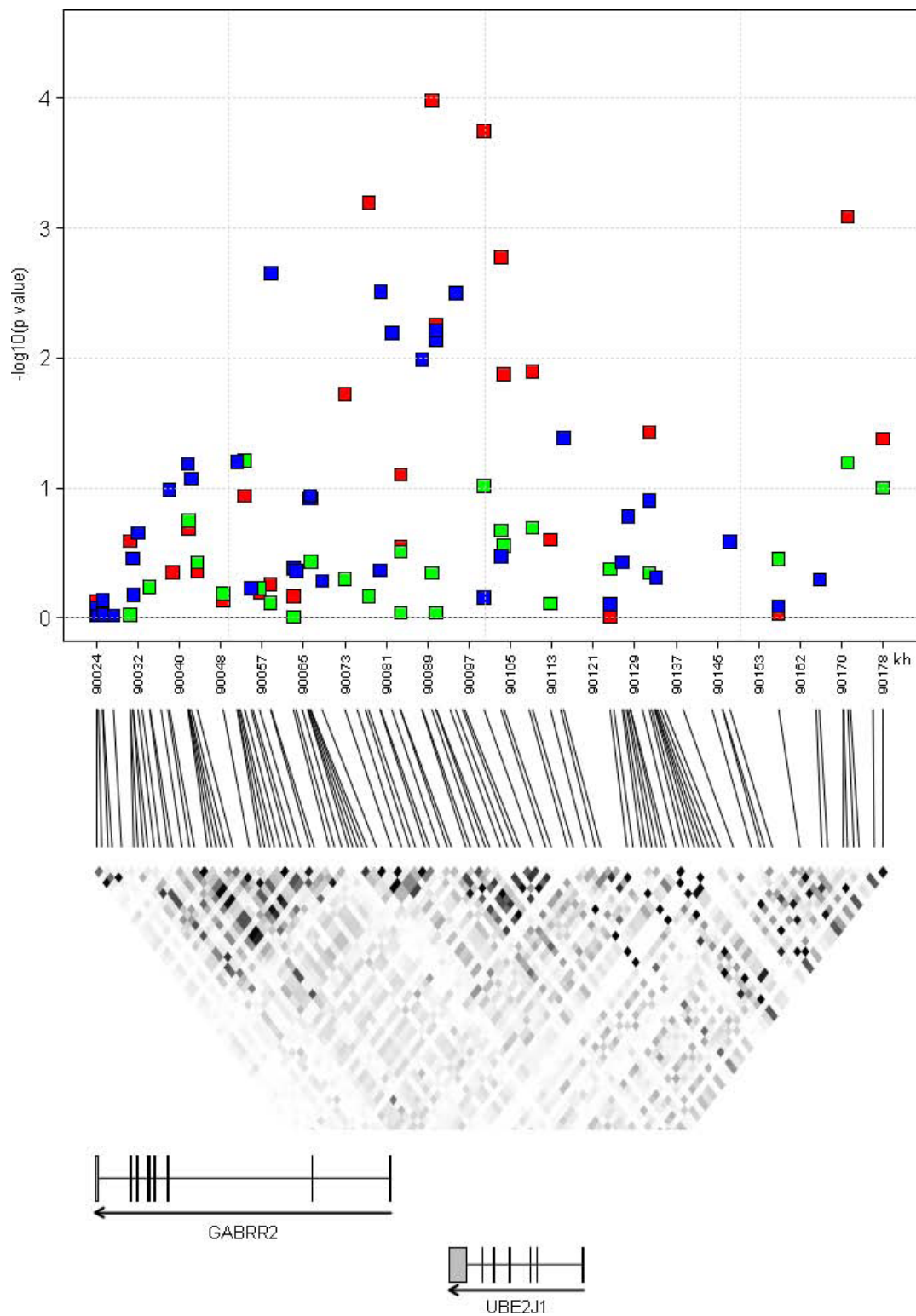


Figure 6 Genomic structure and association results at the *GABRR2-UBE2J1* locus. Upper panel: $-\log_{10}(p \text{ values})$ are plotted by physical position for the EUROSPAN discovery meta-analysis (red squares), the popgen (blue squares) and the Korcula (green squares) replication cohorts. Middle panel: linkage disequilibrium (LD) as quantified by r^2 (the higher the LD, the darker the color, with black indicating perfect LD), based upon the HapMap-CEU database, Phase III/Release 2, (NCBI build 36). Lower panel: genes located in the plotted region, with coding exons indicated by black rectangles and orientation.

