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Article

Novel Metabolites Associated with Decreased GFR: A 12-Year Follow-Up of the METSIM Cohort

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Abstract: Identification of the individuals having impaired kidney function is essential in preventing the complications of this disease. We measured 1 009 metabolites at the baseline study in 10 159 Finnish men of the METSIM cohort and associated the metabolites with estimated glomerular filtration rate (eGFR). A total of 7 090 men participated in the 12-year follow-up study. Non-targeted metabolomics profiling was performed at Metabolon, Inc. (Morrisville, NC, USA) on EDTA plasma samples obtained after overnight fasting. We applied Liquid chromatography-mass spectrometry (LC-MS/MS) to identify the metabolites (the Metabolon DiscoveryHD4 platform). We performed association analyses between the eGFR and metabolites using linear regression adjusted for confounding factors. We found 108 metabolites significantly associated with a decrease in eGFR, and 28 of them were novel including 12 amino acids, 8 xenobiotics, 5 lipids, 1 nucleotide, 1 peptide, and 1 partially characterized molecule. The most significant associations were with five amino acids, N-acetylmethionine, N-acetylvaline, gamma-carboxyglutamate, 3-methylglutaryl-carnitine, and pro-line. We identified 28 novel metabolites associated with decreased eGFR in the 12-year follow-up study of the METSIM cohort.

1. Introduction

Chronic kidney disease (CKD) affects approximately 10% of the Western countries' population [1]. Glomerular filtration rate (GFR) is accepted as the best marker of impaired kidney function, calculated as an estimated GFR (eGFR) [2]. Diabetes is a major risk factor for impaired kidney function [3] but also age, sex, hypertension, obesity, increased total triglycerides, and smoking are risk factors for CKD [4]. During the last few years, genome-wide association studies have identified hundreds of genetic variants for kidney diseases [5–7]. Interestingly, a recent study identified genetic variants in the individuals with and without diabetes and reported that a majority of eGFR loci were similar in individuals with and without diabetes [8].

The first studies aiming to identify metabolites associated with eGFR had a small size and included only a low number of metabolites [9–13]. Grams et al [14] included 587 participants in their study, and identified five metabolites (16-hydroxypalmitate, kynurenate, homovanillate sulphate, N2,N2-dimethylguanosine, hippurate) associated with CKD. Lin et al. performed a large metabolome-wide association study, including 640 metabolites in 3 906 participants of the Hispanic Community Health Study/Study of Latinos. They identified 404 eGFR-metabolite associations and found 79 novel associations [15], where amino acids and xenobiotics were the most frequent metabolites associated with eGFR. Recent two studies have reported substantially increased number of metabolites associated with CKD [16,17].

Early identification of the individuals having impaired kidney function is essential in the prevention of CKD and its complications. However, previous studies aiming to identify metabolites associated with a decrease in eGFR have been mainly cross-sectional and included relatively small number of participants and metabolites. Our population-based study included 10 159 participants having 1 009 metabolites measured at baseline. A total of 7 090 participated in a 12-year follow-up. Our study is the largest study identifying novel metabolites associated with a decline in eGFR during

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a long follow-up. Therefore, our study has a good statistical power to reveal new metabolic pathways for impaired kidney function.

2. Results

2.1. Baseline Characteristics

We included in our study 10 159 METSIM participants. Table 1 shows baseline characteristics of the participants according to their glucose tolerance. These groups differed significantly in age, systolic blood pressure, BMI, total triglycerides, fasting glucose, HbA1c, fasting plasma insulin, eGFR, urine albumin, and high-sensitivity C-reactive protein (hs-CPR). The difference between the three groups was statistically significant but small in eGFR (87.9 in the NGT group, 88.6 and 86.1 in the T2D group).

Table 1. Baseline characteristics of the participants according to glucose tolerance.

