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### Article

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## **Homogentisic acid is not only eliminated by glomerular filtration and tubular secretion but also produced in the kidney in alkaptonuria.**

**Authors:** Ranganath LR<sup>1,14</sup>, Milan AM<sup>1,14</sup>, Hughes AT<sup>1,14</sup>, Khedr M<sup>1</sup>, Davison AS<sup>1,14</sup>, Shweihdi E<sup>1</sup>, Norman BP<sup>13</sup>, Hughes JH<sup>13</sup>, Bygott H<sup>1</sup>, Luangrath E<sup>1</sup>, Fitzgerald R<sup>2</sup>, Psarelli EE,<sup>3</sup> van Kan C<sup>4</sup>, Laan D<sup>4</sup>, Olsson B<sup>5</sup>, Rudebeck M<sup>5</sup>, Mankowitz L<sup>5</sup>, Sireau N<sup>6</sup>, Arnoux JB<sup>7</sup>, Le Quan Sang KH<sup>7</sup>, Jarvis JC<sup>8</sup>, Genovese F<sup>9</sup>, Braconi D<sup>10</sup>, Santucci A<sup>10</sup>, Zatkova A<sup>11</sup>, Glasova H<sup>11</sup>, Stančík R<sup>12</sup>, Imrich R<sup>11</sup>, Rhodes NP<sup>13</sup>, Gallagher JA<sup>13</sup>

Departments of Clinical Biochemistry and Metabolic Medicine<sup>1</sup>, Clinical Pharmacology<sup>2</sup> Royal Liverpool University Hospital, Prescot Street, Liverpool, L7 8XP, UK; Liverpool Cancer Trials Unit, University of Liverpool, Block C, Waterhouse Building, Liverpool L69 3GL, UK<sup>3</sup>; PSR group B.V., Hoofddorp, Netherlands<sup>4</sup>; Swedish Orphan Biovitrum AB (publ), Stockholm, Sweden<sup>5</sup>; AKU Society, Cambridge, UK<sup>6</sup>; Hôpital Necker-Enfants Malades, APHP, Paris Descartes University, Paris, France<sup>7</sup>; School of Sport and Exercise Science, Liverpool John Moores University, Liverpool, UK<sup>8</sup>; Nordic Bioscience, Herlev, Denmark<sup>9</sup>; Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Siena, Italy<sup>10</sup>; Biomedical Research Center, Slovak Academy of Sciences, Bratislava, Slovakia<sup>11</sup>; National Institute of Rheumatic Diseases, Piešťany, Slovakia<sup>12</sup>; Departments of Musculoskeletal Biology, University of Liverpool, L69 7ZX, UK<sup>13</sup>

**Corresponding author:** L Ranganath, Department of Clinical Biochemistry and Metabolic Medicine, Royal Liverpool University Hospital, Prescot Street, Liverpool, L7 8XP; e-mail: [lrang@liv.ac.uk](mailto:lrang@liv.ac.uk)

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## Contributors

All authors contributed to analysis of the data, edited the manuscript and approved the final version.

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**Data sharing statement:** The authors agree to honour any reasonable request by other researchers for materials, methods or data necessary to verify the conclusion of the article.

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## **Abstract**

**BACKGROUND:** The clinical effects of alkaptonuria (AKU) is delayed and ageing influences disease progression. Morbidity of AKU is secondary to high circulating homogentisic acid (HGA) and ochronosis. It is not known whether HGA is produced by or processed in the kidney in AKU.

**METHODS:** Data from AKU patients from four studies were merged to form a single AKU group. A control group of non-AKU subjects was generated by merging data from two non-AKU studies. Data were used to derive renal clearance and fractional excretion (FE) ratios for creatinine, HGA, phenylalanine (**PHE**) and tyrosine (**TYR**) using standard calculations, for comparison between the AKU and the control groups.

**RESULTS:** There were 225 AKU patients in the AKU group and 52 in the non-AKU control group. Circulating HGA increased with age ( $p < 0.001$ ), and was statistically significantly associated with decreased HGA clearance ( $CL_{HGA}$ ) ( $p < 0.001$ ) and  $FE_{HGA}$  ( $p < 0.001$ ).  $CL_{HGA}$  and  $FE_{HGA}$  were increased beyond the theoretical maximum renal plasma flow, confirming renal production and emphasizing the greater contribution of net tubular secretion than glomerular filtration to renal elimination of HGA.

**CONCLUSIONS:** The kidneys are crucial to elimination of HGA. Elimination of HGA is impaired with age resulting in worsening disease over time. The kidney is an important site for production of HGA. Tubular secretion of HGA contributes more to elimination of HGA in AKU than glomerular filtration does.

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## **Introduction**

Increasing age and male gender are associated with a more severe disease manifestation in the rare disease, alkaptonuria (AKU) (OMIM#203500) (1). A genetic deficiency of homogentisate dioxygenase (HGD) (EC:1.13.11.5) results in overproduction of homogentisic acid (HGA) due to an inability to fully metabolise ingested phenylalanine (PHE) and tyrosine (TYR), surplus to daily needs (2) and the TYR that is generated during protein turnover. Increased circulating HGA is directly causal in the disease process known as ochronosis, in which yellow-black pigment is formed (3), and deposited in connective tissues, especially cartilage, causing the connective tissue to become brittle and ultimately breakdown. Circulating HGA is maintained at a relatively low concentration because of efficient renal excretion, to the extent that the concentration of HGA in the urine is more than 1000-fold higher than in the circulation (4). However, other than the fact that renal secretion is crucial to eliminate HGA and minimise progression of disease in AKU, little is known in terms of how HGA is handled by the kidney.

The frequency of AKU is around 1 in 250,000 to 1 in 1,000,000 in most populations worldwide making it a difficult condition to study reliably (5). In unaffected humans, significant expression of HGD has only been shown in the liver and the kidney (6). AKU is present from birth but the onset of morbidity is delayed for reasons that are yet to be fully understood. HGA is water soluble and freely eliminated via the kidney, despite which circulating HGA increases. There has not been a previous study specifically examining the renal handling of HGA in AKU beyond its renal excretion. This lack of data on renal handling of HGA is partly due to the difficulty in generating reliable plasma or serum measurements as HGA is labile and present in low concentrations compared to urine (7, 8).

The debilitating manifestations of AKU include premature arthritis, cardiac valve disease, fractures, and ruptures of muscle and tendon (5); tissue ochronosis in kidneys has not been previously described even though the pigmented proteinaceous tubular casts have been described in the macroscopically grey pigmentation of the medullae (9). The excessive renal elimination of HGA can lead not only to dark urine but also renal stones (10). Renal function declines with ageing in non-AKU subjects, but it is not known if such a decline is present in AKU, although it has been postulated as a cause of the delayed appearance of the musculoskeletal symptoms of the disease despite the biochemical manifestation being present from birth (11). Renal failure has been shown to markedly accelerate ochronosis as well as the morbidity of AKU (12). Intractable fatal haemolysis in renal failure has been described in a

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series of cases (13, 14). Conversely, renal transplantation for end-stage renal failure in AKU has improved the metabolic manifestations such as decreasing serum and urine HGA (12). A recent study in AKU reported changes in urine C3M, a biomarker reflecting the remodelling of the renal tissue, associated with a change in fibrogenesis in the kidneys (15, 16).

Our group has carried out studies in AKU patients in whom similar data were collected at baseline without any HGA-lowering treatment. The studies from which baseline data were extracted to form the dataset presented in this manuscript are SONIA 1 and 2 (Suitability of Nitisinone in Alkaptonuria 1 and 2), SOFIA (Subclinical Ochronosis Features In Alkaptonuria), as well as data collected in the United Kingdom National Alkaptonuria Centre (NAC) (4, 17, 18). This has allowed the generation of the largest dataset in AKU to date and should allow a better understanding of the condition. The objective in this manuscript is to clarify the effects of ageing and gender on metabolism in AKU, as well as to investigate the role of the kidney in AKU.

## **Methods**

### **Patients**

AKU patients, verified by elevated urine HGA concentrations, and at least 16 years old were eligible for inclusion from SONIA 1 (4) and 2, SOFIA (15) and the NAC (17, 18). Two non-AKU control groups were also available and merged to increase patient numbers (one of these was recruited as part of SOFIA, and the other was recruited separately (NRES [United Kingdom National Research Ethics Service] No:07/H1002/111), in parallel with SONIA 1. The United Kingdom NRES granted ethics approval for SONIA 1 (REC No:13/NW/0024; IRAS:121963; EUDRACT No:2012-005340-24), SONIA 2 (REC No:13/NW/0567; IRAS:136411; EUDRACT No:2013-001633-41), and SOFIA (REC N.:15/NW/0749; IRAS:180968). The data collected from the NAC was approved by the Institutional Audit Committee (Audit No:ACO3836). None of the patients in this study received HGA-lowering treatment at the time of participation. The data from the AKU patients in NAC, SONIA 1, SONIA 2 and SOFIA were merged to derive the combined AKU group. Patients from SONIA 1 also then took part in SONIA 2; patients from SONIA 2 and the NAC took part in SOFIA, and therefore duplication of data was avoided by including them once only in the merged group. Similarly, data from the two non-AKU studies were merged to derive a combined non-AKU control group.

## **ASSESSMENTS**

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Study protocols have been described in detail previously for SONIA 1, SOFIA and NAC; a single morning blood and 24-h urine collections from SONIA 2 patients were similar to those collected in SONIA 1. These samples were collected in each patient for measurement of metabolites such as creatinine (CR), PHE, TYR, and HGA. In SONIA 1, 24-h blood samples were collected at baseline (0) and 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 15, 18 and 24 hours after the morning sample, for determination of serum HGA (sHGA), serum TYR (sTYR) and serum PHE (sPHE). All serum and urine samples were acidified on collection as previously described to stabilise the HGA (4, 17). Serum and urine HGA, **PHE and TYR**, were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) methods, again described in detail elsewhere (4, 17, 20). CR, including serum (sCR), was measured by the Jaffe reaction (Roche Diagnostics, Germany) because HGA interferes with the enzymatic measurement of CR (21).

