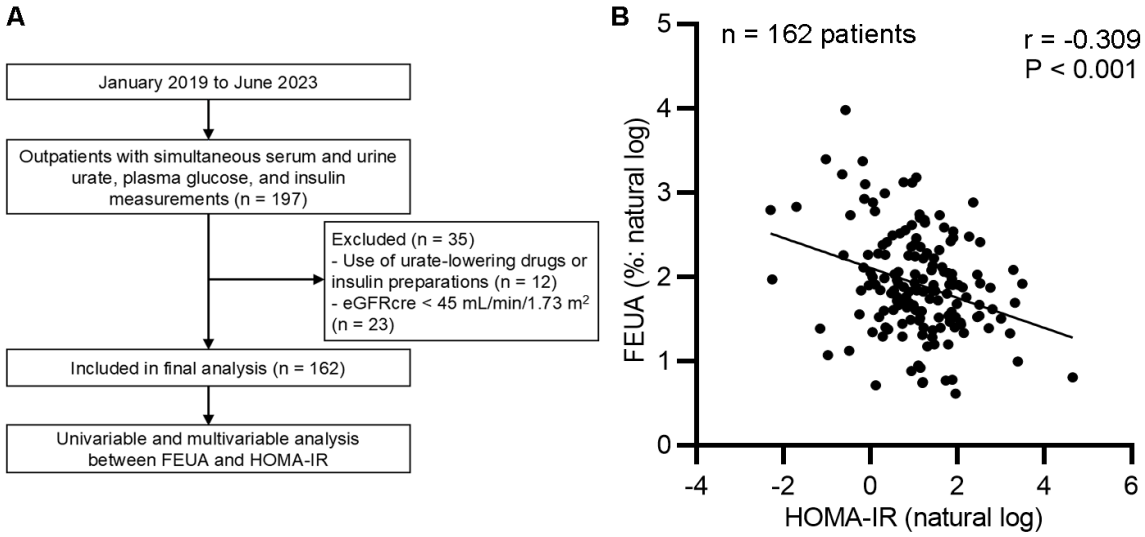
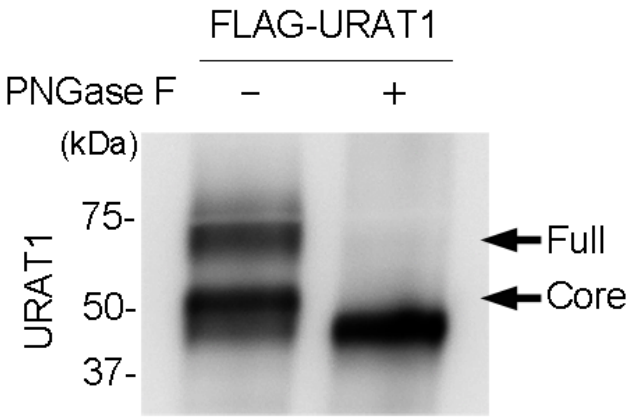
Fujii et al. Supplemental Figures



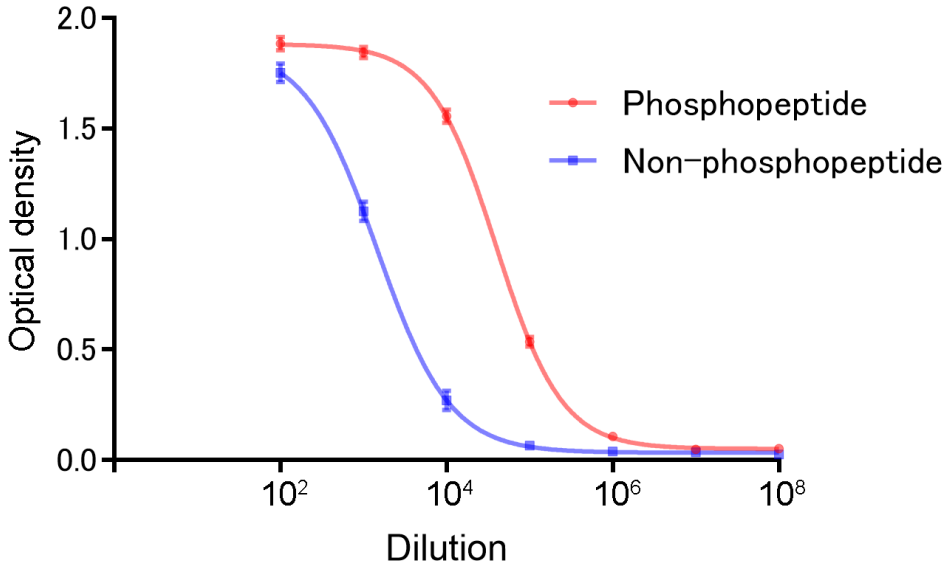
Supplemental Figure S1.