Table 4 SNPs with the highest evidence of association in the region including the *GABRR2* and the *UBE2J1* genes (chromosome 6)[#].

| Study | GENE | name | Position | Genotype (Ref. All.) | Ref. All. Freq. | N | Beta* | SE | p value** |
|----------|------------|------------|----------|----------------------|-----------------|------|---------|--------|------------------------|
| EUROSPAN | GABRR2 | rs6942204 | 90072687 | C/T (C) | 0.34 | 4001 | 0.0497 | 0.0213 | 0.0196 |
| EUROSPAN | GABRR2 | rs3777514 | 90077300 | A/C (A) | 0.20 | 3998 | 0.0855 | 0.0244 | 4.5 × 10 ⁻⁴ |
| EUROSPAN | Intergenic | rs2064831 | 90089661 | C/T (C) | 0.18 | 3995 | 0.1018 | 0.0257 | 7.2 × 10 ⁻⁵ |
| EUROSPAN | Intergenic | rs12195070 | 90090455 | C/T (T) | 0.19 | 3998 | 0.0722 | 0.0254 | 4.5 × 10 ⁻³ |
| EUROSPAN | UBE2J1 | rs1998576 | 90099856 | A/G (A) | 0.18 | 3997 | 0.0993 | 0.0253 | 8.6 × 10 ⁻⁵ |
| EUROSPAN | UBE2J1 | rs12189673 | 90103123 | C/T (C) | 0.30 | 3980 | -0.0653 | 0.0213 | 2.2 × 10 ⁻³ |
| EUROSPAN | UBE2J1 | rs1065657 | 90103732 | C/T (T) | 0.45 | 3999 | 0.0499 | 0.0197 | 0.0115 |
| EUROSPAN | UBE2J1 | rs7760851 | 90109323 | A/G (G) | 0.49 | 3980 | -0.0505 | 0.0196 | 0.0100 |
| EUROSPAN | UBE2J1 | rs1062108 | 90132078 | C/T (T) | 0.42 | 3996 | -0.0435 | 0.0198 | 0.0280 |
| POPGEN | GABRR2 | rs9362633 | 90058261 | A/G (G) | 0.05 | 1136 | -0.2430 | 0.0795 | 2.2 × 10 ⁻³ |
| POPGEN | GABRR2 | rs7764923 | 90079640 | C/T (T) | 0.23 | 1140 | -0.1144 | 0.0387 | 3.1 × 10 ⁻³ |
| POPGEN | GABRR2 | rs2236204 | 90081831 | G/T (T) | 0.17 | 1121 | -0.1190 | 0.0436 | 6.4 × 10 ⁻³ |
| POPGEN | Intergenic | rs10944443 | 90087709 | C/G (C) | 0.15 | 1113 | -0.1203 | 0.0469 | 0.0103 |
| POPGEN | Intergenic | rs12195070 | 90090455 | C/T (T) | 0.18 | 1140 | -0.1155 | 0.0430 | 7.2 × 10 ⁻³ |
| POPGEN | Intergenic | rs12195078 | 90090511 | C/T (T) | 0.15 | 1128 | -0.1261 | 0.0460 | 6.1 × 10 ⁻³ |
| POPGEN | UBE2J1 | rs9048 | 90094300 | C/T (T) | 0.18 | 1139 | -0.1230 | 0.0417 | 3.1 × 10 ⁻³ |
| POPGEN | UBE2J1 | rs9351207 | 90115388 | C/T (T) | 0.24 | 1131 | -0.0791 | 0.0387 | 0.0412 |

[#] association models were adjusted for age and sex; *in standard deviations; **p value: for EUROSPAN, the p value refers to the meta-analysis of the five individual GWA studies, for popgen, the p value is from the linear regression analysis (for details, see Methods).

(UTR), suggesting a regulatory role for this locus. Data on the clones available from public databases clearly show that *SYT1* transcription starts around 77,782 kb and 77,963 kb on chromosome 12 and that the transcripts are spliced in the 5'UTR region (see, among others, the mRNA clones with GI numbers 37589129, 21753708, 34533586, 34366268, 37589129, 164696663). The synaptotagmins are integral membrane proteins of synaptic vesicles thought to serve as Ca(2+) sensors in the process of vesicular trafficking and exocytosis [49]. Of particular interest in the present context is expression of the *SYT1* gene product, synaptotagmin-I, in renal podocytes. Glomerular podocytes possess structures resembling synaptic vesicles and contain glutamate, they co-express *Rab3A* (a GTPase restricted to cell types that regulate exocytosis), synapsin, synaptophysin, and synaptotagmin-I, and undergo spontaneous and stimulated exocytosis and recycling, with glutamate release [50]. Synaptotagmin-I plays a role in neurotransmitter release and neurite outgrowth [51] and is thought to interact with neurotransmitters such as the GABA receptors, expressed in the GBM. It also appears to participate in the regulation of podocyte homeostasis [50].

