Title

Systematic mediation and interaction analyses in an individual population study help characterize kidney function genetic loci

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Abstract

Chronic kidney disease (CKD) is a complex disease affecting >10% of the global population, with large between- and within-continent variability reflecting major environmental effects. To identify molecular targets for treatment, genome-wide association study meta-analyses (GWAMAs) of CKD-defining traits have identified hundreds of genetic loci in aggregated populations. However, while GWAMAs estimate the average allelic effect across studies, single population studies may be relevant to unravel specific mechanisms. To assess whether an individual study from a specific population could extend existing knowledge on kidney function genetics, we selected 147 kidney function relevant loci identified by a large European ancestry GWAMA, assessing their association with the glomerular filtration rate estimated from serum creatinine (eGFRcrea) in the Cooperative Health Research In South Tyrol (CHRIS) study (n=10,146), conducted in an Alpine region where thyroid dysfunction is common. We replicated associations with single nucleotide polymorphisms (SNPs) at 11 loci, showing up-to-5.4 times larger effect sizes than in the corresponding GWAMA, not explainable by minor allele frequency differences. Systematic mediation analysis across 70 quantitative traits identified serum magnesium, the activated partial thromboplastin time, and serum urate as partial mediators of the eGFRcrea associations at *SHROOM3*, *SLC34A1*, and *IGF1R*, respectively. Given that free triiodothyronine and thyroxine were effect modifiers across all loci, we conducted SNP-by-thyroid stimulating hormone (TSH) interaction analyses, identifying significant interactions at *STC1*: SNPs had larger effects on eGFRcrea at higher TSH levels, possibly reflecting stanniocalcin-1 autocrine and paracrine role. Individual population studies can help characterize genetic associations. The interplay between phenotypes at *SHROOM3* and *SLC34A1* and the role of thyroid function as a genetic effect modifier warrant further investigations.

Introduction

Chronic kidney disease (CKD) is a common complex disease that increases the risk of kidney failure, cardiovascular mortality, and all-cause mortality[1]. CKD is predicted to become the 5th leading cause of death by 2040[2]. While affecting >10% of the population globally, CKD prevalence shows marked variability across countries, both between and within continents[3]. Between-continent variability is likely guided by combinations of environmental and genetic differences, such as is the case with sickle cell trait and albuminuria[4]. Within-continent variability could be more related to environmental differences, including lifestyle, public health policies, and assessment methods[4,5].

To unravel the genetic basis of CKD, several genome-wide association study meta-analyses (GWAMAs) were conducted that analyzed the CKD-defining marker glomerular filtration rate (GFR) estimated from serum creatinine (eGFRcrea)[6]. More than 400 associated loci have been identified to date[7]. While GWAMAs estimate the average allelic effect across studies, evidence shows that individual population studies may be extremely relevant to advance discoveries not only in the field of rare diseases[8] but also with regard to markers of common chronic diseases such as triglycerides and low density lipoprotein (LDL) cholesterol[9], age at diabetes onset[10], and mood disorders[11]. The evidence accumulated so far suggests that there is no general rule and that the contribution of an individual study to the dissection the biological basis of common phenotypes can only be assessed on a case-by-case basis and depends on study-specific characteristics.

Here we analyzed genetic associations with eGFRcrea in the Cooperative Health Research In South Tyrol (CHRIS) study, a general population study conducted in a mountainous region of central Europe[12]. A sample from this same population was previously instrumental to identify genetic linkage of serum creatinine with the *APOL1-MYH9* locus[13] and to replicate common variants associated with eGFRcrea[14]. Characteristics of the geographical region where the study was conducted include the demographic stability over time[15], the rural environment, and a high frequency of hypothyroidism, typical of mountainous regions[16]. Aim of our analysis was to assess whether this particular study sample could provide information that extend current knowledge on kidney function genetics, by leveraging on local specific characteristics.

For a given single population study, with a relatively limited sample size, to help expand knowledge on loci already identified by a large consortium, it is necessary and appropriate to limit investigation to the subset of loci associated with the phenotype of interest in the given

study. Thus, we first identified which, among 147 loci found to be associated with kidney function in a large GWAMA from the CKDGen Consortium[17], were specifically associated with eGFRcrea in the CHRIS study. Among the replicated loci, we explored whether there were local determinants enhancing specific genetic associations. Given that several replicated loci showed larger effects in CHRIS than in the discovery GWAMA, we examined the possible reasons of such larger effects and conducted extensive mediation analyses across 70 quantitative biochemical and anthropometric traits, reflecting multiple health conditions. Further, after observing a systematic modification of the genetic association with eGFRcrea when adjusting for thyroid-related traits, we conducted SNP-by-thyroid function interaction analyses. The aims of the mediation and the interaction analyses were to identify the presence of intermediate phenotypes and effect modifiers, respectively, that were specific of the study sample.

Methods (max 1,500 words)

Study sample

The present analyses were based on 10,146 individuals with complete genotype data who were included in the release 3 of the CHRIS study, a population-based study conducted in South Tyrol, Italy, between 2011 and 2018, which has been extensively described elsewhere[12,18]. Briefly, participants underwent blood drawing, urine collection, anthropometric measurements, and clinical assessments early in the morning, after an overnight fast. Medical history was reconstructed through interviewer- and self-administered standardized questionnaires. Drug treatment was identified via barcode scan of the drug containers that participants were instructed to bring at the study center visit, with the drug code linked to an official drug databank[12].

eGFRcrea estimation

Serum creatinine (SCr) was measured with a colorimetric assay on Roche Modular PPE (n=4,176) and Abbott Diagnostic Architect c16000 (n=5,970) instrumentations. According to previous analyses[18], SCr was normalized by instrument (fixed effect) and participation period (random effect) using a linear mixed model implemented in the 'lme4' R package[19]. To reflect the same methods used in the CKDGen Consortium meta-analysis[17], eGFRcrea was estimated

using the 2009 CKD-EPI equation[20] implemented in the R package 'nephro' v1.2[21] and winsorized at 15 and 200 ml/min/1.73m^2. Finally, the natural logarithm transformation was applied.

Genotype imputation

Genotyping was performed in two batches using genotyping array chips based on the Illumina Human Omni Microarray platform. Following quality control analysis, the two data batches were merged, phased with SHAPEIT2 v2.r837, using the duoHMM method (--duohmm -W 5) with 800 states and 30 rounds[22], and imputed based on the TOPMed r2 standard reference panel on the Michigan Imputation Server[23]. We obtained 34,084,280 SNPs with minimum imputation quality index Rsq ≥0.3 and minor allele count (MAC) ≥1, aligned with human genome assembly GRCh38. A genetic relatedness matrix (GRM) was obtained from the autosomal genotyped SNPs using EPACTS v3.2.6 modified for compatibility with assembly GRCh38. Genetic principal components (PCs) were estimated based on the genotyped variants data with minor allele frequency (MAF) >0.05 using GCTA[24].

GWAS and replication of CKDGen results

We conducted a GWAS of age- and sex-adjusted residuals of ln(eGFRcrea) using the EMMAX method[25] implemented in EPACTS v3.2.6, including the GRM to model the sample structure. After the analysis, we removed variants with minor allele frequency (MAF) <0.005, leaving 10,158,100 SNPs for further characterization.

Summary statistics from the CKDGen European ancestry GWAMA[17] were downloaded from the publicly available repository at https://ckdgen.imbi.uni-freiburg.de. Given a subset of 4661 samples from the CHRIS study was previously included in the CKDGen GWAMA, we removed CHRIS data from the CKDGen GWAMA summary statistics using MetaSubtract v1.60[26]. After this process, the 147 variants remained genome-wide significant in CKDGen.

We lifted the CKDGen genomic positions from the GRCh37 to the GRCh38 map, using CrossMap v0.5.3[27]. To ensure consistency of the effect direction, replication of the 147 loci in CHRIS was assessed based on a one-sided test evaluated at the Bonferroni-corrected level of 0.00034 (0.05/147), for any variant in strong linkage disequilibrium (LD) (r^2>0.8) with a lead CKDGen variant. LD was estimated using emeraLD v0.1[28].

Mediation analysis

Replicated ln(eGFRcrea)-SNP associations were submitted to mediation analysis across 70 quantitative anthropometric, blood pressure and biochemical traits (listed in Supplementary Table S1), following the flowchart represented in Fig. 1. SCr was included as a positive control. To prevent potential measurement instrument effects[18], we applied quantile normalization to each trait (Supplementary Methods). Mediation analysis was conducted for each *trait* following the established 4-step framework and fitting linear regression models throughout:

1. ln(eGFRcrea) = β_1SNP +$\sum_i^K \theta_i X_i$ + ε_1
2. ln(eGFRcrea) = β_2SNP + γ_2*trait* + $\sum_i^K \theta_i X_i$ + ε_2
3. *trait* = β_3SNP +$\sum_i^K \theta_i X_i$ + ε_3
4. *trait* = β_4SNP + $\delta_4$ln(eGFRcrea) + $\sum_i^K \theta_i X_i$ +ε_4

where ε_1 to ε_4 refer to Gaussian error terms, and $\sum_i^K \theta_i X_i$ indicate the inclusion of K covariates, namely age, sex, the first 10 genetic PCs and an intercept. Step 1 corresponds to the GWAS results: given we investigated the replicated variants, the estimate of β_1 (b_1) was always significant. We defined two criteria that must be satisfied in order for a trait to be considered at least a partial mediator of the ln(eGFRcrea)-SNP relation: (i) evidence of a substantial alteration of the SNP effect on ln(eGFRcrea) when adjusting for the trait (step 2) and (ii) the SNP is also associated with the trait (step 3) at a multiple-testing corrected level of 6.5×10^{-5} (corresponding to 0.05 / (70 traits × 11 independent loci)). Criterion (i) was assessed by analyzing the distribution of the estimate of β_2 (b_2): for a specific trait, b_2 was classified as an outlier according to the rule $b_2 < P_{10} - 1.5\ (P_{75} - P_{25})$ or $b_2 > P_{90} + 1.5\ (P_{75} - P_{25})$, where P_{10}, P_{25}, P_{75}, and P_{90} indicate the 10th, 25th, 75th, and 90th percentile of the distribution of b_2 across all traits. This is essentially a more stringent version of the Tukey's rule for outlier detection.

To ensure that a lack of association in step 3 was not due to lack of power in the CHRIS study, we interrogated the SNP-trait associations that passed the mediation analysis step 2 on the PhenoScanner v2[29] (http://www.phenoscanner.medschl.cam.ac.uk/, interrogated on 31-Jan-2023), GWAS Catalog[30] (https://www.ebi.ac.uk/gwas/docs/api; 24-Feb-2023), and the ThyroidOmics Consortium summary statistics[31] (https://www.thyroidomics.com), for associations at $P<5.0\times10^{-8}$.