Association between hyperinsulinemia and decreased urate excretion. (**A**) Flowchart of patient selection in Teikyo University Hospital. (**B**) Correlation analysis between Homeostatic Model Assessment for Insulin Resistance (HOMA-IR, a maker of insulin resistance and hyperinsulinemia) and fractional excretion of urate (FEUA) in 162 patients.



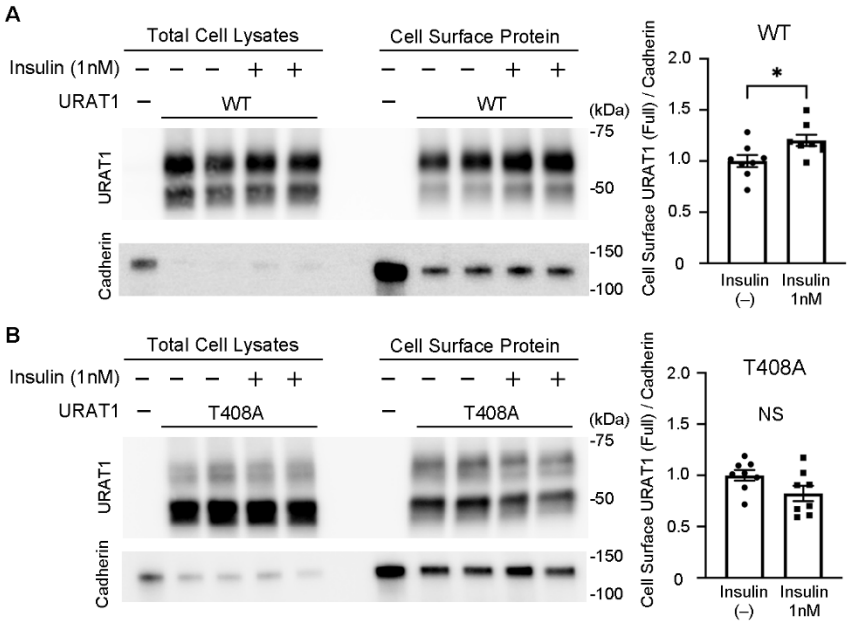
Supplemental Figure S2.

Deglycosylation assay of hURAT1. Flag-tagged, human URAT1 (hURAT1) was expressed in HEK-293 cells and purified by Flag-immunoprecipitation (IP). After incubation in the presence and absence of PNGase F, samples were subjected to Western blotting with anti-Flag antibody. The molecular shift of 65-kDa and 50-kDa bands indicates that both signals represent glycosylated form of hURAT1.



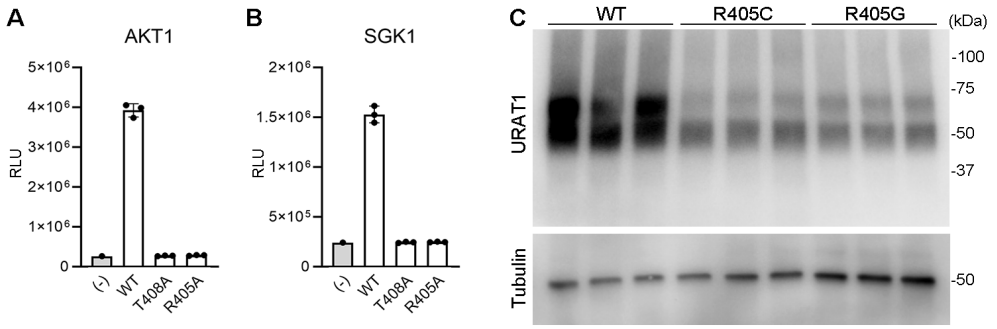
Supplemental Figure S3.

