**Exploiting horizontal pleiotropy to infer new causal pathways**

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## Abstract

It is still a matter of debate whether urate is casually associated with risk of kidney disease represented by the level of estimated glomerular filter rate (eGFR). This study is aimed to evaluate the causal association between urate and eGFR, exploiting pleiotropic variants among the instrument variables to search for the alternative pathway for the progression of the kidney disease. We applied two-sample mendelian randomization (MR) approach using summary statistics from published genome-wide association studies. We used Global Urate Genetics Consortium to obtain the genetic instruments for the effect of urate (n = 110 347). Data on the association of these genetic variants with eGFR were obtained from the CKDGen Consortium (n =133 814). Data on the association of genetic variants with urate and with eGFR were combined to estimate the effect of urate on eGFR. There was a 0.01 ml min per 1.73 m2 decrease (95% CI: -0.03, 0.01) in eGFR per SD (mg/dl) increment in urate level. The results were consistent among the three different sets of instruments for urate, but there was an evidence of pleiotropy in the instruments. We performed two sample MR of possible risk factors (candidate exposures), associated with the pleiotropic variants, against eGFR using pleiotropic SNPs as instruments for each candidate exposure. The MR results suggested that celiac disease, triglyceride and LDL cholesterol can be influenced on eGFR level. Overall, our findings do not support a causal role of urate in eGFR but suggests alternative risk factors for kidney function.

## **Introduction**

Mendelian randomization is now widely used to infer the causal influence of one trait (the exposure) on another (the outcome). It is performed by obtaining instruments for an exposure through genome-wide association studies (GWAS). If the instruments are valid, in that they influence the outcome only through the exposure (vertical pleiotropy), then they will each provide an independent, unbiased estimate of the causal effect of the exposure on the outcome. Meta-analysing these estimates can provide an overall more precise estimate of the causal relationship between the exposure and the outcome. If, however, some of the instruments are invalid, particularly because they influence the outcome through additional pathways that do not go through the exposure (horizontal pleiotropy), then the causal effect estimate will be biased. To-date, MR method development has viewed horizontal pleiotropy as a nuisance that needs to be factored out of the meta-analysis. But here we exploit horizontal pleiotropy as an opportunity, to identify new traits that putatively influence the outcome, and to use this knowledge to improve the original exposure-outcome estimates.

A crucial feature of MR is that it can be performed using only GWAS summary data, where the causal effect estimate can be obtained solely from the association results of the instrumenting SNPs on the exposure and on the outcome. This means that causal inference between two traits can be made even if they have never been measured together in the same samples. Complete GWAS summary results have now been collected from thousands of GWAS analyses, meaning that one can search the database of GWAS results for candidate traits that might be influenced by the outliers. In turn, the causal influence of each of those candidate traits on the outcome can be estimated using MR by identifying their instruments (and excluding the original outlier). Should any of these candidate traits putatively associate with the outcome then this goes some way towards explaining the horizontal pleiotropic effect that was exhibited by the outlier SNP in the initial exposure-outcome hypothesis.

Several methods exist for identifying outliers in MR, each likely to be sensitive to different patterns of horizontal pleiotropy. Cook’s distance can be used as to measure the influence of a particular SNP on the combined estimate from all SNPs, labelling SNPs with large influence as being outliers. Steiger filtering removes those SNPs that do not explain substantially more of the variance in the exposure trait than in the outcome, attempting to guard against using SNPs as instruments that primarily associate with the outcome or confounders. Finally, meta-analysis tools can be used to evaluate if a particular study contributes disproportionately to the heterogeneity between the estimates obtained from the set of instruments, and this has been adapted recently to detect outliers in MR analysis.

Recent large-scale MR scans have indicated that horizontal pleiotropy is widespread, suggesting that there is a tremendous opportunity to identify novel pathways through exploiting outliers. Equipped with automated MR analysis software, outlier detection methods and a database of complete GWAS summary datasets, we developed MR-TRYX (from the phrase coined by William Bateson, “Treasure your exceptions”), a framework for identifying novel putative causal factors when performing a simple exposure-outcome analysis. In this paper we present simulations to show how knowledge of horizontal pathways can be used to discover novel putative causal factors for an outcome of interest, and to also improve the power and reliability of the original exposure-outcome association. We use the effect of urate on chronic kidney disease as an example to showcase the approach.