Measurements	NGT (n=3034)	Prediabetes (n=5715)	T2D (n=1410)	*p*
Age (years)	56.8 ± 6.9	57.4 ± 7.2	60.6 ± 6.7	1.1E-63
Systolic blood pressure (mmHg)	134.3 ± 15.9	138.7 ± 16.2	145.2 ± 18.1	2.1E-93
Body mass index (kg/m^2)	25.8 ± 3.38	27.4 ± 3.9	30.2 ± 5.2	1.1E-247
Current smoking (%)	18.0	18.4	17.2	0.606
Total triglycerides (mmol/l)	1.22 ± 0.65	1.49 ± 1.08	1.90 ± 1.21	1.2E-143
Fasting glucose (mmol/l)	5.24 ± 0.24	5.97 ± 0.37	7.51 ± 2.01	< 1E-250
HbA1C (%)	5.59 ± 0.31	5.71 ± 0.34	6.58 ± 1.13	< 1E-250
Fasting plasma insulin (mU/l)	6.25 ± 4.11	9.32 ± 6.4	19.6 ± 28.5	< 1E-250
Creatinine (umol/l)	84.6 ± 15.9	83.4 ± 12.8	84. 6 ± 22.3	0.0003
eGFR (ml/min/1.73 m2)	87.9 ± 12.3	88.6 ± 12.2	86.1 ± 14.5	4.5E-10
Urine albumin (mg/l)	18.4 ± 110.9	20.6 ± 82.5	93.5 ± 380.1	7.2E-181
hs-CRP (mg/l)	1.82 ± 2.96	2.13 ± 4.5	3.22 ± 6.07	3.4E-40

Abbreviations: NGT, normal glucose tolerance; T2D, type 2 diabetes; HbA1C, hemoglobin A1C; eGFR, estimated glomerular filtration rate; hs-CRP, high sensitivity C-reactive protein

2.2. Metabolites in Participants with Decreased and Normal eGFR

We compared metabolite concentrations between the participants having eGFR < 80 and eGFR ≥ 80 ml/min/1.73 m2, and found statistically significant (p<5.0E-05) differences in 586 metabolites (Table S1). The most significant differences (p <1.1E-350) between the two groups were in 1-methylhistidine, 1-methyl-4-imidazoleacetate, 2,3-dihydroxy-5-methylthio-4-pentenoate (DMTPA), creatinine, hydroxy asparagine, N,N,N-trimethyl-alanylproline betaine, N-acetylalanine, and pseudouridine.

2.3. Effects of Glucose Tolerance on Metabolic Profile

We analysed the associations of eGFR with metabolites in different subgroups of glucose tolerance (n=1 057 in each group matched for age and BMI). Participants with NGT had 379 statistically significant associations with the metabolites, participants with prediabetes had 474 significant associations, and participants with T2D had 378 significant associations. Table S1 presents the 100 most significant metabolites in the participants with T2D, prediabetes and NGT.

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Independently of the glucose tolerance, all metabolites were associated with a decrease in eGFR. Venn diagram (Figure 1) shows that the participants in the different glucose tolerance groups shared 78% of the 100 most significant metabolite associations, 11 of the metabolites were found only in the NGT group, 7 in the prediabetes group, and 12 in the T2D group.

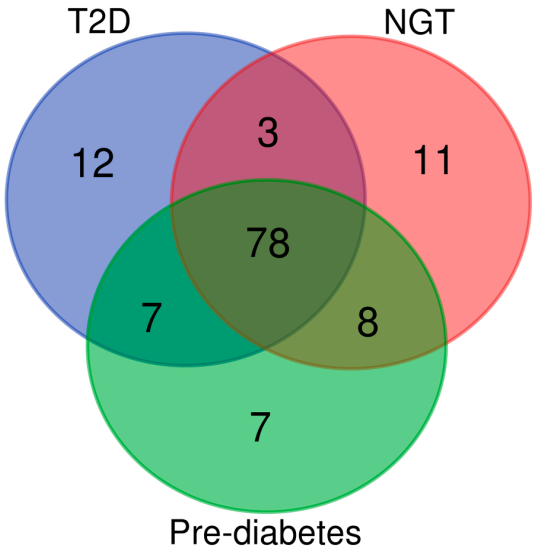

Figure 1. Venn diagram showing the number of the 100 most significant metabolites associated with eGFR, shared between the groups of normal glucose tolerance, prediabetes, and type 2 diabetes. .

