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Metabolic Insights into Urinary Stone Formation: Evidence from Mendelian Randomization, Clinical, and in vivo Studies

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Keywords
Urinary stones · Metabolomics · Mendelian randomization · Gut microbiota · Urolithiasis · Mannose

Abstract
Introduction: The global rise in urinary stone prevalence has become a significant health and economic challenge. Linked to metabolic disorders such as obesity and diabetes, urinary stones represent a complex systemic condition that requires a comprehensive understanding of metabolic profiles for effective management. ***Methods:*** The methodological quality of this study was evaluated in accordance with the STROBE-MR checklist. Using genome-wide association study (GWAS) data for 1,091 blood and 1,172 urine metabolites, we conducted a two-sample Mendelian randomization (MR) analysis, validated by meta-analysis, to explore metabolic influences on stone formation. Multivariable and mediation MR analyses were performed to identify independent metabolite influences and their interaction with gut microbiota and metabolism-related genes. Clinical metabolomic analysis and further animal experiments substantiated our findings. ***Results:*** Univariable MR identified 119 blood and 63 urine metabolites associated with urinary stones, with 16 blood and 2 urine metabolites showing robust associations post-correction. Notably, mannose and 3-aminoisobutyrate emerged as independent influencers of stone formation. Mediation MR suggested these metabolites as potential mediators in the gut microbiota's influence on stone formation. Clinical urine sample analysis indicates higher mannose levels in normal renal sides than stone sides. Animal studies confirmed mannose's protective role by reducing renal calcium oxalate crystal deposition. ***Conclusion:*** Our study establishes causal links between specific metabolites and urinary stones, shedding light on the intricate biological mechanisms of stone formation. The discovery of mannose as a protective factor opens avenues for future research and clinical applications, offering promising directions for the prevention and treatment of stones.

Introduction

Over the past few decades, the prevalence of urinary stone disease has been rising continuously, posing a significant burden on global economy and human health [1]. Recent epidemiological data indicate that the prevalence of kidney stones in the USA and China has reached

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11% and 6.4%, respectively [2, 3]. Although some kidney stones are usually asymptomatic, >50% stone patients will experience recurrence, and recurrent kidney stones can progress to chronic kidney disease (CKD) or even kidney failure [4].

Urinary stones are associated with a variety of metabolic diseases such as obesity, diabetes, and metabolic syndrome and have recently been recognized as a systemic metabolic disease [5–7]. Previous studies have indicated that factors such as oxalate metabolism, reactive oxygen species, and sex hormone metabolism are involved in the formation of stones, but the specific mechanisms by which metabolic factors affect urinary stones remain unknown [8–10]. We all know that the metabolic process of stones is difficult to define as the metabolic changes of one or several specific substances; hence, there is an urgent need for comprehensive metabolomic research on urinary stones.

With the widespread use of nuclear magnetic resonance spectroscopy, especially mass spectrometry and related technologies in recent years, metabolomics methods have begun to be applied to the study of urinary stones [11–13]. These studies discovered significant differences in certain metabolites between patients with stones and those without, for example, the polyphenol metabolite hippuric acid, which is beneficial for preventing kidney stones, is significantly reduced in patients with kidney stones. However, the inherent limitations of observational studies have historically posed a significant challenge in establishing causal relationships between metabolites and urinary stones. Some studies have been hindered by insufficient sample sizes and a lack of rigorous quality control. Although randomized controlled trials (RCTs) represent the gold standard for establishing causal relationships, they are not always feasible for studying the complex interactions between numerous metabolites and stones. The high costs, ethical considerations, and patient compliance issues associated with RCTs can present significant challenges to their implementation in this context. Mendelian randomization studies have recently been employed as a valuable complement to observational studies in the investigation of disease interrelationships and etiology [14, 15]. The fundamental premise of MR is to identify single nucleotide polymorphisms (SNPs) that are associated with exposure as instrumental variables (IVs), with the objective of elucidating the causal relationship between exposure and outcome. SNPs are subject to random combinations during inheritance, in accordance with Mendel's second law. This process emulates the conditions of RCTs [16].

In this study, we conducted a comprehensive MR analysis utilizing genome-wide association study (GWAS) data to explore the causal relationships between 1,091 blood and 1,172 urine metabolites and urinary stones. Furthermore, mediation MR analysis of the gut microbiota and metabolite-related gene expression quantitative trait loci (eQTLs) in blood was conducted to elucidate the potential mechanisms through which metabolites influence urinary stones in the body. Finally, the principal conclusions were validated through animal experiments and clinical samples. This research aims to identify metabolites that influence the development of urinary stones and preliminarily explore their biological processes and related mechanisms.

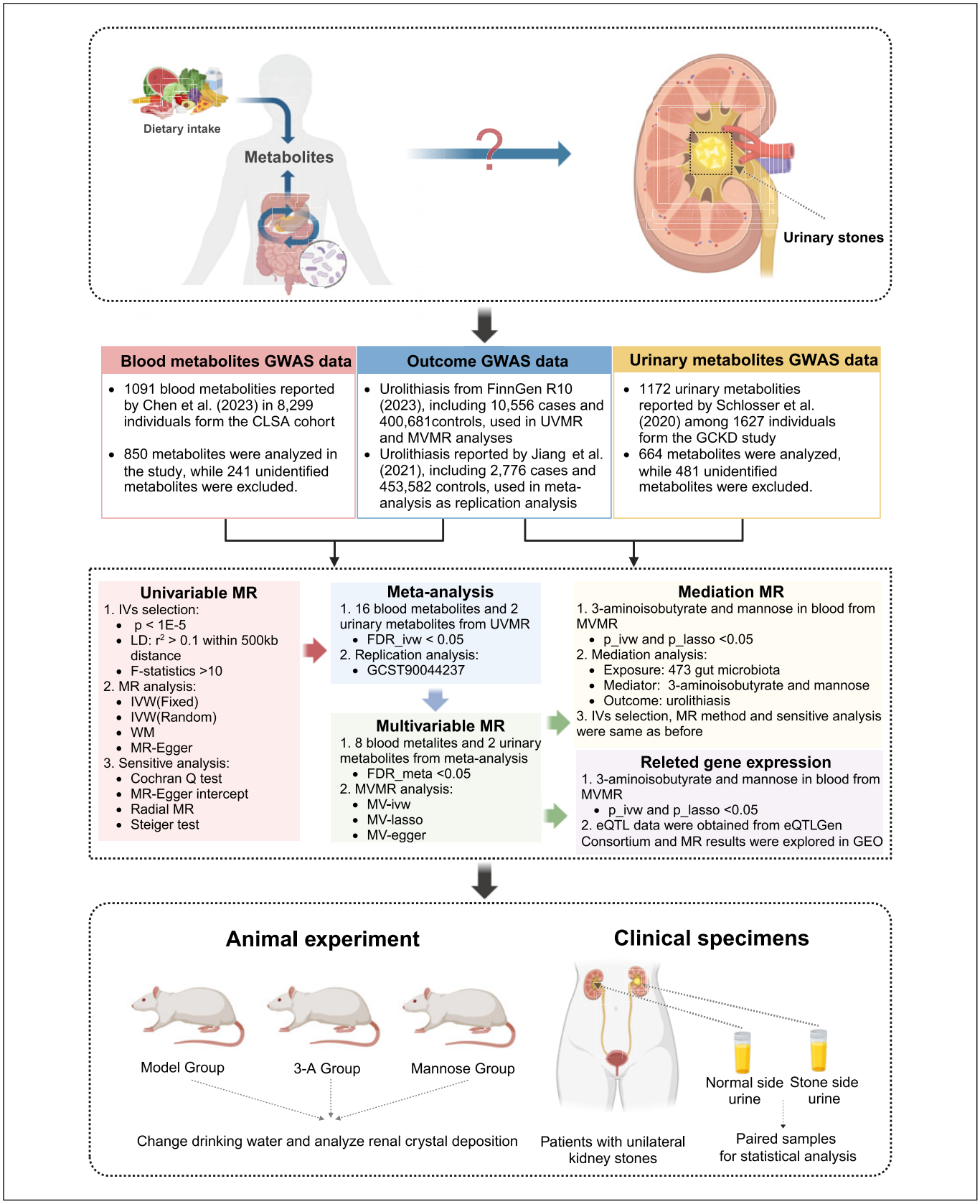
Materials and Methods

Study Design

The veracity of the Mendelian randomization analyses was guaranteed by three fundamental assumptions: (I) genetic IVs have a strong correlation with metabolites; (II) genetic IVs are not influenced by any potential factors that could distort the outcomes; and (III) genetic IVs only affect urinary stones through the metabolites [17]. To enhance the robustness of the conclusions, we conducted MR analyses using outcome dataset from disparate sources and subjected the results to meta-analyses, thereby reinforcing external validation. Multivariate Mendelian randomization was employed to concurrently evaluate the individual causative impacts of the significant metabolites on urinary stones [18]. To provide further insight into the potential mechanisms by which metabolites affect urinary stones, we conducted mediation MR analyses based on GWAS summary data on gut microbiota as well as eQTLs [19]. In response to the findings of the above study, animal experiments as well as clinical samples were collected for further validation. Figure 1 displays a concise depiction of the study design.