Urate is the end product of purine metabolism in humans. Increased levels of urate have been associated with higher risk of chronic kidney disease (CKD)1. Recent observational studies suggest a role for urate as a potential marker of CKD2 and reduced kidney function, such as low estimated glomerular filtration rate (eGFR)3. However, whether urate represents an independent risk factor for the development of kidney diseases is still a matter of debate4. Although experimental studies do support causality suggesting raised urate levels induce elevated renal vascular resistance and reduced renal blood flow in animals, humans metabolise urate differently from most other mammals5. , and genome-wide association studies have found X independent genetic associations

A recent genome wide association study9 and MR study10 found that the variant in the urate level related gene is exclusively associated with increased plasma levels of urate and hyperuricaemia. However, it remains unanswered if these findings implicated a causal effect of urate on eGFR or shared pleiotropic effect as a set of genetic variants more likely to contain invalid instrument variables (IVs). It is due to violations of the assumption where genetic variants affect the outcome via a different biological pathway from the exposure (horizontal pleiotropy)11,12. If the instrumental variable assumptions are violated, the findings of a Mendelian randomization analysis are open to the same criticisms as those levelled at traditional observational epidemiological analyses.

Previous MR studies illustrated that the urate level related variants influences disease outcomes, but conditionally on their relationship with urate. If MR assumptions hold then instruments only influence the outcome through the exposure, which would lead to proportionality of effect sizes between the single nucleotide polymorphism (SNP)-exposure and SNP-outcome effects. However, it is clear that for the 28 variants available for urate several SNPs depart strongly from this expected proportionality. We hypothesised that this is because those SNPs are influencing kidney function through both urate and at least one other pathway. Therefore we aimed to search for novel putative risk factors for declining kidney function by utilising the pleiotropic SNPs in the urate-GFR analysis using MR-Base.

In this study, we examined the causal relationship between urate and eGFR decline within two sample Mendelian randomization framework. A two sample MR approach, whereby data on gene-exposure and gene-outcome associations from different samples obtained from publicly-available summary data from genome-wide association (GWA) study, allows us to assess the causal association between phenotype and outcome with large sample size and sufficient statistical power. One advantage of two sample MR is the availability of data for a range of phenotypes, enabling MR to be performed between a large number of traits. MR-Base has accumulated data for over 1000 traits. Multiple testing prohibits simple exhaustive searches for novel risk factors, so we have devised a novel strategy for identifying risk factors. The findings from this study will suggest exploiting pleiotropy among the instrument variables to search for the factors in the alternative pathway that are involved in the progression of the disease.

## **Methods**

### Study design

Two-sample MR was undertaken using the summary statistics of published GWA studies. SNPs, previously reported to be associated with plasma urate levels were used as instrumental variables for testing the causal effect of urate on eGFR. Summary statistics on the association of SNPs with urate levels and eGFR were combined to estimate the effect of urate on eGFR. In order to investigate the presence of the potential bias such as horizontal pleiotropy or the effect on eGFR via other risk factors (vertical pleiotropy) (Figure 1), we applied sensitivity analysis methods and analysed data on the association of the urate related SNPs with a range of traits using MR-Base. In most studies where we obtained the summary statistics, participants were of European ancestry. To identify other possible factors (candidate exposures) that may influence eGFR and estimate causation between those factors and eGFR, we performed the two-sample MR analyses of candidate exposure against eGFR.

### Outlier detection

* Describe outlier detection method in RadialMR package

### Candidate-trait detection

* Describe the use of MR-Base to detect traits that associate with the outliers, and then to estimate the candidate trait effects on the exposure and the outcome.

### Adjusting exposure-outcome associations for known candidate-trait associations

If a SNP influences a trait, which in turn influences the outcome only or the exposure and the outcome, then the SNP’s influence on the exposure and the outcome will be a combination of its direct effects and indirect effects through the candidate trait. We can obtain adjusted SNP-exposure and SNP-outcome effects conditional on the candidate-trait – exposure and candidate-trait – outcome effects.