Interaction with thyroid function phenotypes

Free triiodothyronine (FT3) and thyroxine (FT4) did not qualify as mediators but caused a major departure of b_2 from its expected distribution (see Results). These traits may reflect thyroid gland issues. Given the relevance of hypothyroidism in the region, we analyzed these traits in detail. Because FT3 and FT4 levels were measured only when the thyroid stimulating hormone (TSH) level was <0.4 or >3.8 µUI/mL, we started with a series of sensitivity analyses to exclude the presence of underlying sample stratification. First, we tested whether the distribution of the genotypes was different between individuals with and without measured FT3 and FT4, using a two-sided Wilcoxon test at a significance level of 0.05. Second, to verify whether there was any geographical cluster of individuals with extreme TSH levels (and so measured FT3 and FT4 levels), which might have implicated population stratification, we further adjusted the ln(eGFRcrea)-SNP association model in step 1 for municipality of residence.

After excluding the presence of sample stratification, we tested the interaction between the SNPs and TSH levels, which were measured in the whole sample. We excluded 416 individuals reporting any condition among thyroid cancer (n=16), kidney cancer (n=1), goiter (n=277), having undergone surgery to the thyroid gland (n=312), or having both missing TSH measurement and therapy information (n=4), leaving 9730 individuals for the interaction analysis. Regression models included both the main and the interaction effect terms and were adjusted for the same covariates used in the step 1 model. In addition to quantitative TSH levels, we also tested interaction with broadly defined hyper- and hypothyroidism. Hyperthyroidism was defined as a TSH level of <0.4 µUI/mL or use of thiamazole (n=3) or propylthiouracil (n=1). Hypothyroidism was defined as TSH levels >3.8 µUI/mL or reported use of levothyroxine sodium (n=512). No other thyroid-related treatment was reported. The statistical significance level for interaction testing was set at 0.0045=0.05/11 loci.

Ethical statement

The CHRIS study was approved by the Ethical Committee of the Healthcare System of the Autonomous Province of Bozen/Bolzano, protocol no. 21/2011 (19 Apr 2011). All participants provided written informed consent. All the methods were performed in strict accordance with the approved protocol.

Results

In the study sample, the mean age was 46 years (standard deviation, SD=16), females were 55%, and the mean eGFRcrea level was 92.00 (SD=16.11) ml/min/1.73m^2 (Table 1; additional clinical characteristics are reported in Supplementary Table S1). After appropriate modeling of the sample structure (genomic inflation factor=1.02; Supplementary Fig. S1), 11 of the 147 CKDGen loci were replicated in CHRIS (1-sided P-value between 3.34×10^{-4} and 4.51×10^{-7}; Table 2; Supplementary Table S2). For each replicated locus we retained all variants in LD (r^2>0.8) with the lead SNP, totaling 163 variants over the 11 loci (Supplementary Table S3). The replicated loci displayed very similar LD pattern in CHRIS as in CKDGen (Supplementary Fig. S2). Among the replicated loci, variants' effect magnitude at *CASZ1*, *DDX1*, *PIP5K1B*, *GAB2*, and *IGF1R* was significantly larger in CHRIS than in CKDGen (Fig. 2; Table 2). The CHRIS-to-CKDGen effect ratio varied between 1 and 5.4 and was not entirely explained by MAF differences (Fig. 3; Supplementary Table S3). Three broad groupings characterized by MAF and effect size differences could be distinguished:

1. at *SLC34A1* and *SHROOM3*, we observed similar MAF and effect magnitude, consistent with the fact that these two loci were amongst the ones identified by the earliest GWAS, when sample size was still relatively limited and the genomic imputation quality was coarser[32,33];
2. at *DAB2*, *TMEM60*, *PIP5K1B*, *GAB2*, and *IGF1R*, MAF was similar or slightly larger in CHRIS (effects in CHRIS were 1.3-to-5 times larger than in CKDGen);
3. at *STC1*, *DDX1*, and *CASZ1*, we observed lower MAF in CHRIS and about 1.8-to-5.4 times larger effects in CHRIS.

Mediation analysis

To identify possible reasons for observed larger effects, we conducted mediation analysis of the association between eGFRcrea and each of the 163 variants at the 11 loci across 70 quantitative health traits available in CHRIS (Supplementary Table S1). Adjusting the eGFRcrea-SNP association for each trait in turn (step 2 of the mediation analysis; see Methods) resulted in a substantial change of the eGFRcrea-SNP association coefficient in between 4 and 12 variant-trait associations per locus (Fig. 4). As expected, adjustment for the positive control SCr resulted in the nearly complete knockdown of the SNP effect at all loci (effect sizes toward null). Often, the effect change happened when adjusting for traits closely related to kidney

function such as urate (*GAB2*, *IGF1R*, *PIP5K1B*, *SHROOM3*, and *STC1*), urinary creatinine (*SHROOM3*), and urinary albumin (*IGF1R*). Serum electrolytes such as magnesium (*SHROOM3*), corrected calcium (*PIP5K1B*), and sodium (*DDX1*, *GAB2*, *RSBN1L*, *SLC34A1*, and *STC1*) were also widely involved. Of note was the widespread effect of FT3, FT4, and the activated partial thromboplastin time (aPTT) across all loci. When adjusting for FT3 and FT4, the SNP effect on ln(eGFRcrea) was often completely altered. This behavior across all loci may suggest the presence of either a measurement artifact or a general related condition affecting the underlying population.

In the 3rd mediation analysis step, we evaluated whether the traits identified as effect modifiers in step 2 were associated with the SNP of which they modified the association with eGFRcrea (Table 3; Supplementary Table S4): SNP rs3812036 at *SLC34A1* was associated with aPTT levels (P=3.54×10^{-6}) and five SNPs at *SHROOM3* were associated with serum magnesium levels (P-values between 5.33×10^{-5} and 6.12×10^{-5}). Three of our associations reproduce previously reported associations found using Phenoscanner: at *SLC34A1*, Tang *et al.*[34] reported association of rs3812036 with aPTT (P=2.88×10^{-18}), and at *SHROOM3*, Meyer *et al.*[35] reported associations of magnesium with rs4859682 (P=2.39×10^{-9}) and rs13146355 (P=6.27×10^{-13}).

All SNP-trait associations that successfully passed the 2nd mediation analysis step were also interrogated against the Phenoscanner, GWAS Catalog, and ThyroidOmics consortium results (Methods) to identify associations that might have been missed by the 3rd step of the mediation analysis in CHRIS due to lack of power (Supplementary Table S4). This analysis additionally identified significant associations between 11 variants at *IGF1R* and serum urate (Table 3) supporting the presence of a mediation mechanism involving *IGF1R*, eGFRcrea and uric acid.

In summary, aPTT, serum magnesium, and urate qualified as partial mediators of eGFRcrea associations at *SLC34A1*, *SHROOM3*, and *IGF1R*, respectively. At *SLC34A1*, the partial mediation of aPTT corresponded to a 21% larger effect of the SNP on ln(eGFRcrea); at *SHROOM3*, the partial mediation of serum magnesium corresponded to an effect attenuation of about 11%; and at *IGF1R* the mediation resulted in up-to-18% attenuation of the SNP effects on eGFRcrea (Table 3).

Interaction with thyroid dysfunction

Despite the pervasive effect change caused by FT3 and FT4 in step-2 analysis, these two traits did not result in significant step-3 associations and so did not qualify as mediators. However, motivated by the fact that hypothyroidism is amongst the leading causes of hospitalization in the region[36], thyroid problems are common in Alpine areas, and the FT3 and FT4 adjustment affected the SNP-eGFRcrea association at all 11 loci, we considered it reasonable to evaluate a potential role of thyroid dysfunction as a modifier of the SNP-eGFRcrea relation.

Preliminarily, given that in the CHRIS study FT3 and FT4 were measured only in individuals with TSH levels above or below specific thresholds, we first conducted sensitivity analyses to exclude the presence of artifacts. We did not observe any genotype stratification by measured versus unmeasured FT3 and FT4 levels (Supplementary Table S5). Adjustment for municipality of residence, to exclude the presence of local clusters of thyroid dysfunction, did not alter the results (Supplementary Fig. 3).

We then tested for the presence of linear interaction with TSH levels, which identified a significant interaction at *STC1* SNPs rs819185 (*P*=0.00177) and rs819196 (*P*=0.00154) both below the multiple testing threshold of 0.05/11 loci (Fig. 5; Supplementary Table S6). Since for most loci the adjustment both for FT3 and for FT4 were causing an increase of the effect magnitude, we also postulated a U-shaped interaction model, with the SNP effects on ln(eGFRcrea) being larger both in hyper- and in hypothyroidism. We classified individuals as healthy (88.5%), with hypothyroidism (9.8%) or with hyperthyroidism (1.7%; Table 1). Our results did not support the presence of interaction with these two conditions at any locus after multiple testing control, despite nominally significant *P*-values observed for interaction between hyperthyroidism and SNPs in *SHROOM3* (*P*=0.03) and between hypothyroidism and SNPs in *PIP5K1B* (*P*=0.03) and *GAB2* (*P*=0.04; Supplementary Table S7).

Discussion

Out of 147 loci known for their association with kidney function, our analysis identified 11 that were more strongly associated with eGFRcrea in the CHRIS study sample than in average European-ancestry population samples. Observed differences in effect magnitude could not be explained by MAF differences. Extensive mediation analysis identified serum magnesium, aPTT, and urate as partial mediators of the association of eGFRcrea with variants at

SHROOM3, *SLC34A1*, and *IGF1R*, respectively. SNP-by-TSH interaction analysis highlighted that the effect of variants at *STC1* on eGFRcrea would vary by TSH levels.

With the exception of *SHROOM3* and *SLC34A1*, for which a relatively small sample size proved to be sufficient for their detection[32], if our replicated loci would had not had a large effect size, they would not have been replicated. But the question remains as to why precisely these and no other loci showed such large effects in CHRIS. SNPs may show a larger effects at lower MAF. This situation was observed at just *DDX1* and *CASZ1*, whose effects got larger as the CHRIS-to-CKDGen MAF ratio got smaller. However, the MAF difference was not related to the larger effects observed at *GAB2*, *TMEM60*, *PIP5K1B*, and *IGF1R*, where the SNP MAF was similar or even smaller in CKDGen than in CHRIS (Fig. 2). The presence of un-modeled sample structure causing genomic inflation can be excluded as relatedness was appropriately modeled. We can also exclude the presence of genetic admixture as the CHRIS study was conducted in a small area, on a local population of homogeneous ancestry[15,37-39]. Differences in LD structure between the local and the overall population can also be excluded as regional association plots showed similar shape of the association results in CHRIS and CKDGen. Epistatic gene-gene interaction would be a further possibility that cannot be easily verified. The most plausible remaining reasons would be the presence of local effect modifiers or gene-by-environment interaction.