Enzyme-linked immunosorbent assay (ELISA) to determine the specificity of antibody against hURAT1 phosphorylated at T408.



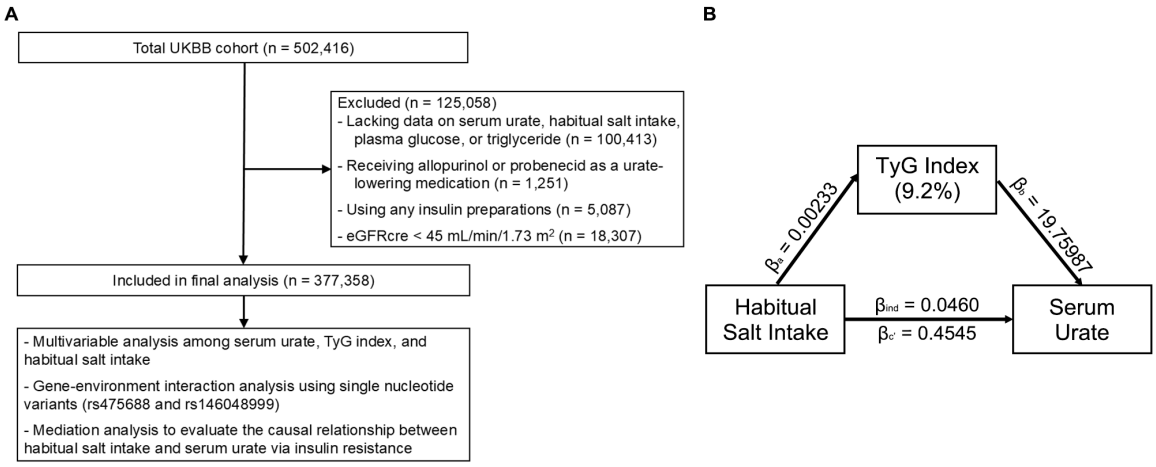
Supplemental Figure S4.

Effects of insulin at 1 nM on hURAT1 cell surface abundance in HEK cells expressing $hURAT1^{WT}$ and $hURAT1^{T408A}$. (**A** and **B**) Wild-type (**A**) and non-phosphorylatable T408A (**B**) hURAT1 were expressed in HEK cells and were incubated with insulin at 1 nM for 3 h. Cell-surface levels of hURAT1 were determined by cell-surface biotinylation assay followed by Western blotting. Bar graphs show the results of quantitation. Data are mean ± SEM (n = 8). Statistical analysis: (**A** and **B**) Unpaired t-test. *$p < 0.05$. NS, not significant.



Supplemental Figure S5.

Arg at –3 position is necessary for Thr408 phosphorylation and maturation of hURAT1. (**A**) Human URAT1 peptide containing Thr408, hURAT1-T408A peptide, and hURAT1-R405A peptide (which lacks an Arg at –3 position) were synthesized and separately incubated with AKT1 in triplicate. Phosphorylation signal was detected by ADP-glo assay. Assays with hURAT1-T408A peptide and without substrate peptide served as negative controls. Phosphorylation of hURAT1 by AKT1 is abolished by R405A substitution. (**B**) Synthesized peptides described in (**A**) were incubated with SGK1 in triplicate, and phosphorylation signal was analyzed by ADP-Glo assay in triplicate. Phosphorylation of hURAT1 by SGK1 is abolished by R405A substitution. (**C**) Comparison of hURAT1 maturation among wild-type hURAT1 and nonsynonymous single-nucleotide variants (rs563239942; hURAT1-R405C and hURAT1-R405G).