#### The candidate trait influences only the outcome trait

In this case the SNP effect on the exposure will not require adjustment. The SNP-outcome effect will be

Where is the total effect of the SNP on the outcome, is the SNP-exposure effect, is the exposure-outcome effect as estimated through MR analysis from the non-outlier SNPs, is the SNP-candidate trait effect and is the causal effect of the candidate trait on the outcome obtained from MR analysis excluding the outlier SNP . Hence, the estimate accounting for the pathway through is simply . We use parametric bootstraps to estimate the standard error of the estimate, where 1000 resamples of , and are obtained based on their respective standard errors and the standard deviation of the resultant estimates represents its standard error.

#### The candidate influences the exposure and the outcome traits

If the candidate putatively trait influences both the exposure and the outcome then to account for the horizontal pleiotropic path correctly we must adjust for the influence of the SNP through the candidate trait on both the exposure and the outcome. This can be achieved in much the same way as the previous case of the candidate trait only influencing the outcome, but we must also obtain an adjusted estimate of , such that . Standard errors can be obtained using the same parametric bootstrap approach as described previously.

### Statistical analysis

All analyses were conducted with the two-sample MR package of MR-Base in R statistical software (ver 3.4.1). The genetic variants were pruned (r2 < 0.001) before analysis. We harmonised the SNP-exposure and SNP-outcome associations using the MR-Base “harmonised\_data” function to ensure that the associations obtain from the exposure and outcome GWAS summary-level data were coded relative to the same effect allele of each SNP. Since the genetic instruments were consisted of multiple genetic variants, inverse-variance weighted (IVW) linear regression was applied that sums the ratio estimates of all variants in a weighted average formula where the intercept is constrained to zero15. this method assumes the gene-exposure association estimates are measured without error. Results are reported as beta coefficient with their 95% confidence intervals (CIs) of eGFR per genetically predicted 1 standard deviation (SD) increase in urate levels. All statistical tests were two-sided considering statistical significance at p <0.05.

### Sensitivity analyses

A number of sensitivity analyses were applied to ensure the robustness of the findings and validity of genetic instrument; the MR-Egger, weighted median and mode approach. The MR-Egger regression was used to test overall directional pleiotropy and provide valid causal estimates, considering the presence of pleiotropy16. MR-Egger assumes no measurement error (NOME) but relaxes the assumption that the effects of genetic variants on the outcome only through the exposure, by not constraining the intercept term to zero. The balanced pleiotropy was visually assessed by using a funnel plot and checking for asymmetry, plotting the causal effect estimates against their precision. The weighted mode method was also applied to obtain valid estimates when the largest number of similar causal effect estimates comes from valid instrument, whilst the majority of instruments are invalid (<50%)17. Finally, we conducted weighted modal regression analyses, which relax the instrumental variable assumptions 18. We also conducted a series of sensitivity analyses to identify potentially influential instruments in the set of urate instruments using different sets of SNPs; a) all SNPs (N SNPs = 28, conservative set), b) SNPs after removing outliers (N SNPs = 9, liberal set), and c) using only one SNP in *SLC2A9* gene (individual SNP set). The Cochran Q test for heterogeneity was applied to the set of instruments without outlier to test for the presence of horizontal pleiotropy. This test assumes that all valid instruments estimate the same effect19. Additionally, a leave-one-out approach was applied to evaluate the influence of each SNP.

### Investigating novel pathway using MR base

We searched MR-Base to investigate whether genetic instruments have pleiotropic associations with other phenotypes. The SNPs had an association with eGFR at p value <0.05 were regarded as pleiotropic SNP. Phenotypes that exceeded the threshold for genome-wide significance (P value < 5 x 10-8) were defined as candidate exposures and used in two sample MR analyses to investigate the causal association with eGFR (Figure 1). To generate the SNP-candidate exposure (i.e. triglyceride, blood pressure, etc) and SNP-outcome (i.e., eGFR) association, effect estimates, and standard errors were obtained from publicly available results of a GWAS analysis (Supplementary Table 2).