Derived data:

From the 24-h profiles of sHGA, sTYR and sPHE, only available for the SONIA 1 cohort, the mean concentration in this period was calculated as the area under the curve, determined by the trapezoidal rule, and divided by 24. The data presented on SONIA 1 (Table S1) represents calculations using mean 24-h serum HGA, TYR and PHE. The combined AKU data likewise includes calculations using mean 24-h serum HGA, TYR and PHE, for SONIA 1, but single fasting values for the NAC, SONIA 2 and SOFIA (in which 24-h profiles were not available).

The estimated glomerular filtration rate (eGFR) was derived from the UK Kidney MDRD equation,  $186 \times (\text{Creatinine}/88.4)^{-1.154} \times (\text{Age})^{-0.203} \times (0.742 \text{ if female}) \times (1.210 \text{ if black})$ , employing sCR and other data pertaining to each patient (22).

Creatinine clearance ( $CL_{\text{CR}}$ ) was derived from the equation  $[\text{urine creatinine (micromoles)} \times \text{urine volume (millilitres)}] / [\text{sCR (micromoles)} \times 1440 \text{ (minutes)}]$ . Clearances for HGA, TYR and PHE ( $CL_{\text{HGA}}$ ,  $CL_{\text{TYR}}$ ,  $CL_{\text{PHE}}$ ) were derived in the same manner by substituting HGA or TYR or PHE for creatinine in the equation in the same manner.

24-hour urinary excretion of HGA, TYR and PHE were derived by multiplying concentrations (micromoles per litre) by urine volume in litres ( $u\text{HGA}_{24}$ ,  $u\text{TYR}_{24}$ ,  $u\text{PHE}_{24}$ ).

The Fractional Excretion of HGA ( $FE_{\text{HGA}}$ ) was calculated by the following equation and expressed as a percentage:  $100 \times [\text{Urine HGA (micromoles per litre)} \times \text{sCR (micromoles per litre)}] / [\text{Urine creatinine (micromoles per litre)} \times \text{sHGA (micromoles per litre)}]$ . The same

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method was used to derive  $FE_{TYR}$  and  $FE_{PHE}$  (23). The formula for FE, for example HGA, consists of two parts: firstly, figuring out how much HGA is excreted in the urine, by multiplying the urine HGA concentration by the urinary flow rate urinary flow rate, the numerator in the equation. Secondly, then finding its ratio to the total amount of HGA that passed through the kidney glomerulus, calculated by multiplying the plasma HGA concentration by the GFR, calculated using creatinine filtration.

The contribution of glomerular filtration, tubular secretion and renal production to total renal HGA elimination was calculated based on the  $CL_{HGA}$ , assuming a theoretical maximum of glomerular filtration of 120 mL/min and renal plasma flow of 600 mL/min. For example, if the  $CL_{HGA}$  was greater than 600, say 900 mL/min, then glomerular filtration contribution would be 120/900, tubular secretion would be 480/900 and renal production would be 300/900 and when expressed as a percentage would be 13.4%, 53.3% and 33.3% approximately. If the  $CL_{HGA}$  was 600 mL/min or less, then filtration was derived as  $(120/CL_{HGA}) \times 100$  as a percentage; the secretion was derived as  $[(CL_{HGA}-120)/CL_{HGA}] \times 100$  as a percentage; production was assumed to be zero. (it was also considered whether to use a 20%/80% estimate when values were 600 and below but it was felt the approach used was better; equally using the GFR from patients themselves seems attractive – in fact  $FE_{HGA}$  – does just that – but since creatinine secretion may possibly be further increased in AKU, this may mislead and lead to underestimation of filtration).

## **STATISTICAL ANALYSIS**

Continuous variables are presented using a mean and a standard deviation (SEM) whereas categorical variables are presented as frequencies. Parametric tests using unpaired 't' test were employed in order to detect differences in demographic, chemical and metabolic characteristics between AKU and control groups as also between male and female AKU patients. Simple linear regressions were the main analyses generated. Outcome variables were plotted against age and other potential predictor data and regression analyses were carried out. Comparisons with controls were made as appropriate. A two-sided significance level of p values less than 0.05 was used throughout. All analyses were conducted using Graphpad InStat 3™ software and figures were generated by Deltagraph 7™ software.

## **Results**

Data from 225 AKU patients (140 male, 85 female) were collated from 37, 90, 30 and 68 patients attending SONIA 1, SONIA 2, SOFIA and NAC, respectively. There were also normal

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control data from 30 subjects in the SOFIA study and 22 subjects from an earlier study where normal data was collected. The demographic data of the individual studies are shown in Supplementary Table 1, including control studies 1 and 2. Data from SONIA 1 included 24-h serum profiles on HGA, TYR and PHE and were used to derive all the indices instead of single morning values (Table S2, Figures S5 and S6). Results described as increased or decreased were all statistically significant with p values shown in tables or text.

Table S1. Demographic, chemical and metabolic data in all studies

	NAC	SONIA 1	SONIA 2	SOFIA AKU	SOFIA CONTROL STUDY 1*	SONIA 1 CONTROL STUDY
Number (M/F)	68 (44/24)	37 (24/13)	90 (57/33)	30 (15/15)	30 (13/17)	22 (11/11)
Age years	48.2±1.9	47.7±2.0	48.3±1.1	40.4±2.8	38.1±2.2	42.7±2.5
sCR $\mu\text{mol/L}$	72±1.5	58±2.1	62.8±1.4	70.4±2.7	75±1.8	88±3.1
eGFR $\text{mL/min}/1.73\text{m}^2$	102±2.3	131±4.2	120±3.1	104±4	93±2.6	78±1.8
CL <sub>CR</sub> mL/min	88±4.1	157±10	117±4.3	106±5.4	NA	103±5.1
sHGA $\mu\text{mol/L}$	29.8±1.8	35.6±1.5	29.0±1.1	29.1±1.4	NA	NA
sTYR $\mu\text{mol/L}$	54.8±4.0	59.6±1.6	63.8±1.7	58.6±2.1	62.8±2.4	58.4±3
sPHE $\mu\text{mol/L}$	64.5±1.6	56.3±1.3	57.6±1.1	54.9±1.3	68.1±2.6	59.5±1.9
uHGA <sub>24</sub> $\mu\text{mol/day}$	22293±1121	33605±1770	32797±1308	37450±2368	NA	NA
uTYR <sub>24</sub> $\mu\text{mol/day}$	101.3±10.4	113.3±19.9	203.2±18.9	144.1±13.2	NA	73.5±13.3
uPHE <sub>24</sub> $\mu\text{mol/day}$	64±6.6	95.8±9.3	133±29.5	85.9±6.3	NA	50.3±6.8
CL <sub>HGA</sub> mL/min	617±44.4	684±42	844±40	932±66	NA	NA
CL <sub>TYR</sub> mL/min	1.44±0.2	1.31±0.2	2.34±0.2	1.7±0.1	NA	0.86±0.15
CL <sub>PHE</sub> mL/min	0.48±0.04	1.2±0.12	1.0±0.18	0.73±0.08	NA	0.42±0.06
FE <sub>HGA</sub> %	699±36	442±15	731±23	884±45	8.4±0.86	0.43±0.05
FE <sub>TYR</sub> %	1.57±0.17	0.32±0.09	2.19±0.24	1.61±0.11	0.88±0.07	0.77±0.1
FE <sub>PHE</sub> %	0.76±0.06	0.11±0.01	1.63±0.46	1.03±0.06	0.46±0.03	0.44±0.07

\*Only random urine was collected and some derived data not available

Data expressed as Mean±SEM

CL<sub>CR</sub> or creatinine clearance; CL<sub>HGA</sub> or HGA renal clearance; CL<sub>PHE</sub> or phenylalanine renal clearance; CL<sub>TYR</sub> or tyrosine renal clearance; FE<sub>HGA</sub> or fractional renal excretion of HGA; FE<sub>PHE</sub> or fractional renal excretion

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of phenylalanine;  $FE_{TYR}$  or fractional renal excretion of tyrosine; sCR or Serum creatinine; sHGA or serum HGA; sPHE or serum phenylalanine; sTYR or serum tyrosine;  $uHGA_{24}$  or daily HGA excretion;  $uPHE_{24}$  or daily phenylalanine excretion;  $uTYR_{24}$  or daily tyrosine excretion.

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Table 1. Comparison of combined AKU and combined non-AKU Controls		
	COMBINED AKU	COMBINED CONTROLS
Number (M/F)	225 (140/85)	52 (24/28)
Age years	47.1±0.9***	40.1±1.7
sCR µmol/L	65.8±0.9***	80.5±1.9
eGFR mL/min/1.73m <sup>2</sup>	114±1.8***	86.6±2
CL <sub>CREAT</sub> mL/min	113.6±3.2	103±5.1
sHGA µmol/L	30.4±0.8***	2.24±0.25
sTYR µmol/L	59.7±1.2	60.9±1.9
sPHE µmol/L	59.1±0.7**	64.7±1.8
uHGA <sub>24</sub> µmol/day	30376±837***	1.94±0.12
uTYR <sub>24</sub> µmol/day	152±9.6*	73.5±13.3
uPHE <sub>24</sub> µmol/day	100±12.4***	50.3±6.8
CL <sub>HGA</sub> mL/min	763±24.7***	0.43±0.03
CL <sub>TYR</sub> mL/min	1.82±0.12***	0.86±0.15
CL <sub>PHE</sub> mL/min	0.84±0.08***	0.42±0.06
FE <sub>HGA</sub> %	694±17.5	5.03±0.74
FE <sub>TYR</sub> %	1.64±0.12	0.83±0.06
FE <sub>PHE</sub> %	1.08±0.19	0.45±0.03
P values: *<0.05; **<0.01; ***<0.001; Parametric unpaired t test was used; Data expressed as Mean±SEM		
CL <sub>CR</sub> or creatinine clearance; CL <sub>HGA</sub> or HGA renal clearance; CL <sub>PHE</sub> or phenylalanine renal clearance; CL <sub>TYR</sub> or tyrosine renal clearance; FE <sub>HGA</sub> or fractional renal excretion of HGA; FE <sub>PHE</sub> or fractional renal excretion of phenylalanine; FE <sub>TYR</sub> or fractional renal excretion of tyrosine; sCR or Serum creatinine; sHGA or serum HGA; sPHE or serum phenylalanine; sTYR or serum tyrosine; uHGA <sub>24</sub> or daily HGA excretion; uPHE <sub>24</sub> or daily phenylalanine excretion; uTYR <sub>24</sub> or daily tyrosine excretion.		