In light of the findings on the *SYT1* gene, the association with the *UBE2J1* and *GABRR2* locus becomes of high interest. The association detected in the EUROSPAN data was corroborated by the popgen data. Moreover, SNPs associated with S_{CR} in popgen enabled us to narrow down the associated region to the *GABRR2* promoter. The same locus was not replicated in Korcula

data. However, while the popgen genotype distribution of the SNPs in question was found to resemble that of the HapMap-CEU samples [52], indicating that popgen can be considered representative of the general Central to Northern European populations, this may not be true for the Korcula sample. In fact, Korcula is a small population located in the Croatian islands, where pronounced genetic isolation is known to have persisted until recently [53]. This could be the reason of the missed replication. The *GABRR2* gene product is a member of the rho subunit family of the transmembrane receptors for gamma-aminobutyric acid (GABA_A), which are linked to potassium channels via G-proteins. GABA_A receptors have been shown to be expressed in the kidney of multiple species, with subunits β_1 and β_2 localized in the proximal tubules [54]. More recently, several subtypes of GABA receptors, including the *GABRR2*, have been shown to be transcribed in normal human glomeruli [50] and it plays a role in retinal neurotransmission [55]. The gene is located close to the *GABRR1* gene in the same transcriptional orientation, suggesting a similar expression and regulatory pattern. Interestingly, the locus falls into the supporting interval for linkage to S_{CR} reported in Mexican Americans [7], and to creatinine clearance as reported in Caucasians [9].

Our discovery analysis was performed in five isolated communities from Southern, Central, and Northern Europe. As has been demonstrated empirically for South Tyrolean villages [56], population isolates provide a reduced within-study heterogeneity of environmental

factors, thereby facilitating the genetic dissection of complex traits. This internal homogeneity of environmental and life style factors could be counterbalanced by enhanced between-study heterogeneity. However, this appears not to have been the case in our study. For SNPs in 27 of the 29 regions selected for replication, the hypothesis of homogeneity of effects' sizes was not rejected, even at a very stringent significance threshold (given the small number of studies involved in the meta-analysis, α was set to 0.10). In particular, homogeneity of association signals was verified for all replicated loci and for the *COL4A3* gene locus. This finding strengthens our results in the sense that, despite the expected between-population heterogeneity, the results of the association analyses were found to be very consistent across studies.

Another advantage of our study has been that S_{CR} values of all EUROSPAN participants were measured in one and the same centralized laboratory using the enzymatic method. In this way, calibration difference could be excluded as a confounder of our genetic association analyses. While the enzymatic method was used to measure S_{CR} in EUROSPAN and popgen participants, the Jaffé rate method was used in the Korcula study. This method is known to be less precise than the enzymatic one. Additional noise introduced by increased measurement error would best explain the reduced evidence for association replication in the Korcula study. However, S_{CR} was standardized in all discovery and replication samples using a transformation based upon the ranks of a standard normal distribution. The standardization of the phenotype measures allowed us to combine data across studies avoiding bias due to technical inter-laboratory differences.

The validity of our analysis was also evidenced by the replication of earlier association findings [18]. Association of S_{CR} with *UMOD* and *SPATA5L1-GATM* was confirmed using the same replication approach used in our own analysis. With our method, however, we could not replicate the association over the *SCHROOM3* locus (the locus would be confirmed if using a classic replication based on the specific SNP or a proxy SNP). This finding could be an indication that our method of replication is more likely to be conservative and, so, less prone to false positive results. In the *COL22A1* and the *SYT1* genes the replicated signals were located in different LD blocks, highlighting the possibility that different polymorphisms at the same locus may be associated with the same phenotype in different populations. In the *GABRR2 - UBE2J1* gene locus, association signals between discovery and replication studies were discordant but clearly overlapping. Whether the discordance of the effect alleles is an indication of a false positive result is very unlikely, given the size of the effects in

EUROSPAN and in popgen. The presence of a flip-flop phenomenon [57] could be hypothesized and should be investigated further.

Conclusions

In conclusion, in addition to confirming earlier findings, our search for genes associated with S_{CR} variation led to the discovery of three novel genes that represent sensible candidates for further functional analysis. The recent hypothesis of an important role of neurotransmitters in the regulation of the GBM at the podocyte level [50] renders our findings particularly relevant for understanding the regulation of renal function, suggesting that the possible interaction between *SYT1* and *GABRR2* warrants further investigation.

Additional file 1: Supplementary materials. The file contains supplementary tables to support the discovery analysis (Table S1), the replication analysis (Table S2) and the confirmation of previous findings (Table S3).
Click here for file
[<http://www.biomedcentral.com/content/supplementary/1471-2350-11-41-S1.DOC>]

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Authors' contributions

SHW, OP, UN, SS, NH, TM, BO, AFW, HC, CMvD, UG, JFW, MK, IR, and PPP conceived and designed the studies. CP, VV, CH, YSA, AJ, AI, DE, and AF performed the data analysis. CP, ADG, SAM, PR, CM, and MK discussed and provided interpretation of the results. CP, ADG, VV, AF, SHW, IK, LZ, TZ, CG, SS, SC, MB, PR, CM, HC, UG, and MK wrote and revised the manuscript. All authors gave final approval of the manuscript to be published.

Competing interests

The authors declare that they have no competing interests. The funders had no role in the study design, the data collection and analysis, the decision to publish, or the preparation of the manuscript.

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