## Results

### Adjusting SNP effects for pleiotropic pathways can improve exposure-outcome causal estimates

When SNPs exhibit horizontal pleiotropy the IVW effect estimates will have a relatively high liability of exhibiting bias, even though asymptotically balanced horizontal pleiotropy (where the pleiotropic effects do not have an average dominant effect). This can also lead to elevated false discovery rates, and as heterogeneity increases the power to detect non-null associations reduces substantially.

Removal of detected outliers tends to improve power, but when the number of SNPs exhibiting horizontal pleiotropy increases, this approach can also elevate false discovery rates and have a drastic influence on increasing bias in the estimates.

Instead of removing outliers, the MR-TRYX approach detects traits that mediate horizontal pleiotropic relationships using the MR-Base database, and adjusts the SNP-exposure and SNP-outcome effects for these estimated pathways. Compared to the raw estimates and those from outlier removal, the exposure-outcome bias is reduced and the false discovery rate is also reduced. Power to detect non-null associations is improved over the raw estimates also.

Theoretical improvements in outlier removal and outlier adjustment could be improved further with improved outlier detection methods: we note that when we assume all outliers are detected correctly in our simulation scenarios the performance of outlier removal and outlier adjustment both improve in terms of FDR, power and bias.

### Causal association between urate and eGFR using Mendelian randomization analysis

Table 1 presents the association between genetically elevated urate and eGFR using conservative urate set, liberal set, and individual SNP set. Using the available 28 SNPs robustly associated with urate, all method revealed a weak association of urate with eGFR. There was a 0.01 ml min per 1.73 m2 decrease (95% CI: -0.03, 0.01) in eGFR per SD (mg/dl) increment in urate level.

While the results of the MR analyses provided weak evidence for a causal effect of urate on decrease of eGFR using the conservative set, we repeated analyses using different set of SNPs. For the conservative approach, the estimate and 95% CI from MR-Egger were opposite to other estimates, but 95% CI from this analysis was included the null (β = 0.006; 95% CIs: -0.020 to 0.032). After exclusion of 20 SNPs that have association with eGFR, the direction of effect estimates obtained from each analysis was consistent (βIVW = -0.004; 95% CI: -0.009 to 0.001) with the results from the conservative urate set. For all of analyses, weighted median and mode estimates were consistent with the IVW estimates. Using the single SNP rs12498742 in *SAL2A9* showed a statistically significant casual effect of βWald ratio = -0.005 (95% CI: -0.0106, -0.0001). The difference between the results from different sets of instruments suggests that some SNPs in the conservative set have pleiotropic effects on the outcome that may bias the result toward null.

### Investigating potential pleiotropy and outliers

The pleiotropy effect of genetic variant violates the MR assumption that genetic instrument influences an outcome only though the effect of exposure. We explored potential pleiotropy applying the sensitivity analyses and heterogeneity tests.

The MR-Egger analysis using the conservative urate set showed no strong unbalanced horizontal pleiotropy (intercept = -0.002, 95% CI: -0.004, 0.001). However, the Cochran Q statistics 574.5 (P value = 5.81 x 10-104) for IVW, indicating that there is some heterogeneity in the estimates, possibly due to pleiotropy. Also, there is a high degree of heterogeneity individual causal estimates obtained for each urate associated variant (Figure 2). When plotting the genetic association with eGFR against the individual estimates in a funnel plot, there was a degree of asymmetry, suggesting some level of directional pleiotropy. We assumed that this was largely driven by the variants related to other phenotypes (e.g. rs1260326 in *GCKR* gene), since the CIs was considerably wider with using multiple instruments. In the leave-one-out MR analyses using the conservative urate set, all beta estimates of eGFR were directionally consistent (Figure 2) whilst the plot indicate the SNP rs12498742 may potentially influence on the association as its removal had effect on the result. This single variant can be suggested to account for most of the strong effect on the association.

To investigate further, we tested whether urate SNPs are associated with eGFR. Among 28 SNPs in the conservative set, 19 SNPs were identified to be potentially pleiotropic since those are associated with eGFR (Table 1). Those are the same SNP that we excluded as a sensitivity analysis.