### Comparison of Combined AKU with Combined Controls

All data from the combined AKU and combined non-AKU controls are shown in Table 1. As expected, all metabolic data such as sHGA, uHGA<sub>24</sub>, CL<sub>HGA</sub>, and FE<sub>HGA</sub> were statistically

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significantly different in the AKU group compared with the non-AKU control group. However, sCR was significantly lower in AKU group compared with the non-AKU controls; conversely, eGFR as well as  $CL_{CR}$  were significantly higher in AKU group compared with the non-AKU controls (Supplementary Figure 1). Further, sPHE was lower, and uTYR<sub>24</sub>, uPHE<sub>24</sub>,  $CL_{TYR}$  and  $CL_{PHE}$  were higher in AKU group compared with non-AKU controls.

### **Gender comparisons**

The male AKU patient group was compared with the female AKU group (Figure 1; Table 2). sCR as well as  $CL_{CR}$  were statistically significantly higher in male AKU compared with female AKU patients. Although sHGA was similar in male and female AKU groups, uHGA<sub>24</sub> was increased in male AKU subjects compared with the female AKU group. sTYR was increased in male AKU subjects compared with female AKU subjects.

### **Renal elimination of HGA**

Figure 2 shows the  $CL_{HGA}$  and  $FE_{HGA}$  in the male and female AKU subjects. It is clear that elimination of HGA by the kidney is through glomerular filtration (smallest component), and through net renal tubular secretion (larger component than glomerular filtration); values noted for  $CL_{HGA}$  and  $FE_{HGA}$  in both male and female AKU patients are above the theoretical maximum of renal plasma flow of 600 mL/min. Renal elimination is greater than the theoretical maximum, namely the elimination of all of the metabolites contained in the total renal plasma flow. The elimination of HGA is greater than the combined total of the glomerular filtration and net tubular secretion. Supplementary figures 5 and 6 show individual studies, including SONIA 1 where 24-h mean serum concentration was used for derived indices instead of single sample values. The contribution of glomerular filtration, tubular secretion and renal production to total renal HGA elimination was calculated as previously described for SONIA 1 (where

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mean 24-h serum HGA values were available), as well as the other AKU studies including the combined AKU group are shown in Table S3 and Fig S7.

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Table 2. Comparison of Male and Female AKU Groups

	Male AKU	Female AKU
Number	140	85
Age years	46.1±1.1	48.8±1.4
sCR $\mu\text{mol/L}$	70.8±1.1***	57.5±1.3
eGFR mL/min/1.73m <sup>2</sup>	116.2±2.0	110.5±3.5
CL <sub>CREAT</sub> mL/min	119±3.4*	105.1±6.1
sHGA $\mu\text{mol/L}$	31.4±0.96	28.7±1.3
sTYR $\mu\text{mol/L}$	62.9±2.1***	54.4±1.5
sPHE $\mu\text{mol/L}$	60.1±0.87	57.5±1.3
uHGA <sub>24</sub> $\mu\text{mol/day}$	32525±976***	26836±1454
uTYR <sub>24</sub> $\mu\text{mol/day}$	161±11.8	137±16.6
uPHE <sub>24</sub> $\mu\text{mol/day}$	95.2±6.1	108.8±31.3
CL <sub>HGA</sub> mL/min	779±30	720±43
CL <sub>TYR</sub> mL/min	1.87±0.14	1.75±0.21
CL <sub>PHE</sub> mL/min	0.81±0.05	0.9±0.19
FE <sub>HGA</sub> %	686±21	708±30
FE <sub>TYR</sub> %	1.56±0.12	1.78±0.24
FE <sub>PHE</sub> %	0.88±0.06	1.39±0.49
P values: * $<0.05$ ; ** $<0.01$ ; *** $<0.001$ ; Parametric unpaired 't' test with Welch Correction (2-tail); Data expressed as Mean±SEM		
CL <sub>CR</sub> or creatinine clearance; CL <sub>HGA</sub> or HGA renal clearance; CL <sub>PHE</sub> or phenylalanine renal clearance; CL <sub>TYR</sub> or tyrosine renal clearance; FE <sub>HGA</sub> or fractional renal excretion of HGA; FE <sub>PHE</sub> or fractional renal excretion of phenylalanine; FE <sub>TYR</sub> or fractional renal excretion of tyrosine; sCR or Serum creatinine; sHGA or serum HGA; sPHE or serum phenylalanine; sTYR or serum tyrosine; uHGA <sub>24</sub> or daily HGA excretion; uPHE <sub>24</sub> or daily phenylalanine excretion; uTYR <sub>24</sub> or daily tyrosine excretion.		

### Linear regression analyses

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Simple linear regression analyses were carried out on data from the combined AKU group and shown in Table 3. Age is a significant positive predictor of sHGA, but  $CL_{CR}$ ,  $uHGA_{24}$ ,  $CL_{HGA}$  and  $FE_{HGA}$  showed the strongest negative relationship to age (Supplementary Figure 2). eGFR also showed a positive linear relationship with creatinine clearance,  $uHGA_{24}$  and  $CL_{HGA}$ ; a negative linear relationship was also seen with sHGA (Supplementary Figure 3). sHGA showed a positive linear relationship with  $uHGA_{24}$  and statistically significant negative linear relationship between  $CL_{HGA}$  and  $FE_{HGA}$ . Serum tyrosine was positively related to  $uHGA_{24}$  as well as to  $CL_{HGA}$ .  $uHGA_{24}$  was positively associated with  $CL_{HGA}$ , as well as with  $uTYR_{24}$ ,  $uPHE_{24}$ ,  $CL_{TYR}$ ,  $CL_{PHE}$ ,  $FE_{TYR}$  and  $FE_{PHE}$ .  $CL_{HGA}$ ,  $CL_{TYR}$ ,  $CL_{PHE}$ ,  $FE_{TYR}$  and  $FE_{PHE}$  were all found to be significant positive predictors of  $uPHE_{24}$ . The linear relationships between sHGA,  $uHGA_{24}$ ,  $CL_{HGA}$  and  $FE_{HGA}$  are graphically represented in Supplementary Figure 4.

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Table 3. REGRESSION ANALYSIS ON COMBINED AKU GROUP

	Age	eGFR	sCR	CL <sub>CR</sub>	sHGA	sTYR	sPHE	uHGA <sub>24</sub>	uTYR <sub>24</sub>	uPHE <sub>24</sub>	CL <sub>HGA</sub>	CL <sub>TYR</sub>	CL <sub>PHE</sub>	FE <sub>HGA</sub>	FE <sub>TYR</sub>	FE <sub>PHE</sub>
Age		-0.17	-0.12	-0.22	0.31	-0.01	-0.02	-0.18	-0.16	-0.03	-0.38	-0.16	-0.02	-0.26	-0.06	0.01
eGFR			-0.75	0.61	-0.16	0.13	-0.11	0.26	0.12	0.01	0.34	0.07	0.03	-0.19	-0.17	-0.11
sCR				-0.41	0.16	-0.02	0.18	-0.07	-0.03	-0.03	-0.16	-0.01	-0.06	0.21	0.12	0.05
CL <sub>CR</sub>					-0.06	0.17	-0.03	0.64	0.28	0.1	0.58	0.21	0.15	-0.23	-0.21	-0.11
sHGA						0.09	0.17	0.23	-0.08	-0.04	-0.47	-0.13	-0.01	-0.57	-0.16	-0.06
sTYR							0.1	0.19	0.18	0.04	0.27	-0.1	-0.01	0.13	-0.15	0.02
sPHE								-0.05	-0.01	-0.04	-0.14	-0.01	-0.14	-0.15	-0.01	-0.07
uHGA <sub>24</sub>									0.27	0.11	0.65	0.16	0.11	0.13	-0.13	-0.02
uTYR <sub>24</sub>										0.45	0.26	0.90	0.43	0.05	0.69	0.33
uPHE <sub>24</sub>											0.09	0.4	0.95	0.03	0.35	0.96
CL <sub>HGA</sub>												0.18	0.06	0.62	-0.04	0.01
CL <sub>TYR</sub>													0.41	0.03	0.85	0.3
CL <sub>PHE</sub>														-0.03	0.33	0.89
FE <sub>HGA</sub>															0.16	0.12
FE <sub>TYR</sub>																0.38
FE <sub>PHE</sub>																