Source of the Metabolites' GWAS Datasets

The GWAS data for blood metabolites were obtained from a study published by Chen et al. [20] in 2023. The study focused on 8,299 unrelated European subjects from the Canadian Longitudinal Study of Aging (CLSA) [21]. The participants were genome-wide genotyped and the levels of 1,458 metabolites in plasma samples were quantified using the ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) platform.



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Chen et al. conducted rigorous quality control of the metabolomics data, retaining only metabolites with missing measurements in less than 50% of the samples (n = 1,091). These 1,091 metabolites included 241 undefined compounds, and the focus of this study was to investigate the relationship between the remaining 850 identified metabolites and urinary stones.

Furthermore, GWAS data for urinary metabolites were obtained from a study by Schlosser et al. [22] in 2020. The study selected 1,627 patients from the German Chronic Kidney Disease (GCKD) cohort diagnosed with CKD [23] and the 1,172 metabolites were quantified using untargeted mass spectrometry [24]. We similarly excluded 481 undefined metabolites and included 664 of them to further investigate the effect of urinary metabolites on urinary stones. We selected the exposure dataset from different cohorts from the outcome dataset to minimize sample overlap.

The GWAS summary data for all blood and urinary metabolites are available for download from the GWAS catalog (https://www.ebi.ac.uk/gwas/). Online supplementary Tables 1 and 2 (for all online suppl. material, see https://doi.org/10.1159/000545550) provided information of these GWAS datasets.

Source of the Urinary Stones' GWAS Datasets

In the discovery phase of investigating the relationship between metabolites and urinary stones, we utilized data with the description of "Calculus of kidney and ureter" from Round 10 of the FinnGen database (https://www.finngen.fi/fi), which is a unique study combining genomic information with digital healthcare data. These data were released in December 2023 and comprised 411,237 participants (10,556 cases and 400,681controls), representing the most recent data currently available in FinnGen.

To further corroborate the reliability of the positive metabolites identified, we also collected data from the UK Biobank (UKB), which was processed by a previous study by Jiang et al. [25] published in 2021. The UKB is a very large, population-based prospective study that aims to examine in detail the genetic and nongenetic determinants of disease in middle-aged and older people [26]. The GWAS dataset selected for analysis was described as "the calculus of the kidney (PheCode 594.1)" with the accession number "GCST90044237" in the GWAS catalog and included 465,358 participants (2,776 cases and 453,582 controls).

Selection Criteria of Instrumental Variables for MR Analysis

In order to ensure the efficacy of the MR analysis, we employed a series of criteria to evaluate the IVs: (I) the IVs were significantly correlated with the exposure factors, i.e., with a p value $<1 \times 10^{-5}$ for genome-wide relevance; (II) in order to eliminate linkage disequilibrium, we set a threshold of $r^2 = 0.1$ and kb = 500, which has been widely applied in previous studies [27–29]; (III) IVs with F-statistics below 10 were excluded from the analysis to mitigate the potential for bias arising from the use of weak IVs [30]. The F-statistics were computed using the following formula [31–33]:

$$F = \frac{R^2 \times (N-2)}{1-R^2}$$

In this formula, N is the sample size in the selected exposure dataset and R^2 represents the degree of variation explained by each SNP, which is calculated as the following formula:

$$R^2 = \frac{2 \times \beta^2 \times \mathrm{EAF} \times (1-\mathrm{EAF})}{2 \times \beta^2 \times \mathrm{EAF} \times (1-\mathrm{EAF}) + 2 \times \mathrm{SE}^2 \times N \times \mathrm{EAF} \times (1-\mathrm{EAF})}$$

where β is the amount of SNP effect on exposure and EAF is the frequency of the effector allele. Detailed information on the selected IVs for the 850 blood metabolites and 664 urine metabolites analyzed in this study is presented in online supplementary Tables 3 and 4, respectively.

Univariable Mendelian Randomization Analysis

In our study, 4 Mendelian randomization methods were adopted to evaluate the causal relationship, including inverse variance weighted (IVW) with fixed-effect model, IVW with multiple random-effect model, MR Egger, and weighted median (WM). The IVW method is derived from the summary analysis of the Wald Ratio and assumes that no horizontal pleiotropy exists, thus providing the most accurate assessment of causal effects [34]. Therefore, we adopted IVW approach as the primary analytical method. If heterogeneity was detected among the genetic instruments, we used IVW with

Fig. 1. Overview of the research hypothesis, data sources, and the entire study process. GWAS, genome-wide association study; CLSA, Canadian Longitudinal Study of Aging; GCKD, German Chronic Kidney Disease cohort; MR, Mendelian randomization; UVMR, univariable Mendelian randomization; MVMR, multivariable MR; IVs, instrumental variables; LD, linkage disequilibrium; IVW, inverse variance weighted; WM, the weighted median approach; FDR, false discovery rate; eQTL, gene expression quantitative trait loci; GEO, Gene Expression Omnibus; 3-A, 3-aminoisobutyrate.

multiple random-effect approach as the primary analysis method. Alternatively, we adopted IVW with fixed-effect analysis as the primary method [35]. Given the considerable number of MR analyses conducted in this study, we applied Benjamini-Hochberg false discovery rates (FDR) correction for the p values under the IVW method to further avoid type I errors. In both cases, we considered FDR-corrected p values <0.05 to be statistically significant [36].

Complementary MR methods, including WM and MR Egger, were employed to further assess the stability and consistency of the findings obtained through the IVW approach. The WM approach allows up to 50% of genetic variants to be invalid, whereas MR Egger can detect and account for horizontal pleiotropy affecting all SNPs used as instruments [34, 37]. The convergence of the results across these different MR methods further strengthened the confidence in the metabolites identified as having causal associations with stones.

Sensitivity Analyses for Univariable Mendelian Randomization

To assess the robustness of our findings, we performed several sensitivity analyses. MR radial identifies and removes potential outlier genetic variants that may violate the MR assumptions [38]. Steiger filtering ensures the validity of the genetic instruments by retaining only those where the exposure significantly predicts the outcome, and not vice versa [39]. We evaluated between-variant heterogeneity using Cochran's Q test [40]. The MR-Egger regression intercept was used to assess the presence of overall horizontal pleiotropy [37], as significant horizontal pleiotropy can indicate violations of the MR assumptions. We also conducted a reverse causation check using the Steiger test [39]. The leave-one-out analysis iteratively removed each genetic variant to ensure the results were not driven by a single instrument [37]. Finally, we visually inspected the funnel plot to detect potential small-study effects or publication bias [41].

External Validation and Meta-Analysis

We performed MR analyses of the FinnGen data as the outcome and initially identified potential positive metabolites. In order to corroborate the reliability and replicability of the initial findings, we reexamined the results using data from UKB as the outcome. Furthermore, we conducted meta-analyses combining the results from the two studies, to reconcile any discrepancies between the different analyses and obtain a more consistent conclusion. I^2 was used to when I^2 was less than 50%, and vice versa, a random-effects model was employed.

Multivariate Mendelian Randomization

In blood and urine, various metabolites coexist, and there are intricate interrelationships between different metabolites. Consequently, the same genetic variant may be significantly associated with multiple metabolites simultaneously. Despite our rigorous efforts to control for horizontal pleiotropy through MR-Egger regression intercept test, we could not entirely rule out the possibility of bias due to the aforementioned reasons. Therefore, we employed multivariate MR (MVMR) analyses for the previously identified positive metabolites to investigate the independent effect of each metabolite on urinary stones, while considering these metabolites simultaneously [42]. In order not to affect the accuracy of the final results, the five metabolites with "*" in the previous meta results were removed, as the authors have noted in their study that the standards for detecting these metabolites are not yet operational. In the MVMR analyses, we used the MR-Lasso method in addition to the IVW and MR-Egger methods mentioned above. MR-Lasso uses lasso regression, which adds a penalty term to the causal effect estimator that shrinks the regression coefficient toward zero and forces the coefficients of individual IVs to be zero [43].