Supplemental Figure S6.

Flowchart showing the participant selection and mediation analysis among serum urate, TyG index, and habitual salt intake in the UK biobank (UKBB) study.

(**A**) A flowchart of participant selection. (**B**) Mediation analysis on the relationship among habitual salt intake, TyG index, and serum urate levels. Whether insulin resistance, as measured by the TyG index, mediates the association between habitual salt intake and serum urate levels described in Table 2 was analyzed by mediation analysis (see Methods). β_a represents the effect of habitual salt intake on the mediator (TyG index); β_b indicates the effect of the mediator on serum urate levels; $\beta_{c'}$ denotes the direct effect of habitual salt intake on serum urate levels. The indirect effect *via* the TyG index (β_{ind}) was calculated as $\beta_a \times \beta_b$, and its proportion relative to the total effect ($\beta_{c'} + \beta_a \times \beta_b$) was quantified. See also Supplemental Table S8.

Table S1. Baseline characteristics of 162 patients according to HOMA-IR.

Characteristics	Total (n = 162)	HOMA - IR < 2.5 (n = 64)	HOMA - IR ≥ 2.5 (n = 98)	*P* value
Serum Urate (μmol/L)	327.5 (107.7)	305.4 (101.3)	342.0 (109.8)	0.034
FEUA (%)	8.24 (6.38)	9.99 (8.45)	7.10 (4.24)	0.005
Age	61.9 (16.2)	63.8 (16.5)	60.7 (16.0)	0.229
Male	100 (62%)	36 (56%)	64 (65%)	0.320
Current Smoker	36 (22%)	12 (19%)	24 (25%)	0.506
Drink ≥ 3 Days/week	44 (27%)	16 (25%)	28 (29%)	0.750
Hypertension	91 (56%)	35 (55%)	56 (57%)	0.884
Diabetes Mellitus	107 (66%)	32 (50%)	75 (77%)	0.001
Hyperlipidemia	72 (44%)	25 (39%)	47 (48%)	0.341
BMI (kg/m^2)	24.5 (5.4)	22.1 (4.7)	26.0 (5.3)	< 0.001
Plasma Glucose (mmol/L)	8.81 (5.02)	6.40 (2.37)	10.38 (5.64)	< 0.001
HbA1c (mmol/mol)	64.6 (29.9)	57.2 (25.3)	69.4 (31.7)	0.011
Total Cholesterol (mmol/L)	5.11 (1.69)	4.85 (1.11)	5.26 (1.95)	0.147
eGFRcre ($mL/min/1.73m^2$)	74.5 (24.7)	76.3 (27.0)	73.4 (23.1)	0.479
Glycosuria	57 (35%)	13 (20%)	44 (45%)	0.002
Proteinuria	36 (22%)	15 (23%)	21 (21%)	0.914

FEUA, fractional excretion of urate; BMI, body mass index; HbA1c, hemoglobin A1c;

eGFRcre, estimated glomerular filtration rate calculated by creatinine;

HOMA-IR, homeostatic model assessment for insulin resistance.

Data are shown as n (%) or mean (SD).

Table S2. Multiple regression analysis of FEUA (natural log) with HOMA-IR as explanatory variable.

Variable	β	95%CI	*P* value
BMI	-0.021	-0.041 − -0.002	0.028
HOMA-IR (natural log)	-0.106	-0.203 − -0.010	0.031
Age, yrs	0.008	0.001 − 0.014	0.016
Male (vs. female)	-0.080	-0.260 to 0.100	0.382
Current Smoker	0.211	-0.003 to 0.426	0.054
Drink ≥ 3 Days/week	-0.162	-0.356 to 0.032	0.100
Hypertension	0.062	-0.149 to 0.273	0.564
Diabetes Mellitus	-0.005	-0.208 to 0.198	0.962
Hyperlipidemia	0.008	-0.192 to 0.209	0.934
eGFRcre (mL/min/1.73m^2)	0.003	-0.001 to 0.007	0.142

Table S3. Multiple regression analysis of FEUA (natural log) with HOMA-IR as explanatory variable (glycosuria and proteinuria were included as additional covariates in model described in Table S2).