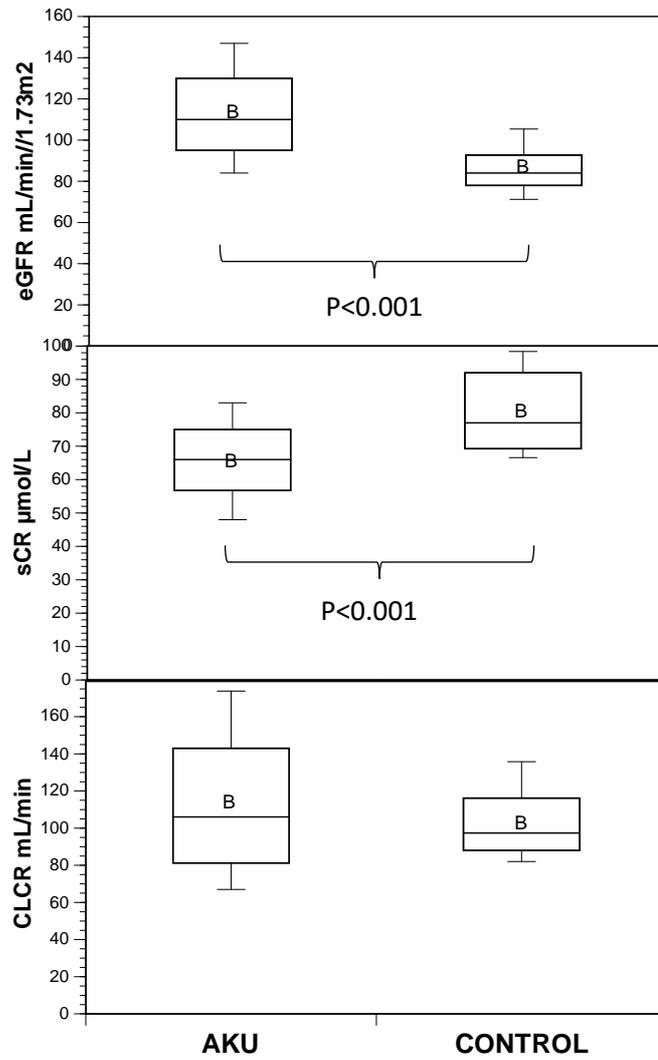
Values in boxes in the table refer to correlation coefficients

p values are colour coded: Yellow <0.05; Green <0.01; Blue <0.001; Red <0.0001

Age – years; CL<sub>CR</sub> or creatinine clearance; CL<sub>HGA</sub> or HGA renal clearance; CL<sub>PHE</sub> or phenylalanine renal clearance; CL<sub>TYR</sub> or tyrosine renal clearance; eGFR mL/min/1.73m<sup>2</sup>; FE<sub>HGA</sub> or fractional renal excretion of HGA; FE<sub>PHE</sub> or fractional renal excretion of phenylalanine; FE<sub>TYR</sub> or fractional renal excretion of tyrosine; sCR or Serum creatinine; sHGA or serum HGA; sPHE or serum phenylalanine; sTYR or serum tyrosine; uHGA<sub>24</sub> or daily HGA excretion; uPHE<sub>24</sub> or daily phenylalanine excretion; uTYR<sub>24</sub> or daily tyrosine excretion.

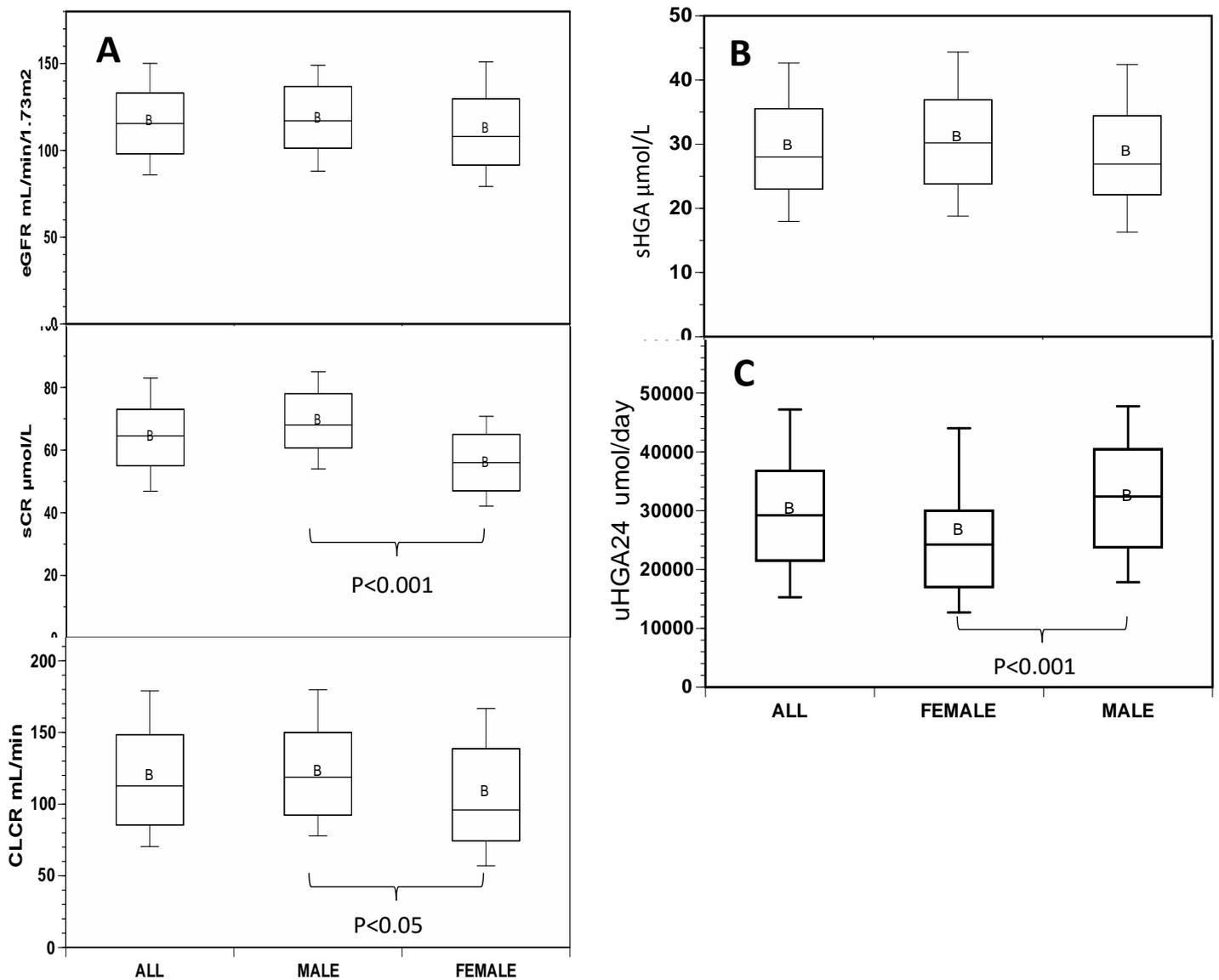
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Figure S1. Comparison of combined AKU and combined non-AKU control groups (Box plots with square (mean) and horizontal line (median) inside the box). Upper panel shows eGFR comparison; middle panel shows sCR comparison; lower panel shows CL<sub>CR</sub> comparison.



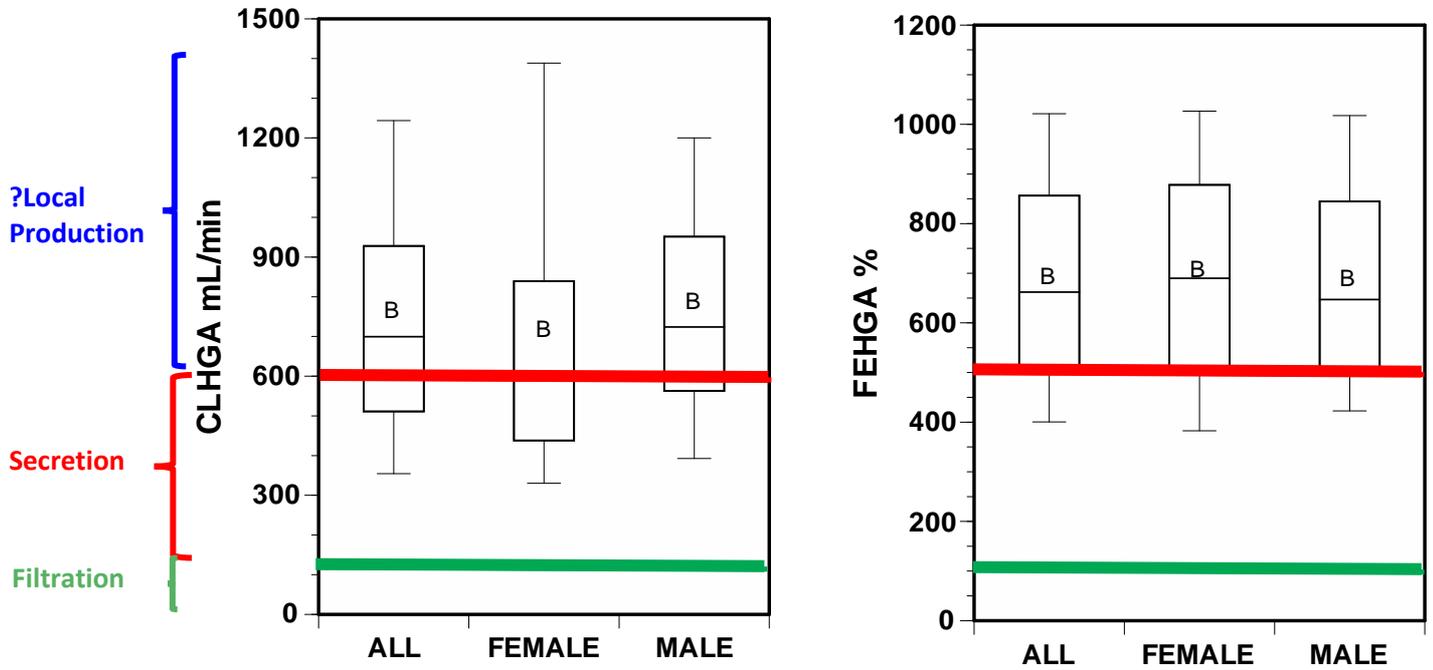
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Figure 1. Comparison of metabolic and other parameters in male and female AKU groups. (A) Upper panel shows eGFR comparison; middle panel shows sCR comparison; lower panel shows  $CL_{CR}$  comparison between All, male and female AKU patients; (B) – comparison of sHGA between All, male and female AKU patients; (C) - comparison of uHGA<sub>24</sub> between All, male and female AKU patients. P values are shown where significant (Box plots with square (mean) and horizontal line (median) inside the box). (eGFR – estimated glomerular filtration rate; sCR – serum creatinine;  $CL_{CR}$  – creatinine clearance; sHGA – serum HGA; uHGA<sub>24</sub> – 24-h urine HGA).