### Associations of the urate associated SNPs with other phenotypes

Given that there was evidence for pleiotropy among genetic variants, eGFR can be decreased through alternative pathway (vertical pleiotropy). One explanation for the level of pleiotropy is the action of the genetic variants in other pathways. To investigate this, we searched for phenotypes that are related to urate SNPs using MR-Base. The two SNPs (rs1260326 near the *GCKR* gene and rs653178 near the *ATXN2*) had strong pleiotropic associations with numerous phenotypes (Table 3). The lipid traits including triglyceride (TG), high density lipoprotein (HDL) cholesterol, Low density lipoprotein (LDL) cholesterol, and unsaturated fatty acids were most frequently observed.

### MR of traits against eGFR

We performed MR analyses of candidate exposures that related to urate SNP against eGFR (Figure 3 and Supplementary Table 1). Celiac disease is causally associated with eGFR with βweighted mode of -0.002 (95% CI: -0.0030 to -0.0002). TG (βweighted mode = -0.005; 95% CI: -0.009 to -0.001) and LDL cholesterol (βweighted mode = 0.008; 95% CI: 0.001 to 0.015) were identified as potential risk factors for decrease or increase eGFR. Haemoglobin concentration (βweighted mode = 0.022; 95% CI: 0.004 to 0.041) and diastolic blood pressure (βweighted mode = -0.004; 95% CI: -0.009 to 0.0001) also have a causal association with eGFR,

## Discussion

We used two sample MR to investigate association between urate and eGFR. Overall, we did not find evidence that urate is causally associated with eGFR. There is an evidence that the association between urate and eGFR might be affected by pleiotropy (where a genetic variant is associated with more than one risk factor). Our results also suggest that celiac disease, TG, and LDL cholesterol play important roles in regulating the level of eGFR.

* Association of urate with eGFR

The findings from this study is broadly consistent with previous findings that urate is a risk factor for renal damage as accumulation of urate within the renal tubules producing a chronic inflammation 5,20. This is further supported by clinical intervention studies that indicate lowering urate levels by treatment improves renal function 21,22. The lack of association also was observed in other large cohorts 23 Consistent with our results, previous MR studies have also shown null effects of urate with kidney function.

* Suggest use of MR Base to exploit outliers to investigate alternative pathway

Several MR studies have previously examined the causal effect of urate on renal function, however, the results were not consistent. The discrepancy between finding a causal effect of urate and kidney function might be attributable to weak instrument bias, which depends on the strength of genetic instrument. Previous instrumental variables explained only a small proportion of variance in urate, meaning that other unidentified factors would be expected to confound the relationship between urate and renal function.

Inference of causality in MR analyses relies on the assumption that the genetic variants are strongly associated with the exposure and that pleiotropic or does not explain the association. Ruling out pleiotropy or an alternative direct causal pathway is a challenge for all MR analyses, particularly for risk factors determined by multiple genetic variants. In this study, we applied for four methods and used three sets of SNPs to instrument the urate levels. Results were similar in the IVW and the three sensitivity analyses within the same set of instruments. All point estimates of eGFR were directionally consistent across the instrument sets. In conservative urate SNP set, the 95% CIs were wider and included the null value of zero whilst there is an evidence that urate is associated with eGFR in the single SNP set. The finding that the statistical significance is different according to instrument used in model provides the evidence for pleiotropy among the instruments.

* Association of candidate exposures with Egfr
* Pleiotropic effect – GCKR / ATXN2
* Biological pathway-celiac disease / lipids

Previous MR study suggested that increased urate is associated with improved renal function 24 suggesting that pleiotropic effect of genetic variants on improving kidney function.

* strength and limitations:

Strength of this study include the use of data from large GWA studies of exposure and outcome, and the MR design. This design allows us to avoid bias from confounding and reverse causation. Potential bias from population stratification was reduced in our study since we restricted the analyses to individuals of European ancestry.

Although we tried to exclude weak instrument bias, there is some bias in our analyses. However, any bias from weak instruments is in the direction of the null as the analyses were undertaken in a two sample MR setting. We modelled a linear association of urate with eGFR. Despite these limitations, our study is large and well powered to detect causal association.

In conclusion, we evaluated association of urate with eGFR using two sample MR approach. Our study also extends use of pleiotropic effect by exploiting pleiotropy to search for the factors and applying a range of sensitivity analyses.

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