Mediation analysis based on commonly measured biochemical and anthropometric traits provided limited answers in this respect but highlighted relevant mechanisms at three loci. The first finding was the identified presence of partial mediation of serum magnesium levels on the association of eGFRcrea with the *SHROOM3* locus. Genetic variants at *SHROOM3* have been associated with CKD[8,9], reduced eGFRcrea[9], increased albumin-to-creatinine ratio[10], and low serum magnesium levels[11]. *SHROOM3* is necessary to maintain the glomerular filtration barrier integrity[40]. Variants in this gene have been shown to be associated with increased risk of CKD[41], likely through disruption of the transcription factor TCF7L2 in podocyte cells. At the same locus, *FAM47E* is highly expressed in the human glomeruli and its transcription levels were associated with eGFRcrea[42]. Additional genes in this locus include *STBD1* and *CCDC158*, for which no connection with kidney function was demonstrated. The kidney is one of the primary regulators of serum magnesium concentration. It has been suggested that *SHROOM3* might increase serum magnesium levels through decreased renal magnesium excretion[43]. At the *SHROOM3* rs13146355 SNP, a previous GWAS on serum magnesium showed that the allele related with lower magnesium levels was associated with higher eGFRcrea levels[35]. The authors observed

that the rs13146355 association with magnesium did not change when adjusting for eGFRcrea, suggesting pleiotropic independent effects. In our analysis, the effect of rs13146355 on eGFRcrea was reduced by 11% when adjusting for magnesium, suggesting partial mediation and thus the presence of partially overlapping mechanisms. Deeper analyses at the molecular level are warranted to identify the relevant mechanism.

A similar situation was observed at *SLC34A1* which, in addition to eGFRcrea[33], was also previously associated with aPTT in a GWAMA of 9240 European ancestry individuals[34] including participants from the same areas where the CHRIS study was sampled. By quantifying the clotting time from the activation of factor XII (*F12*) until the formation of a fibrin clot, aPTT is a measure of the integrity of the intrinsic and common coagulation pathways[44]. The observed mediation, in which adjustment for aPTT increases the SNP-eGFRcrea association by 21%, is likely related to the proximity between *SLC34A1* and the coagulation factor gene *F12*. The LD at this locus is compatible with the presence of common haplotypes tagging both genes, inducing pleiotropic independent effects on eGFRcrea and aPTT.

Finally, at the insulin like growth factor 1 receptor (*IGF1R*) locus, we observed an eGFRcrea-SNP altered association when adjusting for urate levels. Mediation could not be confirmed using CHRIS data only, likely due to lack of power, but it was confirmed through interrogation of publicly available summary statistics: SNPs at *IGF1R* were previously associated with uric acid[45]. The significant mediation probably reflects the involvement of *IGR1R* in urate reabsorption in the proximal tubule of the kidney[46].

An additional relevant finding was the presence of an interaction between two SNPs at *STC1* and TSH levels. *STC1* is chiefly expressed in the thyroid gland (https://gtexportal.org/home/gene/STC1). It is also highly expressed in the kidney, where it is exclusively expressed in the collecting duct[47]. *STC1* is also associated with urate and urea levels and is involved in the physiological response to dehydration and protein-rich diet[47]. The *STC1*-encoded protein, stanniocalcin-1, is involved in phosphate reabsorption in the kidney proximal tubules and in multiple pathophysiological mechanisms including ischemic kidney injury[48]. Stanniocalcin-1 behaves like an endocrine hormone, with both autocrine and paracrine functions. Our observation of a SNP effect on eGFRcrea depending on TSH levels would suggest that altered thyroid function levels might affect Stanniocalcin-1 levels with consequent effect on kidney function phenotypes. While intriguing, this hypothesis should be verified by appropriate studies.

Our study had both strengths and limitations. Using data from a local homogeneous population sample was at the same time the most relevant strength and limitation of our study. While homogeneity may enhance the identification of specific genetic associations, it may also hinder the possibility to explain the underlying reasons of the observed larger effects compared to the average European ancestry population. The availability of a very large number of clinical parameters has allowed us to conduct an exhaustive and unbiased screening on potential mediation mechanisms. However, the analyzed traits lacked molecular specificity to identify underlying mechanisms. Finally, assessment of environmental exposures[47] may also be a tool to explain the observed larger effects and would be ground for further research.

In conclusion, our study has identified loci that have particularly large effect on kidney function in the specific Alpine population sample considered in this study, namely *CASZ1*, *IGF1R*, *GAB2*, and *DDX1*. Allele frequency and LD analyses did not explain the observed larger effects nor did the extensive mediation analyses conducted. On the other hand, we showed that the effect of *SHROOM3*, *SLC34A1*, and *IGF1R* on eGFRcrea is partially mediated by serum magnesium, aPTT, and urate levels, respectively. Finally, the observed *STC1*-TSH interaction implicates thyroid function involvement, which might have been favored by the high burden of thyroid-related diseases in the region. Further investigations are warranted to link the identified loci to environmental and molecular characteristics of specific population samples that may elucidate mechanisms not otherwise identifiable in large genetic meta-analyses.

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Author contributions

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Competing interests

The authors declare no competing financial interests.

Data availability

The data used for the current study and the results are stored in a controlled storage at the Eurac Research Institute for Biomedicine. The data are not openly available due to reasons of sensitivity and can be requested with an application to access.request.biomedicine@eurac.edu.

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Tables

Table 1. Main characteristics of the 10,146 study individuals. Additional clinical characteristics are described in Supplementary Table 1.

Participants' characteristics	Median (IQR) or N (%)
Age, years	46.5 (32.8 – 57.6)
Females	5,585 (55.1%)
eGFRcrea, ml/min/1.73 m^2 (n=10,141)	92.2 (81.3 – 103.2)
Mg, mg/dl (n=10,143)	2.0(1.9 – 2.1)
aPTT, seconds (n=6,369)	29.9 (28.6 – 31.4)
TSH, µUI/mL (n=10,142)	1.38 (0.98 – 1.93)
FT3, pg/ml (n=466)	3.00 (2.70 – 3.30)
FT4, ng/dl (n=469)	0.98 (0.87 – 1.08)
Hyperthyroidism (TSH < 0.4 or drug treatment)	172 (1.7%)
Normal (0.4 < TSH < 3.8 and no drug treatment)	8,972 (88.5%)
Hypothyroidism (TSH > 3.8 or drug treatment)	998 (9.8%)

Abbreviations: IQR, Interquartile range; TSH, Thyroid-stimulating hormone; FT3, Free triiodothyronine; FT4, Free thyroxine; Mg, serum magnesium; aPTT, activated partial thromboplastin time.

Table 2. The 11 loci identified from the European ancestry CKDGen Consortium GWAS that showed significant effects in CHRIS. Loci were considered replicated if any variant in strong LD with the CKDGen lead variant was significantly associated with ln(eGFRcrea) in CHRIS. Consequently, for each locus, reported are either the results on the CKDGen lead variant or the results of the replicated variant in the locus when the lead variant did not replicate itself (see Methods).

				CKDGen[1]			CHRIS			
Locus (gene name)	**CKDGen lead SNP**	**Chr:Pos (build 38)**	**EA/OA**	**EAF**	**b(SE)***	***P*-value**	**EAF**	**b(SE)**	**1-sided *P*-value**	**2-sided *P*-value**
CASZ1	rs74748843	1:10,670,853	T/C	0.021	-0.00598 (0.00123)	1.09×10^{-6}	0.016	-0.02776 (0.00806)	2.85×10^{-4}	5.70×10^{-4}
DDX1	rs807624	2:15,642,347	G/T	0.660	-0.00338 (0.00035)	1.70×10^{-21}	0.701	-0.00886 (0.00221)	3.00×10^{-5}	5.99×10^{-5}
SHROOM3	rs28817415	4:76,480,299	T/C	0.440	-0.00744 (0.00034)	7.32×10^{-109}	0.428	-0.00840 (0.00214)	4.21×10^{-5}	8.42×10^{-5}
DAB2	rs10062079*	5:39,393,631	A/G	0.430	-0.00549 (0.00035)	6.19×10^{-56}	0.453	-0.00721 (0.00211)	3.24×10^{-4}	6.49×10^{-4}
SLC34A1	rs3812036	5:177,386,403	T/C	0.260	-0.00687 (0.00040)	2.31×10^{-67}	0.240	-0.00836 (0.00245)	3.27×10^{-4}	6.55×10^{-4}
TMEM60	rs57514204*	7:77,714,744	T/C	0.410	-0.00332 (0.00034)	1.48×10^{-22}	0.439	-0.00723 (0.00212)	3.34×10^{-4}	6.67×10^{-4}
STC1	rs819196*	8:23,885,208	T/A	0.450	-0.00406 (0.00034)	1.43×10^{-33}	0.437	-0.00718 (0.00210)	3.21×10^{-4}	6.41×10^{-4}
PIP5K1B	rs2039424	9:68,817,258	G/A	0.380	-0.00483 (0.00035)	1.12×10^{-42}	0.421	-0.01042 (0.00212)	4.51×10^{-7}	9.01×10^{-7}
GAB2	rs7113042*	11:78,324,786	A/G	0.830	-0.00271 (0.00045)	1.86×10^{-9}	0.823	-0.01096 (0.00286)	6.41×10^{-5}	1.28×10^{-4}
IGF1R	rs59646751	15:98,733,292	T/G	0.310	-0.00203 (0.00036)	2.47×10^{-8}	0.321	-0.00909 (0.00223)	2.32×10^{-5}	4.63×10^{-5}
PDILT	rs77924615	16:20,381,010	G/A	0.800	-0.00958 (0.00044)	1.52×10^{-104}	0.826	-0.01030 (0.00281)	1.27×10^{-4}	2.53×10^{-4}

[1]European ancestry CKDGen results after subtracting the effect of 4661 CHRIS participants included in the CKDGen meta-analysis.
Abbreviations: Chr, chromosome; Pos, position; EA, effect allele; OA, other allele; EAF, effect allele frequency; b, coefficient of association; SE, standard error of b.
*Replication of a variant in strong LD with the CKDGen lead variant: in these cases, the replicated and not the CKDGen lead variant is reported. See Supplementary Table S2 for lead variant comparisons.

Table 3 Results of the mediation analysis at *SHROOM3*, *SLC34A1* and *IGF1R*. Extensive mediation analysis results for all loci and all traits are reported in Supplementary Table S4.