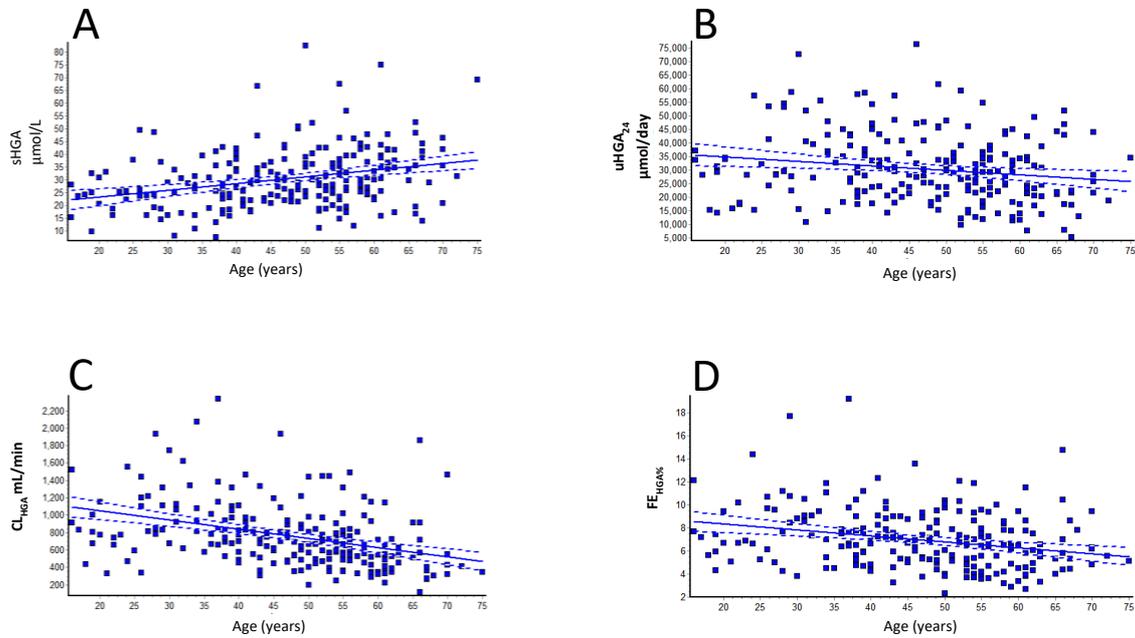
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Figure 2.  $CL_{HGA}$  (A) and  $FE_{HGA}$  (B) comparison in Male and Female AKU patients (Box plots with square (mean) and horizontal line (median) inside the box; GFR is glomerular filtration rate (green line); RPF is renal plasma flow (red line); Filtration: glomerular filtration; Secretion: net tubular secretion; Local production: tubular production).



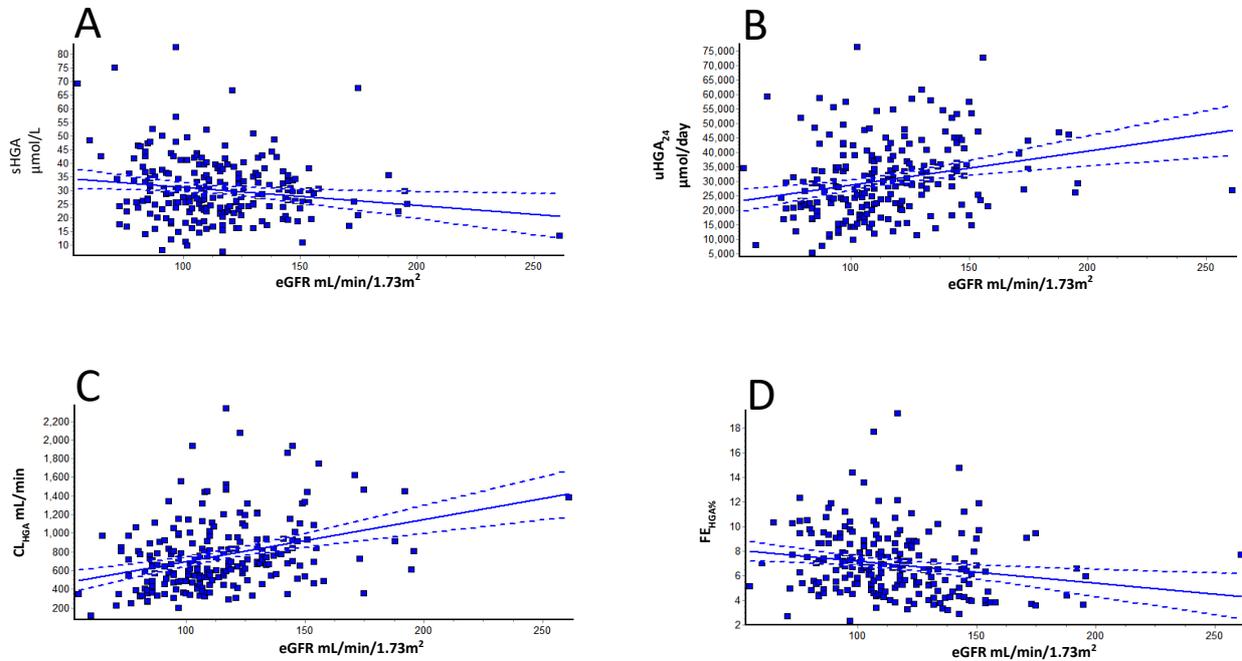
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Figure S2. Linear regression analysis of different parameters vs. age showing (A) – positive linear relationship between sHGA and age; (B) – a negative linear relationship between uHGA<sub>24</sub> and age; (C) – negative linear relationship between CL<sub>HGA</sub> and age; (D) – negative linear relationship between FE<sub>HGA</sub> and age (Solid blue line – linear regression; dotted blue line – 95% confidence interval).



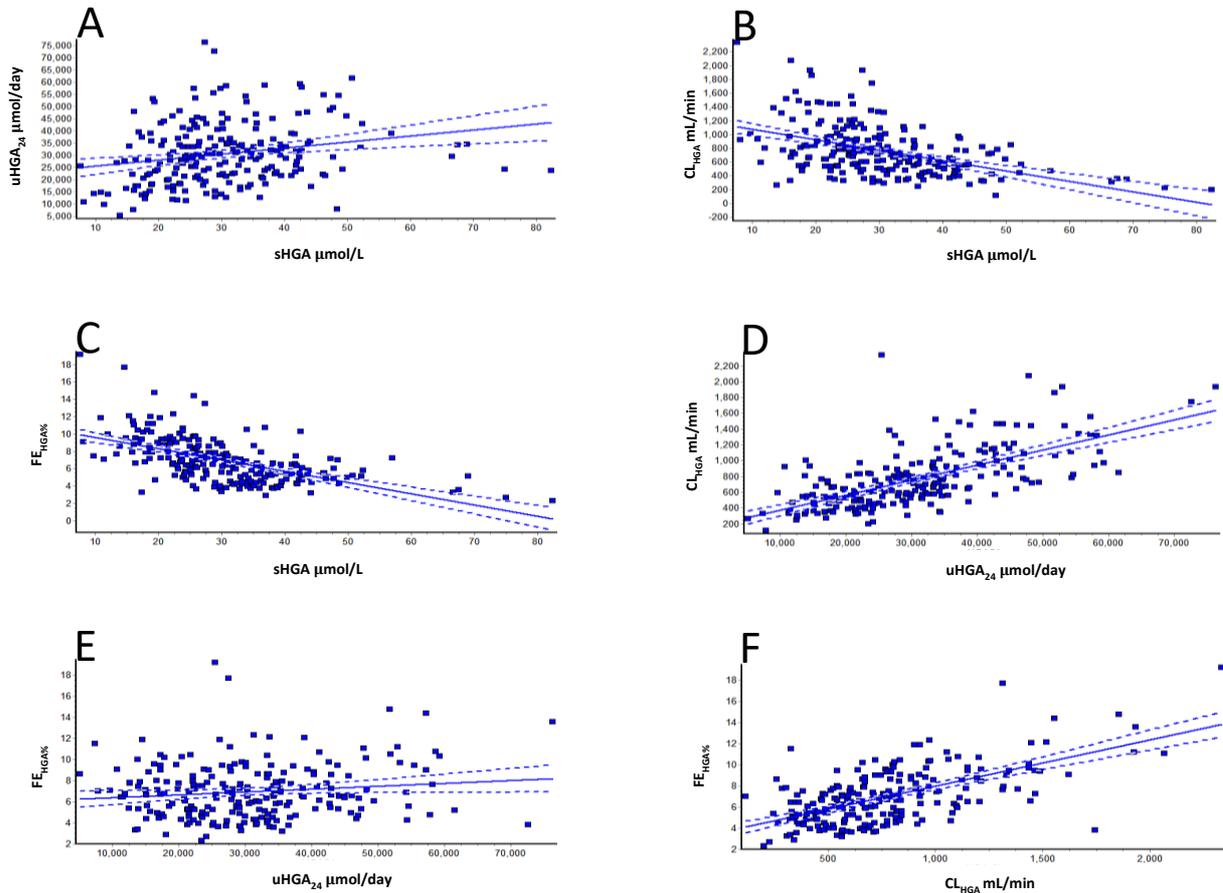
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Figure S3. Linear regression analysis of eGFR with other parameters showing (A) – negative linear relationship between sHGA and eGFR; (B) – positive linear relationship between uHGA<sub>24</sub> and eGFR; (C) - positive linear relationship between CL<sub>HGA</sub> and eGFR ; (D) – negative linear relationship between FE<sub>HGA</sub> and eGFR (Solid blue line – linear regression; dotted blue line – 95% confidence interval).