Mediation MR Analysis Based on Gut Microbiota and eQTLs

After establishing the causal association between metabolites and stones, we hope to further explore the intrinsic mechanisms. A meta-analysis found significant dysbiosis of gut microbiota characteristic among patients with urinary stones, suggesting the importance of gut microbiota in stones formation and prevention [44]. In addition, observations over the past 2 decades suggested that the gut microbiota might contribute to the metabolic health of the human host and promote the pathogenesis of various common metabolic disorders when aberrant [45]. Therefore, we proposed a hypothesis that alterations in gut microbiome composition may influence the production and absorption of metabolites involved in stones pathogenesis. To investigate this hypothesis, we carried out two-step mediation Mendelian randomization analyses [19].

The 473 types of gut microbiota GWAS summary data we included in our analyses were obtained from the study by Qin et al. [46] in 2022. This study used a large single homogeneous population cohort FR02 (N = 5,959) with matched human genotypes and shotgun fecal metagenomes to determine genome-wide associations between human genotypes and gut microbial abundance. By reviewing original studies, we identified genes associated

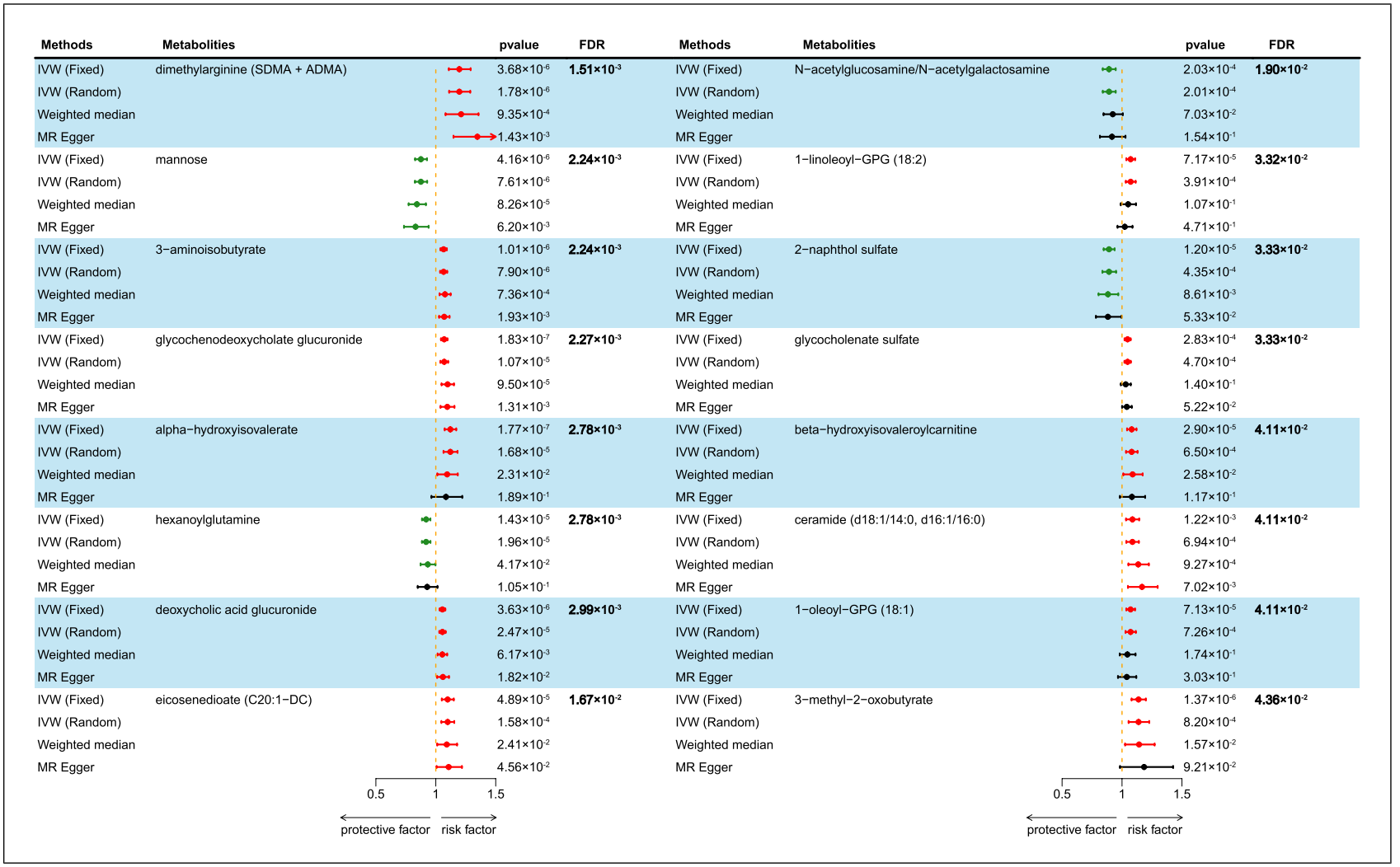

Fig. 2. Forest plot of significant blood metabolites on urinary stones passing FDR correction (FDR <0.05). IVW, inverse variance weighted; FDR, false discovery rate.

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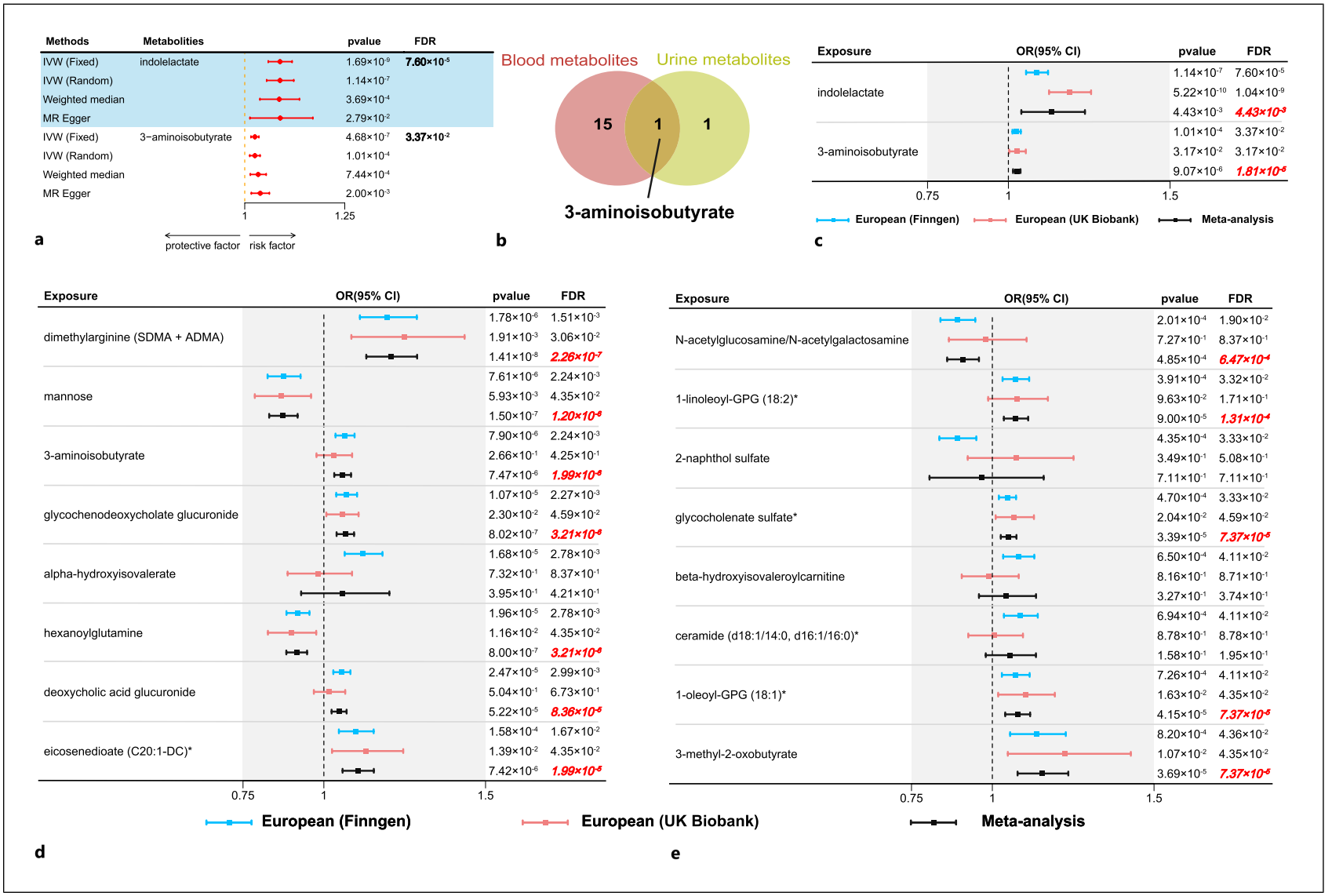

