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## Contribution to the study of uremic toxins in the context of chronic kidney disease

Dan Yi

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## **Contribution to the Study of Uremic Toxins in the Context of Chronic Kidney Disease**

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## List of Publications

- A. Bouchara\*, **Dan Yi**\*, M. Pastural, S. Granjond, J.C. Selag, M. Laville, W. Arkouch, S. Pelletier, D. Fouque, C. O. Soulage, L. Koppe: Serum levels of the adipokine Zinc-Alpha2-Glycoprotein (ZAG) predict mortality in hemodialysis patients. (Kidney International, Under press, 2018)
- **Dan Yi**\*, Elisa Bernardes Monteiro\*, Stéphane Chambert, Julio B. Daleprane, Christophe O. Soulage: Determination of the binding properties of p-cresyl glucuronide to human serum albumin. Biochimie, 2018; 150:1-7
- Nans Florens, **Dan Yi**, Laurent Juillard, Christophe O Soulage: Using binding competitors of albumin to promote the removal of protein-bound uremic toxins in hemodialysis: Hope or pipe dream? Biochimie, 2018; 144:1-8.
- Juliana F. Saldanha\*, **Dan Yi**\*, Milena B. Stockler-Pinto, Hédi A. Soula, Stéphane Chambert, Denis Fouque, Denise Mafra, Christophe O. Soulage: Determination of the binding properties of the uremic toxin phenylacetic acid to Human Serum Albumin. Biochimie, 2016; 125: 53-58.
- Milena B. Stockler-Pinto, Juliana F. Saldanha, **Dan Yi**, Denise Mafra, Denis Fouque, Christophe O. Soulage: The uremic toxin indoxyl sulfate exacerbates reactive oxygen species production and inflammation in 3T3-L1 adipose cells. Free Radical Research, 2016; 50(3): 337-344.
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## Articles under publication

- Line S. Bisgaard, **Dan Yi**, Fitsum Guebre-Egziabher, Markus H. Bosteen, Annemarie A. Pedersen, Christina K. Mogensen, Lars Bo Nielsen, Salka E. Rasmussen, Christophe O. Soulage, Tanja X. Pedersen: Moderate uraemia does not always result in insulin resistance in mice. (Submitted to Clinical and Experimental Nephrology)

## Conferences

- **Dan Yi**, Milena Barcza Stockler Pinto, Fitsum Guebre-Egziabher, Christophe O. Soulage: Indoxyl-sulfate, aryl hydrocarbon receptor (AhR) activation and cardio-metabolic risk in chronic kidney disease.
  - The 24th meeting of G2L2 association (Geneve, Grenoble, Lyon, Lausanne), May 19, 2017, Lyon, France
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Now I am approaching the end of my PhD. I still have a lot of dreams: to be a scientist, to win the Nobel Prize, and World Peace...

PhD is just a start...





*世界和平！*

*World Peace!*

*Paix Mondiale!*



## **Introduction de la thèse (français)**

L'insuffisance rénale chronique (IRC) est une affection caractérisée par une perte progressive de la fonction rénale au fil du temps et associée à l'accumulation de diverses toxines urémiques. Les toxines urémiques ou solutés de rétention urémiques sont des composés qui s'accumulent chez les patients atteints d'IRC en raison d'un défaut de clairance rénale et qui exercent des effets biologiques délétères. Selon les caractéristiques physicochimiques affectant leur comportement de dialyse, les toxines urémiques sont classées en 3 groupes: composés hydrosolubles de faible poids moléculaire, moyennes molécules, et composés liés aux protéines plasmatiques. L'hémodialyse (HD) peut éliminer les petits composés hydrosolubles. Les moyennes molécules peuvent être éliminées par hémodialyse à haut flux. Cependant, les hémodialyses éliminent mal les toxines urémiques liées aux protéines (protein-bound uremic toxins, PBUTs), en raison de leurs interactions étroites avec les protéines plasmatiques, en particulier la sérumalbumine humaine (SAH). En conséquence, les toxines urémiques liées aux protéines s'accumulent chez les patients atteints d'IRC et leur concentration peut difficilement être diminuée chez les patients atteints d'insuffisance rénale terminale (IRT).

Mes travaux sont principalement centrés sur les toxines urémiques, en particulier les toxines urémiques liées aux protéines, comme l'indoxyl sulfate (IS), l'acide phénylacétique (PAA) et le p-crésylglucuronide (p-CG), qui sont des toxines urémiques liées aux protéines; et la zinc-alpha2-glycoprotéine (ZAG) qui est une moyenne molécule. Nous avons étudié le rôle de l'IS dans le développement de la

résistance à l'insuline (IR) et d'autres troubles métaboliques associés à l'IRC, et étudié ses effets sur l'inflammation et le stress oxydant. Pour le PAA et le p-CG, nous avons exploré leurs propriétés de liaison à la sérumalbumine humaine, qui est la plus abondante protéine dans le plasma humain. Pour l'étude clinique, nous avons suivi 252 patients sous hémodialyse pendant 4 ans pour étudier l'impact de l'accumulation de ZAG sur la mortalité et les risques cardiovasculaires. L'hémodialyse n'a qu'un impact limité sur l'élimination des PBUT car ils sont étroitement liés aux protéines plasmatiques, en particulier la sérumalbumine. Ainsi, nous avons essayé de développer une nouvelle stratégie d'élimination des PBUT chez les patients atteints d'IRC, à l'aide de déplaceurs/compétiteurs chimique. Nous avons conçu et synthétisé un certain nombre de composés en fonction de leur affinité de liaison à la sérumalbumine, qui ont été explorés et analysés par docking.

Dans cette thèse sont présentés successivement la maladie rénale chronique (différents stades d'IRC et techniques de suppléance rénale), les toxines urémiques (classification, toxines urémiques liées aux protéines) et la sérumalbumine (y compris sa structure et ses propriétés et sites de liaison), puis résumé les résultats et publications réalisées pendant mon doctorat.

## **Introduction of the thesis (English)**

Chronic kidney disease (CKD) is a condition characterized by a gradual loss of kidney function over time, and associated with the accumulation of variety of uremic toxins. Uremic toxins or uremia retention solutes are compounds that accumulate in CKD patients due to a defect in their renal clearance and exert biological deleterious effects. According to the physicochemical characteristics affecting their clearance during dialysis, uremic toxins are classified into small water-soluble compounds, middle molecules or protein-bound solutes. Hemodialysis (HD) can efficiently remove the small water-soluble compounds. Middle molecules can be removed by high flux hemodialysis. However, protein bound uremic toxins (PBUTs) are poorly removed by hemodialysis, due to their tight interactions with plasma proteins, especially human serum albumin (HSA). As a result, protein bound uremic toxins accumulate in CKD patients and their concentration can hardly be decreased in end-stage renal disease (ESRD) patients.

My study was focused on the uremic toxins especially protein bound uremic toxins and middle molecules in the context of CKD, such as indoxyl sulfate (IS), phenylacetic acid (PAA) and p-cresyl glucuronide (p-CG), which are protein bound uremic toxins; and zinc-alpha2-glycoprotein (ZAG) which is a middle molecule. We investigated the role of IS in the development of insulin resistance (IR) and other metabolic disorders associated with CKD, and studied its effects on inflammation and oxidative stress. For PAA and p-CG, we determined their binding properties to human serum albumin, which is the most abundant protein in human plasma. For clinical

study, we followed 252 patients under hemodialysis for 4 years to study the impact of ZAG accumulation on mortality and cardiovascular risks. HD has only a limited impact on PBUTs removal because they are tightly binding to plasma proteins especially HSA. Thus, we tried to develop a new strategy of PBUTs removal in CKD patients with the use of chemical displacers. We designed and synthesized some innovative compounds according to their binding affinity to HSA, which were explored and analyzed by virtual screening and docking experiment.

This thesis first introduced CKD (different stages of CKD and the therapy), uremic toxins (classification, protein bound uremic toxins) and HSA (including its structure , binding properties and its main binding sites), then, summarized my findings and publications during my PhD, dicussed and interpreted some questions, hypotheses I posed or reviewed in my study, and at last prospected the future study on CKD and uremic toxins.

## List of Abbreviations

	$\alpha$ -h	$\alpha$ -helice
	$\beta$ -h	$\beta$ -helice
A	AAG, AGP	alpha-1-acid glycoprotein
	AAP	antiplatelet agent
	ADH	anti diuretic hormon
	ADMA	asymetric dimethylarginine
	ADPKD	autosomal dominant polycystic kidney disease
	ADR	Annual Data Report
	AGE	advanced glycation end-products
	AhR	aryl hydrocarbon receptor
	Ala	alanine
	ANF	alpha-naphtoflavone
	ANP	atrial natriuretic peptide
	Arg	arginine
	ARNT	aryl hydrocarbon receptor nuclear translocator
	ARRIVE	Animal Research: Reporting of In Vivo Experiments
	Asn	asparagine
	ATP	adenosine triphosphate
	AUC	area under the curve
	AVF	arteriovenous fistula
	AVG	arteriovenous graft
B	BFGF	basic fibroblast growth factor
	BMI	body mass index
	BP	blood pressure
	BSG	N-benzenesulfonyl glycine
C	C8	octanoic acid
	C10	decanoic acid
	CADD	computer-aided drug design
	CC16	clara cell protein
	CGRP	calcitonin gene-related peptide



	CKD	chronic kidney disease
	CMC	Cambridge MedChem Consulting
	CML	N(6)-carboxymethyllysine
	CMPF	3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid
	CNS	central nervous system
	CRDS	Chinese Renal Data System
	CRP	C-reactive protein
	CV	cardiovascular
	CVD	cardiovascular disease
	Cys	cysteine
D	DCT	distal convoluted tubule
	DHOA	2,8-dihydroxyadenine
	DIO	diet induced obesity
	DIP I	degranulation inhibiting protein I
	DST	distal straight tubule
E	eGFR	estimated glomerular filtration rate
	ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
	ESRD	end stage renal disease
	ESRF	end-stage renal failure
	EU	European Parliament
	EUTox	European Uremic Toxin Work Group
	eWAT	epididimal white adipose tissue
F	FA	fatty acid
G	GFR	glomerular filtration rate
	GKHA	Global Kidney Health Atlas
	Glu	glutamic acid
	Gly	glycine
H	HD	hemodialysis
	HDL	high-density lipoprotein
	HIF	hypoxia-inducible factor
	His	histidine
	HOMA-IR	Homeostasis model assessment of insulin resistance
	HR	hazard ratio

	HSA	human serum albumin
	HUVEC	human umbilical vein endothelial cell
I	IGF-1	insulin-like growth factor 1
	IL-10	interleukin-10
	Ile	isoleucine
	iNampt	intracellular nicotinamide phosphoribosyltransferase
	IPGTT	intraperitoneal glucose tolerance test
	IPITT	intraperitoneal insulin tolerance test
	IR	insulin resistance
	IRS-1	insulin receptor substrate-1
	IS	indoxyl sulfate
	ISN	International Society of Nephrology
K	KDOQI	Kidney Disease Outcomes Quality Initiative
L	LDL	low-density lipoprotein
	Leu	leucine
	LOQ	limit of quantification
	Lys	lysine
M	MAC	medial arterial calcification
	MCP-1	monocyte chemo-attractant protein-1
	MDA	malondialdehyde
	Met	methionine
	MW	molecular weight
N	NADPH	Nicotinamide adenine dinucleotide phosphate
	NMR	nuclear magnetic resonance spectroscopy
	nPCR	normalized protein catabolic rate
	NS	not significant
	NSAID	non-steroidal anti-inflammatory drug
	NT-proBNP	N-terminal pro-brain natriuretic peptide
	NX, 5/6 Nx	5/6 nephrectomy
O	OAT	organic anion transporter
	ORM	orosomuroid
P	PAA	phenylacetic acid
	PAAT	phenylalanine aminotransferase

	PAH	phenylalanine hydroxylase
	PAI-1	plasma activator inhibitor-1
	PB-DOPA	dihydroxyphenylalanine
	PBSG	N-phenyl-N-benzenesulfonyl glycine
	PBUT	protein-bound uremic toxin
	p-CG	p-cresyl glucuronide
	p-CP	p-cresol phosphate
	p-CS	p-cresyl sulfate
	PCT	proximal convoluted tubule
	PD	peritoneal dialysis
	PEW	protein energy wasting
	Phe	phenylalanine
	PI3K	phosphatidylinositol 3-kinase
	PKB/Akt	phosphoinositide-3-kinase–protein kinase B
	PST	proximal straight tubule
	PST	phenol sulfotransferase
	PTH	parathyroid hormone
R	RAA	renin-angiotensin-aldosterone
	RBP	Retinol Binding Protein
	RBP-4	retinol binding protein 4
	REIN	French nephrology epidemiology and information network
	rWAT	retroperitoneal white adipose tissue
S	SBP	systolic blood pressure
	scWAT	subcutaneous white adipose tissue
	SD	standard deviation
	SDMA	symetric dimethylarginine
	Ser	serine
	Sirt 1	sirtuin 1
T	T2DM	type 2 diabetes mellitus
	TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
	TNF- $\alpha$	tumor necrosis factor- $\alpha$
	Trp	tryptophan

	Tyr	tyrosine
U	Up4A	uridine adenosine tetraphosphate
	URM	uremic retention molecule
	URS	uremic retention solute
	USRDS	United States Renal Data System
V	Val	valine
	VEGF	vascular endothelial growth factor
	VIP	vasoactive intestinal peptide
W	WAT	white adipose tissue
	WBC	white blood cell
	WCN	World Congress of Nephrology
	WHO	World Health Organization
Z	ZAG	zinc-alpha2-glycoprotein

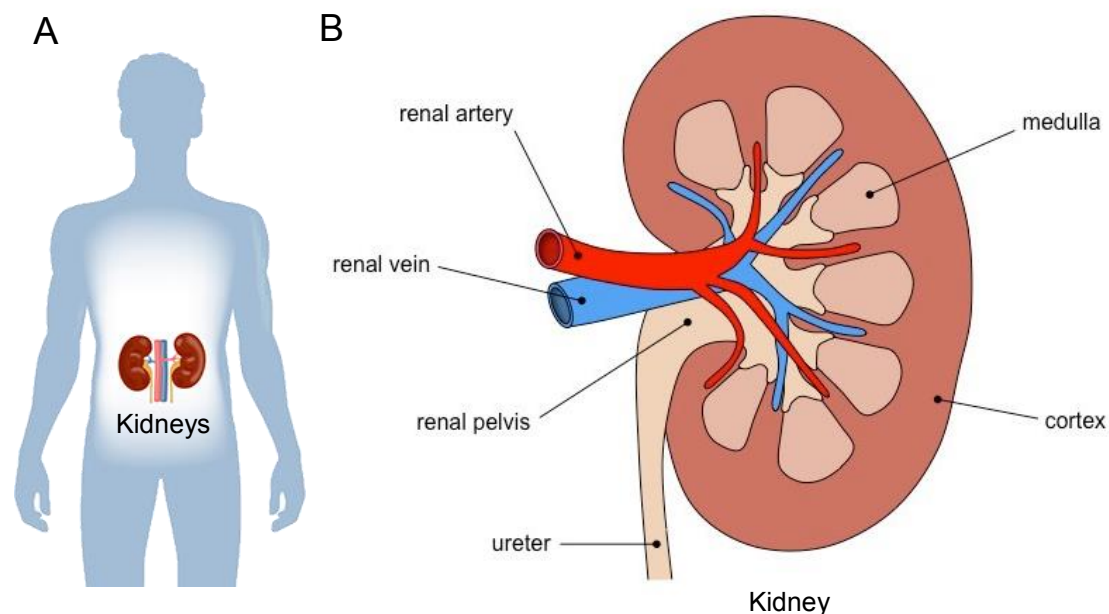


## State of the art

### 1 Chronic kidney disease (CKD)

#### 1.1 Kidney and Nephron

The kidneys are two bean-shaped, fist-sized organs in humans. They are located on the left and right sides at the back of the abdominal cavity in the retroperitoneal space below the rib cage (**Figure 1A**). Kidneys are the main filtration system in the body. They remove non-volatile waste products from the blood and excrete these wastes into urine. **Figure 1B** shows the basic structure of the human kidney. Blood enters the kidneys from the renal arteries and exits into the paired renal veins. Through the kidneys, blood is filtered by specialized structures, **nephrons**, into urine. Each kidney is attached to the ureter that transports excreted urine into the bladder, where the urine is stored prior to excretion.



**Figure 1. A:** The location of the kidneys in human body. (Picture from GW Kidney, <http://www.gwkidney.org>)<sup>2</sup>

**B:** The structure of the kidney. (Picture from BioNinja, <http://ib.bioninja.com.au>)<sup>3</sup>.

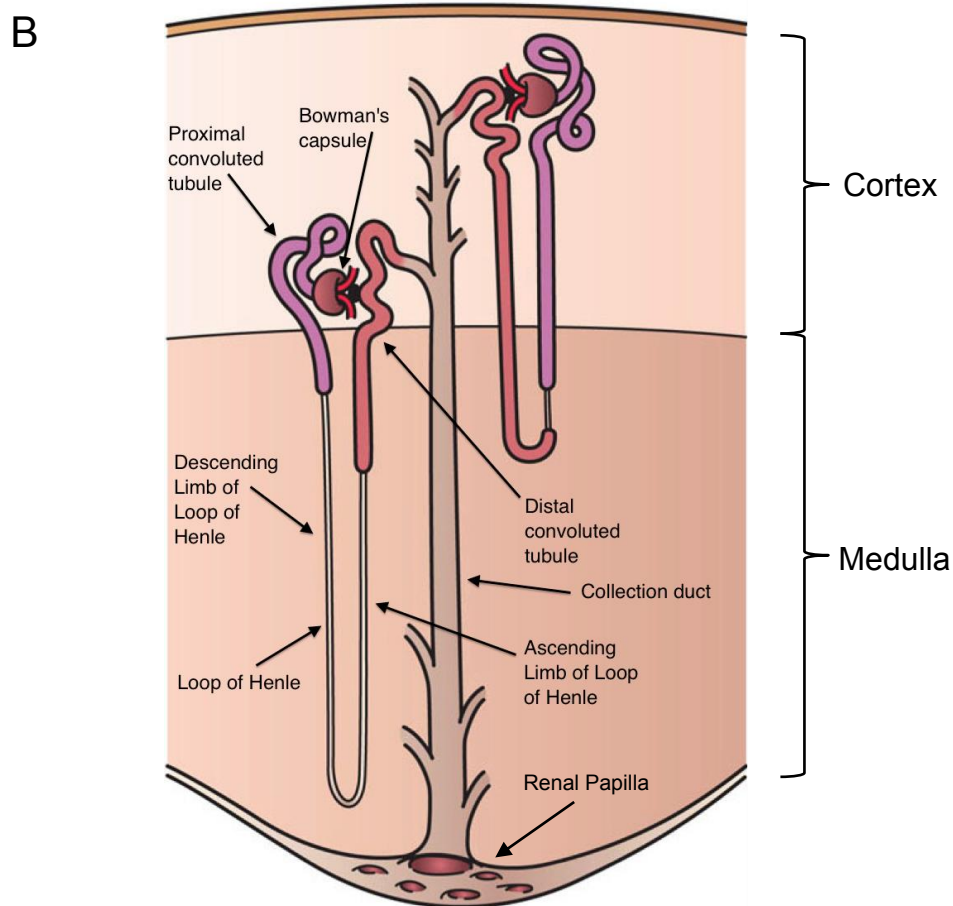
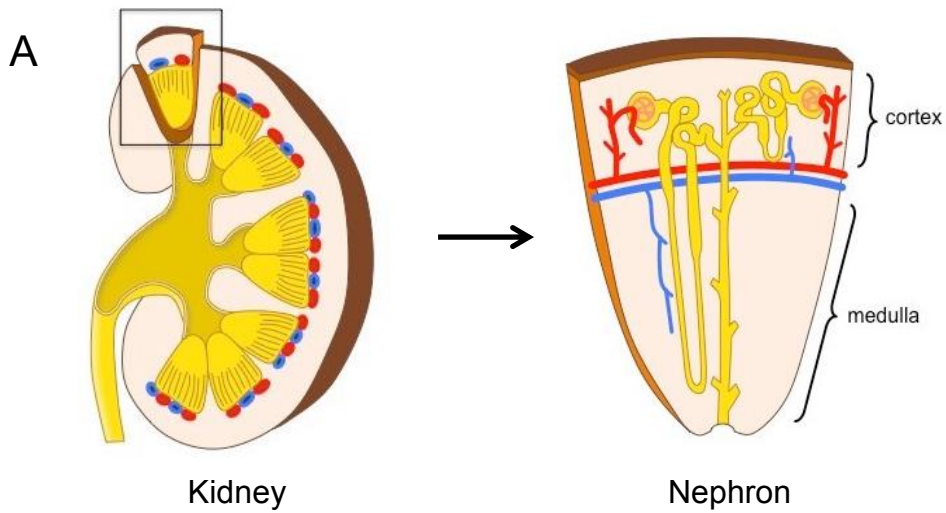
Nephron is the functional and structural unit of the kidney. Each healthy adult human kidney contains 0.8 to 1.5 million nephrons packed in the renal cortex and medulla (**Figure 2A**). The nephron is constituted of renal corpuscle (glomerulus and Bowman's capsule) and renal tubules (proximal tubule, loop of Henle, and distal tubule). The glomerulus is a network of capillaries located in the cortex and enclosed in the cup-like sac that is Bowman's capsule. The proximal convoluted tubule (PCT) leads from Bowman's capsule to proximal straight tubule (PST), which connects to the descending limb of loop of Henlé. The loop of Henlé is a U-shaped selectively permeable tubule loop that descends into the medulla, front connects to the proximal tubule and rear connects to the distal tubule. Distal straight tubule (DST) is adjacent to PST and leads from the ascending limb of loop of Henlé. Distal convoluted tubule (DCT) extends from DST and connects to the collecting duct, which feed into the renal pelvis. There are two configurations of nephrons with different urine concentrating capacities: long juxta-medullary nephron and short cortical nephrons.<sup>4</sup> Only the long juxta-medullary nephron has loops of Henlé in the inner renal medulla (**Figure 2B**).