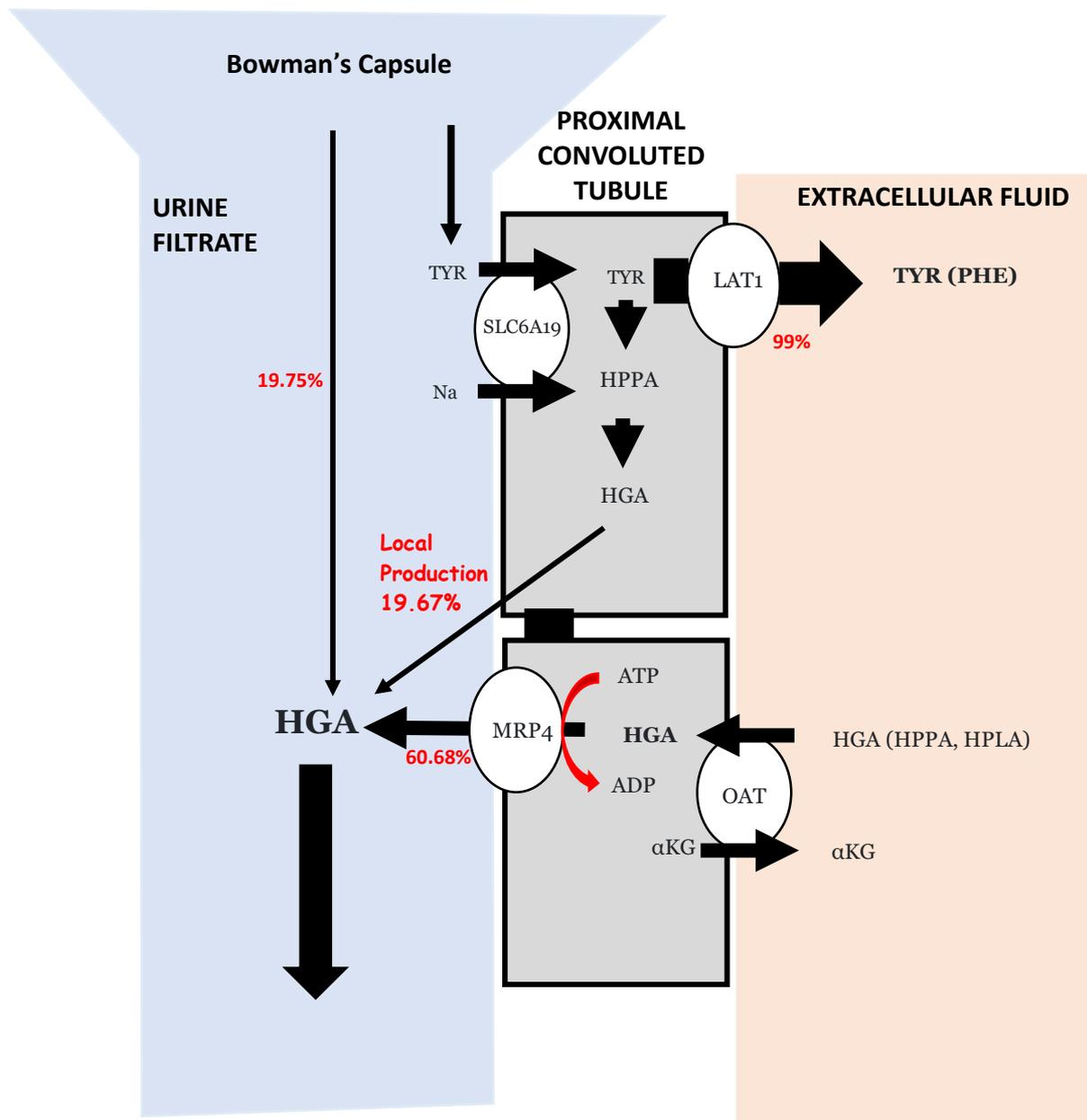
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Figure S4. Linear regression analysis of sHGA, uHGA<sub>24</sub> and CL<sub>HGA</sub> with other parameters showing: (A) – positive linear relationship between sHGA and uHGA<sub>24</sub>; (B) – negative linear relationship between sHGA and CL<sub>HGA</sub>; (C) - negative linear relationship between sHGA and FE<sub>HGA</sub>; (D) – positive linear relationship between uHGA<sub>24</sub> and CL<sub>HGA</sub>; (E) - positive linear relationship between uHGA<sub>24</sub> and FE<sub>HGA</sub>; (F) - positive linear relationship between CL<sub>HGA</sub> and FE<sub>HGA</sub> (Solid blue line – linear regression; dotted blue line – 95% confidence interval).



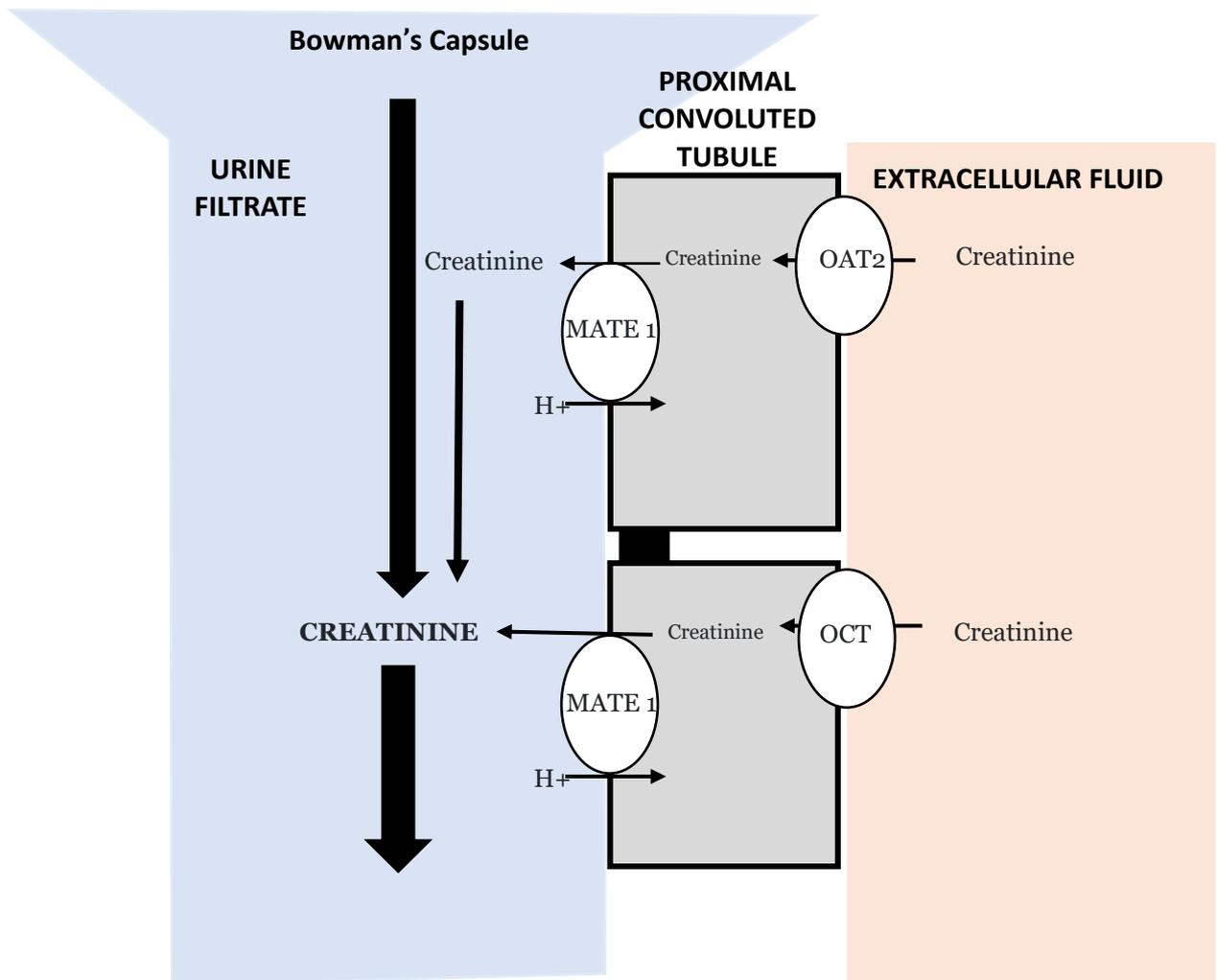
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Figure 3a. Handling of phenylalanine, tyrosine and homogentisic acid in the kidney. Possible transporters are also shown. (TYR: tyrosine; PHE: phenylalanine; HPPA: 4-hydroxyphenylpyruvic acid; HGA: homogentisic acid; Na: sodium; MRP 4: multiple resistance-associated protein 4 (ABC transporter); ATP: adenosine triphosphate; ADP: adenosine diphosphate; SLC6 A19: also known as B<sup>0</sup> or Sodium-dependant transporter or Solute Carrier Family 6 Member 19; OAT: organic anion transporter;  $\alpha$ KG: alpha ketoglutarate; LAT 1: L-type amino acid transporter 1). Glomerular filtration, tubular secretion and tubular production of HGA in Combined AKU group is 15.7%, 62.9% and 27.4% respectively, to total HGA renal elimination.