				GWAS Step 1: association with ln(eGFRcrea)		Step 2: association with ln(eGFRcrea) adjusted for the mediator				Step 3: association with mediator		Step 3b: association with mediator in published literature		Step 4: association with mediator adjusted for ln(eGFRcrea)	
Locus (gene name)	**rsID**	**Chr:Pos (build 38)**	**EA/OA**	**b(SE)**	**P**	**Trait**	**b(SE)**	**P**	**Change %**	**b(SE)**	**P**	**b(SE)**	**P**	**b(SE)**	**P**
SHROOM3	rs28394165	4:76472865	C/T	-0.00841 (0.00214)	8.35×10^{-5}	Mg	-0.00744 (0.00196)	1.51×10^{-4}	-12	0.00826 (0.00204)	5.33×10^{-5}	-	-	0.00773 (0.00204)	1.54×10^{-4}
SHROOM3	rs10025351	4:76472942	T/C	-0.00840 (0.00214)	8.51×10^{-5}	Mg	-0.00743 (0.00196)	1.53×10^{-4}	-12	0.00823 (0.00204)	5.73×10^{-5}	-	-	0.00769 (0.00204)	1.65×10^{-4}
SHROOM3	rs28817415	4:76480299	T/C	-0.00840 (0.00214)	8.42×10^{-5}	Mg	-0.00744 (0.00196)	1.51×10^{-4}	-12	0.00823 (0.00204)	5.68×10^{-5}	-	-	0.00770 (0.00204)	1.64×10^{-4}
SHROOM3	rs4859682	4:76489165	A/C	-0.00840 (0.00210)	6.59×10^{-5}	Mg	-0.00750 (0.00193)	1.02×10^{-4}	-11	0.00812 (0.00201)	5.49×10^{-5}	-	2.39×10^{-9}	0.00758 (0.00201)	1.63×10^{-4}
SHROOM3	rs13146355	4:76490987	A/G	-0.00840 (0.00214)	8.38×10^{-5}	Mg	-0.00749 (0.00196)	1.33×10^{-4}	-11	0.00819 (0.00204)	6.12×10^{-5}	0.00500 (0.00100)	6.27×10^{-13}	0.00765 (0.00204)	1.77×10^{-4}
SLC34A1	rs3812036	5:177386403	T/C	-0.00836 (0.00245)	6.55×10^{-4}	aPTT	-0.01013 (0.00269)	1.64×10^{-4}	21	-0.23110 (0.04980)	3.54×10^{-6}	-0.41999 (0.04810)	2.88×10^{-18}	-0.23046 (0.04986)	3.88×10^{-6}
IGF1R	rs7174918	15:98708127	T/C	-0.00782 (0.00224)	6.55×10^{-4}	Urate	-0.00640 (0.00204)	1.66E-03	-18	0.05446 (0.02086)	0.00906	0.04100 (0.00590)	6.48×10^{-11}	0.03664 (0.02026)	0.07047
IGF1R	rs932071	15:98724801	A/G	-0.00774 (0.00223)	4.80×10^{-4}	Urate	-0.00646 (0.00203)	1.45E-03	-17	0.05143 (0.02077)	0.01331	0.04100 (0.00620)	2.47×10^{-10}	0.03366 (0.02017)	0.09523
IGF1R	rs4616271	15:98725030	T/C	-0.00798 (0.00223)	5.21×10^{-4}	Urate	-0.00668 (0.00203)	9.82×10^{-4}	-16	0.05288 (0.02079)	0.01099	0.04100 (0.00590)	3.39×10^{-11}	0.03446 (0.02019)	0.08779
IGF1R	rs875686	15:98729290	A/T	-0.00906 (0.00223)	3.54×10^{-4}	Urate	-0.00763 (0.00203)	1.68×10^{-4}	-16	0.05380 (0.02078)	0.00965	0.04100 (0.00600)	5.17×10^{-11}	0.03302 (0.02018)	0.10189
IGF1R	rs11633717	15:98729803	C/T	-0.00917 (0.00222)	4.82×10^{-5}	Urate	-0.00769 (0.00202)	1.39×10^{-4}	-16	0.05654 (0.02068)	0.00627	0.04100 (0.00600)	5.62×10^{-11}	0.03546 (0.02009)	0.07750
IGF1R	rs11633294	15:98731779	A/C	-0.00900 (0.00223)	3.58×10^{-5}	Urate	-0.00753 (0.00203)	2.08×10^{-4}	-16	0.05594 (0.02079)	0.00715	0.04000 (0.00590)	1.07×10^{-10}	0.03529 (0.02019)	0.08053
IGF1R	rs907808	15:98737025	G/A	-0.00768 (0.00216)	5.43×10^{-5}	Urate	-0.00652 (0.00196)	8.65×10^{-4}	-15	0.04988 (0.02006)	0.01291	0.04100 (0.00600)	1.46×10^{-10}	0.03195 (0.01948)	0.10100
IGF1R	rs3743263	15:98739329	G/A	0.00756 (0.00219)	3.69×10^{-4}	Urate	0.00642 (0.00199)	0.00127	-15	-0.04744 (0.02041)	0.02013	-0.03900 (0.00590)	5.27×10^{-10}	-0.02991 (0.01982)	0.13122
IGF1R	rs8037467	15:98741423	G/T	-0.00783 (0.00222)	5.66×10^{-4}	Urate	-0.00665 (0.00201)	9.54×10^{-4}	-15	0.04897 (0.02062)	0.01759	0.04000 (0.00600)	3.31×10^{-10}	0.03083 (0.02002)	0.12369
IGF1R	rs4966022	15:98743055	G/A	0.00763 (0.00219)	4.12×10^{-4}	Urate	0.00654 (0.00199)	0.00104	-14	-0.04727 (0.02043)	0.02071	-0.04000 (0.00590)	2.06×10^{-10}	-0.02946 (0.01984)	0.13753
IGF1R	rs11632952	15:98747746	G/A	-0.00767 (0.00222)	5.05×10^{-4}	Urate	-0.00655 (0.00201)	0.00116	-15	0.04870 (0.02065)	0.01840	0.04000 (0.00600)	2.21×10^{-10}	0.03082 (0.02005)	0.12439

Abbreviations: Chr, chromosome; Pos, position; EA, effect allele; OA, other allele; b, coefficient of association; SE, standard error of b; Mg, Magnesium; aPTT, activated Partial Prothrombin Time.

Figures

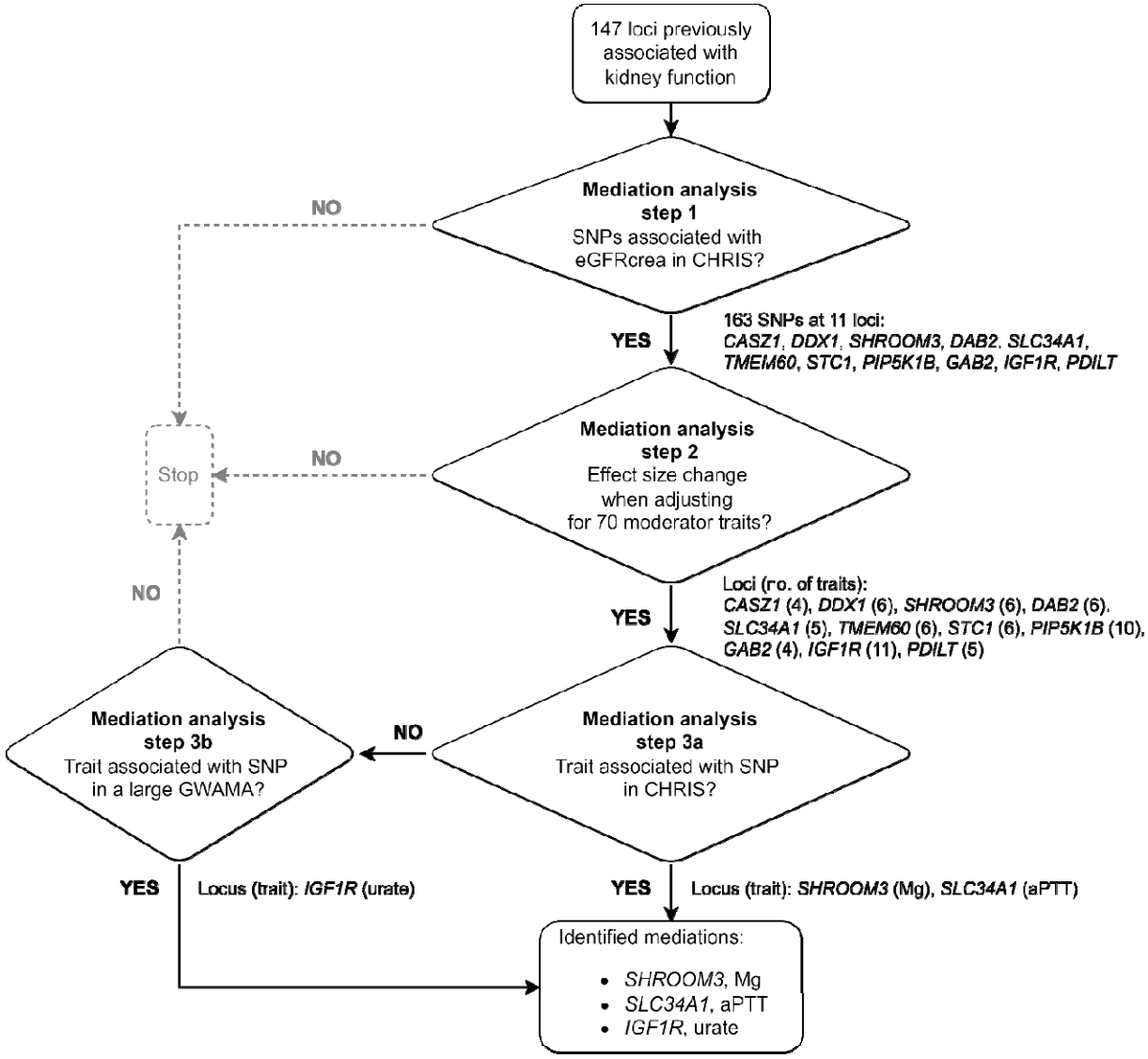


Figure 1. Flowchart of the mediation analysis. Abbreviations: Mg, serum magnesium; aPTT, activated partial thromboplastin time.