As the elementary filtration unit of the kidney, nephron functions to filter the blood and eliminate wastes. There are three processes to alter the blood into urine: **filtration, reabsorption and secretion (Figure 3)**. The glomerulus and its surrounding Bowman's capsule constitute the renal corpuscle, which is the basic initial filtration unit. Blood flows into the glomerulus through the afferent arteriole. In

the renal corpuscle, about 20% of the plasma volume passing through the glomerulus at any given time is filtered.<sup>5</sup> Water and most of the solutes filters out of the blood through the wall of glomerular capillaries into Bowman's capsule and the renal tubule that processes and carries away the filtered fluid (**Figure 3A**). Normally, only components of the blood with molecular weight higher than 60 kDa (such as plasma proteins, red blood cells, white blood cells and platelets) are not filtered into Bowman's capsule. The other 80% of blood exits glomerular capillaries into efferent arterioles instead of directly into venules, then continues into peritubular capillaries. 99% of the water and a variety of solutes (glucose, amino acids, calcium, phosphate, bicarbonate and etc.) from the filtrate are reabsorbed into peritubular capillaries by the cells of the tubule as they flow through the renal tubule and collecting duct. Over 70% the filtrate is reabsorbed in the proximal tubule.<sup>5</sup> These reabsorbed substances are then returned to the blood in the peritubular capillaries and vasa recta. As this filtrate passes through the tubule, the renal tubule and collecting duct continue to secrete additional substances into the tubular fluid. Secretion here refers to the removal of substances from the blood. This enhances the clearance of certain wastes and toxins. It is also essential to the regulation of plasma potassium balance and pH.<sup>6</sup> The final process is the excretion into urine, which is conveyed through the collecting tubule into the renal pelvis (**Figure 3B**). The amount of a particular substance that is excreted can be determined by the following formula:

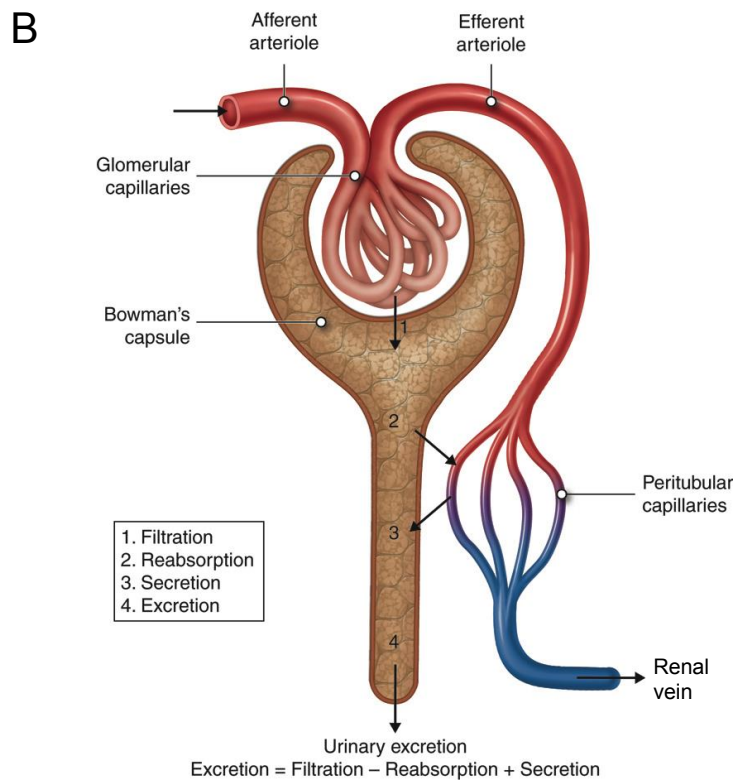
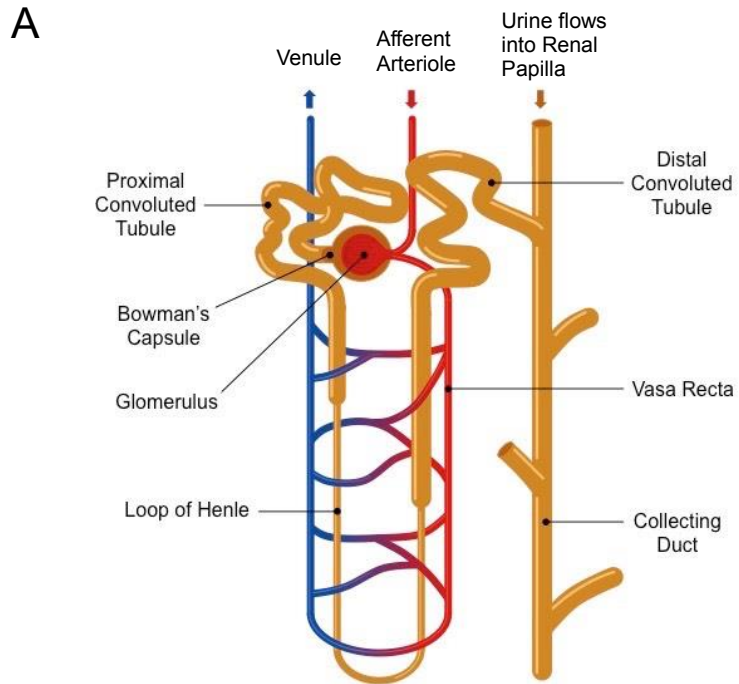
$$\textit{Amount excreted} = \textit{amount filtered} - \textit{amount reabsorbed} + \textit{amount secreted}$$





**Figure 2. A:** Structural organisation of kidney and the location of nephrons. (Picture from BioNinja, <http://ib.bioninja.com.au>)<sup>3</sup>.

**B:** Structure of nephrons: long juxtamedullary nephrons (left) and short cortical nephrons (right). (Picture from Wikipedia, <https://en.wikipedia.org/wiki/Nephron>)<sup>7</sup>.



**Figure 3. A:** The function of nephron and renal blood circulation. (Picture from BioNinja, <http://ib.bioninja.com.au>)<sup>8</sup>

**B:** The renal corpuscle and four processes to convert blood into urine: filtration, reabsorption, secretion, and excretion of numerous substances. (Picture from open learning of Carnegie Mellon University, <http://oil.cmu.edu>)<sup>9</sup>

## 1.2 What is CKD?

Chronic kidney disease (CKD) is the progressive loss of kidney function for a period longer than 3 months. The kidneys are damaged and cannot filter blood the way they should. This damage can cause wastes and uremic toxins to accumulate in the blood and body fluids. CKD is a long-term form of kidney disease. This disease is called “chronic” because the damage of CKD to the kidneys lasts more than 3 months. Thus, CKD is differentiated from acute kidney disease in that the reduction in kidney function must be present over at least 3 months.

Kidney damage is defined as pathologic abnormalities or markers of damage, including abnormalities in blood or urine tests or imaging studies. Blood and urine tests are used to detect and monitor kidney disease. Blood test is a measurement of creatinine to estimate glomerular filtration rate (eGFR, see **1.3** What is GFR?) and diagnose CKD. Urine test is to detect the presence of excess proteins in the urine (i.e. proteinuria). Persistent proteinuria means CKD is present. A persistent reduction of GFR and abnormal urine albumin level are the key markers to identify CKD. Chronic kidney disease is defined as either kidney damage or  $\text{GFR} < 60 \text{ mL/min/1.73m}^2$  for  $\geq 3$  months, according to the criteria listed in **Table 1** (Defined by Kidney Disease Outcomes Quality Initiative (KDOQI) system).<sup>10</sup>

Criteria
1, Kidney damage for $\geq 3$ months, as defined by structural or functional abnormalities of the kidney, with or without decreased GFR, manifest by either: <ul style="list-style-type: none"> <li>· Pathological abnormalities; or</li> <li>· Markers of kidney damage, including abnormalities in imaging tests</li> </ul>
2, $\text{GFR} < 60 \text{ mL/min/1.73m}^2$ for $\geq 3$ months, with or without kidney damage

**Table 1.** Definition of Chronic Kidney Disease based on the KDOQI system.<sup>10</sup>

### 1.3 What is GFR?

The glomerular filtration rate (GFR) is the volume of fluid filtrate from renal glomerular capillaries into Bowman's capsule by both kidneys per unit time. It ranges from 115 to 125 ml/min. The GFR can be regulated by constriction or dilatation of the afferent arterioles.<sup>11</sup> The filtration rate is dependent on the difference between the higher blood pressure created by vasoconstriction of the input or afferent arteriole versus the lower blood pressure created by lesser vasoconstriction of the output or efferent arteriole.

As an index, Glomerular filtration rate is often used to estimate the level of kidney function, detect early kidney damage, determine the stage of kidney disease and help diagnose chronic kidney disease. Specifically, it estimates how much blood passes through the glomeruli each minute. Usually, creatinine level in the blood is tested to estimate GFR, because creatinine is an easily measured by-product of muscle metabolism that it is excreted by the kidneys. Creatinine is a waste that is produced by muscle metabolism via a biological system involving creatine, phosphocreatine, and adenosine triphosphate (ATP). Creatinine is easy to measure and it is only

excreted by the kidneys, so it is often used to estimate GFR.

GFR is calculated based on the results of blood creatinine measurement, age, ethnicity, height, weight and gender. If the GFR is low, it means the kidneys are not working as well as they should. The earlier kidney disease is detected, the better the chance of slowing or stopping the progression of CKD.

GFR equals to the clearance rate when any solute is freely filtered and is neither reabsorbed nor secreted by the kidneys. Clearance rate is the quantity of the substance in the urine that originated from a calculable volume of blood. There are several different equations used to calculate or estimate the glomerular filtration rate (GFR or eGFR). The equation below only applies for GFR calculation when it is equal to the Clearance Rate.

$$\text{Clearance Rate} = \frac{\text{Urine Concentration} \times \text{Urine Flow}}{\text{Plasma Concentration}}$$

#### 1.4 Different stages of CKD

Kidney disease does not happen overnight. It happens slowly over many years and in stages. Based on the level of renal function, chronic kidney disease is divided into 5 stages. For each stage, the kidneys work worse than the stage before. Kidney function decreases from stage 1 to stage 5, as shown in **Table 2**. CKD is a progressive disease, meaning that kidney function can continue to decline over time, eventually resulting in stage 5, the end stage renal disease (ESRD) or kidney failure. The 5 stages are determined through a blood creatinine test to calculate the estimated glomerular

filtration rate (eGFR).

CKD process is commonly not reversible by present medical technology. Kidney function and glomerular filtration rate are lost and cannot be recovered either. With treatment, we may improve GFR and stabilize kidney function. Early detection can help prevent the progression of kidney disease to kidney failure.

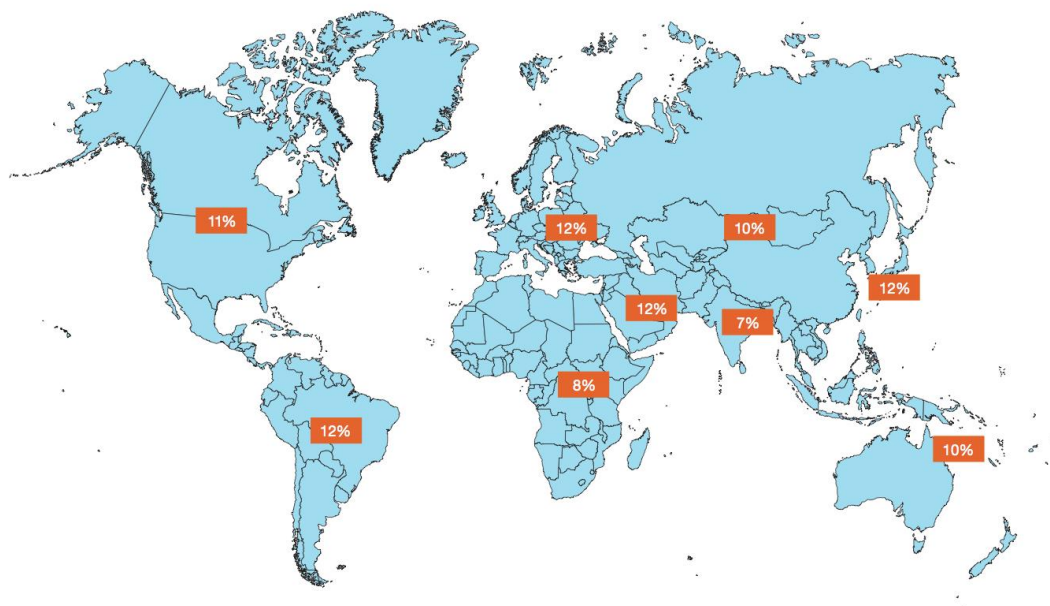
Stage	Description	GFR (mL/min/1.73m <sup>2</sup> )
1	Kidney damage with normal or ↑GFR	≥90
2	Kidney damage with mild ↓GFR	60-89
3	Moderate ↓GFR	30-59
4	Severe ↓GFR	15-29
5	Kidney failure	<15 (or dialysis)

**Table 2.** Chronic kidney disease stages (from KDOQI system)<sup>10</sup>.

### 1.5 Epidemiology of CKD in the world

In 2017, a new global report - the Global Kidney Health Atlas (GKHA) was presented at the World Congress of Nephrology (WCN) in Mexico City (21-25 April 2017). This Atlas is the largest health-related country capacity reviews in history, which is compiled by the International Society of Nephrology (ISN) and kidney health experts worldwide and highlights the huge gaps in kidney disease care and prevention in both developed and developing countries, with many countries not prioritizing kidney health. The multinational cross-sectional survey received the responses of the questionnaire from 125 out of 130 countries (96%), representing an estimated 93% (6.8 billions) of the world's population of 7.3 billions. Overall,

approximately **10% of the global population has CKD**. The prevalence of CKD worldwide varies from 7% to 12% with the geographical region. The prevalence of CKD was highest in Latin America, Europe, East Asia and the Middle East, where approximately 12% of the population has CKD<sup>12</sup>. The lowest prevalence was reported in South Asia (7%) and Sub-Saharan Africa (8%) (**Figure 4**).<sup>13</sup> Although an estimated 10% of people worldwide have CKD, 9 out of 10 of them are unaware of their condition.<sup>14</sup> The global silent epidemic of CKD is ongoing growing, as the risk factors of CKD such as obesity, diabetes, smoking and hypertension continue to grow in many regions.<sup>13,14</sup>

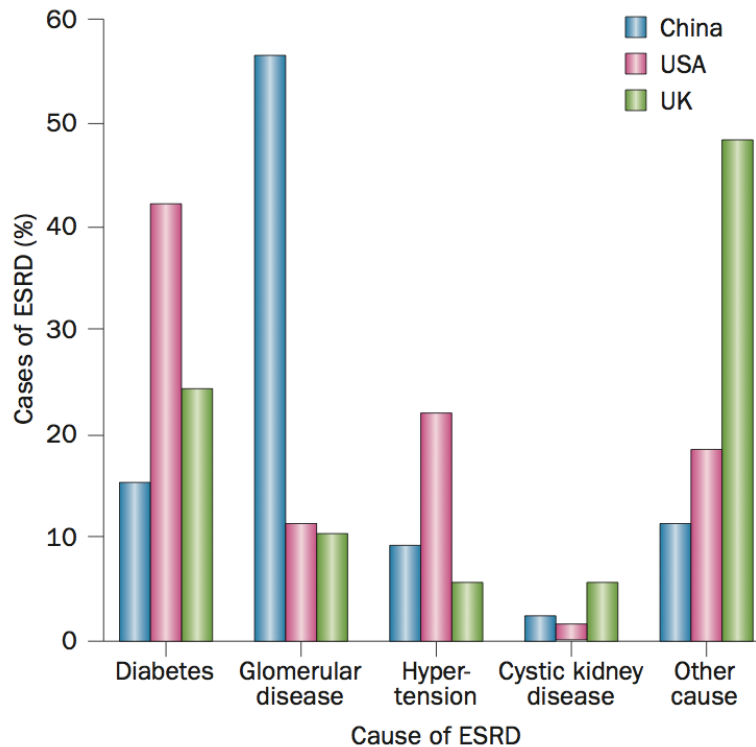


**Figure 4.** Estimated global prevalence of CKD (Picture from ISN Global Kidney Health Atlas<sup>13</sup>; Data source: Nathan R. Hill et al., Plos One, 2018<sup>12</sup>).