Fig. 3. Meta-analysis of significant blood and urinary metabolites on stones. **a** Forest plot of significant urinary metabolites on urinary stones passing FDR correction (FDR <0.05). **b** The Venn diagram shows that there is an intersection between 16 significant blood metabolites and 2 significant urinary metabolites, 3-A. **c** Replication and meta-analysis of 2 urinary metabolites. **d**, **e** Replication and meta-analysis of 16 blood metabolites. OR, odds ratio; CI, confidence interval; FDR, false discovery rate.

with our significant metabolites, including the closest protein coding genes, colocalized genes, and biological relevant genes. For these genes, we selected the corresponding GWAS datasets in the eQTLGen database [47]. Using gut microbiota and eQTL data, we initiated further mediation analyses, in which we focused on the β-values, *p* values, and mediation proportions of the mediation effects while metabolites were used as mediators.

Transcriptome Validation and Differential Expressed Gene Analysis

Based on the results of eQTLs Mendelian randomization, we obtained potential genes that simultaneously affect metabolites and urinary stone formation. We accessed the Gene Expression Omnibus (GEO) database and downloaded the GSE73680 dataset, which collected renal papillary tissues from kidney stone patients and healthy controls [48, 49]. We evaluated the expression levels of these genes in the patients' kidney tissues to further screen for genes with significant differences. The KRTCAP3 expression levels were used to divide patients into two populations. Differential expressed gene (DEG) analysis was performed on these populations, and the resulting DEGs were subjected to gene set enrichment analysis, as well as gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. "*Metascape*" online tool was used to visualize the results of the enrichment analysis [50].

Metabolomic Analysis of Urine from Clinical Patients

A total of 18 patients with unilateral calcium oxalate stones were recruited from the Department of Urology at Tongji Hospital. To ensure the accuracy of the results, patients with confounding factors such as urinary tract infections (UTIs), a history of major urological surgery, were excluded from the study. All patients were diagnosed by computed tomography and underwent percutaneous nephrolithotomy. The stones removed during the endoscopic surgery were sent for chemical composition analysis. The collection of renal pelvic urine was approved by the Ethics Review Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (TJ-IRB20231210). Informed consent was obtained from all patients for the use of their samples. Prior to surgical intervention, the bladder was emptied using a urethral catheter. A ureteroscope was gently inserted into the renal pelvis with stones, and a ureteral catheter was placed within the ureteroscope to collect renal pelvic urine. A new ureteral catheter was then employed to perform the procedure once more to collect urine from the renal pelvis of the contralateral kidney without stones in the same patient. Urine samples were flash-frozen in liquid nitrogen immediately after collection and stored at −80°C. The entire process was conducted under sterile conditions. The collected urine samples were analyzed for relative metabolite content using the LC-ESI-MS/MS system (UPLC, ExionLC AD; MS, QTRAP® System) for metabolomic analysis.

Animal Experimental Design

The rats were maintained on a normal day-night cycle with free access to food and water. All the subsequent animal experiments were approved by the Tongji Hospital Animal Care and Use Committee (IACUC Number: 2746). A total of 18 male Sprague-Dawley rats (6–8 weeks old) were randomly assigned to three groups: a control group, a 3-A group, and a mannose group. The rats in the control group were provided with pure water, while the rats in the 3-A group were provided with 3-aminoisobutyrate (3-A) and the rats in the mannose group were provided with D-mannose. The 3-A solution (1.25 mg/mL) was prepared by dissolving 3-aminoisobutyric acid crystals (217,794, Sigma-Aldrich) in saline and adjusting the pH to 7.0 with NaOH solution. The concentration of the 3-A solution was estimated based on an intake of 200 mg/kg and the normal water consumption of rats [51]. D-Mannose (5%, w/v) (M6020, Sigma-Aldrich) was prepared for the mannose treatment. After 14 days of free access to water, each rat was given intraperitoneal injections of glyoxylic acid monohydrate (GAM; G10601, Sigma-Aldrich) at a dose of 100 mg/kg for 8 days, thereby establishing a rat model of CaOx nephrocalcinosis [52]. Von Kossa staining was employed to facilitate the visualization of calcium oxalate deposits.

Statistical Analysis

All statistical analyses were performed in R software (version 4.2.2). R packages utilized for data collation, MR analysis, charting include "*TwoSampleMR*," "*dplyr*," "*tidyverse*," "*data.table*," "*ggpubr*," "*grid*," "*forestploter*," "*foreign*," "*ieugwasr*," "*limma*," "*ggplot2*," "*clusterProfiler*," "*GseaVis*," and "*meta*." A bilateral $p < 0.05$ was considered statistically significant.

Results

Preliminary Two-Sample MR Analysis

Based on the previous criteria mentioned, IVs for 850 blood metabolites as well as 664 urine metabolites were selected for further analysis. The number of IVs ranged from 11 to 148 for blood metabolites and from 4 to 753

Table 1. The sensitivity analysis results of blood and urinary metabolites with a significant association with stones (FDR <0.05)

Metabolites	Super pathway	*N*	Heterogeneity		Pleiotropy		Steiger test	
			Q	*p* value	intercept	*p* value	direction	*p* value
Blood metabolites								
Dimethylarginine (SDMA + ADMA)	Amino acid	24	21.8	0.470	−0.015	0.116	True	<0.001
Mannose	Carbohydrate	33	29.4	0.550	0.008	0.356	True	<0.001
3-A	Nucleotide	55	45.0	0.775	−0.001	0.755	True	<0.001
Glycochenodeoxycholate glucuronide	Lipid	61	41.6	0.958	−0.006	0.289	True	<0.001
Alpha-hydroxyisovalerate	Amino acid	33	21.4	0.902	0.005	0.544	True	<0.001
Hexanoylglutamine	Lipid	55	52.2	0.504	−0.002	0.818	True	<0.001
Deoxycholic acid glucuronide	Lipid	79	64.7	0.841	−0.001	0.858	True	<0.001
Eicosenedioate (C20:1-DC)	Lipid	32	26.8	0.633	−0.002	0.860	True	<0.001
N-Acetylglucosamine/N-acetylgalactosamine	Carbohydrate	23	21.7	0.415	−0.004	0.598	True	<0.001
1-Linoleoyl-GPG (18:2)	Lipid	40	27.4	0.899	0.012	0.061	True	<0.001
2-Naphthol sulfate	Xenobiotics	25	15.5	0.877	0.002	0.843	True	<0.001
Glycocholenate sulfate	Lipid	68	62.0	0.617	0.002	0.688	True	<0.001
Beta-hydroxyisovaleroylcarnitine	Amino acid	54	35.2	0.964	0.000	0.969	True	<0.001
Ceramide (d18:1/14:0, d16:1/16:0)[a]	Lipid	40	40.5	0.359	−0.011	0.146	True	<0.001
1-Oleoyl-GPG (18:1)	Lipid	41	28.0	0.904	0.007	0.345	True	<0.001
3-Methyl-2-oxobutyrate	Amino acid	20	8.9	0.962	−0.006	0.651	True	<0.001
Urinary metabolites								
Indolelactate	Amino acid	33	24.8	0.777	0.000	0.987	True	<0.001
3-A	Nucleotide	29	14.6	0.975	−0.010	0.163	True	<0.001

for urine metabolites, which indicated that we had included sufficient IVs to effectively ensure the stability and reliability of the results. The F-statistics of all IVs were greater than 10, which guaranteed that there was no bias caused by weak IVs. All IVs for blood and urine metabolites were shown in online supplementary Tables 3–4, respectively.