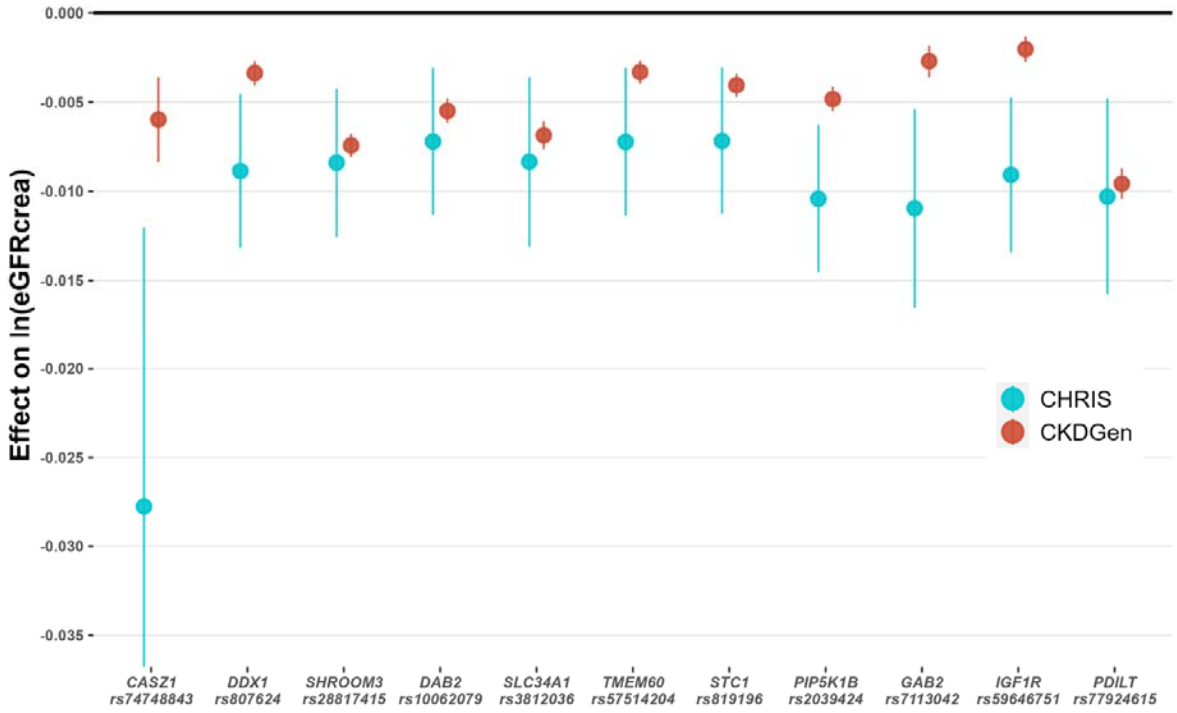


Figure 2. Effect sizes with 95% confidence intervals for the most associated variants at each locus in CKDGen and CHRIS following harmonization to the eGFRcrea-lowering allele. The effect is meant per copy of the effect allele on the natural logarithm of eGFRcrea in ml/min/1.73m^2 (y-axis). For *CASZ1*, *DDX1*, *PIP5K1B*, *GAB2*, and *IGF1R* the magnitude of the effect in CHRIS was larger than in CKDGen.

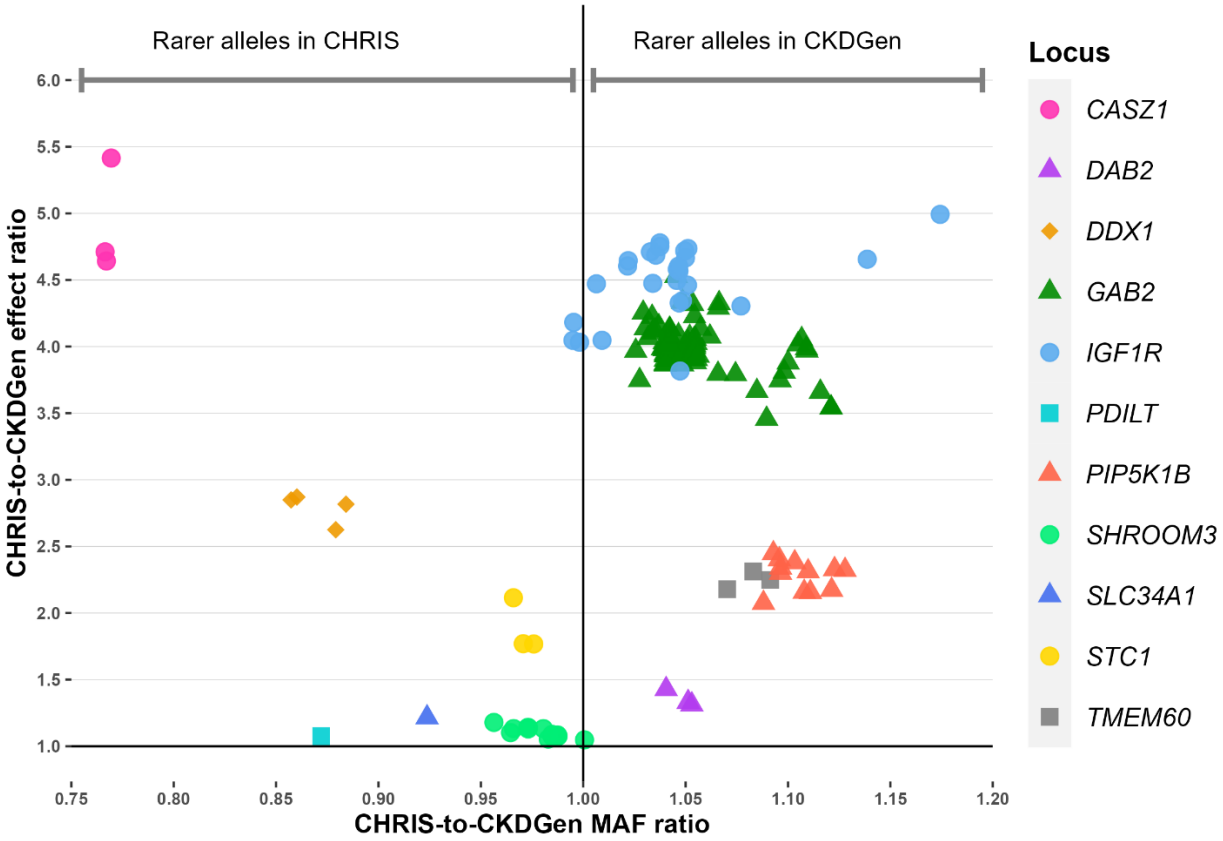


Figure 3. Comparison of the characteristics of all 163 variants in the 11 replicated loci in CKDGen versus CHRIS after alignment of the effect alleles. The ratio between CHRIS and CKDGen minor allele frequencies (MAF, x-axis) is plotted against the ratio between CHRIS and CKDGen effect estimates (y-axis). Effects are expressed in terms of change of the natural logarithm of eGFRcrea in ml/min/1.73m^2 per copy of the effect allele.

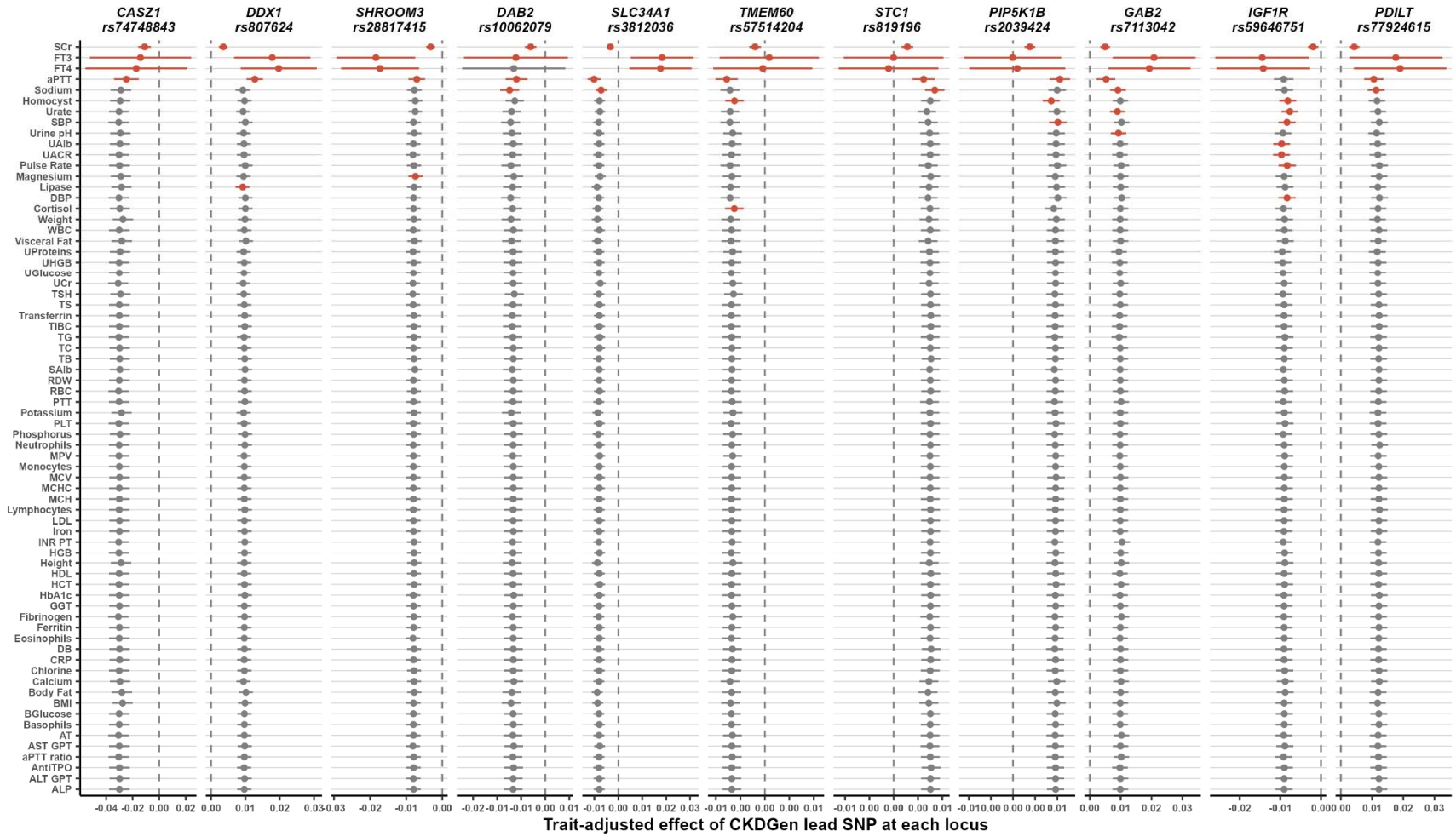


Figure 4. Results of the 2nd step of the mediation analysis for the most associated variants at each locus. SNP effect size and their 95% confidence intervals are reported. Effects are expressed in terms of change of the natural logarithm of eGFRcrea in ml/min/1.73m^2 per copy of the effect allele. Large departures from the expected effect size ('outlier', see Methods) are indicated in red. The vertical dashed lines identify the null. Large effect size changes were observed when adjusting for SCr (positive control; all loci), FT3 (all loci) and FT4 (10 loci), aPTT (10 loci), sodium (5 loci), homocysteine (3 loci), SBP (2 loci), urate (2 loci), and cortisol, DBP, lipase, magnesium, pulse rate, UACR, urinary albumin and urine pH (1 locus each).