In France, 10% of the French population is affected kidney disease that may lead to kidney failure; around 50% cases of kidney failure are due to diabetes or hypertension. The French nephrology epidemiology and information network (REIN) 2015 annual report listed 82,295 patients treated for end-stage chronic renal failure at the end of 2015, where there were 45,862 dialysis patients (6.2% of whom benefit from peritoneal dialysis) and 36,433 recipients of a renal transplant (10.3% of them were obtain from a living donor) in France.<sup>15</sup>

In China, the national survey of CKD from 2009 to 2010 including 47,204 patients shows the overall prevalence of CKD in China was 10.8%; therefore the number of CKD patients in China is estimated to be approximately 119.5 million.<sup>16</sup> The estimated prevalence of CKD in China (10.8%) is similar to that reported in developed countries, but the aetiology is very different from Western countries, such as the USA and the UK. Data from the Chinese Renal Data System (CRDS) revealed that glomerular disease was the major cause of ESRD (57.4%), followed by diabetic nephropathy (16.4%), hypertension (10.5%), and cystic kidney disease (3.5%). These rates differ to that reported in developed countries where the main cause of ESRD is diabetic nephropathy (**Figure 5**).<sup>17</sup>



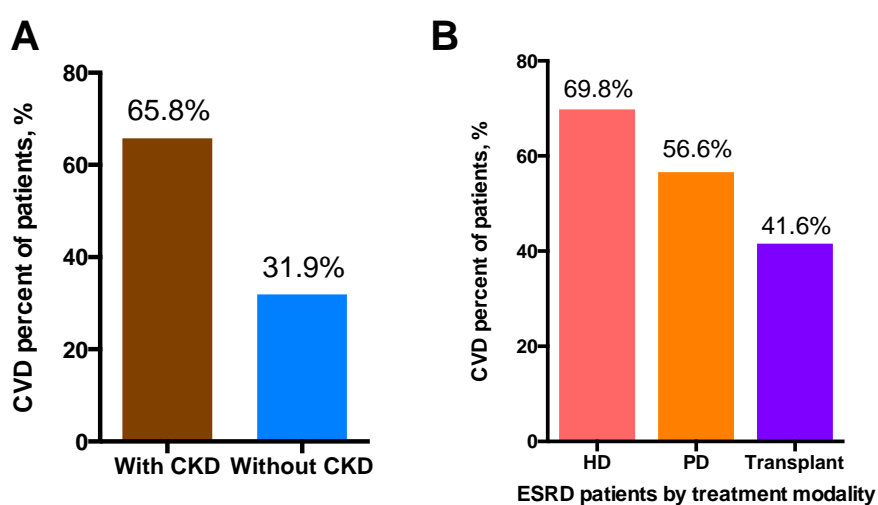


**Figure 5.** Etiology of ESRD in China, the USA and the UK (Zhi-Hong Liu, Nature Reviews Nephrology, 2013).<sup>17</sup>

### 1.6 CKD is associated with cardiovascular disease (CVD)

CKD can cause many health problems like insulin resistance, high blood pressure, anemia, bone disease, malnutrition and nerve damage. These complications may happen slowly over a long period of time. At the same time, **CKD sharply increases the risk of cardiovascular diseases (CVD)**. Some studies shows CKD is a potent harbinger for adverse cardiovascular (CV) outcomes, incorporating both conventional and nonconventional cardiovascular risk factors.<sup>18</sup> In turn, CKD may also be caused by many disorders. Two leading causes of CKD in adults are diabetes and hypertension, which are responsible for up to 2/3 of the cases.<sup>19</sup> Patients with CKD in any stage have a higher risk of cardiovascular diseases than the general

population. **Figure 6** is drawn with the data from United States Renal Data System (USRDS) 2017 Annual Data Report (ADR). The prevalence of cardiovascular diseases in CKD patients is twice as in the patients without CKD (**Figure 6A**). In the end stage of CKD, although the ESRD patients are treated by different therapies (See 1.7 Renal suppletion therapies of CKD), the risk of CVDs is still in the range of 40~70% varies in the treatment modality (**Figure 6B**).



**Figure 6.** Cardiovascular risk in CKD. Figures are drawn with the data from U.S. Renal Data System (USRDS) 2017 Annual Data Report (ADR). (<https://www.usrds.org>)<sup>20</sup>

**A:** Prevalence of cardiovascular diseases in patients with or without CKD, 2015. (USRDS 2017 Annual Data Report Volume 1 CKD, Chapter 4)<sup>21</sup>

**B:** Prevalence of cardiovascular diseases in adult ESRD patients, by treatment modality, 2015. (USRDS 2017 Annual Data Report Volume 2 ESRD, Chapter 8)<sup>22</sup>

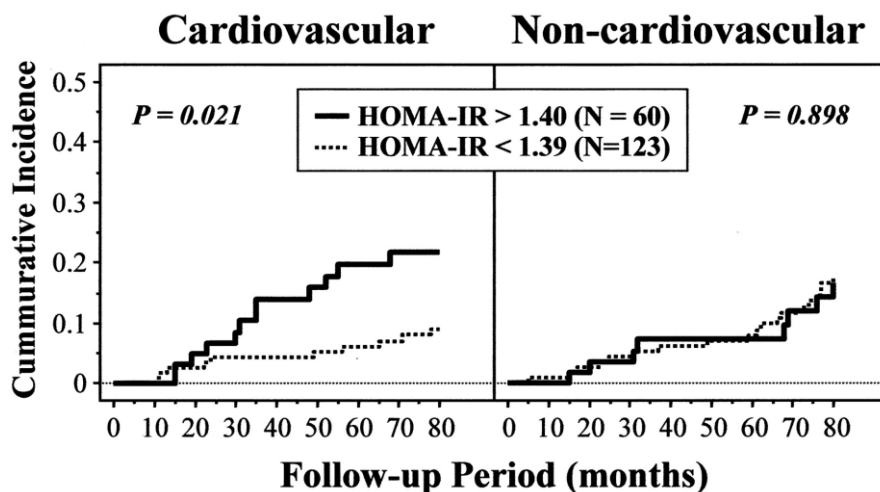
**Abbreviations:** CKD, chronic kidney disease; CVD, cardiovascular disease; HD, hemodialysis; PD, peritoneal dialysis; ESRD, end stage renal disease.

Cardiovascular diseases are the number one cause of death globally. The World Health Organization (WHO, [www.who.int](http://www.who.int)) indicates more people die annually from

CVDs than from any other cause.<sup>23</sup> CVDs also represent the main causes of morbidity and mortality in CKD patients.<sup>24,25</sup> Over 90% of CKD patients will develop cardiac hypertrophy and approximately half of them will die from CVD.<sup>26</sup> Insulin resistance (IR) is an independent risk factor and predictor for cardiovascular mortality and morbidity in CKD patients.<sup>27, 28</sup> Homeostasis model assessment of insulin resistance (HOMA-IR) is an index to assess IR, and calculated using the following formula:

$$HOMA-IR = \text{fasting glucose (mmol/L)} \times \text{fasting insulin (\mu U/ml)} / 22.5$$

The survival curves estimated by the Kaplan-Meier method from Shinohara et al. showed that the patients in higher tertile of HOMA-IR had a significantly higher risk of cardiovascular mortality (**Figure 7**).



**Figure 7.** Kaplan-Meier curves showing association between HOMA-IR and outcomes (Shinohara et al., Journal of the American Society of Nephrology, 2002).<sup>27</sup> Survival curves were estimated by the Kaplan-Meier method and compared between the subjects with and without increased HOMA-IR of  $\geq 1.40$  or higher. P values by log rank test. Insulin resistance was assessed using HOMA-IR.

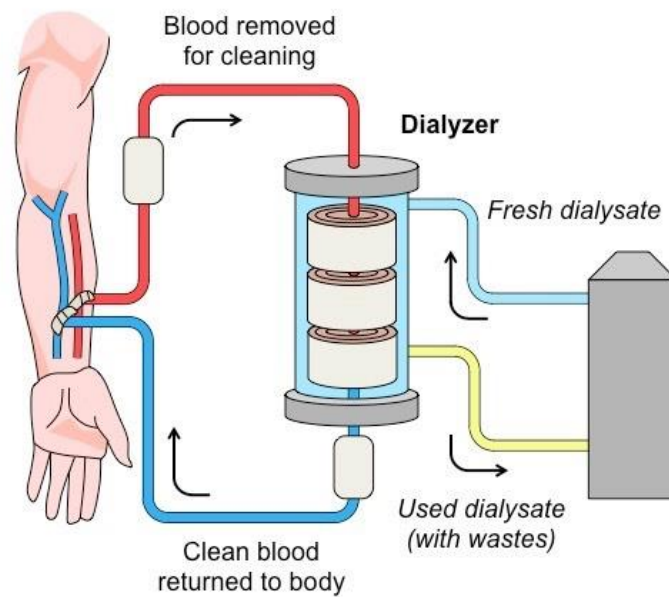
As CKD is a long-term disease, early detection and treatment is very important to keep kidneys from getting worse. If CKD continues progressing, it may eventually lead to kidney failure, which requires dialysis or kidney transplant to maintain life. Chronic kidney disease can be diagnosed with a routine blood or urine test that indicates the kidneys may not work normally. CKD, hypertension, diabetes, obesity and cardiovascular diseases have a complex relationship and affect each other. Many diseases that cause kidney failure may have their origins in childhood. Regular tests for CKD is recommended for people with family history of advanced CKD or an inherited kidney disease protein or blood in their urine where there is no certain cause, and patients with hypertension, diabetes, acute kidney injury, cardiovascular disease and other conditions that can affect the kidneys such as kidney stones, an enlarged prostate or lupus. Early detection and appropriate treatment may improve prognosis of CKD. With periodic examinations of blood pressure, urine albumin and serum creatinine, high-risk people could detect CKD as early as possible.

### **1.7 Renal suppletion therapies of CKD**

CKD is irreversible. While there is no typical cure for CKD, with treatment and lifestyle changes, kidney disease can be prevented from getting worse. Some therapies can reduce the risk of complications. Patients with chronic kidney disease typically need to take a large number of medications. Treatments for CKD includes: Anemia treatment, avoid phosphate unbalance, control the blood pressure, attention to skin

itching, anti-sickness medications, avoid non-steroidal anti-inflammatory drugs (NSAIDs) and end-stage treatment.<sup>29</sup>

For end-stage CKD patients, there are two types of treatment for kidney failure—dialysis and kidney transplant. Dialysis is the process of removing excess water, solutes and toxins from the blood in those whose native kidneys have lost the ability to perform those functions. There are two kinds of dialysis: hemodialysis (HD) and peritoneal dialysis (PD). Hemodialysis is a treatment for kidney failure that uses an artificial machine to work as kidneys and filter the blood. A pump on the hemodialysis machine draws the blood into a tube, a few ounces at a time. The blood flows through the tube to the dialyzer. Inside the dialyzer, blood flows through thin fibers that filter out wastes, extra salt and extra fluid. After the dialyzer filters the blood, a different tube carries blood back to the patients' body (**Figure 8**). This is referred to as renal replacement therapy. In peritoneal dialysis, the inside lining of belly (i.e. peritoneum) acts as a natural filter. Wastes are taken out by means of dialysate, which is washed in and out of the belly in cycles.



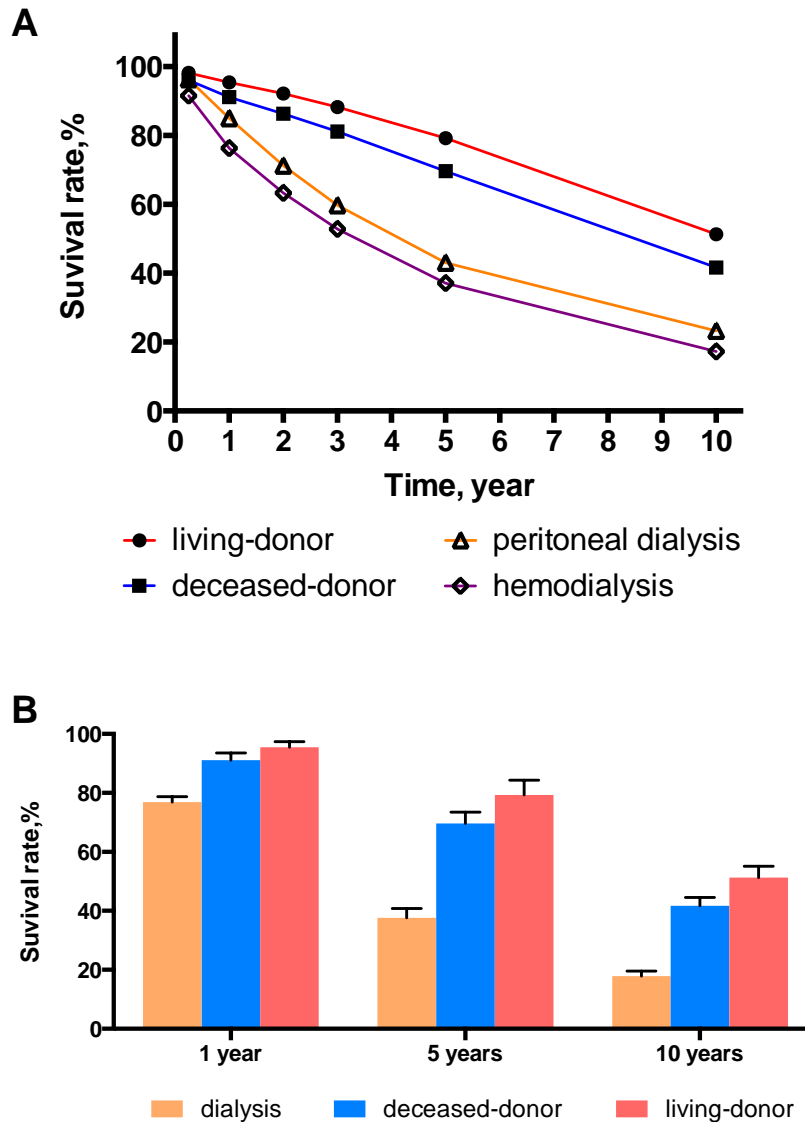
**Figure 8.** Overview of the procedure of hemodialysis. (Picture from BioNinja, <http://ib.bioninja.com.au>)<sup>30</sup>

For the long-term treatment of kidney failure, kidney transplant is the best choice for the majority of patients with end-stage renal disease (ESRD) at present. Kidney transplant is a renal replacement therapy using surgical method to transplant a healthy kidney into the patient that has little or no kidney function, mostly at the end-stage of CKD. Kidney transplantation is typically classified as deceased-donor and living-donor transplantation, depending on the source of the donor organ. Unlike many other types of organ donation, it is possible to obtain a kidney from a living donor because normally there are two kidneys in the human body. This is known as a living donation. Kidney transplantation is a major surgical procedure that has risks both during and after the surgery. The transplant recipients take the risk of blood clots, bleeding, infection, cardiac disease, stroke, graft loss, and some other complications.<sup>31</sup>

Rejection is the most common and important complication that may occur after any transplant surgery including kidney transplant. The patients must keep a lifetime regimen of immunosuppressant medications to prevent rejection.

ESRD patients are among the highest risk populations for cardiovascular diseases, a major cause of death in ESRD patients. The prevalence of CVDs is different with different therapies. Kidney transplant has about 42% risk of CVDs, which is the lowest among all the treatments today, while the risk of CVDs is about 70% in the patients with hemodialysis (**Figure 6B**).<sup>22</sup> The survival of the transplant recipients has considerably improved over the past decades. **Figure 9** is drawn with the data from U.S. Renal Data System (USRDS) 2017 Annual Data Report (ADR), including all ESRD patients in the United States. For ESRD patients with different treatments of transplantation and dialysis, the survival rates of both living- and deceased- donor renal transplantation are higher than peritoneal dialysis and hemodialysis (**Figure 9A**). The ESRD patients who receive a kidney transplant have a survival rate of more than 90% after 1 year, and 70%~80% at 5 years. While the survival rate of those patients under dialysis is about 77% at 1 year and sharply decreased to 38% after 5 years. At 10 years, patients of kidney transplant still have 40%~50% survival rate, while the survival rate of dialysis remains only 18% (**Figure 9B**). The data collected by USRDS 2017 Annual Data Report show that dialysis treatment costs an average of about \$80,000 per patient annually from 2003 to 2015, while the average cost of a kidney transplant is about \$30,000 for the transplant surgery and post-surgery care in the US (**Figure 10**). A successful kidney transplant

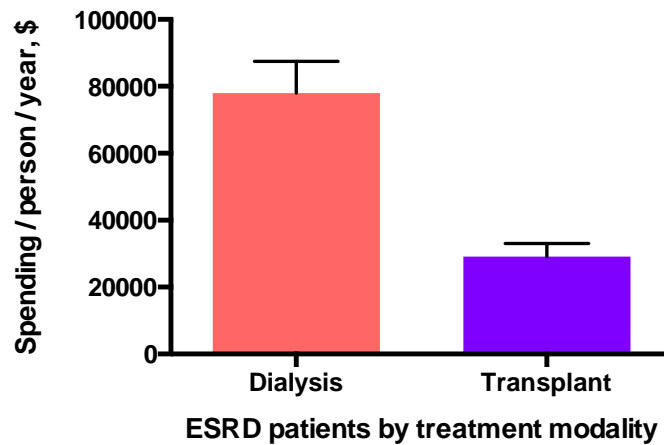
offers a good quality of life, an improved survival and health-care cost savings compare to dialysis.<sup>32</sup>



**Figure 9.** Patient Survival Statistics of Dialysis and Transplant. Figures are drawn with the data from USRDS 2017 Annual Data Report. Data are mean  $\pm$  SD including all ESRD patients in the United States, and adjusted by age, sex, race, ethnicity, and primary cause of ESRD. (<https://www.usrds.org>)<sup>20</sup>

**A:** The tendency of survival of ESRD patients who received a living- or deceased- donor kidney transplant, and the patients under peritoneal dialysis or hemodialysis. **B:** Comparison of one-year, five-year and 10-year survival rates for patients who undergo dialysis or have transplant surgery from deceased or living donors.





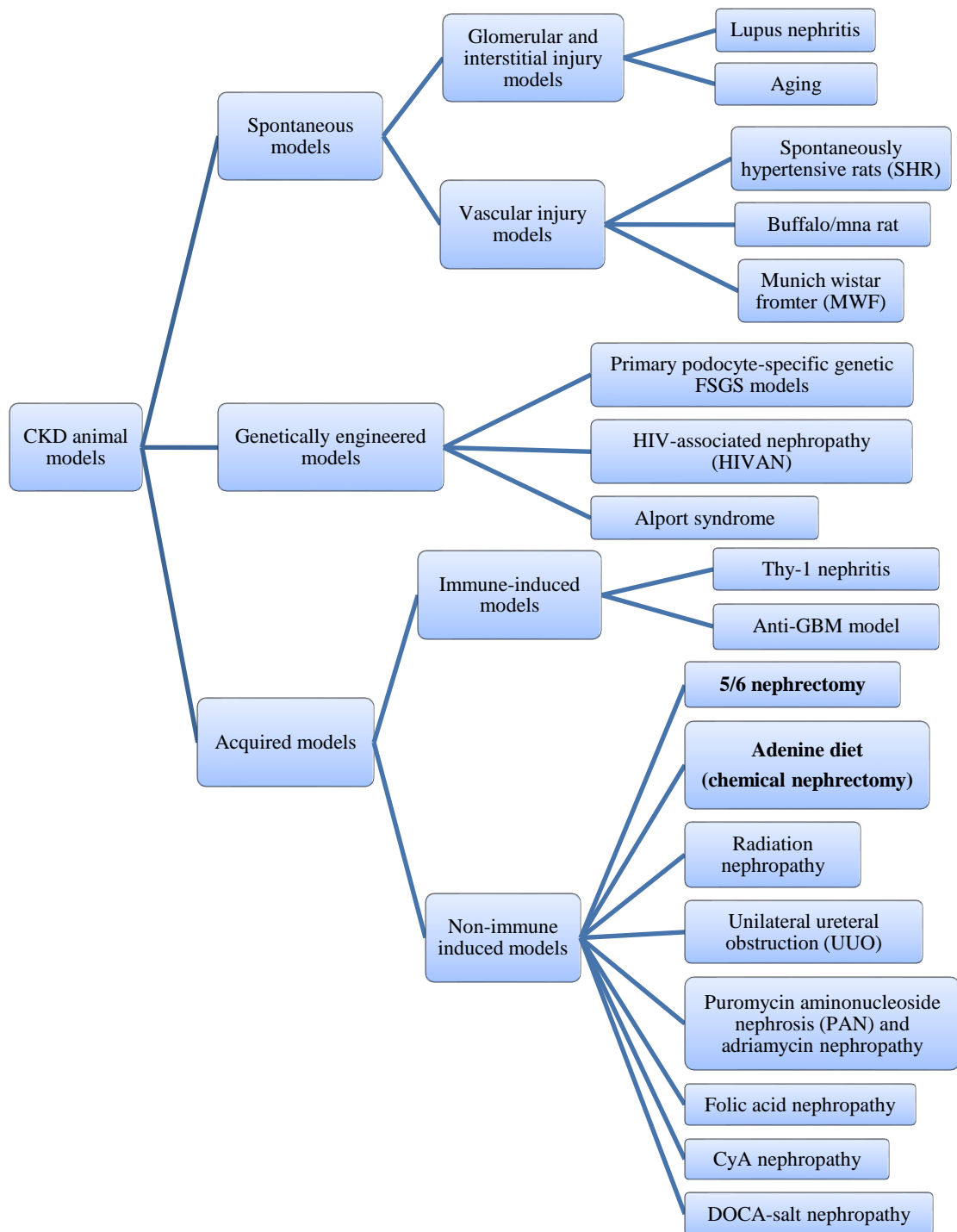
**Figure 10.** Spending of dialysis and transplant patients per person per year (2003~2015) in the US. Figure is drawn with the data from USRDS 2017 Annual Data Report (<https://www.usrds.org>).<sup>20</sup> Data are mean  $\pm$  SD using as-treated actuarial model, the standard USRDS as-treated economic model in which all spending are attributed to the patient's modality at the time when they are incurred. In the case of a modality change, the spending incurred after the change is attributed to the new modality.