For 119 blood and 63 urine metabolites with an IVW *p* value below 0.05, the detailed outcomes of Mendelian randomization are presented in online supplementary Tables 5 and 6. The results were corrected for FDR and subsequently identified 16 significant blood metabolites and two positive urine metabolites. Risk blood metabolites included dimethylarginine (SDMA + ADMA) (OR: 1.20, 95% CI: 1.11–1.29, $p = 3.68 \times 10^{-6}$), 3-A (OR: 1.07, 95% CI: 1.04–1.09, $p = 1.01 \times 10^{-6}$), glycochenodeoxycholate glucuronide (OR: 1.07, 95% CI: 1.04–1.10, $p = 1.83 \times 10^{-7}$), alpha-hydroxyisovalerate (OR: 1.12, 95% CI:1.07–1.17, $p = 1.77 \times 10^{-7}$), deoxycholic acid glucuronide (OR: 1.06, 95% CI: 1.03–1.08, $p = 3.63 \times 10^{-6}$), eicosenedioate (OR: 1.10, 95% CI: 1.05–1.15, $p = 4.89 \times 10^{-5}$), 1-linoleoyl-GPG (OR:1.07, 95% CI: 1.04–1.11, $p = 7.17 \times 10^{-5}$), glycocholenate sulfate (OR: 1.05, 95% CI: 1.02–1.07, $p = 2.83 \times 10^{-4}$), beta-hydroxyisovaleroylcarnitine (OR: 1.08, 95% CI: 1.04–1.12, $p = 2.90 \times 10^{-4}$), ceramide (OR: 1.09, 95% CI: 1.03–1.14, $p = 1.22 \times 10^{-3}$), 1-oleoyl-GPG (OR: 1.07, 95% CI: 1.04–1.11, $p = 7.13 \times 10^{-5}$), and 3-methyl-2-oxobutyrate (OR: 1.14, 95% CI: 1.08–1.20, $p = 1.37 \times 10^{-6}$), while the protective blood metabolites included mannose (OR: 0.88, 95% CI: 0.83–0.93, $p = 4.16 \times 10^{-6}$), hexanoylglutamine (OR: 0.92, 95% CI: 0.88–0.95, $p = 1.43 \times 10^{-5}$), N-acetylglucosamine/N-acetylgalactosamine (OR: 0.89, 95% CI: 0.84–0.95, $p = 2.03 \times 10^{-4}$), and 2-naphthol sulfate (OR: 0.89, 95% CI: 0.85–0.94, $p = 1.20 \times 10^{-5}$) (Fig. 2). Both urinary metabolites were risk factors for stones, including indolelactate (OR: 1.09, 95% CI: 1.06–1.12, $p = 1.69 \times 10^{-9}$) and 3-A (OR: 1.03, 95% CI: 1.02–1.04, $p = 4.68 \times 10^{-7}$) (Fig. 3a). We found that 3-A was identified as a significant metabolite in both blood and urine, suggesting its potential crucial role in stone development (Fig. 3b). The results for significant metabolites maintained a high degree of consistency across different Mendelian randomization methods (online suppl. Tables 5–6; Fig. 1).

Sensitivity Analysis

Table 1 demonstrated the results of the Cochran Q test, the MR-Egger intercept test, and the Steiger test for significant metabolites. *p* values for the Cochran Q test

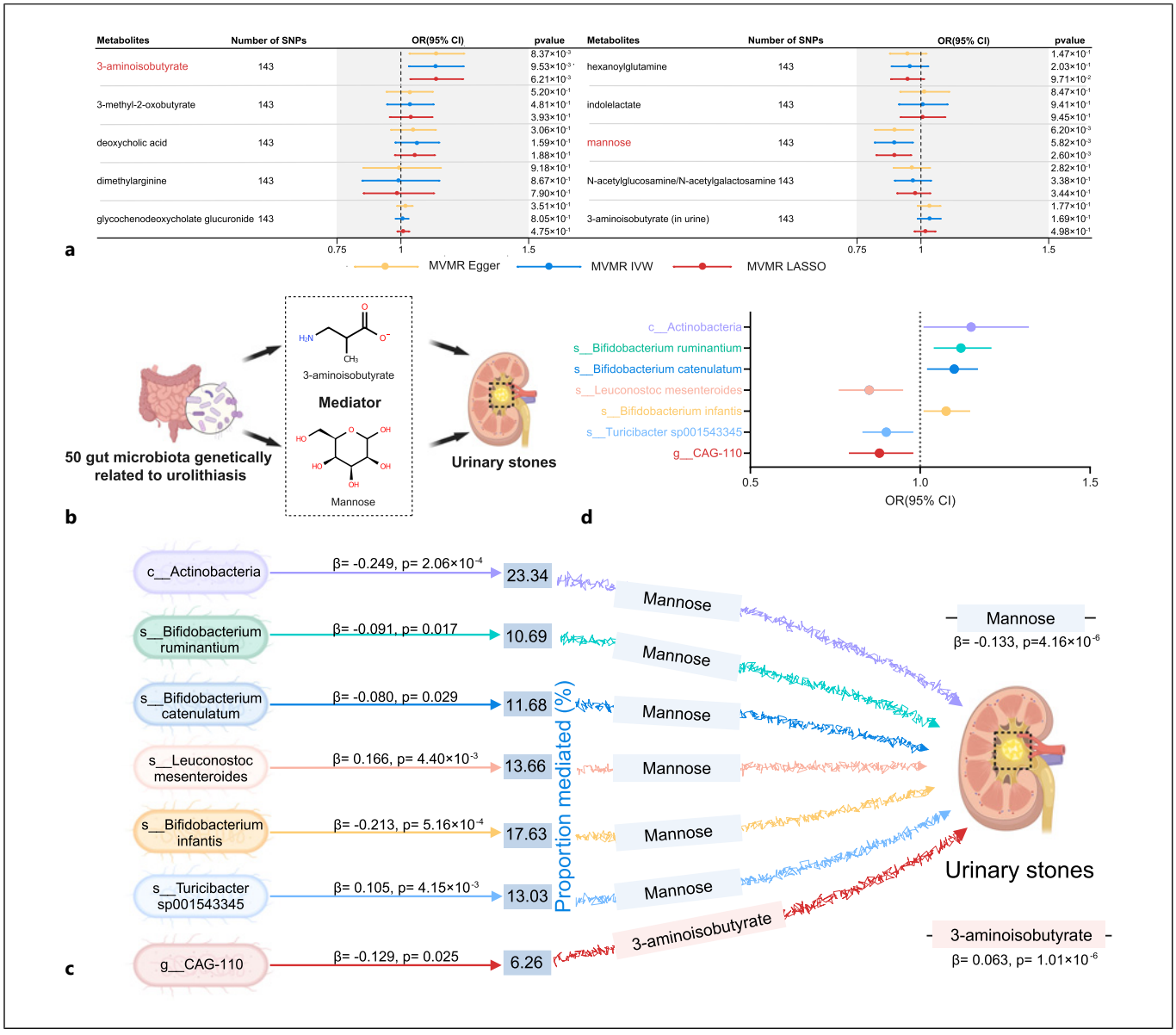

Fig. 4. MVMR analysis and mediation MR analysis of the mediating effects of metabolites between the gut microbiota and stones. **a** MVMR analysis of ten principal blood and urinary metabolites. **b** Schematic diagram of mediation MR analysis for gut microbiota and urinary stones. **c** Mannose and 3-A exert mediating effects between the relationships of seven types of gut microbiota and stones. β represents the causal association between exposure and outcome. The numbers in the blue squares represent the proportion of the mediating effect of the metabolites. **d** The direct causal relationship between the aforementioned seven types of gut microbiota and urinary stones. SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval. The chemical structure schematic of mannose and 3-A originates from https://pubchem.ncbi.nlm.nih.gov/.

and the MR-Egger intercept test were greater than 0.05, indicating that there is no heterogeneity and horizontal pleiotropy in the interference. The *p* values of the Steiger test were all less than 0.05, suggesting that the direction of the causal effect from exposure to outcome was unidirectional and the bias from the reverse causal effect was excluded. The results of the above analyses for the other positive metabolites that did not meet the FDR adjustment criteria were presented in online supplementary Tables 5–6.

The leave-one-out test tested the effect on the results after removing each IV, and we found that the identified positive metabolites all demonstrated a high degree of stability, suggesting that our conclusions could not be ascribed to effect of a few SNPs (online suppl. Fig. 2). The funnel plot test mainly observed publication bias, and most of our funnel plots showed high symmetry, with a few results showing slightly less symmetry due to the limited number of SNPs; thus, our conclusions were less affected by publication bias (online suppl. Fig. 3).