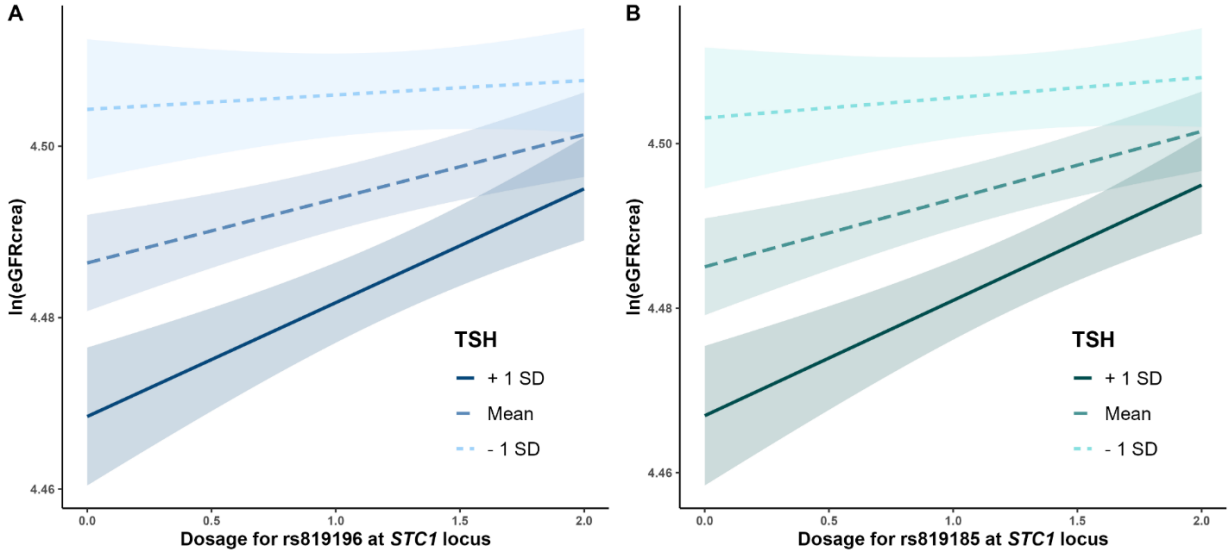


Figure 5. Interaction of ln(eGFRcrea)-associated variants with TSH levels. Effects of *STC1* SNPs rs819185 (P=0.00177; **Panel A**) and rs819196 (P=0.00154; **Panel B**) are larger at lower TSH levels.

Supplementary Material

Contents

Supplementary method. Quantile normalization of the 70 health traits used in the mediation analysis.

We used *normalize2Reference* function in the caret R package version 6.0-34 (the function is available in the later versions here: https://github.com/topepo/caret/tree/master/deprecated). Each trait was measured partially with an older method and partially with a newer, most recent method. The observations from the newer method (method = 1) were set as the reference to derive the quantiles of the trait distribution on which to standardize the observations from the older method (method = 0). We then replaced the observation from the older method with the standardized ones and included together with the values from the newer method.

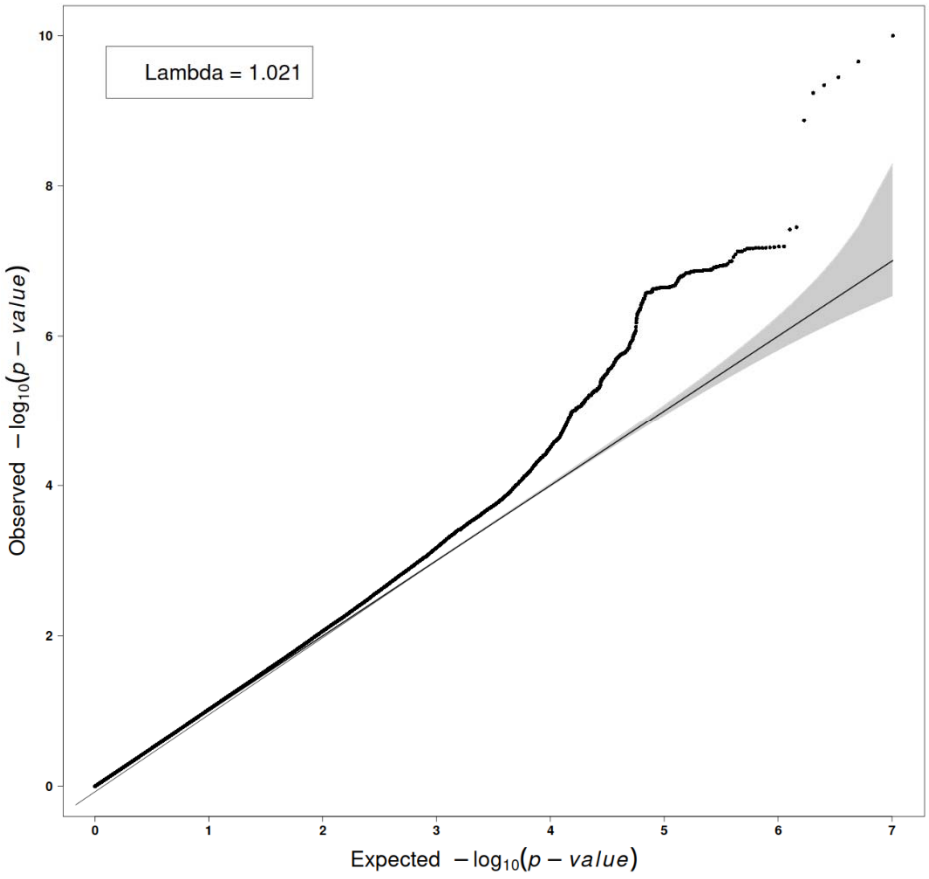
Notation:

- **trait**: the quantitative variable to which quantile normalization was applied;
- **method:** the newer method for measuring the trait (1); the older method (0);
- **df:** dataframe; the data in which the trait of interest is available

```
library(caret)
quantile_norm  <-
  normalize2Reference(
    data = df[df$method == 0, "trait"],
    refData = quantile(
      df[df$method == 1, "trait"],
      probs = seq(0, 1, length.out = length(df[df$method == 0, "trait"])),
      na.rm = TRUE,
      names = TRUE,
      type = 7,
      digits = 7),
    ties = TRUE)

# Creating a new variable for the quantile normalized trait
df$trait_std <- df$trait
# Replacing non-transformed values with the quantile-normalized values
df[df$method == 0, "trait_std"] <- quantile_norm
```

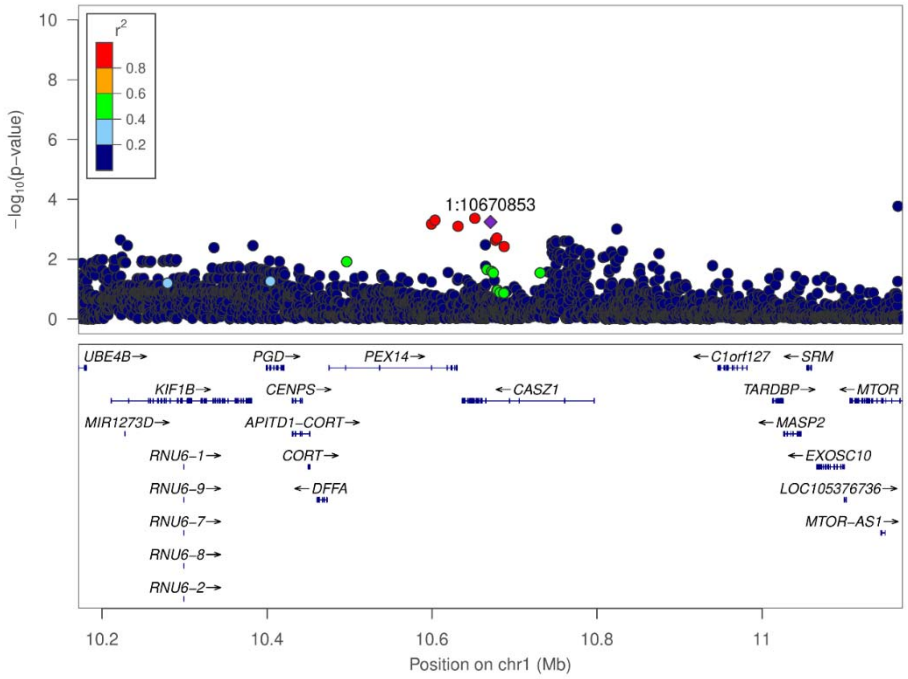
Supplementary Figure S1. Quantile-quantile (QQ) plot of the P-values from the GWAS of ln(eGFRcrea) in the CHRIS study.



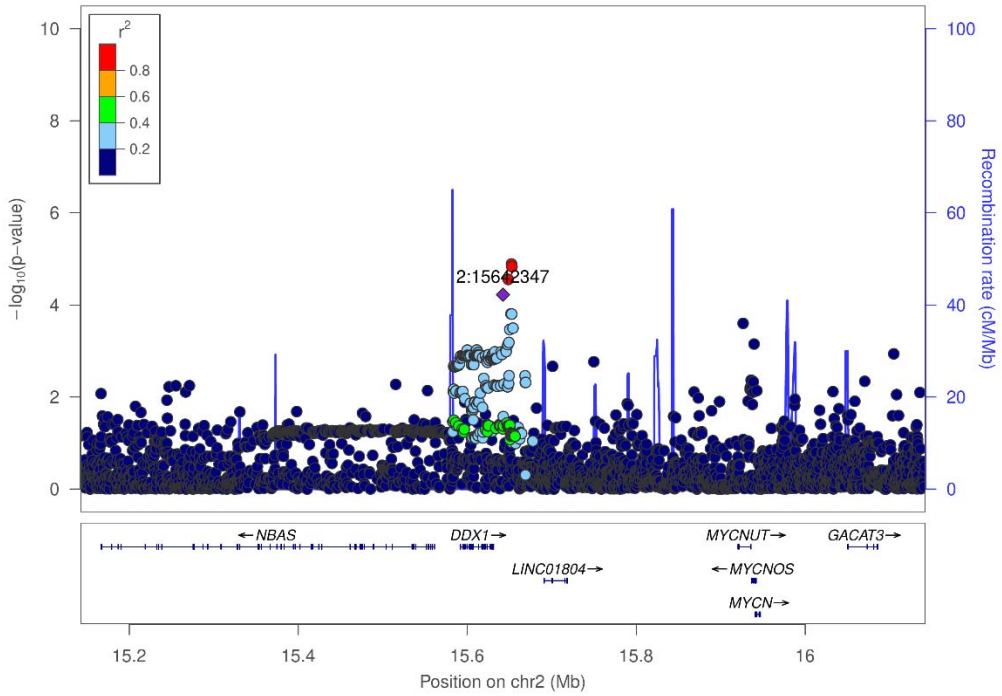
Supplementary Figure S2. Regional association plots for the 11 replicated loci. Highlighted in purple is the most associated SNP in the CKDGen GWAS meta-analysis. All SNP positions are referred to the NCBI Build 38. Plots were generated with LocusZoom version 1.4 (Pruim RJ, et al., Bioinformatics, 2010. 26(18): 2336-7)**.**

* indicates cases where not the CKDGen lead SNP but a proxy of it was replicated in the CHRIS study.