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Figure 3b. Creatinine excretion and the kidney. (MATE 1: Multidrug and toxin extrusion protein 1; OAT 2: Organic anion transporter 2; OCT: Organic cation transporter).



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## **Discussion**

Our group has carried out assessments in AKU from four different cohorts, and by combining the data from these cohorts we have obtained the largest dataset in this rare disease. The individual data from the four separate cohorts are shown in Table S1. Merging data in this way also generated data with sufficient power to reliably compare gender and age differences in metabolism in AKU patients.

**Kidney and HGA, TYR and PHE:** The HGA concentration in the body fluid compartments is the most important determinant of the evolution of AKU. The kidney is crucial in AKU as it has significant effects on tyrosine metabolism including handling of HGA thus maintaining low HGA levels within the body. Renal failure leads to accelerated disease in AKU while renal transplantation decreases serum and urine HGA (12, 13, 14). Glomerular filtration, tubular reabsorption and secretion are involved in dealing with circulating PHE, TYR and HGA, although their relative contribution in AKU is unknown. The kidney also expresses HGD (6) and should be able to metabolise phenylalanine and tyrosine via HGA to fumarate and acetoacetate in healthy and to HGA in AKU subjects; but this renal production of HGA has never been previously shown. However, an active tyrosine pathway causing nephropathy in hereditary tyrosinaemia type 1 (HT 1) is previously described, although the magnitude of the activity in the renal tyrosine pathway remains unknown (24).

**Glomerular filtration in AKU:** Glomerular filtration in this dataset was assessed by eGFR (22) and traditional  $CL_{CR}$ . There is a strong positive relationship between eGFR and  $CL_{CR}$  confirming the robustness of using these parameters to assess renal glomerular function in the present analysis.  $CL_{CR}$ , like eGFR, showed the expected decline with age (25). Contrary to expectations, eGFR was significantly increased, and serum creatinine significantly decreased,

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in AKU compared with non-AKU controls, even though the AKU group was older;  $CL_{CR}$  showed a trend to be higher in AKU than in non-AKU controls (single tail unpaired 't' test  $p < 0.04$ ). This could be due to sarcopenia in AKU, a severely debilitating disease of spine and joints, in relation to eGFR. However, this cannot be the full explanation since  $CL_{CR}$  is also trending to be higher in AKU subjects than in non-AKU controls. Further, eGFR is likely to be less useful in estimating glomerular filtration than in normal.

Even in normal human subjects,  $CL_{CR}$  does not only reflect glomerular filtration because there is also net tubular secretion of creatinine (26), suggesting that  $CL_{CR}$  overestimates glomerular filtration. In terms of tubular secretion, creatinine has been shown to enter the renal tubular cell through the basolateral route via a number of transporters including organic anion transporter 2 (OAT2) and organic cation transporters (OCT 1 and 2). Intracellular creatinine is then secreted into the urinary filtrate via the multidrug and toxin extrusion protein 1 and 2 (MATE 1 and 2 or SLC47A1 transporter) apical transporters (Figure 3a) (27). With chronically increased serum levels of organic anions in AKU from birth (overproduction of HGA), we postulate that this leads to chronic compensatory upregulation of renal OAT 2 and possibly cation transporters, to increase HGA excretion (28, 29). HGA as an anion can, like creatinine, not only be filtered at the glomerulus, but also be secreted by the tubular cells. HGA tubular secretion is expected to follow a similar process to that for creatinine, being taken up into the tubular cell via basolateral OATs before the intracellular HGA is secreted into the urinary filtrate via the ABC-transporter protein, multiple resistance-associated protein 4 (MRP 4) (30) (Fig 3a, b; drawn based on current knowledge). It is therefore possible that the need to efficiently eliminate HGA also leads to more efficient creatinine elimination, lower serum creatinine, higher eGFR as well as a trend to increased  $CL_{CR}$  in AKU patients. It is noteworthy that there is a significant amount of literature on factors decreasing creatinine tubular secretion

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(31, 32) but not with respect to those increasing creatinine tubular secretion.

**Ageing and gender and AKU:** AKU symptoms worsens with age (1). It is worth noting that besides a decreased  $CL_{CR}$  and eGFR with age, older patients had increased sHGA, and decreased  $uHGA_{24}$ ; all of these findings can be explained by the observed decreased  $CL_{HGA}$  and decreased  $FE_{HGA}$  with ageing in the AKU group. Interestingly, decrease in  $uTYR_{24}$  and  $CL_{TYR}$  with age may point to a common mechanism such as impaired tubular secretion or reduced blood flow with age.

Female sex is associated with less severe and delayed disease progression compared to males (1). sCR was increased in male AKU compared with female AKU patients as one would expect; however,  $CL_{CR}$  was increased in male AKU compared with female AKU consistent with the idea of more efficient creatinine secretion with higher HGA urinary excretion as already described. Although circulating HGA is similar in males and females,  $uHGA_{24}$  was increased in males; interestingly circulating TYR was increased in males without being accompanied by increased  $uTYR_{24}$  or  $CL_{TYR}$  or  $FE_{TYR}$ , suggesting greater possible conversion of filtered TYR to HGA in the renal tubular cell in males before being excreted as HGA into the urinary filtrate.

**Role of kidney in tyrosine metabolism:** With increasing  $CL_{CR}$ , there is increased  $uTYR_{24}$ , higher sTYR, greater  $CL_{TYR}$  and more  $FE_{TYR}$ , which is consistent with the important role of the kidney in tyrosine metabolism. Higher sHGA associates with lower  $CL_{TYR}$  and  $FE_{TYR}$ , as well as higher sPHE, possibly due to a common denominator of decreasing renal function. The higher sTYR was associated with greater  $uHGA_{24}$  suggesting renal tubular conversion to HGA prior to elimination in the urine; the higher sTYR was also related to higher  $uTYR_{24}$  and higher  $CL_{HGA}$ , as expected due to greater tyrosine load in the kidney. Also, higher  $uHGA_{24}$  was

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associated with greater  $uTYR_{24}$ , increased  $CL_{TYR}$ , consistent with the presence of an active tyrosine pathway in the kidney and renal tubular conversion of tyrosine to HGA.

Greater  $uTYR_{24}$  was seen with higher  $uPHE_{24}$ , higher  $CL_{PHE}$ , and higher  $FE_{PHE}$ , consistent with a renal tubular conversion of PHE to TYR; this was also supported by the finding of an association between higher  $uPHE_{24}$  linked to higher  $CL_{TYR}$ , higher  $CL_{PHE}$ , higher  $FE_{TYR}$  and higher  $FE_{PHE}$ . While these relationships are suggestive and supportive of active renal tubular conversion of phenylalanine and tyrosine to HGA, it is realised that this is not proof of causal relationships.

**Components of renal HGA handling:** If the circulating HGA increases, there is greater likelihood of ochronotic damage. Development of ochronosis is slow in AKU and is apparent around the second decade (1). The severity of AKU both in terms of ochronosis and its consequences of breakdown of tissues, increases with age. Therefore, elimination of HGA through the renal route as already mentioned is crucial (30). The observed  $CL_{HGA}$  is very efficient, much higher than predicted by complete clearance of HGA from renal plasma in one pass, and cannot be explained solely by glomerular filtration and the quantitatively more important net tubular secretion; glomerular filtration and tubular secretion comprise 20% and 80% of total elimination respectively, if these two were the only factors contributing to urinary HGA. The renal tubular cell can also take up HGA from the renal extracellular fluid via the organic anion transporters, such as OAT 1, OAT 2 and OAT 3 (33, 34, 35). Renal tubular HGA can then be eliminated via the ABC Transporter, MRP 4 (30), into the urine. Interestingly, creatinine is also handled via the OAT 2 in the basolateral membrane followed by excretion by the apical multidrug and toxin extrusion protein 1 (MATE 1) or SLC47A1 transporter, as already mentioned (32). In AKU due to the need to excrete large quantities of HGA anions from

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birth, it is reasonable to postulate compensatory chronic over-expression of the OAT/MRP 4/MATE 1 transporters (28, 29). The clearance of HGA greater than the theoretical renal plasma flow is unphysiological (36), and can only be explained on the basis of local production of HGA through the presence of the full tyrosine pathway in the renal cells. The  $CL_{HGA}$  data is also strongly supported by similar  $FE_{HGA}$  data. This is the first time the magnitude of the renal contribution to the PHE/TYR pathway has been clearly demonstrated by data. It can also then be presumed that PHE and TYR filtered along with HGA in the glomerulus is reabsorbed via luminal solute transporter SLC6A19 (37, 38, 39) into the renal cell; the PHE and TYR can then be pumped out basolaterally into the extracellular fluid through the complex transporter, a glycoprotein termed CD98 that is a heterodimer composed of SLC3A2 and SLC7A5 (40).

However, PHE and TYR can also be metabolised within the renal tubular cells to HGA in AKU and excreted in the urine; the deamination of tyrosine, by providing nitrogen, could contribute to ammonium generation by the renal tubular cells to maintain acid base balance, besides providing energy substrates to the cells through fumarate and acetoacetate.