However, the demand for donated kidneys is far higher than the available supply of donors. 84% of the patients are awaiting their first kidney transplant,<sup>33</sup> and the waiting time may last too long. So most patients with kidney failure have to use dialysis, the only alternative way to kidney transplantation today, to clear the wastes in the body while they are waiting for an available donated kidney.

## 1.8 Main animal models of CKD

Animal models have been the mainstay of experimental means to study chronic kidney disease. An ideal experimental animal model should be technically simple to

produce and provide stable uremic milieu and a reproducible degree of glomerular filtration rate reduction to allow experimental manipulation. The model should also be close mimicry of CKD on human beings without any unwanted additional physiologic changes.<sup>34</sup> Hai-Chun Yang et al. provided a brief overview of the most widely used CKD animal models (**Figure 11**)<sup>35</sup>. There is no model that can exactly mimic or predict response in human renal disease. Different models have different weaknesses and strengths. Judicious choice of model is vital to the understanding of the mechanisms and pathogenesis in CKD. Being aware of the specialty of each CKD model could offer valuable insights to choose the appropriate model for the study of CKD depending on the research orientation. The most frequently used animal models studying CKD are 5/6 nephrectomy models and adenine diet models, which are in the group of non-immune induced models.



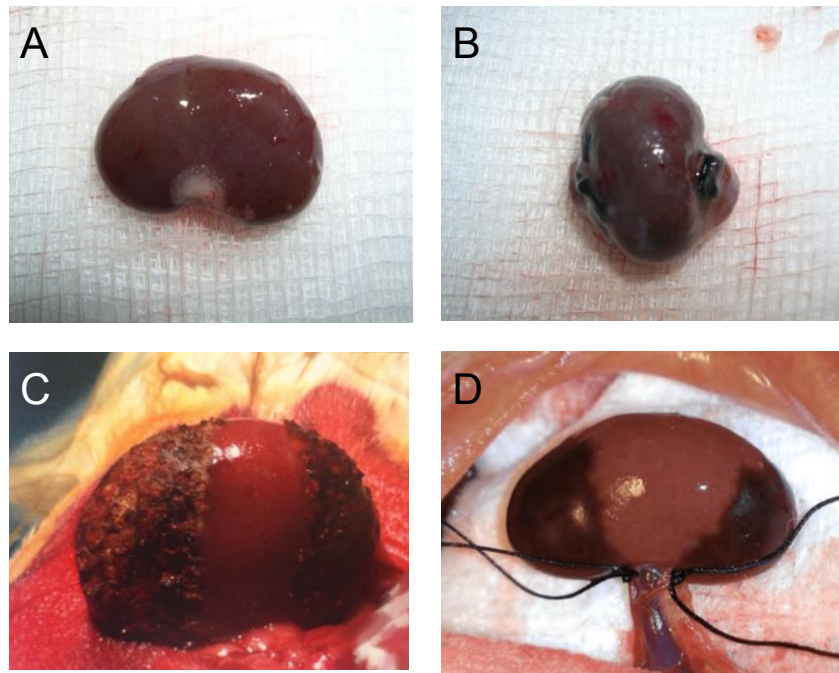
**Figure 11.** Main CKD animal models (Hai-Chun Yang et al., Drug discovery today: Disease models, 2010).<sup>35</sup>

**Abbreviations:** FSGS, focal and segmental glomerulosclerosis; GBM, glomerular basement membrane; CyA, Cyclosporine A; DOCA, deoxycorticosterone acetate.

### 1.8.1 Surgical models of CKD (subtotal nephrectomy)

In 1932, Chanutin and Ferris first showed that the removal of five-sixths of the renal mass caused rats to become uremic and to remain so for a prolonged period of time.<sup>36</sup> Since then, experimental model of 5/6 nephrectomy or (5/6 Nx) the so-called remnant kidney model has been the most frequently used animal model to induce chronic progressive renal disease. The procedure of 5/6 nephrectomy model involves the surgical removal of one kidney and then removal of 2/3 of the remnant kidney.<sup>37</sup> The second step is performed 5-7 days after stage one.

There are some different methods to remove 2/3 of the remnant kidney in order to avoid bleeding and increase the survival rate of animal, such as electrocoagulation, nitrogen freezing, renal artery branch ligation, etc. before the resection. **Figure 12** shows the kidney before and after 5/6 nephrectomy (**Figure 12A, B**), the electrocoagulation (**Figure 12C**) and ligation (**Figure 12D**) methods before the resection of the 2/3 remaining kidney.



**Figure 12.** Pictures of control and 5/6 nephrectomized kidneys.

**A:** An intact kidney of mouse before 5/6 nephrectomy. **B:** The 1/3 remnant kidney of mouse after 5/6 nephrectomy. **C:** Electrocoagulation of the cortex of the 2/3 remnant kidney of rabbit. **D:** Infarction of 2/3 of the left kidney of the mouse after ligation of both polar arteries (Iliescu et al.)<sup>37</sup>

The mechanism under 5/6 nephrectomy model is to reduce nephron number.<sup>38</sup> After reduction of kidney mass to 1/6 of its original size leads to the loss of a critical mass of nephrons, the workload for the remaining nephrons results in progressive renal damage and loss of remnant kidneys. The pathogenic mechanisms for progression become manifest and lead to the terminal stage of CKD renal failure. This model enables to investigate the influence of pharmacological, nutritive and other factors on functional and morphological renal parameters<sup>38</sup>.

### 1.8.2 Chemical nephrectomy (Adenine diet)

In 1982, Yokowaza et al proposed a new CKD model on rats based on the oral intake of adenine.<sup>39</sup> They found diets with high adenine increased serum uric acid, creatinine and urea nitrogen by decreasing the urinary excretion of uric acid, creatinine and urea. In 1986, they performed this model and suggest that long-term adenine feeding provides a model which would be useful to study chronic renal failure.<sup>40</sup> The adenine is metabolized to 2,8-dihydroxyadenine (DHOA), which is a constituent of the stones, precipitates crystals in the microvilli and apical epithelial region of the proximal tubule<sup>41</sup>, causing degenerative changes in renal tubules and the interstitium. Adenine diet CKD model have been shown to develop hyperphosphatemia, secondary hyperparathyroidism, bone disease, and vascular calcification.<sup>42</sup>

Most animal models do not mimic the complexity of the human disease as many do not develop CKD associated cardiovascular disease. The adenine diet model of CKD in rodents is an exception.<sup>43</sup> Adenine diet model is a non-surgical model, easy performed and reproducible. Animal are fed with 0.25%~0.75% (w/w) adenine depending on the type of rodent and the stage of CKD for 4 weeks. In our lab, mice are fed with 0.25% (w/w) adenine while rats are fed with 0.75% (w/w) adenine to induce CKD. CKD caused by adenine diet model is always associated with cardiovascular disease<sup>43</sup> and a growth retardation in uremia<sup>44</sup>. Animals develop advanced CKD, hyperphosphataemia severe hyperparathyroidism despite normocalcaemia, and medial arterial calcification (MAC) within 4 weeks when on a

0.75% (w/w) adenine diet.<sup>45,46,47</sup> Oral intake of adenine is simple and can induce CKD rapidly, but this method to induce kidney failure causes substantial weight loss and mineral bone disorder,<sup>42,47</sup> which should be taken into account when performing adenine diet CKD model.

## 2 Human serum albumin (HSA)

### 2.1 The role of HSA

Plasma Proteome Database (PPD) shows there are 10,546 proteins in human plasma or serum, and 509 of them have been documented in the literature.<sup>48</sup> Among these plasma proteins, human serum albumin (HSA) is the most abundant protein in human plasma.<sup>49</sup> HSA, molecular mass 66,438 Da, constituted by 585 amino acids, is a single chain, non-glycosylated monomeric multidomain protein secreted by the liver. Accounting for 50% ~ 60% of the total plasma proteins, HSA represents up to 80% of the plasma colloid oncotic pressure (25-33 mmHg) and plays an important role in modulation of fluid distribution.<sup>50,51</sup> In the serum of human adults, the concentration of HSA can reach 40 g/L. HSA exhibits an extraordinary ligand binding capacity. Indeed, HSA binds a wide variety of drugs and carries for many endogenous and exogenous compounds.

HSA infusions has been widely used for more than 70 years since it was introduced as a plasma expander during World War II.<sup>50</sup> Since 1940s, considerable studies on plasma expanders have been developed. This allowed a better understanding of biochemical properties and potential clinical benefits of HSA, which showed it to be a multifunctional protein with antioxidant, immunomodulatory and detoxification functions.<sup>52</sup>

Clinically, HSA has been widely used essentially as a plasma volume replacement in the treatment of several medical and surgical problems, including hypovolemia, hemorrhagic shock, serious burn injuries, surgical blood loss, trauma,



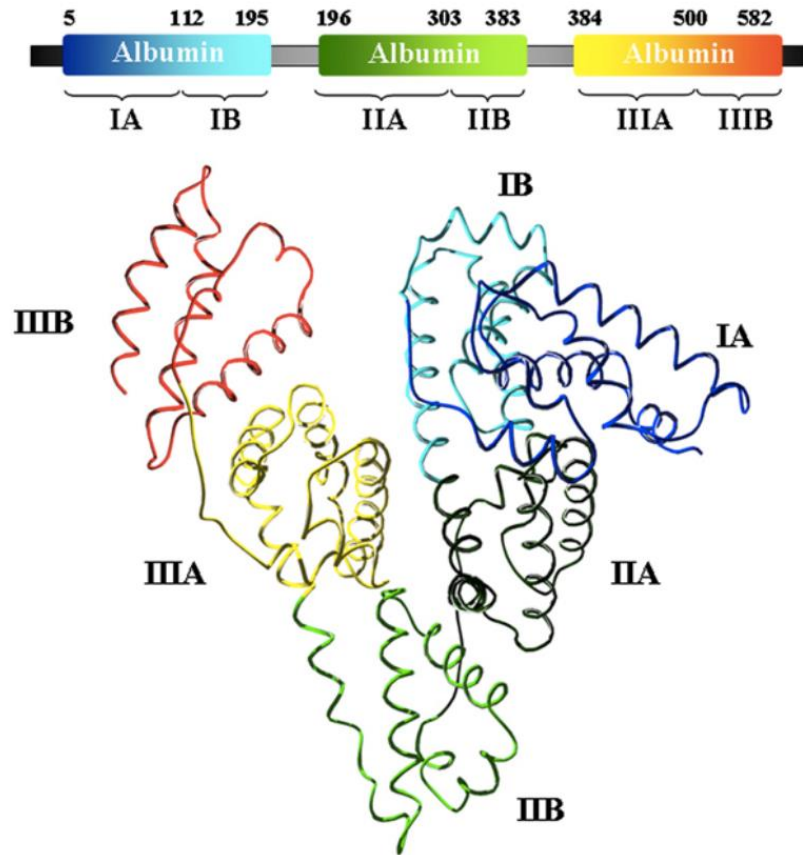
hemorrhage, acute respiratory distress syndrome, hemodialysis, nutrition support, resuscitation, and hypoalbuminemia, hypoproteinemia, fetal erythroblastosis, and ascites caused by liver cirrhosis.<sup>50</sup>

## 2.2 Structure of HSA

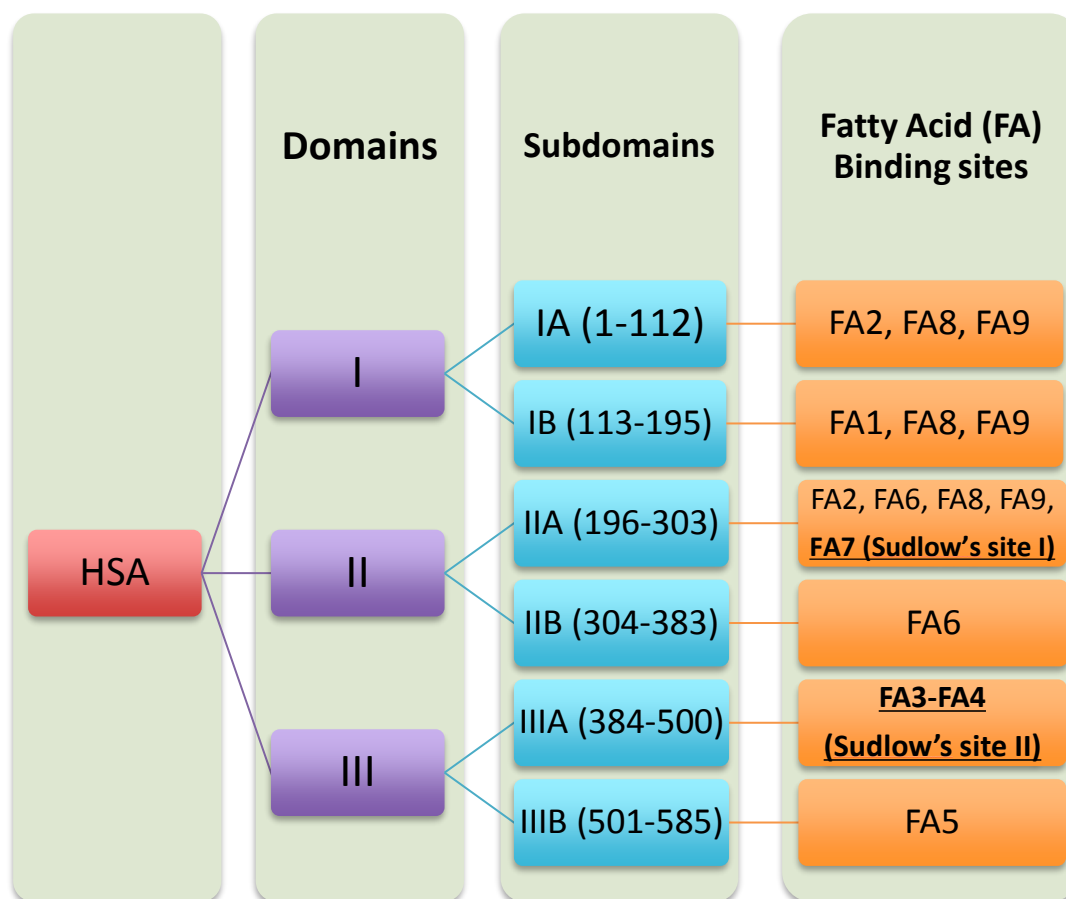
X-ray crystallography study shows HSA has a globular heart-shaped conformation, with approximate dimensions of  $80 \times 80 \times 30 \text{ \AA}$ .<sup>53,54</sup> The secondary structure of HSA is dominated with about 68%  $\alpha$ -helix without any  $\beta$ -sheet. HSA contains three homologous domains: domain I (1~195), domain II (196~383) and domain III (384~585). The three domains are comparable in the amino acid sequence, secondary structure and tertiary structure as well. All three domains of the HSA molecule have similar three-dimensional structures, however, their assembly is highly asymmetric.

Each of the three domains is further divided into two helical subdomains: subdomain IA (1-112), IB (113-195), IIA (196-303), IIB (304-383), IIIA (384-500) and IIIB (501-585). Each domain of HSA has two long loops and one shorter loop, including ten helices. The first two loops, named subdomain A, are constituted of a cluster of four  $\alpha$ -helices ( $\alpha$ -h1 to  $\alpha$ -h4) flanked by two short  $\alpha$ -helices ( $\alpha$ -h5 and  $\alpha$ -h6). The remaining loop, composed of another cluster of four  $\alpha$ -helices ( $\alpha$ -h7 to  $\alpha$ -h10), is referred to as subdomain B. A long extended loop traverses the two subdomains to link them together. As the last helices ( $\beta$ -h4) of domains I and II are fused with the first helices ( $\alpha$ -h1) of the next domains, thus the total number of  $\alpha$ -helices in a HSA molecule is 28 rather than 30.

The polypeptide chain folding and disulfide bond topology are similar between the subdomains. The helix clusters in subdomains A and B are also similar in 3-dimensional structure. Despite their structural similarity, each domain interacts with their neighbor domains in different ways. Terminal regions of sequential domains contribute to the formation of inter domains, 9-turn-long helices connect domain IB to IIA (residues 173–205) and IIB to IIIA (residues 336–398).<sup>50</sup> The orientation of domains I to II with respect to domains II to III constitutes a highly asymmetric environment where a variety of ligand binding sites are located. Therefore, the three domains of HSA assemble asymmetrically and resemble a heart shape (**Figure 13**). The actual shapes of domains II and III look more similar than those of I and II. Domains I and domains II are almost perpendicular to each other to form a T-shaped assembly.<sup>54</sup> The tail of subdomain IIA attaches to the interface region between subdomains IA and IB by hydrogen bonds and hydrophobic interactions. In contrast, domain III protrudes from subdomain IIB at an angle of about 45° to form a V-shaped assembly for domains II and III. Domain III interacts only with subdomain IIB. Domains I and III are connected by few contacts and separated by a big channel created by subdomains IB, IIIA, and IIIB. These features make the HSA molecule heart-shaped. The HSA conformation is highly maintained even in the presence of a wide variety of ligands. Most compounds bind to one of two principal binding sites commonly referred to as **Sudlow's binding sites I and II**.<sup>55</sup> Sudlow's site I is located in subdomain IIA and Sudlow's site II is located in subdomain IIIA (**Figure 14**).



**Figure 13.** Modular domain organization of HSA (Fanali et al., *Molecular Aspects of Medicine*, 2012).<sup>50</sup> The upper panel shows the architecture of the HSA sequence, with three repeated conserved domains. The lower panel shows the three-dimensional structure of HSA with the subdomains rendered with different colors (domain IA, in blue; domain IB, in cyan; domain IIA, in dark green; domain IIB, in light green; domain IIIA, in yellow; domain IIIB, in red).