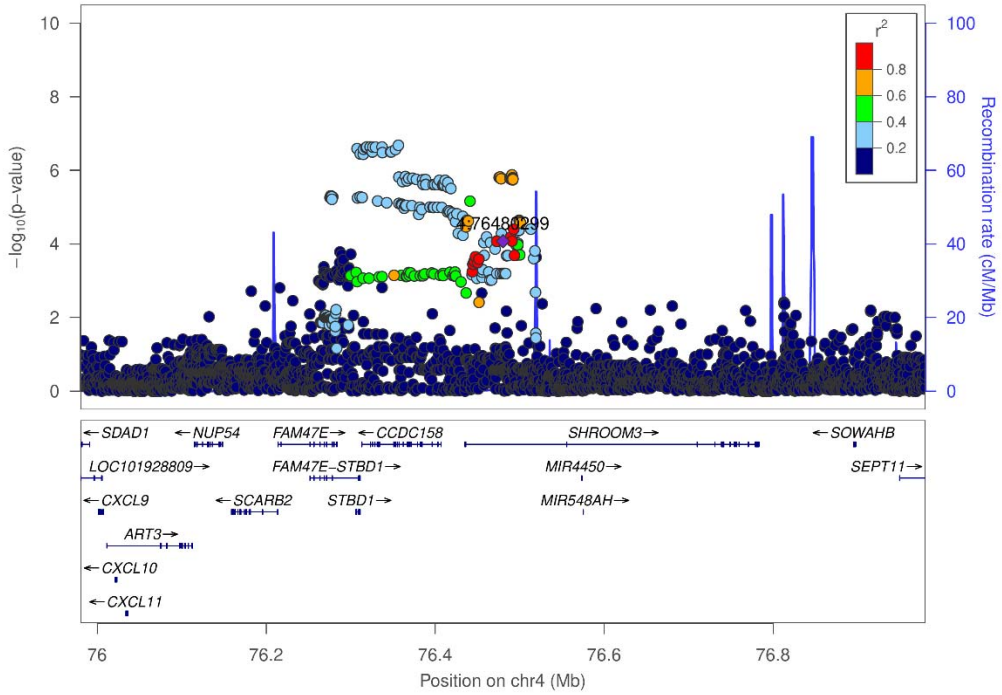
Top CHRIS SNP: rs74748843 (1:10,670,853) at ***CASZ1***



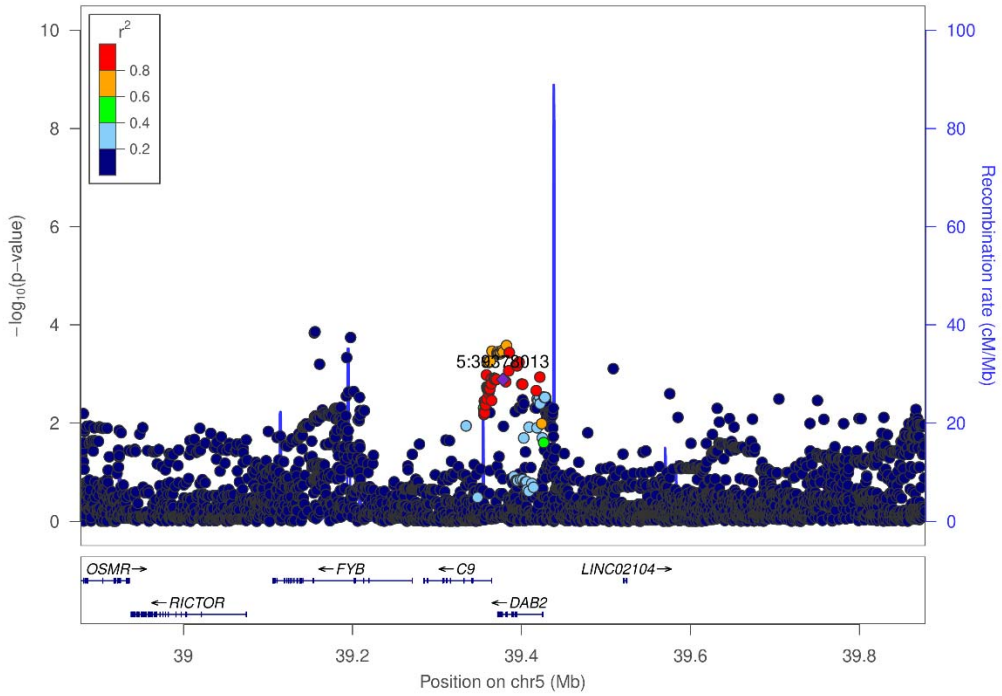
Top CHRIS SNP: rs807624 (2:15,642,347) at ***DDX1***



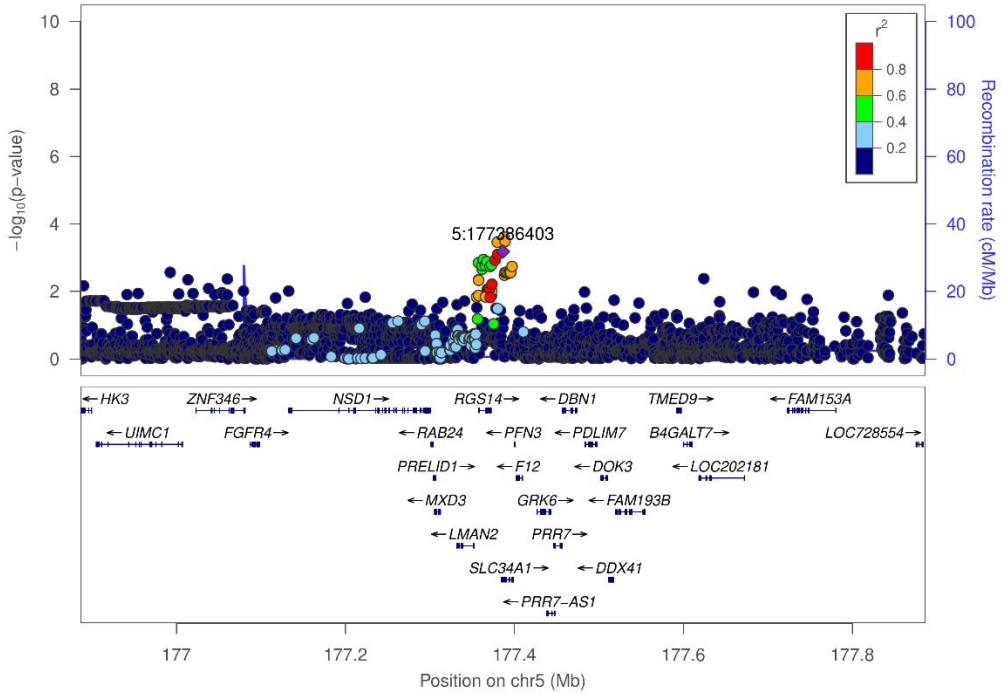
Top CHRIS SNP: rs28817415 (4:76,480,299) at ***SHROOM3***



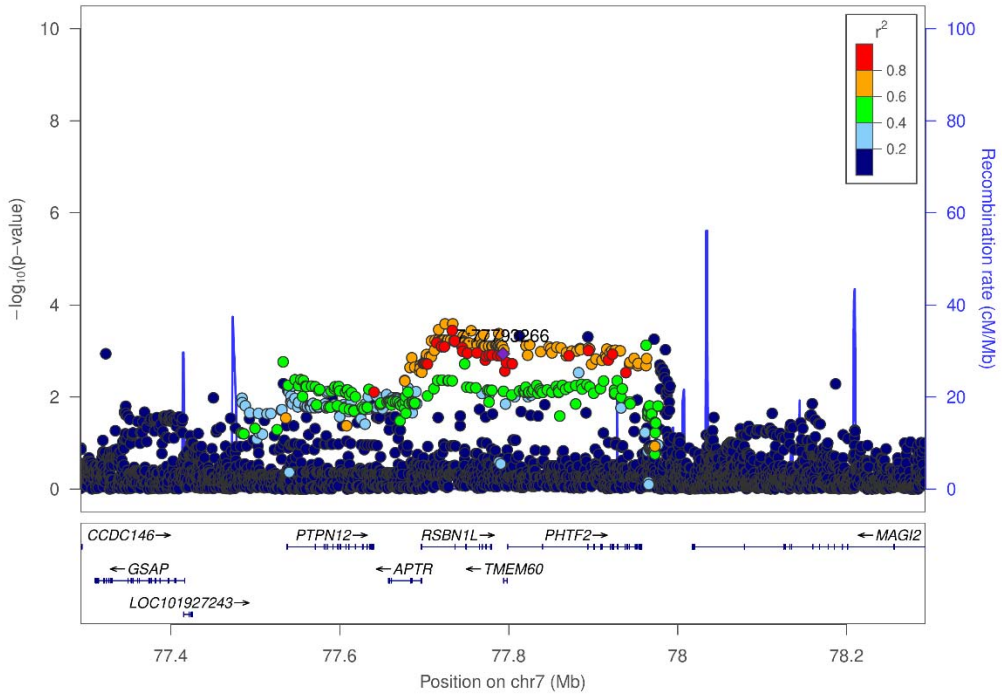
Top CHRIS SNP: rs1362800* (5:39,378,013) at ***DAB2****