2.4. Metabolites Associated with a Decrease in eGFR

We performed linear regression to associate 1 009 metabolites with eGFR at baseline without adjustment for confounding factors, adjusted for baseline eGFR (Model 1), and adjusted for baseline eGFR, age, BMI, smoking, fasting glucose, total triglycerides, and systolic blood pressure (Model 2) (Table S2). All metabolites listed in Table S2 had $p < 5x10^{-5}$ in all models. Adjustment for the baseline eGFR (Model 1) decreased substantially beta and p values. In Model 2 beta and p value further deceased but the decreases were relatively small.

We found 108 metabolites significantly associated with a decrease in eGFR, and 28 of them were novel (Table 2). The 10 most statistically significant metabolites associated with decreased eGFR were six amino acids, creatinine, hydroxyasparagine, N,N,N-trimethyl-alanylproline betaine, N-acetylalanine, N-acetylserine, C-glycosyltryptophan, and N-formylmethionine; a nucleotide pseudouridine; xenobiotics erythritol; and carbohydrate erythronate.

Among the novel 28 metabolites decreasing eGFR, 12 were amino acids, 5 lipids, 1 nucleotide, 1 peptide, 8 xenobiotics, and 1 partially characterized molecule. Among the amino acids the three most significant associations with a decrease in metabolites were for N-acetylmethionine (beta -0.087, p= 5.5E-24), N-acetylvaline (beta -0.082, p = 2.6E-21), and γ-carboxyglutamate (beta -0.065, p= 2.6E-14). Among the lipids the two most significant associations with a decrease in metabolites were for 11beta-hydroxyetiocholanolone (beta -0.050, p = 4.0E-7), and 2-methylmalonylcarnitive C4-DC (beta -0.042, p = 3.1.0E-6), and among the xenobiotics for 2,3-dihydroxyisovalerate (beta -0.048, p = 6.8E-06, and (S)-a-amino-omega-caprolactam (beta -0.050, p = 1.0E-8).

Table 2. Novel metabolites associated with a decrease in eGFR.

Metabolite	Sub-class	N	Beta	*p* *	Beta	*p* **
Amino acids						
N-acetylmethionine	Methionine, cysteine, taurine metab.	7080	-0.334	**1.4E-183**	-0.087	**5.5E-24**

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N-acetylvaline	Leucine, isoleucine, valine metab.	7082	-0.343	**1.0E-194**	-0.082	**2.6E-21**
γ-carboxyglutamate	Glutamate metab.	6929	-0.295	**1.1E-138**	-0.065	**2.6E-14**
3-methylglutaryl-carnitine (2)	Leucine, isoleucine, valine metab.	7001	-0.257	**1.1E-105**	-0.058	**5.8E-12**
Proline	Urea cycle; arginine proline metab.	7081	-0.107	**1.3E-19**	-0.048	**3.9E-9**
Pro-hydroxy-pro	Urea cycle; arginine proline metab.	7079	-0.155	**1.9E-39**	-0.047	**5.2E-9**
4-guanidinobutanoate	Guanidino acetamido metab.	7049	-0.158	**1.7E-40**	-0.049	**2.3E-9**
N-acetyltaurine	Methionine, cysteine, taurine metab.	7048	-0.208	**1.4E-69**	-0.041	**7.6E-7**
Hydantoin-5-propionate	Histidine metab.	6154	-0.211	**3.6E-63**	-0.043	**1.1E-6**
N-lactoyl valine	Lactoyl amino acid	6781	-0.182	**2.5E-51**	-0.043	**3.1E-6**
N-lactoylisoleucine	Lactoyl amino acid	5437	-0.189	**4.4E-45**	-0.043	**1.6E-5**
N-lactoyl phenylalanine	Lactoyl amino acid	7033	-0.233	**2.7E-87**	-0.037	**4.4E-5**
Lipids						
11beta-hydroxy etiocholanolone glucuronide	Androgenic steroids	4891	-0.204	**2.9E-47**	-0.050	**4.0E-7**
3-decenoylcarnitine	Fatty acid metab.	5395	-0.217	**2.9E-58**	-0.042	**9.2E-6**
Cis-3,4-methylene heptanoylglycine	Fatty acid metab.	6825	-0.161	**5.2E-41**	-0.038	**4.8E-6**
2-methylmalonyl carnitine (C4-DC)	Fatty acid metab.	5827	-0.235	**8.0E-74**	-0.042	**3.1E-6**
Propionylglycine	Fatty acid metab	3960	-0.119	**4.9E-14**	-0.049	**1.3E-5**
Nucleotide						
5-methyluridine(ribothymidine)	Pyrimidine metab.	7082	-0.134	**6.8E-30**	-0.038	**3.1E-6**
Peptide						
Pyroglutamylvaline	Modified peptides	6398	-0.202	**7.7E-60**	-0.051	**2.6E-9**
Xenobiotics						
2,3-dihydroxyisovalerate	Food component/plant	6998	-0.206	**3.8E-68**	-0.048	**6.8E-9**
(S)-a-amino-omega-caprolactam	Food component/plant	7007	-0.296	**1.3E-141**	-0.050	**1.0E-8**
3-methoxycatechol sulfate (2)	Benzoate metab.	5379	-0.185	**2.0E-42**	-0.044	**1.9E-6**