**Figure 14.** Domains, subdomains and fatty acid binding sites on HSA.

HSA has a single tryptophan (Trp) residue at the position of 214 and contains 35 cysteine (Cys) residues forming 17 disulfide bridges, with the only free Cys residue, located at position 34 in a loop between helices Ia-h2 and Ia-h3, which is the only cysteine residue that does not participate in any disulfide bridge.<sup>54</sup> The disulfide bridges significantly contribute to the stability of HSA and lead to its long biological life-time (28–36 days)<sup>49</sup>, during which HSA may undergo several modifications that could affect ligand binding and anti-oxidant properties.<sup>56</sup>

Methionine (Met), glycine (Gly), and isoleucine (Ile) residues on HSA are low,

whereas cysteine (Cys), leucine (Leu), glutamic acid (Glu), and lysine (Lys) are abundant. Due to the large number of ionized residues (i.e., 215 ions per molecule at pH 7.0), HSA has a high total charge, which contributes to its solubility.<sup>50</sup> Moreover, the acidic amino acid residues outnumber the basic ones in HSA, resulting in a negative net charge per molecules of about -15, at pH 7.0,

## **2.3 Binding properties**

### **2.3.1 Binding sites on HSA**

The structural multi-domain organization of HSA results in its extraordinary ligand-binding capacity. HSA provides a variety of ligand binding sites: Fatty acid binding sites (FA), thyroxine binding frequent sites, bacterial protein binding site, metal binding sites, less frequent binding sites,... etc.<sup>50</sup> HSA is able to bind up to nine equivalents of long chain fatty acids, which are the primary physiological ligands at multiple binding sites from FA1 to FA9.<sup>50</sup> The pioneering work of Sudlow *et al* shows most drugs bind with high affinity to one or two sites on human serum albumin, which are called Sudlow's sites I and II.<sup>55,57</sup>

### **2.3.2 Binding site I**

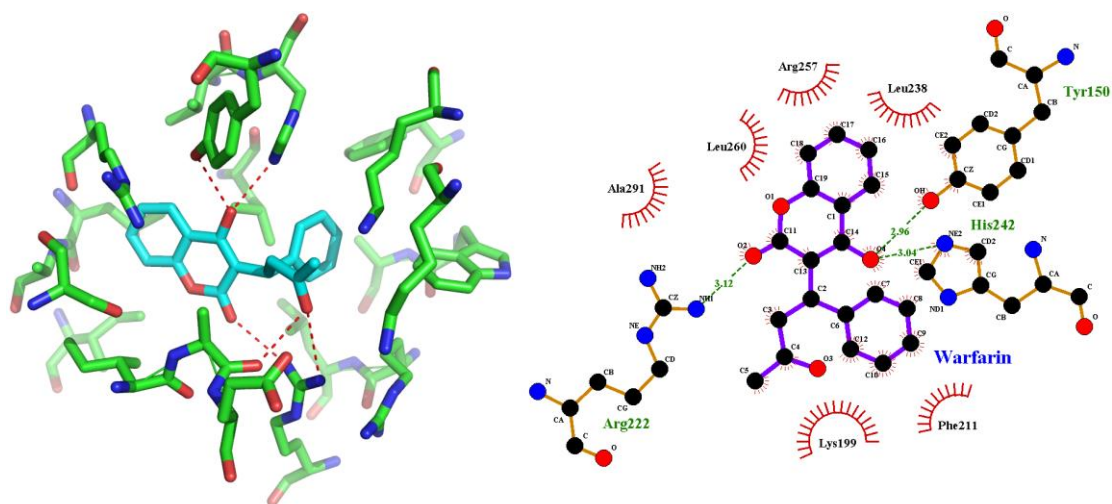
The pocket-shaped cavity hosting FA7 in subdomain IIA represents the **Sudlow's site I**, which includes the only tryptophan at position 214. The entrance of the cavity is surrounded by residues with positive charge, i.e., Arg 257, Arg 222, Lys 199, His 242, Arg 218 and Lys 195, and the inside of the pocket is formed by hydrophobic side

chains.<sup>54</sup> Due to its hydrophobic core, ligands of site I seem to be the molecules with dicarboxylic acids or bulky heterocyclic and a negative charge in the middle of the ligand.<sup>58</sup> This site is capacious, flexible and harbours a large number of individual ligand-binding sites that interact each other.<sup>59,60</sup>

The prototypical ligand of Sudlow's site I is warfarin, so site I is also commonly referred to as the **warfarin binding** site. We examine the binding mode of warfarin via docking experiments targeting the site I of HSA. **Figure 15** shows the interaction between warfarin and HSA. Warfarin fits very well with several hydrogen bonds with Tyr 150, Arg 222 and His 242. The phenyl group of warfarin interacts tightly with hydrophobic residues Lys 199, Phe 211, Leu 238, Arg 257, Leu 260 and Ala 291. Besides, 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF), aspirin, and benzylpenicillin also bind to this site on HSA.

Probe displacement method is commonly used to explore the binding sites of molecules on HSA with a fluorescence spectrometer. The binding of racemic mixtures of warfarin to HSA is associated by an increase in the fluorescence quantum yield of these compounds.<sup>61</sup> This property made warfarin suitable to be used as a specific probe to identify the binding site of drugs or uremic toxin on site I. The fluorescence yield of warfarin highly enhances on binding to HSA. The binding of warfarin and the tested molecule are in a competitive manner. The decreased intensity of fluorescence indicates the displacement of warfarin. When warfarin is used as site I specific probe, fluorescence is measured at 380 nm with excitation at 320 nm. Binding of warfarin to human serum albumin results in a red shift of the fluorescence emission peak at the

excitation wavelengths of 280nm, which is the absorbance of aromatic amino acid residues that is the only Trp214 on HSA.



**Figure 15.** Proposed binding mode of warfarin in the binding site I of HSA: interacting with warfarin colored in blue. The binding mode of warfarin obtained as a result of docking experiment was generated with PyMol and the 2D-representation was generated using LigPlot+.

### 2.3.3 Binding site II

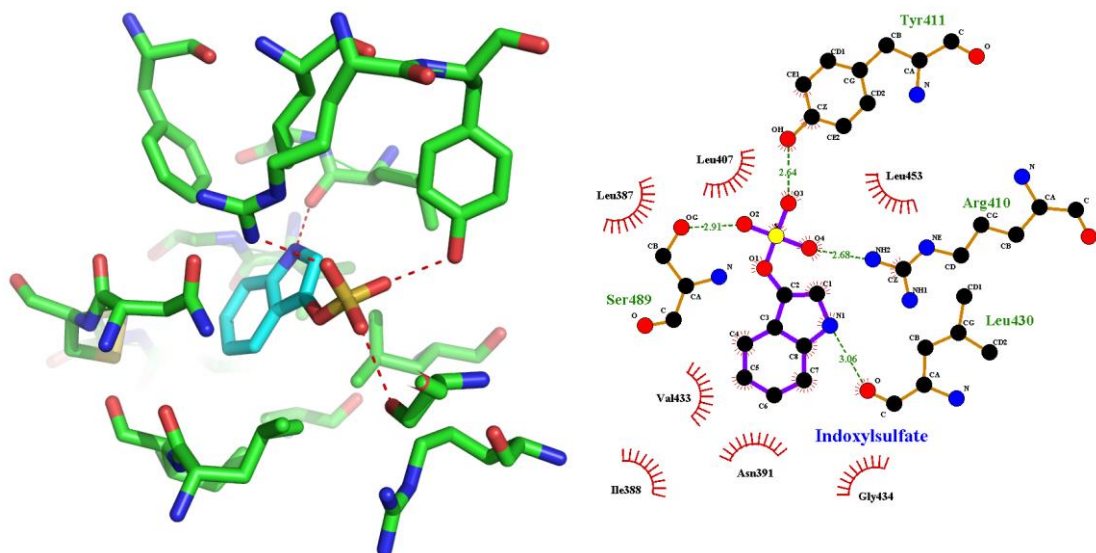
FA3 and FA4 are composed of all six helices and located in an analogous preformed hydrophobic cavity with distinct polar features in subdomain IIIA, which constitutes **Sudlow's site II**.<sup>62</sup> Site II is a hydrophobic cleft that is 12-16 Å deep and 6-8 Å wide with a cationic group located near the surface of HSA. Due to its cationic center carrying a positive charge, ligands with a negative charge or strongly electrophobic forces appear to provide the main binding energy.<sup>63</sup> FA3 makes salt bridges with Arg348 and Arg485, and a hydrogen bond with Ser 342, while FA4

forms a salt bridge with Arg410 and hydrogen bonds with Tyr411 and Ser489.<sup>64</sup> Site II is topologically similar to but smaller or more narrow than site I.<sup>62</sup>

Ligands binding to site II are often aromatic carboxylic acids with a generally extended shape carrying the negatively charged carboxyl group at one end of the molecule away from the extended hydrophobic center.<sup>63</sup> **Diazepam and non-steroidal anti-inflammatory drug ibuprofen** are prototypical ligands for site II.<sup>57</sup> Renal toxins indoxyl sulfate and p-cresyl sulfate also bind to site II. So Sudlow's site II is also called the **indole-benzodiazepine binding site**. We examine the binding mode of indoxyl sulfate via docking experiments targeting the site II of HSA. **Figure 16** shows the interaction of indoxyl sulfate with HSA. Indoxyl sulfate fits very well with several hydrogen bonds with Arg 410, Tyr 411, Leu 430 and Ser 480, and interacts tightly with hydrophobic residues Leu 387, Ile 388, Asn 391, Leu 407, Val 433, Gly 434 and Leu 453.

As warfarin is used as a probe for site I, **Dansylsacosine** is used as a specific probe for site II.<sup>65</sup> The fluorescence intensity highly increases when dansylsacosine binds to HSA. The decreased intensity of fluorescence shows the competitive displacement. When dansylsacosine is used as site II probe, the excitation/emission wavelength is set at 350nm/475nm.





**Figure 16.** Proposed binding mode of indoxyl sulfate in the binding site II of HSA: interacting with indoxyl sulfate colored in blue. The binding mode of indoxyl sulfate obtained as a result of docking experiment was generated with PyMol and the 2D-representation was generated using LigPlot+.

## 2.4 Drugs and the binding sites on HSA

### 2.4.1 Plasma protein binding rate of drugs

Drugs in blood exist into two forms: unbound and bound to plasma proteins. Depending on the binding affinities of different drugs and plasma proteins, a proportion of the drug binds to plasma proteins, and the remnant part being unbound. If the protein binding is reversible, the chemical equilibrium between the bound and unbound states can be written as follow:



Drug-protein binding may be a reversible or an irreversible process. Irreversible drug-protein binding is usually a result of chemical activation of the drug, which

attaches strongly to the protein or macromolecule by covalent chemical binding. This binding type accounts for certain types of drug toxicity that may occur over a long time period. Reversible drug-protein binding implies that the drug binds the protein with weaker chemical bonds, such as electrostatic or hydrophobic interactions, hydrogen bonds or Van der Waals forces.

Plasma protein binding strongly affects the pharmacokinetic behavior of drugs with consequences on their overall pharmacological action.<sup>66</sup> Only the unbound fraction or free fraction of the drug undergoes metabolism in the liver and other tissues and exhibits pharmacologic effects.

For example, the bound fraction of the anticoagulant drug warfarin is about 97%. It means that 97% of warfarin in the blood is bound to plasma proteins (ie. biologically inactive). The remaining 3% is the free fraction that is actually active and could be metabolized and/or excreted.

The binding of a drug to plasma proteins in the blood plays an important role in its distribution, elimination, and pharmacological effect. The more a drug is proteins bound to plasma, the less efficiently it can traverse cell membranes or diffuse.

The major plasma proteins that can interact with drugs are: human serum albumin (HSA), alpha-1-acid glycoprotein (AAG, AGP) or orosomucoid (ORM), lipoprotein,  $\alpha$ ,  $\beta$ , and  $\gamma$  globulins, steroid binding globulin, fibrinogen etc. HSA is the most abundant plasma protein that has a pronounced affinity for acidic and neutral compounds, while AGP has been reported to have a pronounced affinity to basic and

neutral drugs. **Table 3** shows the major proteins in human plasma and their concentrations.

Plasma protein	Molecular Weight (kDa)	Plasma concentration (g/dl)	Protoitypoe drug binding to protein
Serum albumin	69	3.5-5.0	Acidic
Alpha-1-acidic glycoprotein	44	0.04-0.1	Basic
Lipoproteins	200-3400	Varies	Basic
Globulins	140	2.0-2.5	/
Steroid Binding globulin	53	0.003-0.007	Steroids
Fibrinogen	400	0.2-0.4	/

**Table 3.** Major proteins in human plasma and their drug binding properties. Table from Cambridge MedChem Consulting (CMC, <https://www.cambridgemedchemconsulting.com>).<sup>67</sup>

The extent of binding of drugs to protein is determined by drug and protein concentration, the affinity of drug for the protein, and the number of binding sites on each molecule of protein.<sup>68</sup>

#### 2.4.2 The relationship between binding rate and toxicity

Drug toxicity refers to the level of damage that a compound can cause to an organ or a tissue.<sup>69</sup> The toxic effects of drugs are dose-dependent and can affect an entire system as in the central nervous system (CNS) or a specific organ such as the liver and kidney. Drug toxicity may occur with doses that exceed the therapeutic

efficacy of a drug, or in the case of the disease of liver or kidneys that are unable to remove the drug from the bloodstream, allowing it to accumulate in the body and reach to a high concentration, leading to poisonous and harmful effects on the body.

The kinetics of drug binding and drug residence time are recognized to be important in the clinical effectiveness of drug candidates.<sup>70</sup> The binding of drugs to serum proteins strongly affects the pharmacokinetic behavior of drugs due to their overall pharmacological action. High binding affinity, especially above 95%, can cause drug safety issues or several adverse effects, such as low clearance, low brain penetration, drug–drug interactions, loss of efficacy, etc.<sup>66</sup>

However, toxic and therapeutic effects of drug can occur simultaneously.<sup>69</sup> And the binding affinity of drug can also affect the drug residence time. In most cases, long residence time of the drug-protein complex results in an extended duration of pharmacodynamic activity, even when systemic concentrations of drug have been notably reduced through elimination routes,<sup>70</sup> leading to the increase of the drug efficacy duration and the diminishment of the potential for off-target-mediated toxicities, if the target of the drug has been selective.

### **2.4.3 The competition between drugs and uremic toxins**

Protein-bound uremic toxins are poorly removed by current dialysis techniques and their concentration can therefore hardly be decreased in end-stage renal disease (ESRD). These uremic toxins exist in the bloodstream into two forms: bound (non-diffusible) and free (i.e. unbound) fractions. Depending on the affinity of the

uremic toxin for plasma proteins, a variable proportion of the toxin may bind to plasma proteins, with the remaining fraction being free and thus diffusible. In most cases, this protein binding is reversible (excepted for instance for advanced glycation end-products or AGEs).

Drugs in blood also exist in two forms: bound and unbound. Therefore, there is a competition between drugs and uremic toxins. Notably, for both drugs and uremic toxins, the biological action of the molecules is exerted by their free fraction. The free fraction is also the sole that can be metabolized and excreted. The bound fraction may act as a storage from which the toxin is slowly released as the unbound form. When the unbound form is being metabolized and excreted from the body, the bound fraction is released to maintain the equilibrium.

Protein bound drug and protein bound uremic toxin in the same context at the same time may affect the bound / free fraction to each other. For example, a protein bound drug is given in the circumstance with protein bound uremic toxin. If the affinity of the drug binding to plasma protein is higher than the uremic toxin, the drug will displace the uremic toxin from the protein, thereby increasing the free fraction of the uremic toxin. This makes it possible to use common hemodialysis to remove the protein bound uremic toxins.

### 3 Uremic toxins

#### 3.1 Uremic toxins and uremic syndrom

Uremic syndrome, also referred to as uremia, is attributed to the progressive retention of uremic toxins. Uremic syndrome can be defined as a deterioration of biochemical and physiologic functions, in parallel with the progression of terminal clinical manifestation of renal failure, thereby resulting in complex and variable symptomatology.<sup>71</sup> Uremic syndrome results from a malfunctioning of various organ systems due to the retention of compounds. Under normal conditions, healthy kidneys can metabolize or excrete uremic toxins in urine. But in CKD patients, uremic retention solutes (URS) accumulate in the blood. One of the important toxic effects of uremic toxins is cardiovascular damage.

Uremia is a serious complication of chronic kidney disease and acute kidney injury. It occurs when urea and other waste products accumulate in the body fluids because the kidneys are unable to eliminate them. These compounds can become toxic if they reach high concentrations in the body. These putative toxic substances are called uremic toxins. In order for a substance to be recognized as an uremic toxins, a necessary criterion is that it is excreted by the kidney and present in increased concentration in body fluids and/or tissues in patients with renal failure. An “authentic” uremic toxin should fulfill specific conditions as follows<sup>72, 73</sup>:

- (1) The toxin must be identified and characterized as a unique chemical entity.
- (2) Quantitative analysis of the toxin in biological fluids must be possible.
- (3) The level of the putative toxin must be elevated in biological fluids of

subjects with kidney failure.

(4) A relationship between the level of the putative toxin in biological fluids and one or more of the manifestations of the uremic syndrome must be present.

(5) A reduction in the levels (or total body burden) of the putative toxin in biological fluids must result in some measurable amelioration of uremic manifestations.

(6) Administration of the putative toxin to achieve levels similar to that observed in the uremic syndrome must reproduce the uremic manifestations in otherwise normal animals or human.

(7) A plausible patho-biological mechanism should be demonstrated to explain the linkage between the putative toxin and the uremic manifestation.

The uremic syndrome is attributed to the progressive retention of a large number of uremic toxins, which under normal conditions are excreted by the healthy kidneys. The toxicity of substances accumulating in uremia is influenced by the following factors: <sup>72</sup>

(1) The rapidity of changes in the levels in biological fluids.

(2) Fluctuations in levels over time (time averaged vs. peak levels).

(3) Penetration into sites of action.

(4) Intrinsic toxicity versus dependency of metabolism to more (or less) toxic compounds.

(5) Distribution in body fluids (protein binding, lipophilicity, hydrophilicity).

(6) Presence and activity of naturally occurring inhibitors or promoters.

(7) Rate of metabolism at active sites.

### 3.2 Operational classification of uremic toxins

The current understanding of the issue of uremic toxicity allowed for a widely accepted classification of uremic toxins based on the physicochemical characteristics affecting their clearance during dialysis, which is still the main therapeutic option for their removal nowadays. Molecular weight and plasma protein-binding characteristics are the two main factors that influence the removal pattern and the clearance of uremic toxins by dialysis or other methods of extracorporeal elimination.<sup>73</sup> A preferential classification of putative uremic solutes focuses on three types of molecules:<sup>74,75</sup>

(1) The small water-soluble compounds, with urea as a prototype (**Table 4**).