External Validation and Meta-Analysis

We replaced the outcome dataset with GWAS summary data from UKB and reconducted MR analysis for the previously obtained positive metabolites. Furthermore, we performed meta-analysis of the results from both FinnGen and UKB, which identified more consistent positive metabolites and provided more accurate estimates of the effect of each metabolite on urinary stones. Two urinary metabolites, indolelactate and 3-A, remained significant risk factors for stones after meta-analysis (Fig. 3c). In the meta-analysis of blood metabolites, dimethylarginine, 3-A, glycochenodeoxycholate glucuronide, deoxycholic acid glucuronide, eicosenedioate, 1-linoleoyl-GPG, glycocholenate sulfate, 1-oleoyl-GPG, and 3-methyl-2-oxobutyrate were proven to be risk factors, whereas mannose, hexanoylglutamine, and N-acetylglucosamine/N-acetylgalactosamine were identified as protective factors (Fig. 3d, e).

MVMR and Gut Microbiota Mediation MR Analysis

We included 10 metabolites in the MVMR analysis to further screen for metabolites that had the most significant effects when multiple metabolites were interacting simultaneously. The results of MVMR were shown in Figure 4a. We found that only 3-A and mannose presented as significant risk and protective factors for stones in MVMR, respectively. This finding was highly consistent and significant within three different MR methods, further suggesting a robust and steady causal relationship between these two metabolites and urinary stones.

Details of the 473 types of gut microbiota were included in online supplementary Table 7. The causal relationship between 473 gut microbiota and urinary stones was explored using the univariable Mendelian randomization method previously described. The results indicated that there is a causal link between 50 gut microbiota and urinary stones (online suppl. Table 8). To demonstrate that the metabolites indeed play a mediating role in the link between gut microbiota and urinary stones, and to determine the proportion of this mediating effect, we used a two-step mediation MR analysis for 3-A and mannose (Fig. 4b). We discovered that 3-A mediated the protective effect of g__CAG-110 against stones (proportion mediated = 6.26%, $\beta = -0.008$, $p = 0.041$) (Fig. 4c). Additionally, mannose also mediated the risk effect of c__Actinobacteria (proportion mediated = 23.34%, $\beta = 0.033$, $p = 3.86 \times 10^{-3}$), s__*Bifidobacterium ruminantium* (proportion mediated = 10.69%,$\beta = 0.012$, $p = 0.034$), s__*Bifidobacterium catenulatum* (proportion mediated = 11.68%, $\beta = 0.011$, $p = 0.048$), and s__*Bifidobacterium infantis* (proportion mediated = 17.63%, $\beta = 0.028$, $p = 5.57 \times 10^{-3}$) against stones, and the protective effect of s__*Leuconostoc mesenteroides* (proportion mediated = 13.66%, $\beta = -0.022$, $p = 0.015$) and s__Turicibacter sp001543345 (proportion mediated = 13.03%, $\beta = -0.014$, $p = 0.015$) against urinary stones (Fig. 4d–i).

Results of eQTLs Mendelian Randomization and Transcriptome Analysis

We included 5 mannose-related genes (IFT172, C2orf16, KRTCAP3, NRBP1, and KHK) and 2 3-A-related genes (KDM5A and SLC6A13). Mediation MR analysis was conducted while utilizing these genes as exposure factor, corresponding metabolites as mediator, and urinary stones as outcome. The results of the mediation analysis, expressed as odds ratios, were presented in Figure 5a. Among these results, we discovered that KRTCAP3, NRBP1, and SLC17A3 demonstrated significant effects on stones through mannose (Fig. 5b).

In GSE73680, we evaluated the expression level of these three genes. Our findings indicated that KRTCAP3 expression levels were downregulated in patients with calcium oxalate stones compared with controls, while NRBP1 and SLC17A3 did not show significant differences (Fig. 5c, online suppl. Fig. 4a, b). This was consistent with our previous observation of a protective mediating effect of KRTCAP3. Based on the median expression level, the patients with calcium oxalate stones in GSE73680 were divided into two groups for DEG analysis (Fig. 5d). The volcano plot of DEGs and their GO and KEGG enrichment results are displayed in online supplementary Figure 4c–e. Gene set enrichment analysis demonstrated an upregulation trend on Epithelium Development and Cellular Metabolic Process pathway (Fig. 5e). Figure 5f presents the Metascape enrichment network showing the intracluster and intercluster similarities of different enrichment terms, including GO terms, KEGG pathways, Reactome, and other relevant data.

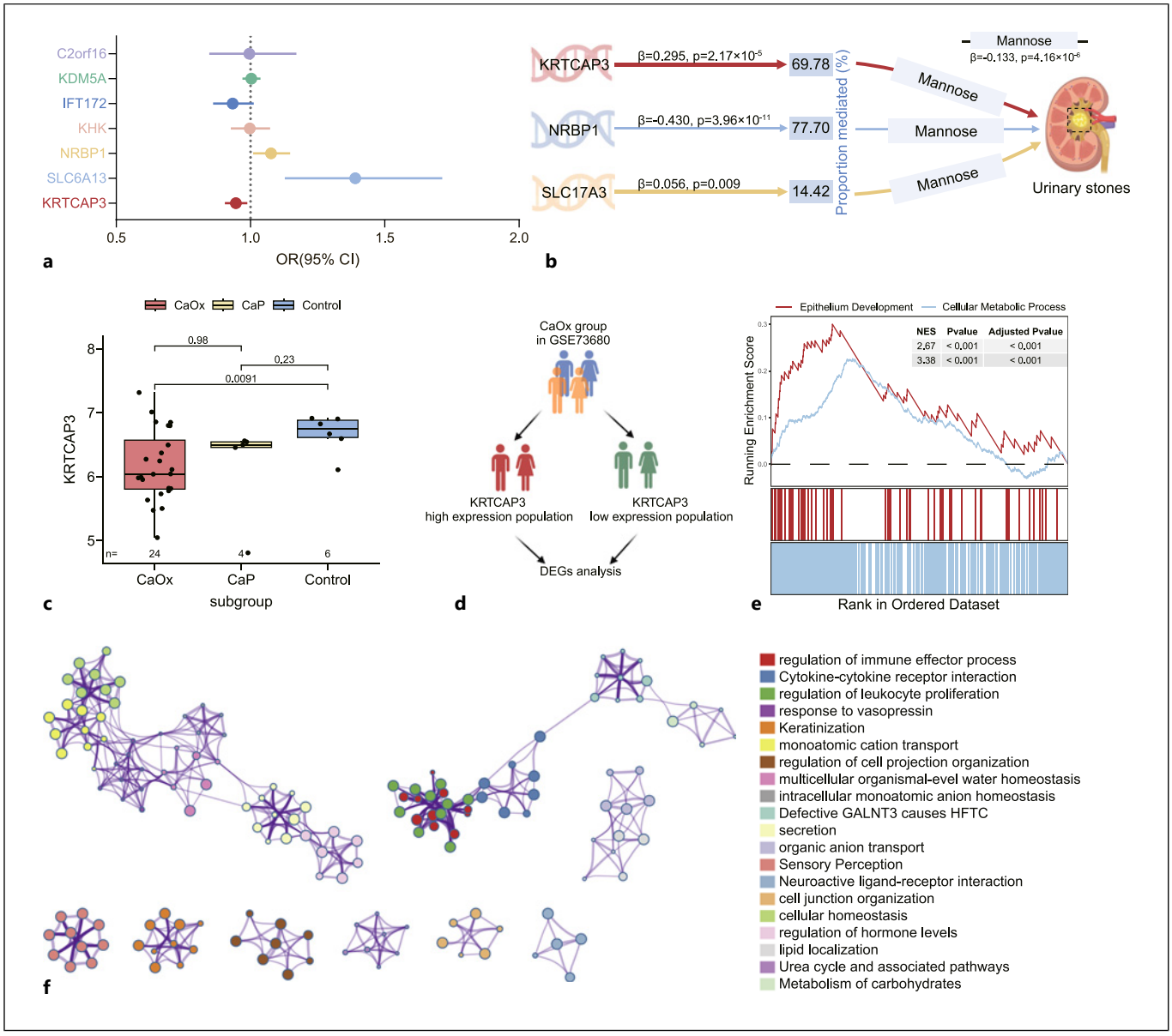

Fig. 5. Blood eQTL MR analysis, mediating MR analysis, and transcriptome analysis of metabolite-associated genes. **a** The MR analysis results between the blood eQTLs for genes associated with mannose and 3-A and the development of urinary stones. **b** The mediating effects of mannose between the significantly related gene eQTLs and stones. **c** The expression of the KARCAP3 in renal papillary tissues of different stone groups from GSE73680. **d** Schematic diagram of the DEG analysis categorized based on the expression status of the KARCAP3 gene. **e** Some significant GSEA enrichment analysis results of the DEGs. **f** Metascape enrichment network visualization showing the intracluster and intercluster similarities of enriched GO terms, KEGG pathways, Reactome, and other relevant data. The color code is used to indicate the cluster annotations. OR, odds ratio; CI, confidence interval.