The limitation of the clearance analyses of HGA, TYR and PHE, due to lack of mean 24-h values needs to be pointed out, since single sample morning values were used instead for the data from NAC, SONIA 2 and SOFIA; obtaining mean 24-h values for all subjects is impractical. However, 24-h profiles from SONIA 1 were used to calculate mean serum 24-h values and shown in Table S2 and Fig S5. The mean 24-h serum values for TYR and PHE were very similar to single sample TYR and PHE values; however, mean 24-h serum values for HGA were higher than the single sample HGA, despite which  $CL_{HGA}$  remained greater than the theoretical renal plasma flow (Figure S6). It is therefore believed that the analyses using single sample values of  $sHGA$ ,  $sTYR$  and  $sPHE$  for  $CL_{HGA}$ ,  $CL_{TYR}$  and  $CL_{PHE}$  in the NAC, SONIA 2

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and SOFIA is still meaningful. It is worth pointing out that the renal production of HGA was greatest in SOFIA, where the majority of patients were younger than 35 years of age (15). The 24-h profiles for sHGA, sTYR and sPHE are also shown as Figure S5. Calculation of the contributions from glomerular filtration, tubular secretion and tubular production to renal elimination of HGA in the combined AKU group was estimated as percentages which were 19.75, 60.68 and 19.67 respectively (Fig 3a, Table S3).

In summary, we present data on tyrosine metabolism in AKU in the largest number of patients ever studied. As expected, the amount of HGA in the body fluids and urine is increased in AKU. The data shows the crucial part played by the kidneys in slowing down the progression of symptoms in AKU. The roles played by glomerular filtration, tubular secretion and local production of HGA are documented in this dataset. Possible molecular mechanisms for these processes are described for the first time. The filtered amino acids PHE and TYR may also contribute to the metabolic picture in AKU.

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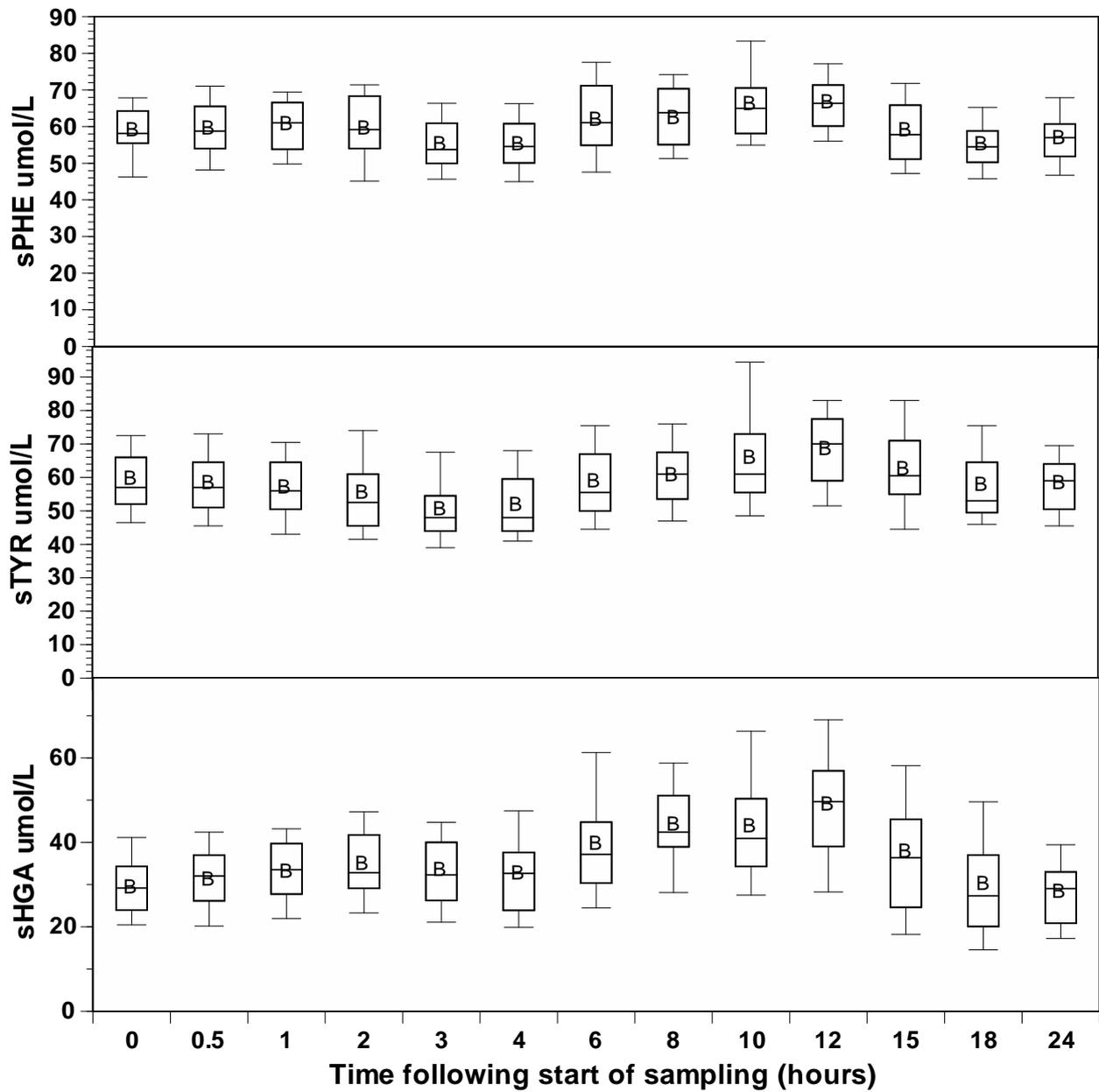
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	Fasting serum value	Mean 24-h serum value
sHGA (μmol/L)	29.2±1.3	36.5±1.5
sTYR (μmol/L)	59.7±2.1	59.9±1.5
sPHE (μmol/L)	58.9±1.4	59.7±1.0

	Glomerular filtration %	Tubular secretion %	Renal production %
Combined AKU group (n=225)	19.75±0.74	60.68±1.04	19.67±1.41
NAC (n=68)	25.86±1.92	61.59±1.85	12.64±2.33
SONIA 1 (n=37)	19.56±1.08	67.14±1.91	13.30±2.62
SONIA 2 (n=90)	17.03±0.79	59.14±1.70	23.82±2.32
SOFIA (n=30)	14.87±1.07	54.80±2.82	30.33±3.82
Values expressed as Mean±SEM			

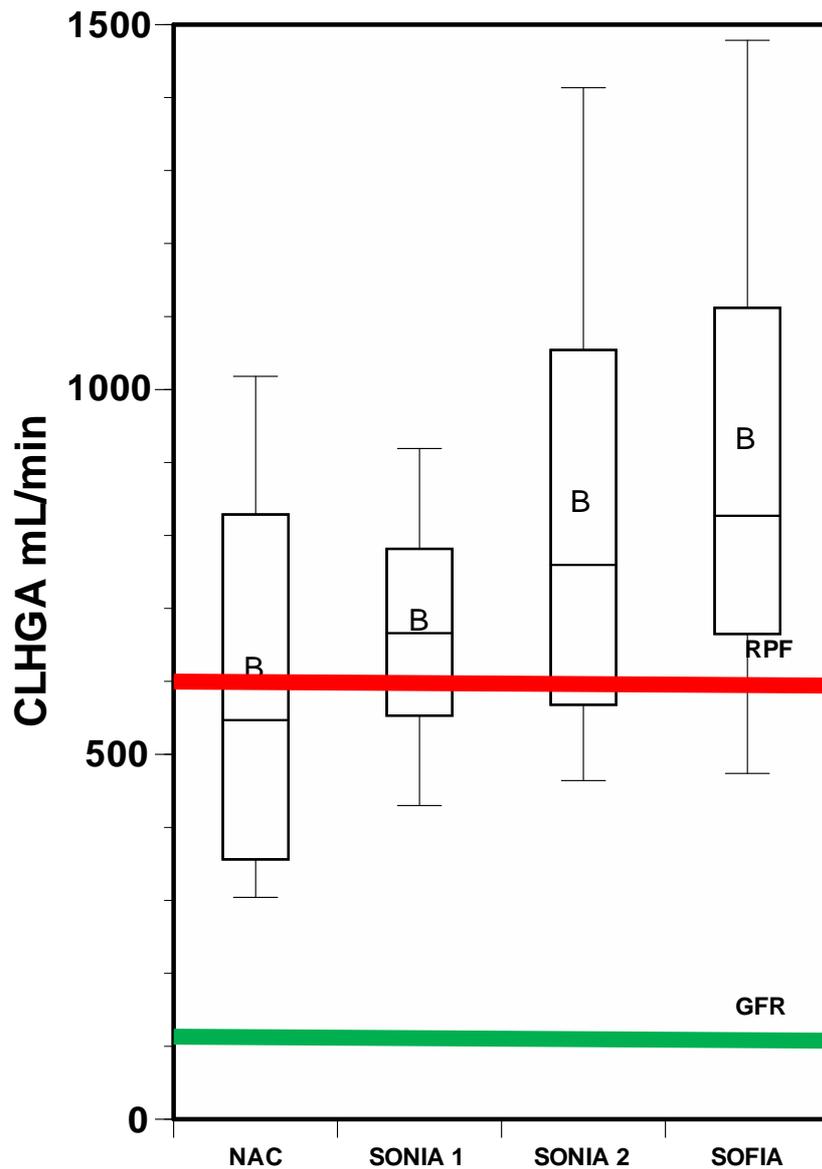
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Figure S5 showing 24-hour serum profiles of PHE, TYR and HGA in SONIA 1 pre-nitisinone (n = 40)  
(Box plots with square (mean) and horizontal line (median) inside the box).



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Figure S6 showing  $CL_{HGA}$  using mean 24-h serum HGA profile in SONIA 1 but single morning sHGA in the NAC, SONIA 2 and SOFIA showing comparable clearances of HGA in all 4 groups. Highest clearances in SOFIA containing the youngest patients. (Box plots with square (mean) and horizontal line (median) inside the box; GFR is glomerular filtration rate (green line).



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Fig S7. Proportions of renal tubular filtration, secretion and production contributing to renal HGA elimination (ALL: combined AKU group; NAC: National AKU Centre; SN1: SONIA 1; SN2: SONIA 2; SOF: SOFIA)