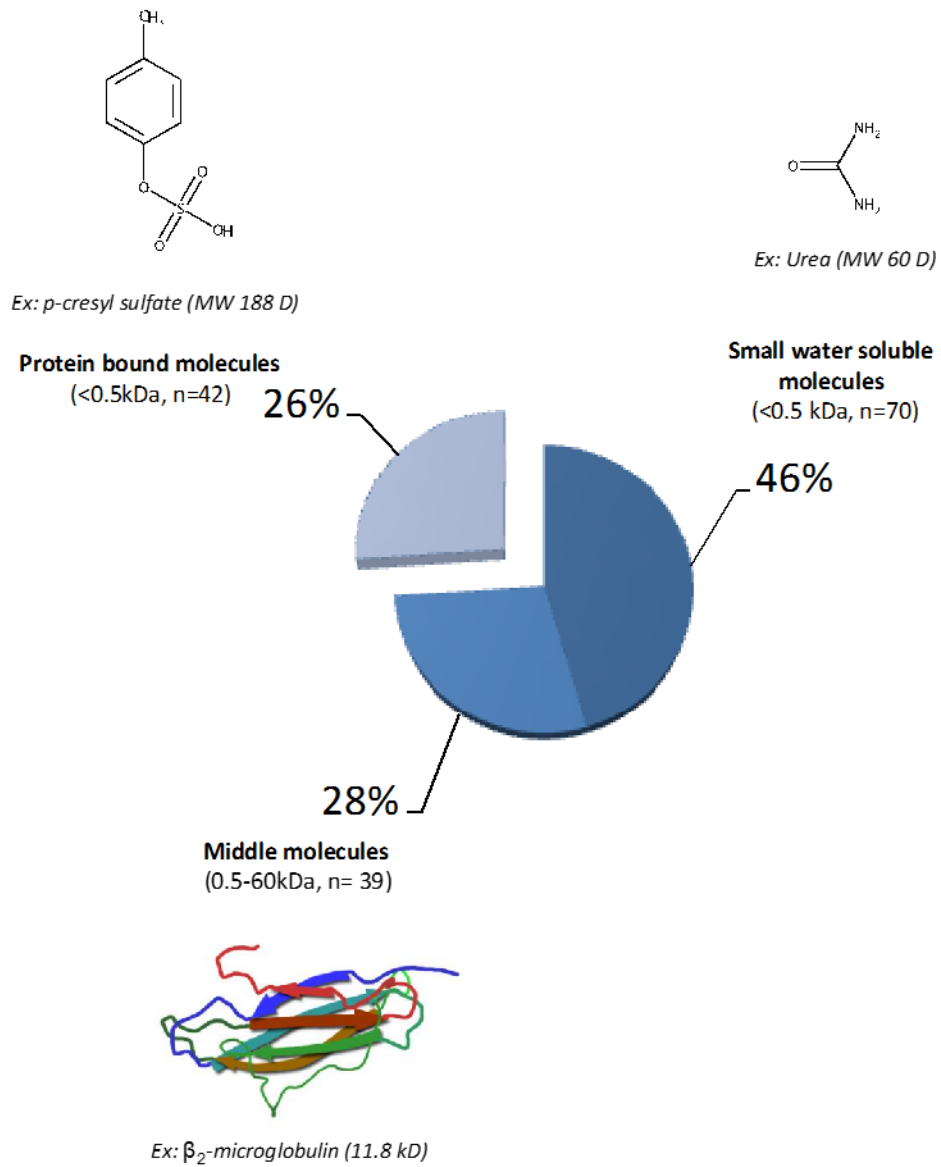
(2) The larger peptides (molecular weight: MW>500 Da), also named middle molecules, with beta2-microglobulin as a prototype (**Table 5**).

(3) The protein-bound solutes, with the groups of indolic and phenolic compounds as prototypes (**Table 6**).

Currently, 151 solutes and compounds have ever been identified as uremic toxins in the literature and this number is rapidly growing. **Figure 17** shows the classification of the 151 uremic toxins. **Table 4**, **Table 5** and **Table 6** list all the uremic toxins in each group from the database of European Uremic Toxin Work



Group (EUTox, <http://www.uremic-toxins.org>)<sup>76</sup>, “Uremic Toxins” (Niwa et al., 2012)<sup>74</sup>, and “Nutritional Management of Renal Disease” (Kopple et al., 2013)<sup>75</sup>.



**Figure 17.** Classification of uremic toxins according to the physicochemical characteristics affecting their clearance during dialysis.

Small Water-Solute Compounds (<500 Da)		
1-Methyladenosine	Ethylamine	Orotidine
1-Methylguanosine	Guanidine	Oxalate
1-Methylinosine	Guanidinosuccinic acid	Phenylacetylglutamine
2-Heptenal	Heptanal	Pseudouridine
2-Hexenal	Hexanal	S-Adenosylhomocysteine
2-Nonenal	Hypoxanthine	Sorbitol
2-Octenal	Inosine	Symetric Dimethylarginine (SDMA)
4-Decenal	Malondialdehyde (MDA)	Taurocyamine
4-HO-Decenal	Mannitol	Threitol
4-HO-Hexenal	Methylguanidine	Trimethylamine
4-HO-Nonenal	Monomethylamine	Trimethylamine-N-oxide
4-HO-Octenal	Myoinositol	Uracil
4-Pyridone-3-carboxamide-1- $\beta$ -D-ribo- nucleoside	N-methyl-2-pyridone-5-carboxamide	Urea
8-Hydroxy-2'-deoxyguanosine	N-methyl-4-pyridone-3-carboxamide	Uric Acid
Arab(in)itol	N2, N2-Dimethylguanosine	Uridine
Argininic Acid	N4-Acetylcytidine	Xanthine
Asymetric Dimethylarginine (ADMA)	N6-Methyladenosine	Xanthosine
Creatine	N6-Threonylcarbamoyl-adenosine	$\alpha$ -keto- $\delta$ -Guanidinovaleric Acid
Creatinine	Neopterin	$\alpha$ -N-Acetylarginine
Cytidine	Nicotinamide	$\beta$ -Guanidinopropionic Acid
Decanal	Nitrosodimethylamine	$\beta$ -Lipotropin
Dimethylamine	Nitrosomethylamine	$\gamma$ -guanidinobutyric Acid
Dimethylglycine	Nonanal	Orotidine
Erythritol	Orotic Acid	

**Table 4.** List of 70 small water-solute compounds of uremic toxins (From the database of EUTox, <http://eutoxdb.odeesoft.com/soluteList.php>).<sup>77</sup>

Middle Molecules (>500 Da)		
Adiponectin	Endothelin	Parathyroid hormone
Adrenomedullin	Guanylin	Resistin
Atrial Natriuretic Peptide (ANP)	Ghrelin	Substance P
Basic fibroblast growth factor (BFGF)	Hepcidin	Uridine adenosine tetraphosphate (Up4A)
Calcitonin gene-related peptide (CGRP)	Hyaluronic acid (Hyaluronan)	Uroguanylin
Cholecystokinin	Interleukin-18	Vasoactive intestinal peptide (VIP)
Clara cell protein (CC16)	Interleukin-1 $\beta$	Vasopressin (ADH)
Complement Factor D	Interleukin-6	Zinc- $\alpha$ 2-glycoprotein (ZAG)
Cystatin C	Methionine-Enkephalin	$\beta$ -2-Microglobulin
Degranulation Inhibiting Protein I (DIP I)	Motiline	$\beta$ -Endorphin
Delta-sleep Inducing Peptide	Neuropeptide Y	$\delta$ -Sleep-inducing peptide
Desacylgrelin	Octopamine	$\kappa$ -Ig Light chain
Dinucleoside polyphosphates	Orexin A	$\lambda$ -Ig Light Chain

**Table 5.** List of 39 middle molecules of uremic toxins (From the database of EUTox, <http://eutoxdb.odeesoft.com/soluteList.php>).<sup>77</sup>

Protein-Bound Uremic Toxins		
2-Methoxyresorcinol	Indoxyl sulfate	Pentosidine
2-Deoxyglucosone	Indoxyl- $\beta$ -D-glucuronide	Phenol
3-Carboxy-4-Methyl-5-Propyl-2-Furanpropanoic Acid (CMPF)	Insulin-like growth factor 1 (IGF-1)	Phenylacetic acid
3-Deoxyglucosone	Interleukin-10	Phenylethylamine
Angiogenin	Kinurenine	p-OH-hippurate
Dihydroxyphenylalanine (PB-DOPA)	Kynurenic Acid	Putrescine
Dimethylguanosine	Leptin	Quinolinic Acid
Fructoselysine	Melatonin	Retinol Binding Protein (RBP)
Glyoxal	Methylglyoxal	Spermidine
Hippuric acid (total)	N(6)-Carboxymethyllysine (CML)	Spermine
Homocysteine	Osteocalcin	Thiocyanate
Hydroquinone	p-Cresyl	Tumor Necrosis Factor $\alpha$ (TNF)
Indican	p-Cresyl glucuronide	Vascular endothelial growth factor (VEGF)
Indole-3-acetic acid	p-Cresyl sulfate	$\alpha$ 1-Acid glycoprotein

**Table 6.** List of 42 protein-bound uremic toxins (From the database of EUTox, <http://eutoxdb.odeesoft.com/soluteList.php>).<sup>77</sup>

Molecule	MW, Da	Protein binding, %	Binding site
CMPF	240	>99	High affinity, site I
Hippuric acid	179	42-48	High affinity, site II
Homocysteine	135	80-95	unknown
Indole-3-acetic acid	175	86-94	High affinity, site II
Indoxyl-sulfate	212	86-98	High affinity, site II
Indoxyl-glucuronide	309	60	unknown
p-Cresylsulfate	31	91-95	High affinity, site II
p-Cresyl glucuronide	284	8-32	Weak affinity, neither I nor II
Phenylacetic acid	136	26-60	High affinity, neither I nor II

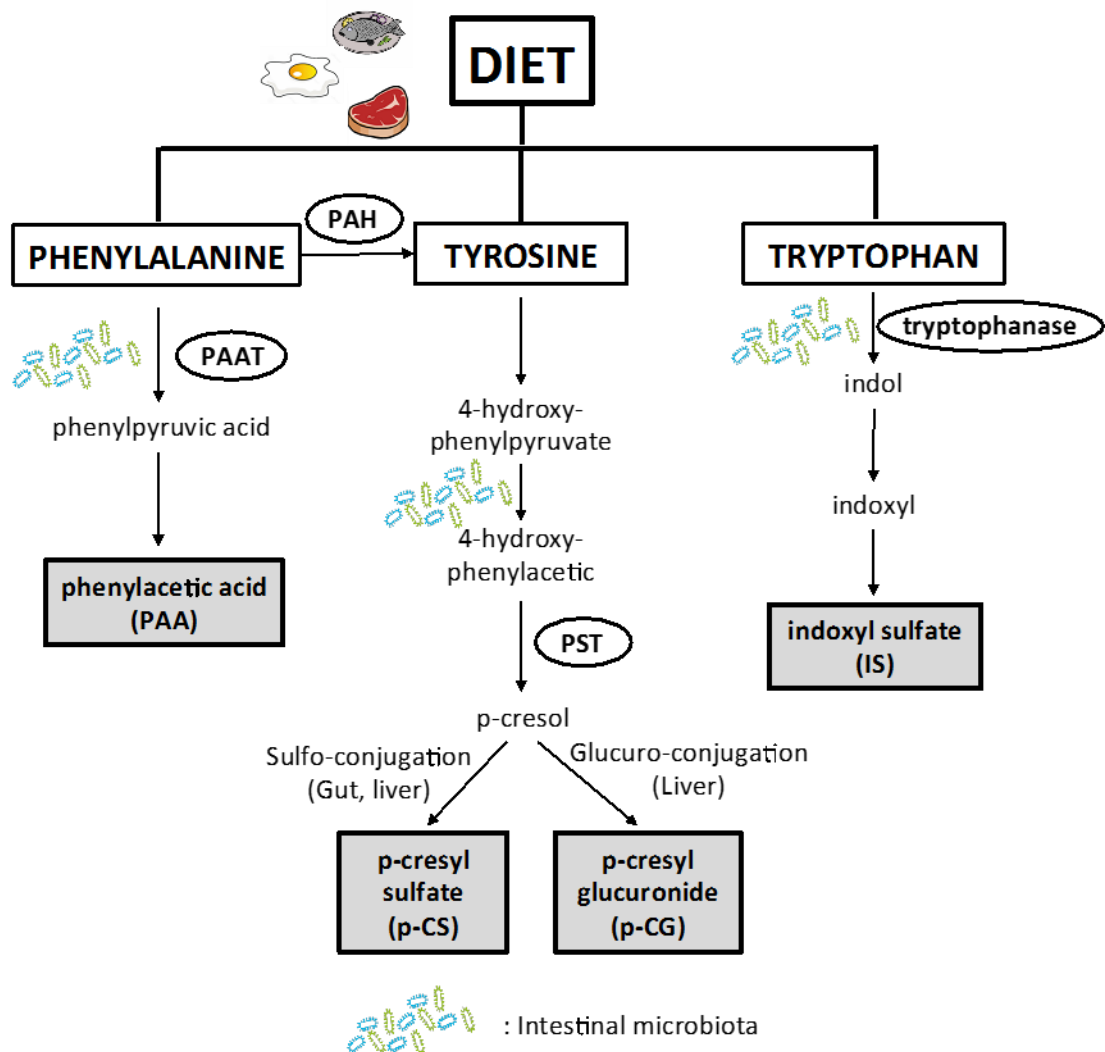
**Table 7.** The binding percent of main protein-bound uremic toxins to plasma proteins and their binding site(s) on Human serum albumin (Florens et al., *Biochimie*, 2018).<sup>78</sup> Note that uremic toxin covalently (i.e. noncompetitive binding) bound to proteins were not included in the present Table (AGE, pentosidine, acrolein,...). Data of p-Cresyl glucuronide are updated with our new results.

**Abbreviations:** CMPF, 3-Carboxy-4-methyl-5-propyl-2-furan-propanoic acid; MW, molecular weight.

It has been reported by EUTox Group that many solutes with different chemical structures belong to the protein-bound classe including indoles, phenols, amines, hippuric acid, peptides and advanced glycation end products. **Table 7** shows the main protein-bound uremic toxins with their binding percent and binding site(s) on HSA<sup>78</sup> (See **2.3.1** Binding sites on HSA). Among them, indoxyl sulfate (IS) and p-cresyl sulfate (p-CS) are the two-prototypical solutes both binding tightly to site II on HSA. In the present matter, we determined the binding properties of phenylacetic acid (See **2.1** Phenylacetic acid (PAA)) and p-cresyl glucuronide (See **2.2** p-cresyl glucuronide (p-CG)).

### 3.3 Protein-bound uremic toxins (PBUTs)

The uremic syndrome is attributed to some progressive retention of numerous compounds, which are normally excreted or metabolized by the kidneys in healthy individuals. These uremic retention molecules (URMs) can originate from endogenous metabolism, microbial metabolism or exogenous intake. Indoxyl sulfate (IS) and p-cresyl sulfate (p-CS), two kinds of protein-bound uremic toxins increasing significantly during CKD, are generated in part in the gastrointestinal tract and poorly removed by common dialysis. Many protein-bound uremic toxins (PBUTs) are derived from dietary proteins (**Figure 18**). Phenylalanine is metabolized into tyrosine and phenylpyruvic acid by phenylalanine hydroxylase (PAH) and phenylalanine aminotransferase (PAAT) respectively. Phenylpyruvic acid through putrefaction yields phenylacetic acid (PAA), while tyrosine undergoes analogous decomposition. p-cresyl is the main product of tyrosine biotransformation under the effect of the enzyme of phenol sulfotransferase (PST) by anaerobic intestinal bacteria and then transformed into p-cresyl sulfate and p-cresyl glucuronide (p-CG). Indoxyl sulfate is derived from tryptophan. By tryptophanase in intestinal bacteria, tryptophan is fermented into indole, which is then metabolized into indoxyl sulfate.



**Figure 18.** The metabolism of main protein-bound uremic toxins from dietary protein in human body.

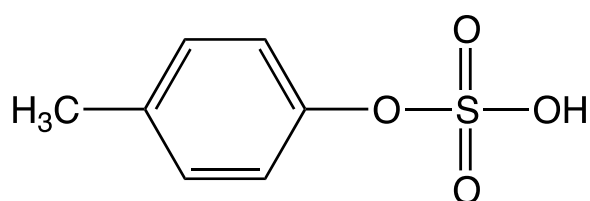
**Abbreviations:** PAAT, phenylalanine aminotransferase; PAH, phenylalanine hydroxylase; PST, phenol sulfotransferase.

### 3.3.1 *p*-cresyl sulfate (p-CS)

*p*-cresyl sulfate (**Figure 19**) is one of the main protein bound uremic toxins, which especially exert major toxic effects due to a poor removal by the common dialysis techniques. *p*-Cresol is the mother compound of an important group of protein-bound retention solutes. It is produced in the gut from the metabolism of



aromatic amino acids (especially tyrosine) by putrefactive bacteria of the gut microbiota. Intestinal fermentation of the amino acid tyrosine generates 4-hydroxyphenyl-acetic acid, which is decarboxylated to p-cresol or demethylated to phenol.<sup>79</sup> As it crosses through the intestinal mucosa, p-cresol is then metabolized by a cytoplasmic sulfotransferase and therefore mainly circulates in blood as its sulfate conjugate, p-cresyl sulfate.



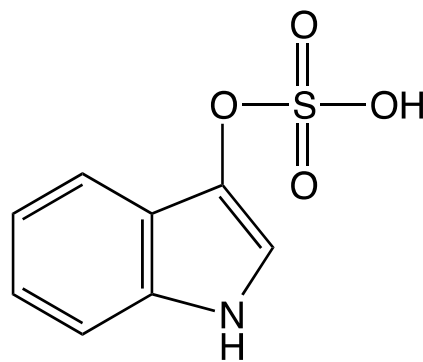
**Figure 19.** Chemical structure of p-cresyl sulfate.

p-cresyl sulfate is excreted by the kidney mainly through proximal tubular secretion and therefore accumulates in serum of patients with renal dysfunction.<sup>80</sup> p-CS is associated with mortality and cardiovascular diseases in patients with CKD. There are now accumulating in vitro data on the harmful effects of p-cresol/p-CS and related protein-bound retention solutes. p-CS has been shown to promote insulin resistance in CKD mice<sup>81</sup> and to be a valuable predictor of clinical outcomes in pre-end stage renal disease (ESRD) patients.<sup>82</sup>

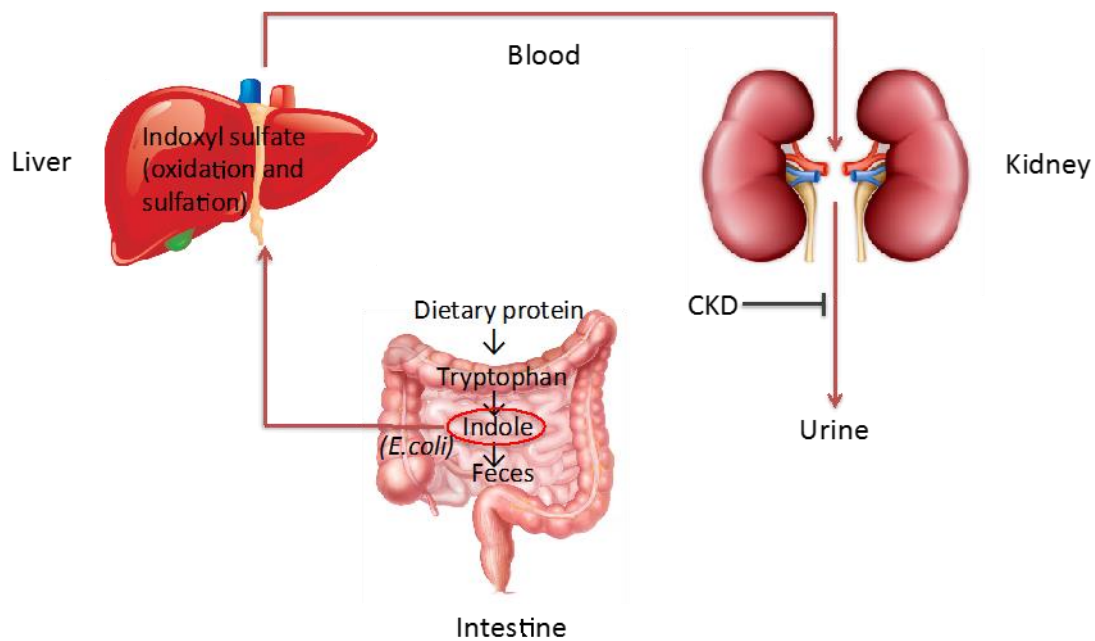
### 3.3.2 Indoxyl sulfate (IS)

Indoxyl sulfate (**Figure 20**) is derived from dietary proteins. Tryptophan is metabolized into indole by tryptophanase in intestinal bacteria such as *Escherichia*

coli. Indole is then absorbed into the blood from the intestine and metabolized to indoxyl sulfate in the liver. Normally, indoxyl sulfate is excreted into urine. In CKD patients, because of the reduced function of kidneys, indoxyl sulfate accumulates in the blood. Approximately 95% of indoxyl sulfate is bound to human serum albumin. The metabolism of indoxyl sulfate is shown in **Figure 21**.