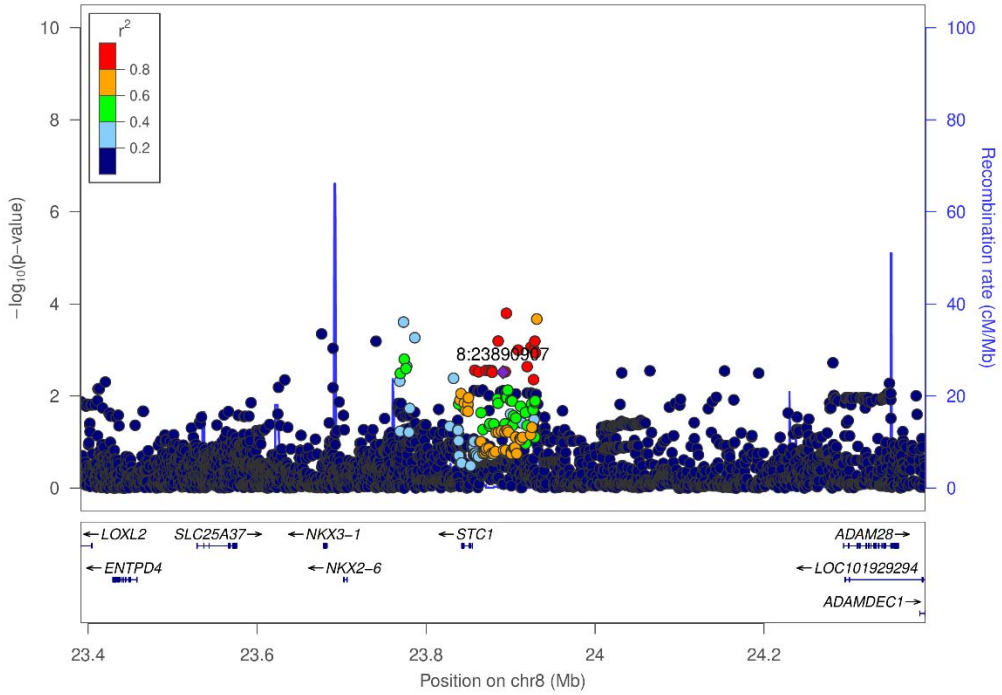
Top CHRIS SNP: rs3812036 (5:177,386,403) at ***SLC34A1***



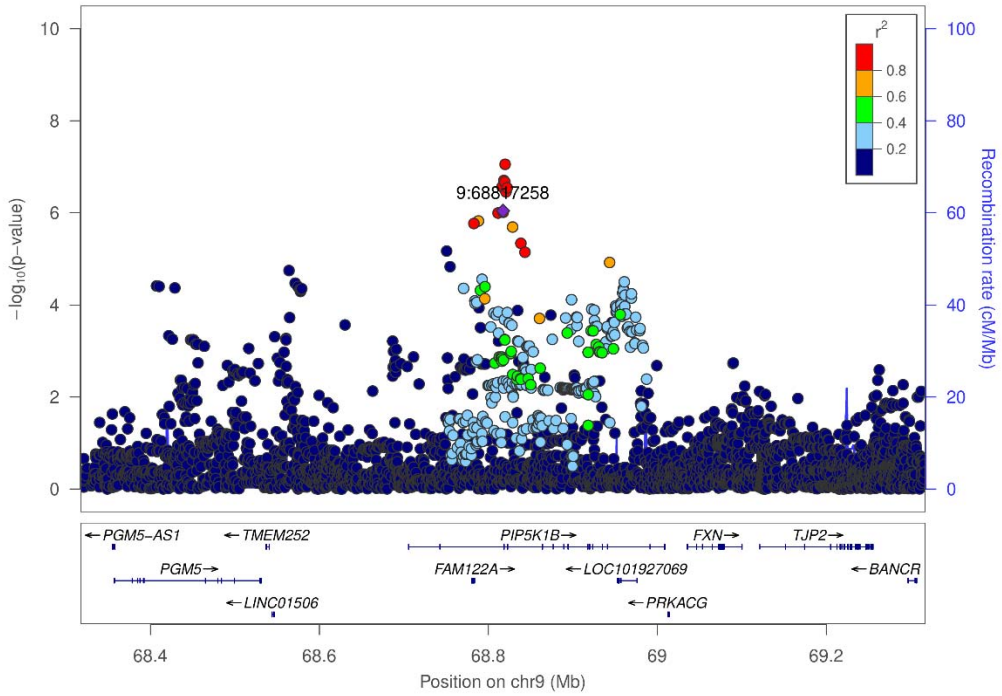
Top CHRIS SNP: rs6973656* (7:77,793,266) at ***TMEM60****



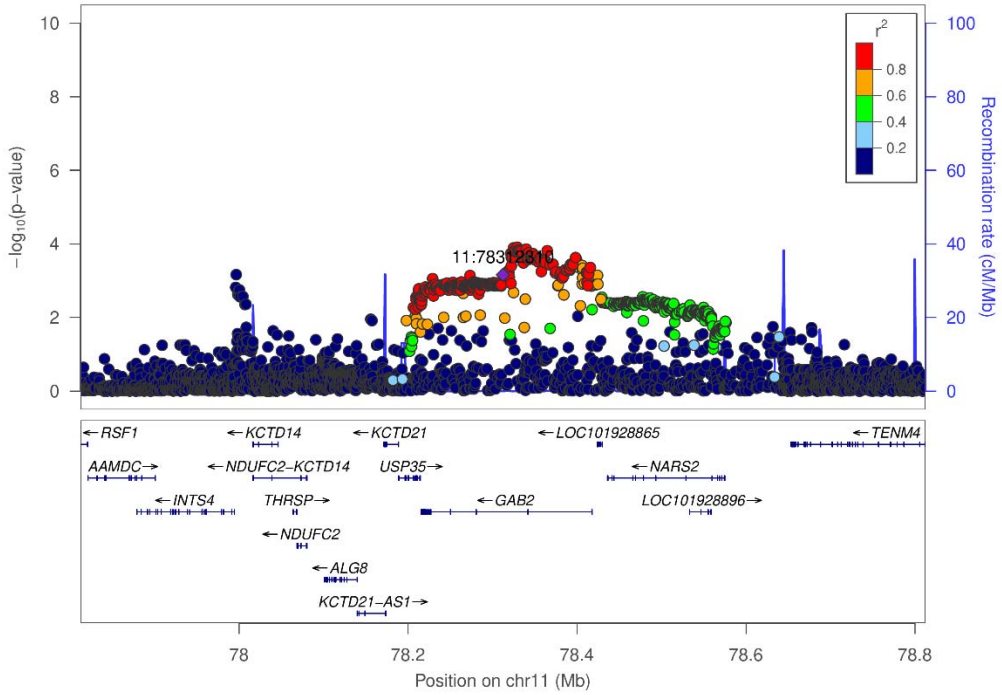
Top CHRIS SNP: rs34861762* (8:23,890,907) at ***STC1****



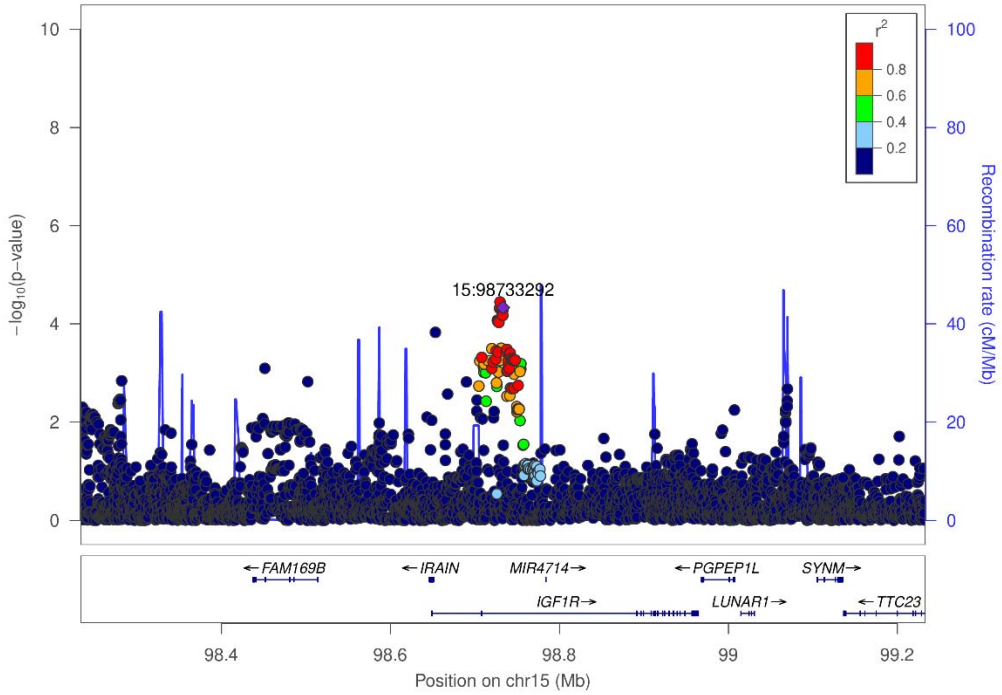
Top CHRIS SNP: rs2039424 (9:68,817,258) at ***PIP5K1B***



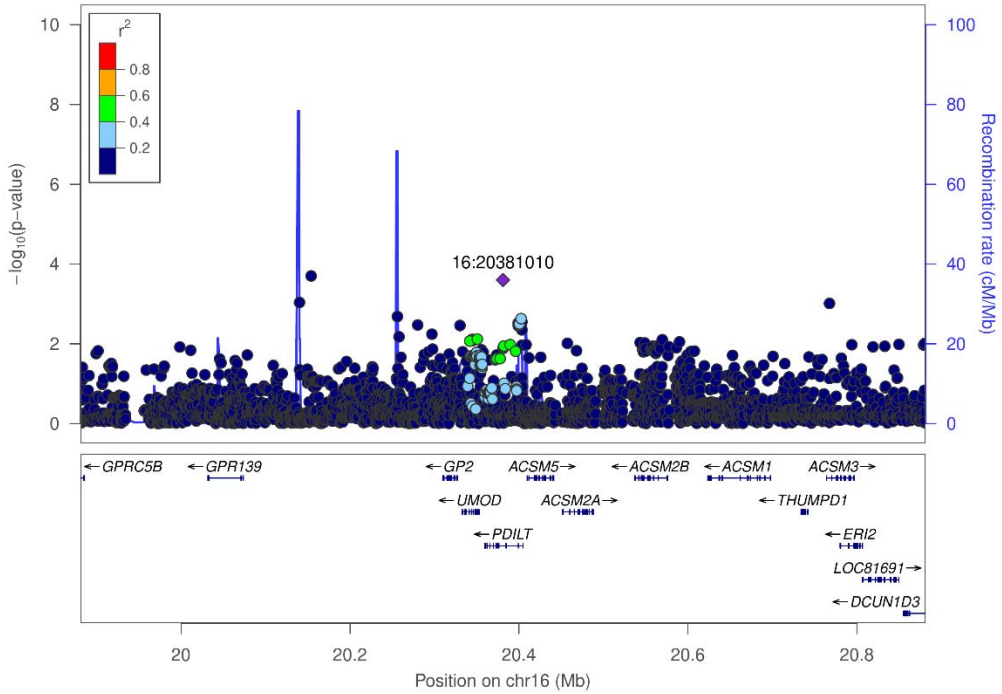
Top CHRIS SNP: rs11237450* (11:78,312,310) at ***GAB2****



Top CHRIS SNP: rs59646751 (15:98,733,292) at ***IGF1R***



Top CHRIS SNP: rs77924615 (16:20381010) at ***PDILT***



Supplementary Figure S3. Comparison of effect size and standard error ratio for the variants in the model regressing eGFRcrea on age, sex, FT3 (or FT4), genetic variants with and without adjusting for municipality.