Validation of Clinical Urine Specimens and Animal Experiments

The collection and analysis workflow of renal pelvic urine samples is shown in Figure 6a. The metabolomics data reveal that the mannose level in the urine of the stone side in patients with unilateral kidney stones was significantly lower than that of the normal side ($p = 0.0078$), while the level of 3-A showed no significant difference compared to the normal side (Fig. 6b). The animal experiments showed the same results. The design and grouping of the animal experiment are illustrated in Figure 7a. Rats in three groups with different drinking

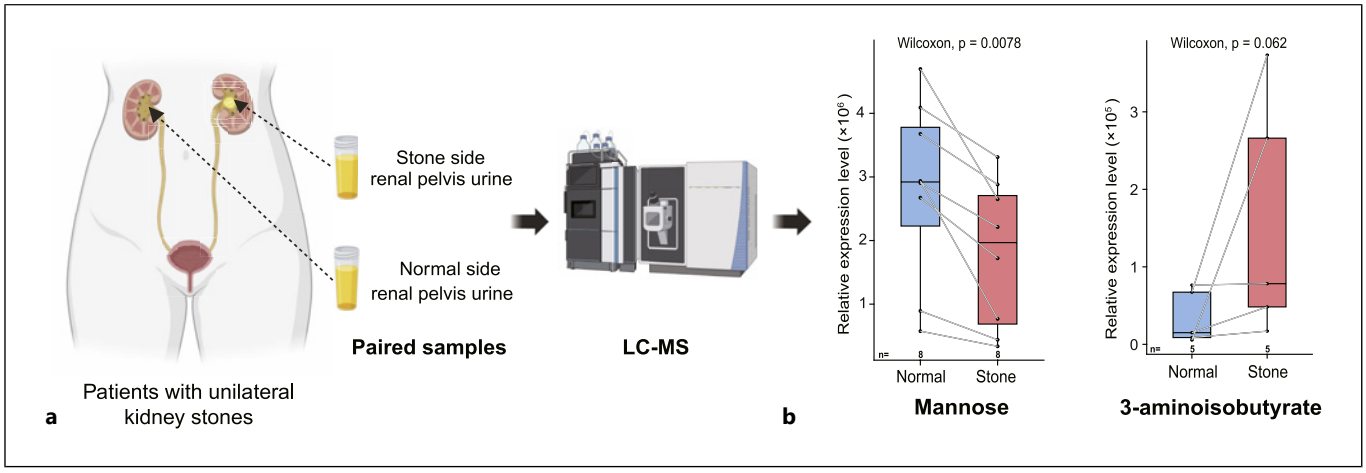

Fig. 6. Mannose levels are higher in the normal side than in the stone side of patients with unilateral kidney stones. **a** Illustration of the process for urine sample collection and analysis. **b** Comparative levels of mannose and 3-A metabolites between the stone and normal sides. LC-MS, liquid chromatography-tandem mass spectrometry.

water had a slow increase in body weight during the first 14 days, which then turned into a slow decline after GAM treatment (Fig. 7b). We obtained the maximal longitudinal section of the kidney for Von Kossa staining, observing a notable reduction in calcium salt deposition within the kidneys of rats from the 5% mannose consumption group. Group 3-A exhibited a large number of kidney crystal formations, but no significant statistical difference was found compared to the GAM group (Fig. 7c, d).

Discussion

In this comprehensive study, we integrated two large-scale metabolite GWAS datasets and employed a rigorous MR design to investigate the causal effects of 1,091 blood metabolites and 1,172 urinary metabolites on the development of urinary stones. The causal relationships between 119 blood metabolites and stones, and between 63 urinary metabolites and stones, were initially identified through the use of univariable Mendelian randomization. Among the identified metabolites, 16 blood metabolites and 2 urinary metabolites exhibited statistical significance following multiple corrections and were deemed to have robust associations, as confirmed by sensitivity analyses. The results indicate that genetically determined high levels of mannose, hexanoylglutamine, N-acetylglucosamine/N-acetylgalactosamine, and 2-naphthol sulfate are associated with a lower risk of stones, while a genetic predisposition to high levels of dimethylarginine (SDMA + ADMA), 3-A, glycochenodeoxycholate glucuronide, alpha-hydroxyisovalerate, deoxycholic acid glucuronide, eicosenedioate, 1-linoleoyl-GPG, glycocholenate sulfate, beta-hydroxyisovaleroylcarnitine, ceramide, 1-oleoyl-GPG, and 3-methyl-2-oxobutyrate increases the risk of stones. MVMR estimates indicate that mannose and 3-A can directly affect urinary stones independently of other metabolites. Subsequently, we utilized mediation MR analysis to explore the mediating effects of gut microbiota and metabolite-related genes on the causal relationship between these two metabolites and stones. To our knowledge, this is the first study to use large-scale blood and urine metabolite GWAS data to comprehensively explore the causal relationships between metabolites and urinary stones. It also attempts to investigate the specific mechanisms using gut microbiota and blood gene eQTLs and validates key conclusions through animal experiments and clinical specimens. Our study provides new insights into the metabolic research of kidney stones, revealing the role of gene-metabolite interactions in the pathogenesis of stones, and identifying potential therapeutic targets for the treatment of urinary stones.

Mannose, a six-carbon monosaccharide widely present in nature and human metabolism, has been found in our study to reduce the risk of kidney stones. In recent years, mannose has been found to play a role in the prevention and treatment of UTIs, and many studies have found that mannose plays an important role in tumor treatment and intestinal homeostasis [53–55]. Xiao et al. [54] discovered that mannose can stabilize intestinal homeostasis by preserving the integrity of the intestinal epithelium and

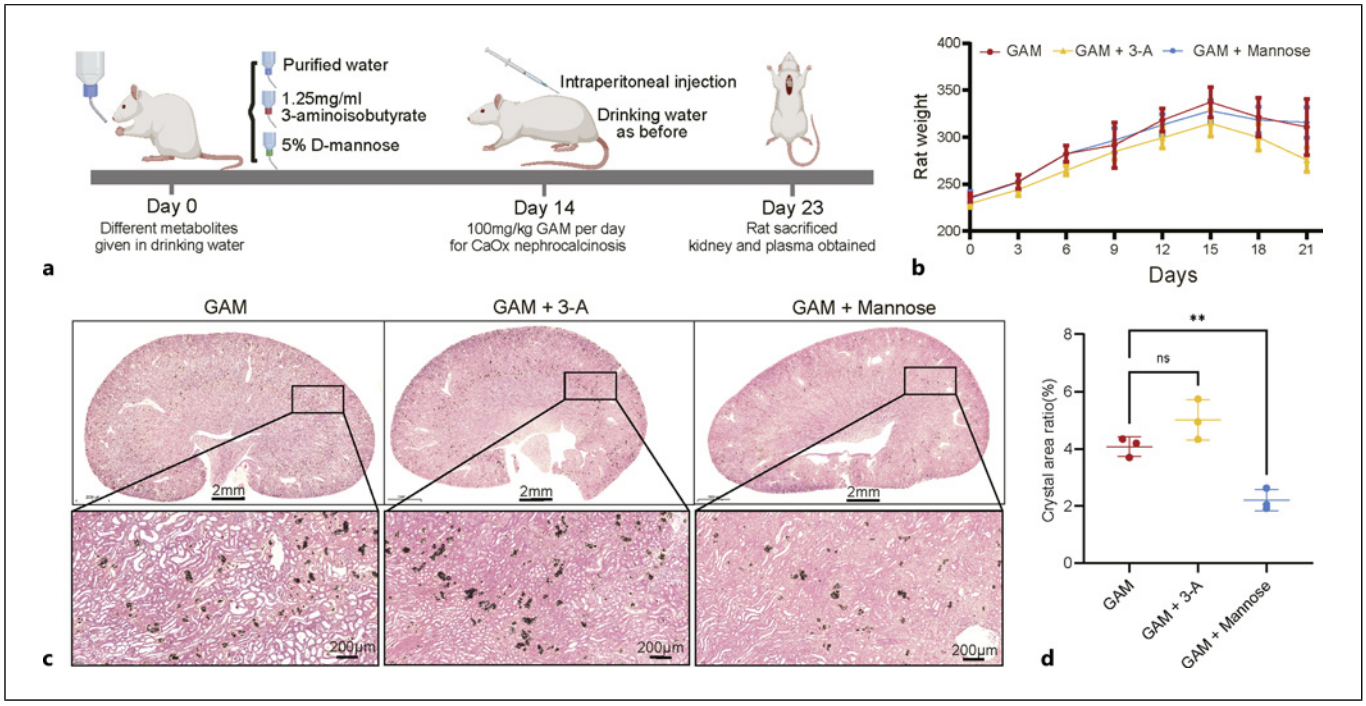

Fig. 7. The addition of D-mannose to drinking water can reduce renal crystal deposition in rats. **a** The overflow of animal grouping and experimental procedure. **b** Weight changes of three groups of rats from day 0 to 21. **c** Panoramic and locally magnified images of crystal deposition in rat kidney tissue under different treatments observed by Von Kossa staining. **d** Quantitative analysis of kidney crystals using the ratio of crystal area. 3-A, 3-aminoisobutyrate.