**Figure 20.** Chemical structure of indoxyl sulfate.



**Figure 21.** The metabolism of indoxyl sulfate in human body.

### 3.3.3 Indoxyl sulfate and Aryl hydrocarbon receptor (AhR)

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcriptional factor that mediates adaptive and toxic responses in cells and now recognized as being the receptor mediating the effects of some persistent man-made organic pollutants such as dioxins. Recent studies represent IS, as well as other tryptophan metabolites, to be the first identified relatively high effect endogenous agonist for AhR and plays a key role in human disease progression.<sup>83</sup> Some studies provide evidence that IS can lead to AhR activation and altered drug metabolism. It was also observed in kidney dialysis patients that prolonged activation of the AhR by IS may contribute to toxicity and thus represent a possible therapeutic target.<sup>83</sup>

IS can stimulate the expression of AhR target genes in a time-dependent manner, as well as translocation of AhR into the nucleus, indicating AhR activation. IS directly activates AhR as an endogenous agonist and induces monocyte chemo-attractant protein-1 (MCP-1) expression to generate oxidative stress through reactive oxygen species production nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-4 in human umbilical vein endothelial cells (HUVECs).<sup>84</sup> This finding gives a new understanding of physiological effect of IS on cardiovascular system indicates a possible way to prevent cardio-renal syndrome by controlling IS level in plasma.

IS regulates sirtuin 1 (Sirt1) through impairing the intracellular nicotinamide phosphoribosyltransferase (iNampt)-NAD<sup>+</sup>-Sirt1 system and affects endothelial senescence via AhR activation.<sup>85</sup> This result indicates that blocking the effect of AhR

in the endothelium may provide a new therapeutic method for treating cardio-renal syndrome.

### **3.3.4 Uremic toxicity of indoxyl sulfate**

Indoxyl sulfate was recently identified as a novel cardiovascular factor for CKD.<sup>86</sup> IS is derived from tryptophane metabolism by intestinal microbes and there is evidence suggesting that besides the passive accumulation of bacterial uremic toxins, increased colonic generation or absorption may contribute to the high level of these uremic toxins in CKD.<sup>87,88</sup> IS has been shown to increase the risks of cardiovascular diseases and cognitive impairment in patients with CKD.<sup>89</sup> Cognitive impairment is common in patients with chronic kidney disease (CKD), but the etiologies are not clear and no study has investigated the associations of p-CS and IS with cognitive dysfunction in patients with CKD. Recent studies have showed that in the early stage of CKD, a higher serum IS level is associated with poor executive function.<sup>89</sup>

Patients with chronic kidney disease display an impairment of neovascularization in ischemic tissues.<sup>90</sup> Studies on the involvement of the uremic toxin indoxyl sulfate have demonstrated that indoxyl sulfate impairs endothelial progenitor cells function via the inhibition of hypoxia-induced hypoxia-inducible factor (HIF), interleukin-10 (IL-10), and vascular endothelial growth factor (VEGF) signaling.<sup>91</sup> In addition, as losartan is widely used in patients with end-stage renal failure (ESRF), Some studies found decreased the metabolic clearance of losartan in patients with ESRF is partly due to high concentrations of IS and p-CS.<sup>92</sup>

### 3.4 Zinc-alpha2-glycoprotein (ZAG)

Zinc-alpha2-glycoprotein (ZAG) is a secreted soluble single-chain protein which is synthesized in adipocytes and a variety of other cell types.<sup>93</sup> In 1961, ZAG was first reported in human serum and subsequently purified by Bürgi et K. Schmid.<sup>94</sup> Many recent studies dealing with the potentially exciting and proposed functions of ZAG shows that ZAG may be considered as a multidisciplinary functional protein.<sup>93</sup> ZAG is a new adipokine that acts as a lipid-mobilizing factor to stimulate lipolysis in adipocytes. ZAG is involved in body weight control through its lipid-mobilizing activity and the up-regulation of cancer cachexia and overexpressed in certain human malignant tumors.<sup>95</sup>

With a molecular weight of 43 kDa, ZAG is referred to the group of middle molecule uremic toxins. It has been shown that plasma ZAG concentration was increased in CKD and patients with hemodialysis.<sup>96</sup> In CKD patients, ZAG is overproduced in white adipose tissue (WAT) through increased lipolysis and decreased lipogenesis.<sup>97</sup> Recent study shows ZAG is regulated by growth hormone and strongly positively associated with whole-body and adipose tissue insulin sensitivity.<sup>98,99</sup> ZAG is also identified as a new candidate factor involved in the pathogenesis of insulin resistance in type 2 diabetes mellitus (T2DM).<sup>100</sup> These results indicate a potential role of ZAG in CVD and metabolic disease. ZAG was shown to accumulate in CKD patients.<sup>101</sup> Our new study was focus on the impact of ZAG accumulation on mortality and cardiovascular risks (See 4 Zinc-alpha2-glycoprotein as an uremic toxin).

### 3.5 Methods to remove uremic toxins

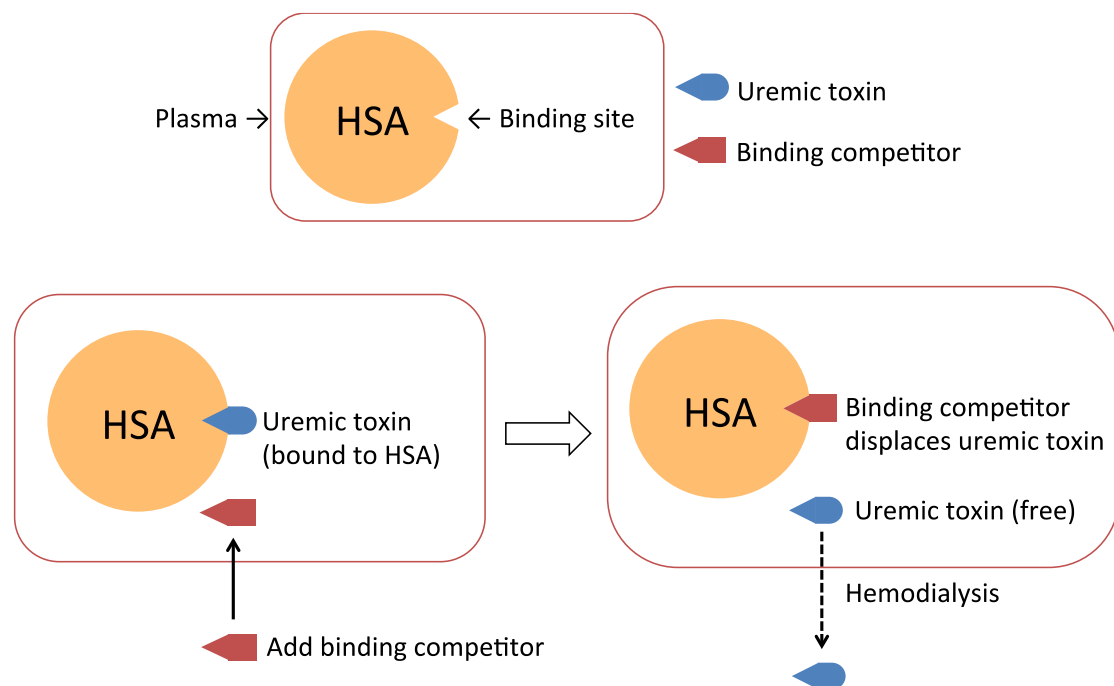
Dialysis is still the main therapeutic option for the removal of uremic toxins today. There are two methods of dialysis in clinic: Hemodialysis (HD) and peritoneal dialysis (PD). Dialysis is needed at the end stage of renal disease, usually by the time of the loss about 85 to 90 percent of the kidney function and have a GFR of  $<15$  ml/min/1.73m<sup>2</sup>. Dialysis can remove the waste, electrolytes and extra water to prevent them from building up in the body, and also keep a safe level of certain chemicals in the body. Dialysis can help to control blood pressure and keep body in balance.

Removal pattern of dialysis depends on the different physicochemical characteristics of uremic toxins. Small water-soluble compounds are easily removed by conventional hemodialysis. However, it is not the case for many other molecules.<sup>102</sup> Middle molecules can be removed better by dialyzers containing membranes with a larger pore size, namely High flux HD. It is not clear whether this removal is sufficient to prevent the related uremic complications.

Removal of protein bound uremic toxins by HD is not efficient due to its albumin binding. Larger pore size has virtually no effect on the removal of PBUTs. Therefore, nowadays, the current dialytic methods do not offer many possibilities to thoroughly remove protein-bound compounds. Nutritional and environmental factors as well as the residual renal function may influence the concentration of uremic toxins in the body fluids.

### 3.6 Future perspectives of removal of uremic toxins

Human serum albumin (HSA) can bind various ligands on different sites. Protein bound uremic toxins (PBUT) are widely bound to albumin. Therefore, some efficient displacers are studied to displace the PBUT on albumin and increase the free fraction of those toxins, which could then be removed by hemodialysis. Thus, these displacers could be a valuable option to improve PBUT clearance in hemodialysis. The probable process of the binding competitor displacing PBUTs is shown in **Figure 22**.



**Figure 22.** The probable process of the binding competitor displacing PBUTs.

As the main strategy of this method is displacement, the displacers need to be not toxic and easy to handle. Once they displace the PBUTs, free toxins can rapidly bind on other sites on HSA, and the phenomenon of “cascade displacement” is difficult to

evaluate. For an efficient strategy of displacement, the selected compound should displace the binding on both sites and need to be safe for a chronic IV use in HD. Despite some promising candidates, none of them fulfilled all the characteristics of safety, efficiency and cost-effectiveness yet.<sup>78</sup>

We designed and synthesized some compounds using this strategy. Some of these candidates showed the capacity to be displacers with high affinity binding to HSA (See **1.2** Discovery of the potential candidates of displacers for binding site II on human serum albumin (HSA)).





## Objective of the present work

The binding characteristics of p-CS and IS to HSA have been fully studied.<sup>103</sup> However, very limited data is available in the literature regarding to the binding properties of phenylacetic acid (PAA) and p-cresyl-glucuronide (p-CG) to HSA (**Table 8**). The first goal of this thesis was to characterize and fully explore the interactions between PAA or p-CG and HSA i.e. to determine their binding affinity, binding site(s), and competition with other uremic toxins.

	p-CS <sup>103</sup>	IS <sup>103</sup>	PAA	p-CG
$K_I, M^{-1}$	$1 \times 10^5$	$0.98 \times 10^5$	n/a	n/a
HSA bound	80%	78%	n/a	n/a

**Table 8.** Research status of binding properties of some uremic toxins.

Protein-bound uremic toxins are hardly removed by common hemodialysis because their tight binding to the proteins in human plasma. The second aim of this work was to find a strategy to enhance the dialytic removal of protein-bound uremic toxins. To this end, we found using a virtual screening and synthesized some candidates as potential displacers of uremic toxins on serumalbumin to improve the dialytic removal of PBUT.

At last, we focused on a middle molecule: Zinc alpha2 glycoprotein (ZAG). From the previous papers of our lab, ZAG concentration is significantly increased in the end stage CKD patients and the patients under dialysis.<sup>101</sup> To be classified as an uremic toxin, ZAG should exhibit deleterious effects. We collected abundant clinical data in order to explore whether ZAG could be a predictor of mortality in HD patients?



## Publications

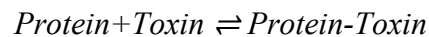
### 1 New strategy to remove protein bound uremic toxins (PBUTs)

#### 1.1 Using binding competitors of albumin to promote the removal of protein-bound uremic toxins in hemodialysis: Hope or pipe dream?

Nans Florens, Dan Yi et al, *Biochimie* 2018; 144:1-8.

Chronic kidney disease is associated with the progressive retention of a large range of uremic solutes, which are called uremic toxins. According to their physicochemical characteristics affecting their clearance during dialysis, uremic toxins are categorized into three types of molecules: small water-soluble compounds, middle molecules and protein bound uremic toxins (PBUTs).<sup>104 73</sup> Hemodialysis (HD) is efficient to get rid of the small water-soluble compounds while middle molecules can only be removed by high flux hemodialysis. Unfortunately, Protein Bound Uremic Toxins (PBUTs) are poorly removed by most hemodialysis techniques, due to their tight interactions with plasma proteins, especially human serum albumin (HSA). As a result, protein bound uremic toxins accumulate in CKD patients and their concentration can hardly be decreased in end-stage renal disease patients. This review introduces hemodialysis, PBUTs and HSA, summarizes and discusses a new strategy of PBUTs removal in CKD patients, which are chemical displacers.

Protein bound uremic toxins in the bloodstream exist into two forms as the equation below: bound fraction (non-diffusible) and free fraction (unbound). In most cases, this protein binding is reversible.



As only the free fraction is biologically active and available to removal by dialysis techniques, protein-bound uremic toxins are therefore recognized as important targets of therapeutic removal. However, The improvement of convective and diffusive properties of hemodialysis (HD) has only a limited impact on PBUTs removal. Depending on the affinity of the uremic toxin binding to serum albumin, especially onto Sudlow's sites I and II, it is theoretically possible to design a **chemical displacer** with a higher affinity to HSA and displaces the binding fraction of PBUTs. We have designed and tested some potential candidates. Some of them exhibited similar binding affinity to HSA as p-CS and IS. However, the toxicity of these candidates needs to be tested before the *in vivo* experiment.

Free fraction of the toxins can rapidly bind on other sites, even if this site is not the “predilection site “ for those toxins. Thus, for an efficient strategy of displacement, displacement could be the use of mix of multiple displacers together at the same time. The selected compounds must be safe, efficient and cost-effectiveness. A better understanding of the interactions of PBUTs with HSA and plasma protein is mandatory to develop new dialysis strategies aimed at the removal of PBUTs. Current technics of hemodialysis hardly remove PBUTs efficiently due to their strong interaction with plasma proteins. Thus novel dialysis methods should be developed to more efficiently remove these protein bound uremic toxins in order to prevent uremic complications. With a better knowledge of the structure of HSA and the binding sites

on HSA and the binding properties of protein bound uremic toxins, to remove the PBUTs in CKD patients will be easy and convenient with new strategies and technics of hemodialysis.





## Review

# Using binding competitors of albumin to promote the removal of protein-bound uremic toxins in hemodialysis: Hope or pipe dream?



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## ABSTRACT

Chronic kidney disease is associated with the accumulation of a large range of uremic retention solutes as referred to as uremic toxins. Some of these compounds belong to the group of Protein Bound Uremic Toxins (PBUT) due to their tight interactions with plasma proteins and especially serum albumin. These PBUT therefore exist in the bloodstream into two forms: a major bound (and non-diffusible) fraction and a minor free fraction. As a result, these compounds are poorly removed by most of the renal replacement therapies (such as hemodialysis) and their concentration can hardly be decreased in end-stage renal disease patients. An increase of the free fraction of PBUT could be achieved using chemical displacers that could compete with PBUT for binding to serum albumin. This review summarizes and discusses the interest of chemicals displacers as a valuable option to enhance PBUT removal in CKD patients.

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**Abbreviations:** CKD, chronic kidney disease; CMPF, carboxy-4-methyl-5-propyl-2-furanpropionic acid; DHA, docosaheptaenoic acid; ESRD, end-stage renal disease; FA, fatty acids; FPAD, Fractional plasma separation and adsorption; HD, hemodialysis; HDF, hemodiafiltration; HIPA, hippuric acid; HSA, human serum albumin; IAA, indole acetic acid; IS, indoxyl sulfate; MW, molecular weight; NEFA, non-esterified fatty acids; PBUT, protein bound uremic toxins; PD, peritoneal dialysis; PUFA, polyunsaturated fatty acids; p-CS, p-cresyl sulfate; RRF, residual renal function; UT, uremic toxins.

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## 1. Introduction

Chronic kidney disease is associated with the accumulation of a large range of uremic retention solutes. Plenty of them can be considered as uremic toxins (UT) as their accumulation leads to noxious biological effects. The “operational” classification of the uremic toxins was originally based on the main characteristics that dictate their behaviors (and thus influence their removal pattern) during hemodialysis/peritoneal dialysis processes. The classification of the uremic toxins was therefore based i) on their molecular weight (i.e. their size) and ii) their relative affinity for protein binding. The European uremic toxin working group (EuTox, <http://www.uremic-toxins.org>) divided UT into three physico-chemically relevant sub-classes: the low molecular weight water-soluble molecules (MW < 500Da, prototype being urea), the large and middle molecules (MW > 500 Da but with no strict upper limit, prototype of which being Beta<sub>2</sub>-microglobulin) and the protein-bound uremic toxins (PBUT) [1,2]. To date, 88 compounds were identified as belonging to uremic toxins [2]. Amongst these 88 compounds, 40 belong to the free water-soluble low MW compounds (<0.5 kDa) (i.e. 46%), 25 to the “middle molecules” (0.5–60 kDa) (i.e. 28%), and to 23 the protein-bound solutes (i.e. 26%). Among PBUT, and because of their high toxicity, p-cresyl sulfate and indoxyl sulfate received extensive attention. PBUT removal in end-stage renal disease (ESRD) patients undergoing renal replacement therapies remains a complex clinical challenge. Because of a low circulating free fraction, convective and diffusive techniques have limited effects on PBUT removal. Indeed, because of biochemical properties, they widely remain bound onto plasma proteins and especially onto albumin [3].

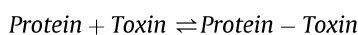
With a better knowledge about their generation and metabolism, solutions for PBUT removal have emerged but remain unsatisfactory from a clinical point of view. As only the free fraction of PBUT is available for removal by dialysis techniques, the improvement of diffusive and convective properties in hemodialysis (HD) has only a limited impact on PBUT removal. The increase of the free fraction of PBUT can be achieved using chemical displacers that could compete with PBUT onto albumin binding sites. These molecules could be valuable solutions for the improvement of PBUT removal but some issues still need to be addressed (selectivity of the displacement, toxicity of the molecules and safety of use). In this review, we will discuss about the interest of displacers as a valuable option to enhance PBUT removal in CKD patients.