Variable	β	95%CI	*P* value
BMI	-0.020	-0.040 − -0.001	0.038
HOMA-IR (natural log)	-0.110	-0.210 − -0.010	0.031
Age, yrs	0.007	0.001 − 0.014	0.035
Male (vs. female)	-0.079	-0.261 to 0.100	0.389
Current Smoker	0.224	0.007 to 0.440	0.043
Drink ≥ 3 Days/week	-0.182	-0.382 to 0.017	0.073
Hypertension	0.073	-0.143 to 0.289	0.503
Diabetes Mellitus	0.011	-0.216 to 0.238	0.925
Hyperlipidemia	-0.004	-0.207 to 0.200	0.972
eGFRcre (mL/min/1.73m^2)	0.002	-0.002 to 0.007	0.288
Glycosuria	-0.013	-0.238 to 0.212	0.911
Proteinuria	-0.121	-0.348 to 0.106	0.294

Table S4. Kinase screening assay of 53 AGC kinases.

Kinase	Signal Ratio (WT vs T408A)
AKT1	35.9740079
AKT2	3.32342976
AKT3	26.28964623
CDC42BPG	4.821177914
DMPK	1.08980649
GRK1	1.055577285
GRK2	1.033572491
GRK3	1.031960293
GRK5	1.072406516
GRK6	1.026160795
GRK7	1.034224972
LATS1	1.228488103
LATS2	1.047736067
MAST3	1.084295917
MASTL	1.064729878
MRCKalpha	5.290781272
MRCKbeta	4.305585278
MSK1	8.38225172
MSK2	1.481949101
NDR	1.191687683
NDR2(STK38L)	1.250226176
p70S6K	1.103142685
p70S6Kb	1.383486065
PKAc-alpha	48.67296296
PKAc-beta	19.066839
PKAc-gamma	3.354892549
PKCalpha	3.318294075
PKCbetaI	8.116876246
PKCbetaII	3.647305855
PKCdelta	1.70033364
PKCepsilon	1.334921737
PKCeta	2.14918051
PKCgamma	1.368075862
PKCiota	1.369963881
PKCtheta	16.62783688
PKCzeta	2.119428894
PKN1/PRK1	4.065815335
PKN2/PRK2	2.181856663
PKN3/PRK3	1.137933847
PRKG1	28.61257712
PRKG2	10.4298703
PRKX	14.64817893
ROCK1	3.807741649
ROCK2	2.975303438
RSK1	2.183700935
RSK2	1.636480672
RSK3	1.197147071
RSK4	2.046223864
SGK1	26.80118706
SGK2	5.369436074
SGK3	4.500632892
STK32B(YANK2)	1.030386529
STK32C(YANK3)	1.053317144

Table S5. Gene expression of *Slc22a12* in disected rat nephron as analyzed by RNA-seq.

Data are desribed by Lee JW et al (J Am Soc Nephrol 26:2669-2677, 2015; https://esbl.nhlbi.nih.gov/helixweb/Database/NephronRNAseq/All_transcripts.html).

Values indicate median RPKM.

Gene Symbol	S1	S2	S3	SDL	LDLOM	LDLIM	tAL	mTAL	cTAL	DCT	CNT	CCD	OMCD	IMCD
Slc22a12	1.6	21.6	120.5	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.2	0.0	0.0	0.0

S1, first segment of the proximal tubule; S2, second segment of the proximal tubule; S3, third segment of the proximal tubule; SDL, short descending limb of the loop of Henle; LDLOM, long descending limb of the loop of Henle in the outer medulla; LDLIM, long descending limb of the loop of Henle in the inner medulla; tAL, thin ascending limb of the loop of Henle; mTAL, medullary thick ascending limb of the loop of Henle; cTAL, cortical thick ascending limb of the loop of Henle; DCT, distal convoluted tubule; CNT, connecting tubule; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct.

Table S6. Baseline characteristics of 377,358 individuals of UKBB cohort according to habitual salt intake.