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3-methyl catechol sulfate (1)	Benzoate metab.	7065	-0.209	**3.0E-70**	-0.040	**2.1E-6**
3-methoxycatechol sulfate (1)	Benzoate metab.	6318	-0.174	**4.0E-44**	-0.039	**5.5E-6**
2-acetamidophenol sulfate	Food component/plant	5939	-0.153	**2.9E-32**	-0.042	**3.6E-6**
N-(2-furoyl)glycine	Food component/plant	5025	-0.235	**5.0E-64**	-0.042	**2.4E-5**
2-aminophenol sulfate	Food component/plant	7066	-0.147	**2.8E-35**	-0.036	**1.1E-5**
Other metabolite						
Glutamine_degradant	Partially characterized molecules	7060	-0.222	**7.3E-80**	-0.071	**2.2E-17**

*p**: non-adjusted; *p***: adjusted for eGFR at baseline, age, BMI, smoking, fasting glucose, total triglycerides and systolic blood pressure.

2.5. Genetic Variants Associated with Novel Metabolites

We identified nine genetic variants significantly associated with the novel metabolites (Table 3). The most significant associations were with 5-methyluridine, glycine, proline and N-acetylmethionine. Each of the nine genetic variants was associated with at least three different metabolites suggesting pleiotropy of these genes. Importantly, none of these genetic variants was significantly associated with a decrease in eGFR indicating that the effects of the metabolites on eGFR were not explained by genetic factors.

Table 3. The association of genetic variants of nine genes with novel metabolites association with a decline in eGFR the METSIM cohort.

Gene-variant	Metabolite	*p*
KLHDC7B-rs470118	5-methyluridine	9.9E-199
CPS1-rs715	Glycine	8.1E-90
AC007326.4-rs5992344	Proline	2.0E -63
DOCK3- rs138144932	N-acetylmethionine	1.3E -44
AOX1-rs7562507	Hydantoin-5-propionate	1.4E-17
COLEC10-rs13264172	Pro-hydroxy-pro	3.5E-10
MAGI1-rs264676	2.3-dihydroxy-5-methylthio-4-penenoate	2.9E-8
DCBLD2- rs192423025	Pyroglutamylvaline	3.4E-8
CNTNAP2-rs533473709	γ-carboxyglutamate	5.3 E-8

3. Discussion

We measured 1 009 metabolites with LC-MS/MS in 10188 participants of the METSIM study. Our study reports several novel findings. We found that glucose tolerance did not have a major effect on the metabolite profile at baseline. Among the top 100 metabolites associated with eGFR, 78 were identical in participants with normal glucose tolerance, pre-diabetes, and diabetes (Figure 1). Our results suggest that the metabolic pathways leading to a decrease in eGFR are largely independent of glucose tolerance. This observation agrees with a previous study reporting that the majority of the eGFR loci were similar in the individuals with and without diabetes [8].