inhibiting the production of TNF-α by macrophages. Another study showed that D-mannose can inhibit macrophage IL-1β production, suggesting that mannose may affect the state or polarization of macrophages [56]. Recent studies on nanomaterials have shown that utilizing mannose and mannose receptors can promote the polarization of macrophages from M1 to M2 [57, 58]. Therefore, we speculate that mannose may affect kidney stones by altering the polarization state of macrophages, which has been shown to play a key role in the pathogenesis of kidney stones [59]. Our study revealed that six distinct types of gut microbiota exert an influence on the formation of urinary stones through the intermediary of mannose levels in the body. These include members of the *Bifidobacterium*, *Leuconostoc*, and *Actinobacteria* genera. Specifically, S_*L. mesenteroides* has been demonstrated to diminish the likelihood of developing urinary stones and prior research has indicated that it can reduce blood glucose levels and alleviate abdominal fat accumulation, suggesting potential clinical applications [60, 61]. The expression levels of certain genes in the blood, particularly the KRTCAP3 gene, may also contribute to the alleviation of stones through mannose. Gene enrichment studies have indicated that the KRTCAP3 gene may influence immune processes and epithelial development in stone patients. To sum up, mannose seems to be a metabolite with potential for both the prevention and treatment of urinary stone.

3-A, the conjugate base of 3-aminobutyric acid, is associated with a high risk of urinary tract stones in MR studies. Its significance is evident in both blood and urine metabolites. However, the differences in 3-A between animal experiments and urine samples are not statistically significant; thus, we regard the results of 3-A with caution. Nevertheless, this does not imply that 3-A has lost its significance in stone research. Rather, it suggests that further studies may reveal its role in human metabolic diseases.

Dimethylarginine includes symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA), both of which have been shown to be associated with a high risk of stones. ADMA can affect the progression of cardiovascular diseases by inhibiting the synthesis of nitric oxide, while SDMA can influence the glomerular filtration rate and is primarily involved in some pathological physiological processes of the kidney

[62]. The production of alpha-hydroxyisovalerate from 3-methyl-2-oxobutyrate via LDH catalysis has been demonstrated in the previous study, and both metabolites have been identified as causal factors in the development of stones [63]. Moreover, research has demonstrated a correlation between alpha-hydroxyisovalerate and body mass index, which is a significant contributing factor in stone formation [20]. The MR study also identified three additional blood metabolites (hexanoylglutamine, N-acetylglucosamine/N-acetylgalactosamine, and 2-naphthol sulfate) that have a protective effect against stone formation. It is probable that N-acetylglucosamine plays a role in the posttranslational modification of O-linked N-acetylglucosamine, and that this process is associated with the progression of chronic diseases [64]. 2-Naphthol sulfate has been demonstrated to have a negative correlation with the consumption of red meat over the long term [65]. Unfortunately, there has been scanty literature on the effects of hexanoylglutamine in the past, and the precise mechanisms of these protective metabolites deserve more research.

The extensive prior research establishing a correlation between specific metabolites and the formation of urinary stones has been further substantiated by the findings of this study. The majority of the results did not meet the FDR correction criteria, suggesting that the positive outcomes that did not pass the correction also have notable implications for further research. Our study found that urinary hippuric acid and blood trigonelline levels are associated with a lower risk of stones. Hippuric acid, a biomarker of polyphenol-rich foods in urine, has been found to be significantly reduced in the urine of stone patients in previous studies [11, 13]. Trigonelline, a metabolite derived from coffee consumption, has been demonstrated in studies, along with coffee itself, to mitigate the risk of forming kidney stones [66, 67]. Additionally, the present study has revealed that 5 of the 20 amino acids present in the body are causally linked to stone formation. Arginine, histidine, and glycine act as protective factors, whereas threonine and lysine are considered risk factors. Similarly, prior research has demonstrated that glycine can reduce the formation of calcium oxalate crystals in the kidneys [68]. The online supplementary Tables 5 and 6 still contain a considerable number of uninvestigated blood and urinary metabolites related to stones. However, the current research lacks reports on these metabolites, which indicates a need for further investigation. Zhu et al. [69] investigated the genetic causal relationship between blood metabolites and stone disease, but their outcome data from the IEU was multiethnic, resulting in unreliable positive findings. One of their reported findings, 4-hydroxychlorothalonil, appears in our online supplementary Table 5 with an OR of 1.07 (1.02–1.12), but it was omitted from the main text as its FDR did not meet our threshold [70].

The study has several advantages. First, it is the most thorough and systematic investigation to date of the causal relationships between blood and urinary metabolites and urinary stones, as we analyzed a larger amount of precise metabolite data identified by UPLC-MS/MS in this study. Second, rigorous MR analysis coupled with meaningful sensitivity analysis was used to circumvent the common problems of reverse causality and confounding bias in observational research. Furthermore, the robustness of the findings was strengthened by applying FDR correction. Third, we corroborated our findings with additional stone GWAS data, obtained results from meta-analysis, and then performed multivariate MR analysis to identify independent effectors. Forth, we sought to investigate the mechanisms of how key metabolites influence stone formation by utilizing data from the gut microbiome and blood gene eQTLs. Finally, we confirmed our findings through animal experiments and sample analysis, a feature that is lacking in most MR studies.

We must acknowledge some limitations. First, due to the limitations of data sources and racial differences, the GWAS data in this study come from individuals of European descent, so our results are worth further investigation in other ethnic groups. Furthermore, to obtain more meaningful results to reveal possible mechanisms, the results of the 50 positive gut microbiotas were not subjected to FDR correction. In addition, due to the lack of relevant research, 3-A was prepared using NaOH titration, and the dosage administered to the rats may not have been accurate, which may also be one of the reasons for the lack of statistical results. Lastly, we only explored the effects of the two main metabolites using animal experiments, and there are still meaningful results that require future RCTs and basic research for validation.

Conclusion

This study provides evidence for the causal relationship between multiple blood and urine metabolites and urinary stones and indicates that mannose and 3-A may act as independent factors affecting the occurrence of stones. By integrating gut microbiota, blood gene eQTL data, and transcriptomics, the study explored the mechanisms affecting metabolite levels. Animal experiments found that mannose can alleviate calcium oxalate

crystallization in rat kidneys, which represents a potential therapeutic direction for stones. This MR study, which integrates genetics and metabolomics and is validated by basic experiments, provides a reference direction for exploring the etiology and mechanisms of urinary stones.

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We sincerely thank the authors who shared the original dataset of this study. We also thank the patients who participated in our study. Venn diagram was plotted by https://www.bioinformatics.com.cn, an online platform for data analysis and visualization. The flowchart presented in our figures was created using the online platform BioRender (https://biorender.com/).

Statement of Ethics

The research proposal of this study was approved by the Ethics Review Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (TJ-IRB20231210). Written informed consent was obtained from all participants. The animal experiment in this study was approved by the Animal Care and Use Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (IACUC No. 2746).

Conflict of Interest Statement

The authors declare no competing interests.

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Author Contributions

L.M., J.X., and Q.X. contributed in the conception and design of the study. L.M. and J.X. conceived and drafted the manuscript. L.M., S.H., and Y.G. collected patient samples and clinical information. L.M. and Y.Y. were in charge of conducting the animal experiments, while J.S. and S.Z. participated in data processing, analysis, and interpretation. Q.X. and S.W. supervised the project and revised the manuscript. All authors have read and approved the final manuscript.

Data Availability Statement

The original source of the datasets analyzed in the study is included in the article and its online supplementary materials. Further inquiries can be directed to the corresponding authors.

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