Characteristics	Overall (n = 377,358)	Never/Rarely (n = 209,630)	Sometimes (n = 105,915)	Usually (n = 43,830)	Always (n = 17,983)
Serum Urate (μmol/L)	308.3 (79.2)	305.1 (78.3)	310.2 (79.6)	316.3 (80.4)	314.9 (82.3)
Age	56.5 (8.1)	56.5 (8.1)	56.4 (8.1)	57.0 (8.0)	55.9 (8.3)
Male (%)	46.3	44.6	46.7	51.9	49.3
White British (%)	89.2	90.9	88.3	86.9	81.6
Current Smoker (%)	10.5	7.9	11.3	15.3	23.9
Drink ≥ 3 Days/week (%)	44.1	42.5	45.2	49.0	43.6
Waist to Hip Ratio	0.87 (0.09)	0.87 (0.09)	0.87 (0.09)	0.88 (0.09)	0.89 (0.09)
TyG Index	8.71 (0.56)	8.68 (0.55)	8.72 (0.56)	8.75 (0.57)	8.76 (0.58)
Mean Blood Pressure (mmHg)	120.7 (15.7)	120.9 (15.8)	120.4 (15.6)	120.5 (15.5)	119.7 (15.7)
Plasma Glucose (mmol/L)	5.08 (1.08)	5.07 (1.06)	5.09 (1.12)	5.08 (1.07)	5.09 (1.13)
HbA1c (mmol/mol)	35.8 (6.0)	35.7 (5.8)	36.0 (6.3)	36.0 (6.2)	36.5 (7.32)
Total Cholesterol (mmol/L)	5.72 (1.13)	5.69 (1.13)	5.74 (1.13)	5.75 (1.14)	5.72 (1.15)
eGFRcre (mL/min/1.73m^2)	86.8 (15.9)	86.9 (15.8)	86.7 (16.0)	86.4 (15.8)	85.9 (16.2)
Physical Activity (MET minutes per week)*	2696.6 (2613.9)	2703.3 (2620.5)	2679.8 (2599.4)	2711.2 (2640.9)	2681.8 (2556.3)
Fruit Intake (serving per day)**	3.27 (2.26)	3.42 (2.27)	3.14 (2.20)	2.97 (2.22)	2.68 (2.38)

Data are shown as %, or mean (SD).

*Physical activity observations, n = 300,277. **Fruit intake observations, n = 160,981.

Table S7. Additional multiple regression analysis of serum urate levels with TyG index and habitual salt intake.

Variable	β	95%CI	*P* value
Salt Added to Food (Sometimes) (vs. Rarely)	0.87	0.37 − 1.36	< 0.001
Salt Added to Food (Usually) (vs. Rarely)	0.78	0.09 − 1.47	0.026
Salt Added to Food (Always) (vs. Rarely)	1.37	0.35 − 2.39	0.008
TyG Index	19.86	19.40 − 20.32	< 0.001

Adjusted for age, sex, ethnicity, smoking habits, drinking habits, waist-to-hip ratio, mean blood pressure, HbA1c, total cholesterol, eGFRcre, and physical acticity.

Variable	β	95%CI	*P* value
Salt Added to Food (Sometimes) (vs. Rarely)	0.67	0.01 − 1.33	0.046
Salt Added to Food (Usually) (vs. Rarely)	0.96	0.01 − 1.90	0.047
Salt Added to Food (Always) (vs. Rarely)	2.07	0.49 − 3.66	0.010
TyG Index	19.25	18.63 − 19.88	< 0.001

Adjusted for age, sex, ethnicity, smoking habits, drinking habits, waist-to-hip ratio, mean blood pressure, HbA1c, total cholesterol, eGFRcre, and fruit intake.

Table S8. Mediation analysis of the effect of habitual salt intake on serum urate levels via insulin resistance.