We found several statistically significant associations of the metabolites with a decrease in eGFR in the 12-year follow-up of the METSIM cohort. Of the 108 metabolites associated with a decrease in eGFR 28 were novel (Table 2). We also replicated metabolite associations with decreased eGFR reported in previous studies [13,18–27].

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We found three novel associations of N-acetylated amino acids (N-acetylmethionine, N-acetylvaline, and N-acetyltaurine) with a decrease in eGFR. N-acetylated amino acids are uremic toxins [28]. Aminoacylase-1 (ACY1) enzyme converts acetylated amino acids into free amino acids, and therefore the individuals having impaired activity of ACY1 or a mutation in the ACYL1 gene have increased concentrations of acetylated amino acids in blood and urine [29–34].

Amino acid γ-carboxyglutamate was significantly associated with a decrease in eGFR. γ-carboxyglutamate is a calcification inhibitor [35]. Atherosclerotic and vascular calcification are closely linked to the vitamin K–dependent protein matrix γ-carboxyglutamate. Vitamin K antagonists, including warfarin, are associated with increased calcification of renal and other arteries [35,36]. Coronary artery calcification has been previously associated with a decline in eGFR [37]. Our results suggest that γ-carboxyglutamate increases arterial damage leading to a decline in eGFR.

We report three novel associations of N-lactoyl-amino acids (N-lactoylvaline, N-lactoylisoleucine, N-lactoylphenylalanine) with a decrease in eGFR. N-lactoylphenylalanine concentrations are increased in patients with phenylketonuria [38]. These patients have increased oxidative stress leading to tubulointerstitial disease, impaired kidney function, proteinuria, and arterial hypertension [39,40]. N-lactoylvaline and N-lactoylisoleucine have been found in the urine of a patient with maple syrup urine disease [41], which is associated with nephrotic syndrome [42].

We also found that the nucleoside 5-methyluridine (ribothymidine), an endogenous methylated nucleoside, decreased eGFR. This finding has been previously reported in rats with CKD [43]. Altered DNA methylation modulates the expression of pro-inflammatory and pro-fibrotic genes, stimulating renal disease progression [44]. High concentrations of homocysteine, hypoxia, and inflammation alter the epigenetic regulation of gene expression in CKD, impacting eGFR [44].

Eight of the 28 novel metabolites impairing eGFR were xenobiotics, chemical substances within an organism that are not naturally produced. Xenobiotics are food components, plant constituents, pesticides, industrial chemicals, environmental pollutants, or benzoate metabolites. An organic compound (S)-a-amino-omega-caprolactam is a uremic solute previously shown to impair kidney function [45]. 3-methyl catechol sulphate, a marker of current smoking and coffee consumption [46], decreased eGFR in our study. We also showed that genetic variants were not associated with xenobiotics suggesting that decreased eGFR is largely regulated also by lifestyle and environmental factors.

Our findings highlight multiple metabolic pathways associated with a decrease in eGFR. We identified 28 novel metabolites among amino acids, lipids, nucleotides, peptides, and xenobiotics associated with decreased eGFR. Eight xenobiotics were associated with a decrease in eGFR show that non-genetic factors, including benzoate pathway, food components, and plants play an important role in kidney dysfunction demonstrating the influence of environmental factors on eGFR. Additionally, the effects of N-lactoyl-amino acids and 5-methyluridine show a potential for epigenetic regulation of kidney function. Overall, our novel findings provide valuable insights into the complex biochemical interactions affecting kidney function and pave the way for future studies to explore metabolic pathways on kidney function in diverse populations.

The strength of our study is that the METSIM study is the largest randomly selected population-based cohort identifying metabolites associated with a decrease in eGFR applying the LC-MS/MS analysis method. Additionally, we followed our cohort for 12 years, and at baseline and at follow-up the metabolites identified were inversely associations with eGFR increasing the credibility of our findings. We applied a conservative statistical significance threshold in all analyses to obtain reliable conclusions. The limitations of our study are that our study included only middle-aged and elderly Finnish men. Therefore, the replication of our findings in non-European populations, including both men and women, is needed.