Exposure	Mediator	Outcome	Pathway	Estimate	P value	Mediation Effect (%)
Salt Added to Food	TyG Index	Serum Urate	Salt → TyG (β_a)	0.00233	0.008	9.2
			TyG → UA (β_b)	19.75987	< 0.001	
			Salt → UA ($\beta_{c'}$)	0.45450	< 0.001	
			Indirect Effect	0.0460	0.008	
			Total Effect	0.5005	< 0.001	

Table S9. Information on candidate URAT1 loss-of-function mutations from previous studies.

rs number	Site (GRCh37)	Ref	Alt	Mutation Type	AA Change	MAF (%)	INFO Score
rs141570522	64359252	T	C	Missense	I75T	0.057	0.503
rs150255373	64366298	C	T	Missense	R325W	0.084	0.757
rs147647315	64367854	G	A	Missense	R434H	0.063	1

Ref, reference allele; Alt, alternative allele; AA, amino acid; MAF, minor allele frequency.

Table S10. Baseline characteristics of 377,358 individuals of UKBB cohort according to rs147647315 risk allele.

Characteristics	Total (n = 377,358)	URAT1-R434H mutation (-) (n = 376,887)	URAT1-R434H mutation (+) (n = 471)
Serum Urate (μmol/L)	308.3 (79.2)	308.3 (79.2)	262.6 (70.7)
Age	56.5 (8.1)	56.5 (8.1)	54.6 (8.4)
Male (%)	46.3	46.3	46.5
White British (%)	89.2	89.3	49.3
Current Smoker (%)	10.5	10.5	10.6
Drink ≥ 3 Days/week (%)	44.1	44.1	33.1
Waist to Hip Ratio	0.87 (0.09)	0.87 (0.09)	0.87 (0.08)
TyG Index	8.71 (0.56)	8.71 (0.56)	8.59 (0.61)
Mean Blood Pressure (mmHg)	120.7 (15.7)	120.7 (15.7)	120.3 (17.1)
Plasma Glucose (mmol/L)	5.08 (1.08)	5.08 (1.08)	5.13 (1.56)
HbA1c (mmol/mol)	35.8 (6.0)	35.8 (6.0)	37.3 (8.5)
Total Cholesterol (mmol/L)	5.72 (1.13)	5.72 (1.13)	5.55 (1.26)
eGFRcre (mL/min/1.73m^2)	86.8 (15.9)	86.8 (15.9)	89.4 (16.4)

Table S11. Baseline characteristics of 377,358 individuals of UKBB cohort according to rs4529048 allele number.

Characteristics	Total (n = 377,358)	rs4529048 A allele = 0 (n = 23,922)	rs4529048 A allele = 1 (n = 140,551)	rs4529048 A allele = 2 (n = 212,885)
Serum Urate (μmol/L)	308.3 (79.2)	268.3 (80.4)	299.4 (78.4)	318.6 (77.4)
Age	56.5 (8.1)	56.2 (8.1)	56.4 (8.1)	56.5 (8.1)
Male (%)	46.3	46.4	46.3	46.3
White British (%)	89.2	85.4	88.4	90.2
Current Smoker (%)	10.5	10.6	10.4	10.5
Drink ≥ 3 Days/week (%)	44.1	42.7	43.9	44.3
Waist to Hip Ratio	0.87 (0.09)	0.87 (0.09)	0.87 (0.09)	0.87 (0.09)
TyG Index	8.71 (0.56)	8.70 (0.56)	8.71 (0.56)	8.71 (0.56)
Mean Blood Pressure (mmHg)	120.7 (15.7)	120.3 (15.7)	120.6 (15.7)	120.7 (15.7)
Plasma Glucose (mmol/L)	5.08 (1.08)	5.07 (1.06)	5.08 (1.07)	5.08 (1.09)
HbA1c (mmol/mol)	35.8 (6.0)	35.8 (5.9)	35.8 (6.1)	35.8 (6.0)
Total Cholesterol (mmol/L)	5.72 (1.13)	5.70 (1.13)	5.71 (1.13)	5.72 (1.14)
eGFRcre (mL/min/1.73m^2)	86.8 (15.9)	87.0 (15.9)	86.9 (15.9)	86.7 (15.9)