4. Materials and Methods

4.1. Study Population and Laboratory Measurements

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The METSIM (METabolic Syndrome In Men) study includes 10 197 men, aged from 45 to 73 years at baseline, and randomly selected from the population register of Kuopio, Eastern Finland. The METSIM study was approved by the Ethics Committee of the Kuopio University Hospital, Finland. All participants provided written informed consent.

The design and methods of the METSIM study have been previously described in detail [47,48]. A total of 10 159 men were included in the current study, 3034 had normal glucose tolerance (NGT, fasting glucose < 6.1 mmol/l, 2-hour glucose < 7.8 mmol/l), 5 715 prediabetes [impaired fasting glucose (6.1-6.9 mmol/l) or impaired glucose tolerance (7.8 to 11.0 mmol/l) or both], and 1 410 T2D, [fasting glucose ≥ 7.0 mmol/l, or 2-hour glucose ≥ 11.1 mmol/l or glycated hemoglobin A1c (HbA1c) ≥ 6.5 %] according to the American Diabetes Association classification [49]. BMI was calculated as weight divided by height squared. Smoking status was defined as current smoking (yes/no). All participants excluding participants with T2D at baseline underwent a 2-hour oral glucose tolerance test (75 g of glucose), and samples for plasma glucose and insulin were drawn at 0, 30, and 120 minutes. Other laboratory measurements have been previously explained [47]. eGFR was calculated using the CKD-Epi equation [50].

4.2. Metabolomics

Non-targeted metabolomics profiling was performed at Metabolon, Inc. (Morrisville, NC, USA) on EDTA plasma samples obtained after overnight fasting, as previously described in detail [48,51]. We applied Liquid chromatography-mass spectrometry (LC-MS/MS) to identify the metabolites (the Metabolon DiscoveryHD4 platform). All samples were processed together for peak quantification and data scaling. We quantified raw mass spectrometry peaks for each metabolite using the area under the curve, and evaluated overall process variability by the median relative standard deviation for endogenous metabolites present in all 20 technical replicates in each batch. We adjusted for variation caused by day-to-day instrument tuning differences and columns used for biochemical extraction by scaling the raw peak quantifications to the median for each metabolite by the Metabolon batch.

4.3. Selection of genetic variants decreasing glomerular filtration rate

We identified genetic variants associated with a decrease in eGFR from previously published studies and from the GWAS Catalog (The NHGRI-EBI Catalog of human genome-wide association studies (https://www.ebi.ac.uk/gwas/) in the individuals of European ancestry. Altogether 117 genes were found to be associated with impaired eGFR.

4.4. Statistical Analysis

We conducted statistical analyses using IBM SPSS Statistics, version 29. We log-transformed all continuous variables except for age and follow-up time to correct for their skewed distribution. We performed association analyses between the eGFR and metabolites using linear regression adjusted for confounding factors (Model 1, adjustment for eGFR at baseline, Model 2, adjustment for eGFR at baseline, age, BMI, smoking, systolic blood pressure, fasting glucose, and total triglycerides). We give the results as standardized beta coefficients and *p* values with the metabolite as a dependent variable. We used one-way ANOVA to assess the differences in clinical traits and metabolites between the two groups at baseline. We applied the Bonferroni correction to determine statistical significance for the metabolites identified ($p<5.0 \times 10^{-5}$).

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1, and Table S2.

Author Contributions: Conceptualization: LFS and ML; Methodology: AO, JV, LFS and ML; Investigation: LFS, JV, AO and ML; Visualization: LFS and AO; Funding acquisition: ML; Project administration: ML; Supervision: ML; Writing – original draft: LFS, JV, AO, and ML.

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Institutional Review Board Statement: The METSIM study was approved by the Ethics Committee of the Kuopio University Hospital, Finland. All participants provided written informed consent.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding authors, [ML] upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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