## 2. Protein-bound uremic toxins: why should we focus on their removal?

### 2.1. Definition and main members

Protein-bound uremic toxins are a class of 23 compounds characterized by their tight interaction with plasma proteins and especially serum albumin. As a result, these compounds are poorly removed by most of the dialytic procedures and their concentration

can hardly be decreased in end-stage renal disease (ESRD). These uremic toxins exist in the bloodstream into two forms: a bound (non-diffusible) and a free (i.e. unbound) fractions. Depending on the affinity of the uremic toxin for plasma proteins, a variable proportion of the toxin may bind to plasma proteins, with the remaining fraction being free and thus diffusible (see Fig. 1). In most cases, this protein binding is reversible (involving non-covalent binding such as for instance electrostatic or hydrophobic interactions, Van de Waals forces or hydrogen bonds) obeying the “law of mass action”, and then a chemical equilibrium can be reached between the bound and unbound states, according to the following equation:

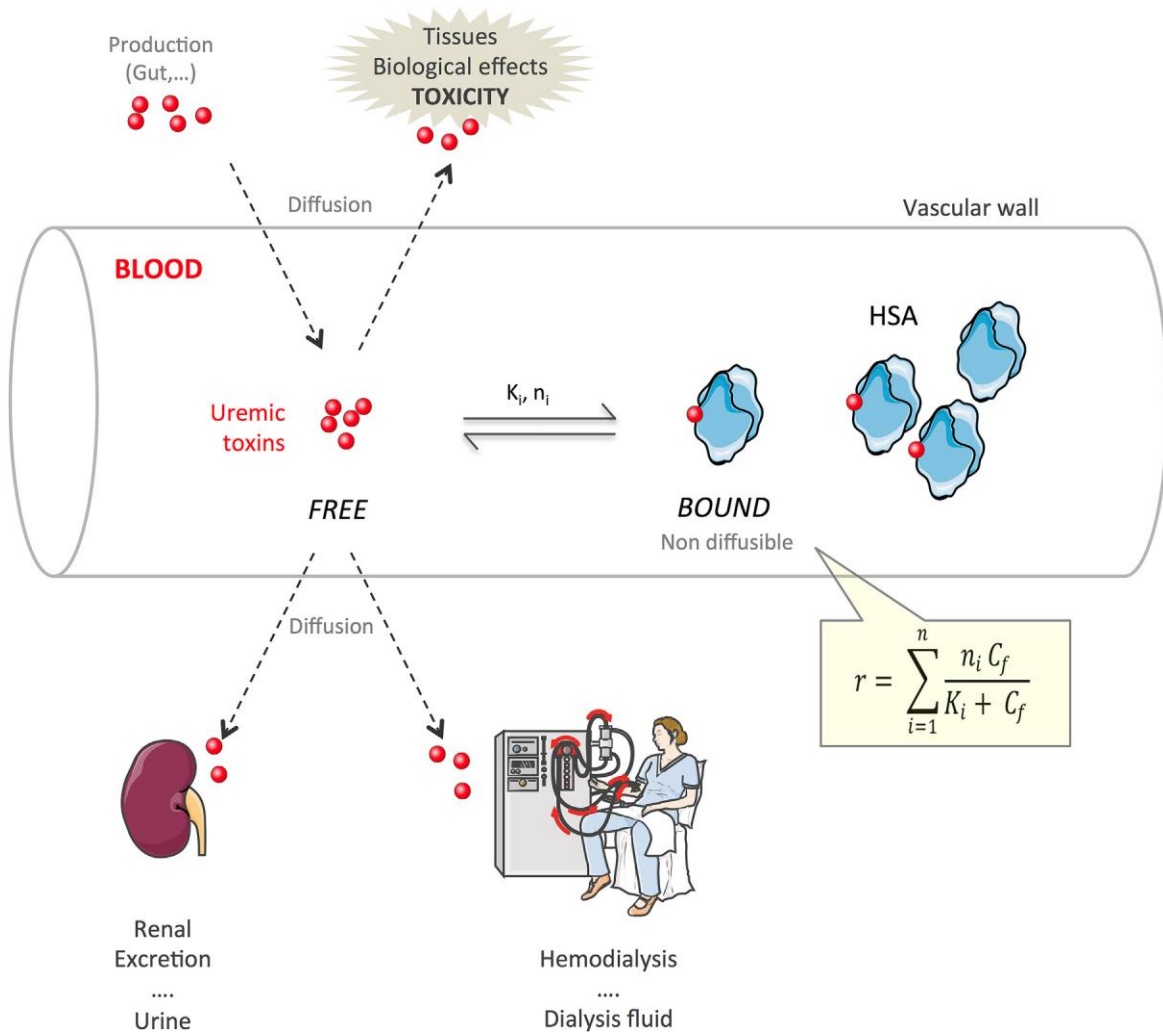


Notably, the biological action of the protein-bound molecules is exerted by their free fraction (i.e. the fully diffusible fraction). The free fraction is also the sole that can be metabolized and/or excreted. For example, the bound fraction of the p-cresyl sulfate is reported to be 95%. This means that only the remaining free fraction (i.e. 5% of the total plasmatic concentration) is biologically active and available for excretion. The bound fraction may act as a storage from which the toxin is slowly released as the unbound form. When the unbound form is being metabolized and/or excreted from the body, the bound fraction is released to maintain the equilibrium.

EuTox Group [1,2] reported many chemically different solutes to belong to this class gathering phenols, indoles, hippuric acid, amines, peptides and advanced glycation end products (see Table 1). Among those, two prototypical compounds gained a large interest because of a well-documented toxicity: Indoxyl sulfate (IS) and p-Cresyl sulfate (p-CS). The first is an indole with a molecular weight of 213 g/mol, the second is a phenol with a molecular weight of 188 g/mol. They are both roughly 90%-bound to plasma proteins. Their generation results from a sulfatation by the liver and/or enterocytes of indole and p-cresol, two solutes generated in the gut by the microbiota metabolism of respectively tryptophan, phenylalanine and tyrosine. Phenylacetic acid (PAA) is a by-product of the phenylalanine metabolism, identified by Jankowski et al. [4] and shown to be increased >300-fold in the plasma of end stage renal disease (ESRD) patients compared to healthy people [4]. Indole-3 acetic acid (IAA) is a protein-bound uremic solute issued from tryptophan metabolism. 3-Carboxy-4-methyl-5-propyl-2-furan-propanoic acid (CMPF), a toxic of unknown origin, was shown to markedly accumulates in the plasma of CKD patients [5]. Normally excreted by the kidneys, all of those toxins exert pleiotropic toxic biological effects. Thus, their removal became a major concern in ESRD patients.

### 2.2. Evidences for toxicity of PBUT

A profuse literature widely describes IS and p-CS toxicity [6–8]. p-CS and IS levels are both correlated to CKD progression [9] and p-CS level is correlated with mortality in hemodialysis patients [10]. IS has been associated with cardiovascular outcomes [7]. p-CS is



**Fig. 1. The protein bound uremic toxins (PBUTs).** The PBUTs exist in the bloodstream into two forms: a bound and a free fraction. Depending on the affinity of the uremic toxin for plasma proteins, a variable proportion of the toxin may bind to plasma proteins, with the remaining fraction being free and thus diffusible. The protein binding is reversible and a chemical equilibrium can be reached between the bound and unbound states. The binding of PBUT to albumin ( $r$  indicating the number of mole of PBUT bound per mole of albumin) depends on the affinity (i.e. evaluated by constant  $K_i$ ) and the number of binding sites (i.e.  $n_i$ ). Note that only the diffusible fraction of PBUT (i.e. the unbound fraction) is accessible for excretion or for removal by hemodialysis.

**Table 1**  
The main protein-bound uremic toxins.

Molecule	MW, Da	Protein binding, %	Binding site	References
CMPF	240	>99	High affinity, site I	[23,28]
Hippuric acid	179	42–48	High affinity, site II	[2,28,70]
Homocysteine	135	80–95	unknown	[71,72]
Indole-3-acetic acid	175	86–94	High affinity, site II	[2,28,68]
Indoxyl-sulfate	212	86–98	High affinity, site II	[2,28]
Indoxyl-glucuronide	309	60	unknown	[23,28]
p-Cresylsulfate	31	91–95	High affinity, site II	[23,28,73]
p-Cresyl glucuronide	284	8–32	unknown	[74]
Phenylacetic acid	136	26–60	High affinity, neither I nor II	[28,75]

Abbreviations: CMPF, 3-Carboxy-4-methyl-5-propyl-2-furan-propanoic acid. Note that uremic toxin covalently (i.e. noncompetitive binding) bound to proteins were not included in the present Table (AGE, pentosidine, acrolein, ...).

also involved in cardiovascular morbidity [6] and plays a role in the onset of metabolic dysfunction as insulin resistance [11]. In many in vitro studies, toxicity of p-CS and IS were tested in the presence of normal concentration of albumin demonstrating that those toxicities only involve the free fraction of those toxins [8]. Moreover, Viaene et al. demonstrated that albumin is the main plasma

binding protein for p-CS and IS [3]. In vitro, IAA induces endothelial inflammation and oxidative stress and activates inflammatory pathways. Furthermore, serum IAA concentration is an independent predictor of mortality and cardiovascular events in patients with CKD [12]. PAA exerts many deleterious biological activities and especially it impairs inducible NO synthase (iNOS) regulation in

many cell types [4]. CMPF was shown to promote pancreas dysfunction leading to diabetes [13]. Unfortunately, all these toxins are tightly bound to plasma and thus, poorly removed by hemodialysis. Taken together, these observations render relevant a focus on albumin binding sites and the strategies of displacement of those toxins.

### 3. Human serum albumin and its binding sites

Human serum albumin (HSA), molecular mass 66,500 Da, constituted by 585 amino acid residues, is a single chain, non-glycosylated protein secreted by the liver. Standing for 50–60% of the total plasma proteins, HSA is the most abundant protein in human plasma [14]. With an extraordinary ligand binding capacity, HSA binds a wide variety of drugs and carries many endogenous and exogenous compounds. Thus, it accounts for 80% of the plasma colloid oncotic pressure (25–33 mmHg) and plays an important role in modulation of fluid distribution [15,16]. Clinically, HSA is used in the treatment of severe hypoalbuminemia and as a plasma expander [16].

#### 3.1. Structure overview

X-ray crystallography study shows HSA is a heart-shaped protein with about 68%  $\alpha$ -helix without any  $\beta$ -sheet and approximate dimensions of  $80 \times 80 \times 30 \text{ \AA}$  [17,18]. HSA contains three homologous domains: domain I (residues 1–195), domain II (residues 196–383) and domain III (residues 384–585). Each domain has two long loops and one shorter loop. The first two loops in each domain are named subdomain A, and the remaining loop is donated as subdomain B. Thus, each domain is divided into two helical subdomains: subdomain A and subdomain B. Most compounds bind to one of two principal binding sites commonly referred to as Sudlow's binding sites I and II [19]. Sudlow's site I is located in subdomain IIA and Sudlow's site II is located in subdomain IIIA (See Table 2). In addition, HSA contains a single Trp residue at the position of 214 and only free Cys residue located at 34.

#### 3.2. Binding properties

The structural multi-domain organization of HSA results in its extraordinary ligand-binding capacity. HSA provides a variety of ligand binding sites: Fatty acid binding sites (FA), thyroxine binding frequent sites, bacterial protein binding site, metal binding sites, less frequent binding sites [15]. HSA is able to bind up to nine equivalents of long chain fatty acids, which are the primary physiological ligands at multiple binding sites from FA1 to FA9 [15]. Different classes of ligands bind to HSA at multiple sites. The pioneering work of Sudlow et al. shows most drugs bind with high affinity to one or two sites on human serum albumin, which are called Sudlow's sites I and II [19].

**Table 2**  
Domains, subdomains and fatty acid binding sites on HSA.

Domain	Subdomain	Binding sites
I (1–195)	IA(1–112)	FA2, FA8, FA9
	IB(113–195)	FA1, FA8, FA9
II (196–383)	IIA(196–303)	FA2, FA6, FA8, FA9, <b>FA7 (Sudlow's site I)</b>
	IIB(304–383)	FA6
III (384–585)	IIIA(384–500)	<b>FA3-FA4 (Sudlow's site II)</b>
	IIIB(500–585)	FA5

Abbreviations: FA, fatty acids, HSA, human serum albumin.  
Bold characters represent the two major sites of binding for the uremic toxins.

#### 3.2.1. Sudlow's site I (FA7)

The pocket-shaped cavity hosting FA7 in subdomain IIA represents the Sudlow's site I, which includes the only tryptophan at 214. The entrance of the cavity is surrounded by residues with positive charge, i.e., Arg257, Arg222, Lys199, His242, Arg218 and Lys195, and the inside of the pocket is formed by hydrophobic side chains [18]. Due to its hydrophobic core, ligands of site I seem to be the molecules with dicarboxylic acids or bulky heterocyclic and a negative charge in the middle of the ligand [20]. This site is capacious, flexible and harbors a large number of individual ligand-binding sites that interact each other [21,22].

The prototypical ligand of Sudlow's site I is warfarin, so site I is also called the warfarin binding site. Besides, aspirin, benzylpenicillin bind to site I on HSA. The uremic toxin 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF) is described to bind to this site on HSA [23]. The binding of racemic mixtures of warfarin to HSA is associated by an increase in the fluorescence quantum yield of these compounds [24]. This property made warfarin to be used as a specific probe to identify the binding site of drugs or uremic toxin on site I.

#### 3.2.2. Sudlow's site II (FA3-FA4)

FA3 and FA4 are composed of all six helices and located in an analogous preformed hydrophobic cavity with distinct polar features in subdomain IIIA, which constitute Sudlow's site II [25]. Site II is a hydrophobic cleft that is 12–16  $\text{\AA}$  deep and 6–8  $\text{\AA}$  wide with a cationic group located near the surface of HSA. Due to its cationic center carrying a positive charge, ligands with a negative charge or strongly electrophobic forces appear to provide the main binding energy. FA3 makes salt bridges with Arg348 and Arg485, and a hydrogen bond with Ser 342, while FA4 forms a salt bridge with Arg410 and hydrogen bonds with Tyr411 and Ser489. Site II is topologically similar to but smaller or more narrow than site I.

Ligands binding to site II are often aromatic carboxylic acids with a generally extended shape carrying the negatively charged carboxyl group at one end of the molecule away from the extended hydrophobic center [26]. Diazepam and non-steroidal anti-inflammatory drug ibuprofen are the prototypical ligands for site II [19]. The major uremic toxins indoxyl sulfate and p-cresyl sulfate are reported to bind to this site. So Sudlow's site II [23] is also called the indole-benzodiazepine binding site. As warfarin is used as probe for site I, Dansylsarcosine is used as specific probe for site II [27].

## 4. Methods for reducing PBUT levels in CKD

### 4.1. PBUT removal: state of the art

Conventional hemodialysis does not achieve a satisfactory removal of PBUT and especially for p-CS and IS. Indeed Itoh et al. reported that after an HD session the reduction rate were 32%, 29% and 0% for IS, p-CS and CMPF, respectively [28]. As the generation of those toxins is localized in the gut, rather than promoting their clearance, multiple strategies to reduce their generation have been tested so far.

#### 4.1.1. Limit PBUT generation

First, the modification of the diet by the increase in the carbohydrate and fiber compounds and a reduction of the protein intake (especially tryptophan, phenylalanine and tyrosine rich food) has been associated with a significant reduction of p-CS and IS levels in CKD patients [29,30]. Nevertheless, such a diet is difficult to undergo in CKD patients. Indeed, especially in HD patients, protein restriction lead to malnourishment and recent recommendations suggest not less than 1.1 g/kg/day [31]. Moreover, as these patients

need to control their water and potassium intake, the intake of fruits and other vegetables that contain fiber and carbohydrates must be limited. As a point for reflection, Garneata et al. showed that this diet is effective for delaying CKD progression in ESRD non-dialysis patients. Unfortunately, neither IS nor p-CS levels have been monitored in this study [32].

Second, the administration of pro-, pre- or synbiotics has been tested. Probiotics as *Lactobacillus acidophilus* showed positive effects on the serum p-CS and IS accumulation [6,33]. Prebiotics as inulin also showed interesting results in PBUT reduction. Moreover, synbiotics also showed reduction of p-CS levels in HD patients in a randomized study [34]. However, the real benefit of those therapies is not known yet as there is no evidence of a strong and undebatable effect on mortality and morbidity. Laxatives have also been considered for reducing PBUT levels as PCS and IS concentrations were correlated with colonic transit time [35].

Third, in-situ sorbents (such as AST-120) have been tested for reducing PBUT circulating levels. This strategy was a success in animal model with a significant reduction in both p-CS and IS concentration in rats [36] and also in HD patients [37]. Nevertheless, two randomized control trials failed to show a significant effect on CKD progression and spread the seeds of doubt on the relevance of this strategy for reducing PBUT levels [38,39].

#### 4.1.2. Role of residual renal function

The urine output in ESRD patients is altered. In HD and peritoneal dialysis (PD), the save of a residual diuresis can improve the tolerance and management of the water-balance. Moreover, even if glomerular filtration is anecdotal, tubular transport of such toxins is maintained. PBUT levels in ESRD patients have been linked with the residual renal function (RRF) in those patients [40]. Therefore, sparing of RRF in HD patients can be a valuable option to increase PBUT removal but many of them suffer from a quick and irreversible total loss of diuresis. Thus, enhancing PBUT removal in HD is still a central issue for the nephrologist.

#### 4.1.3. Enhance PBUT removal in renal replacement therapies

Because of a 90% binding on plasma proteins, PBUT and especially IS and p-CS are hardly removed during HD session. Combination of diffusive and convective techniques as pre- or post-dilution hemodiafiltration (HDF) seems to increase the PBUT removal rate in HD [41] but the difference between pre-, post- and mid-dilution HDF is not set [42,43]. Nevertheless, this observation is not unanimously found in the literature as some data do not suggest any differences between HDF and high-flux HD [44]. Moreover, even if the removal was significant, the clinical relevance of this reduction remains to be demonstrated.

The use of sorbent during the HD procedure can improve PBUT clearance. Fractional plasma separation and adsorption (FPAD) significantly increased the PBUT removal in different studies [45,46] but the clinical relevance and the safety of this procedure are not achieved. Other sorbent devices as nanoporous monolith [47,48], highly porous microparticles [49] or an activated carbon sorbent [50] showed their ability of removing large amounts of PBUT. HDF combined with the perfusion of hypertonic NaCl solution for increasing the ionic strength (HDF-IPIS) showed its ability of increasing the PBUT removal in-vitro [51] but this observation was qualified in a pilot study including HD patients in which only free fraction of IS was significantly increased by this technique [52].

Beyond their performance, these techniques question about the safety and easiness of use in routine HD. Indeed, FPAD induced a higher incidence of clotting events and HDF-IPIS is quite hard to settle in HD centers. Moreover, these techniques are expansive and their spread in worldwide HD centers is illusory. Thus, displacers could combine these two advantages namely: i) a facility of use and

ii) cost effectiveness.

## 5. Albumin displacers to improve PBUT clearance

### 5.1. PBUT binding on albumin

As discussed in section 3, albumin can bind various ligands on numerous sites. Uremic toxins and particularly PBUT mainly bind on Sudlow site I (e.g. CMPF) or II (e.g. IS or p-CS). IS and PCS bind both on Sudlow site II and in normal and CKD conditions, there is a competitive binding between these two toxins [23]. For PBUT, the binding affinity on albumin is variable. Indeed, IS and PCS exhibit tight binding properties requiring high affinity and concentrated displacers. Hippuric acid, CMPF or phenylacetic acid are weakly bound on albumin respectively on Sudlow site I for the two firsts and site II for the latter allowing an easier displacement. Nevertheless, specific displacers are needed for each of these two binding sites. Improving the clearance of all PBUT will involve the concomitant use of multiple displacers.

### 5.2. Enhancing PBUT clearance with displacers: proof of concept

#### 5.2.1. Non-esterified fatty acids

Non-esterified fatty acids (NEFAs) are well-known endogenous ligands for HSA which represents a major carrier for NEFAs in plasma (as discussed in section 3). Free NEFAs can therefore be used to displace the binding of uremic toxins, especially those bound to site II. This method was proved to be efficient in vitro to extract p-CS and IS from plasma proteins to allow their quantification. Octanoate (C8:0) could be an interesting displacer as its display a strong affinity for site II of albumin. A 0.24 M solution of sodium octanoate triggered a complete displacement of IS and p-CS allowing the dosage of the total fraction by liquid chromatography [53]. To our knowledge, there is no available data on the properties of enhancement of IS and p-CS clearance in the context of HD with a sodium octanoate or another short-chain NEFA. The use of NEFA in vivo has however some limitations. First, as NEFA are lipophilic compounds, their dilution into a water-based solution can be difficult, leading to the formation of emulsion. Moreover, long chain fatty acids melting point is quite high limiting their use at room temperature. For example, the melting point of decanoic acid (C10:0) is 33 °C requiring a heater to be perfused or prepared as a solution. However, the use of short chain NEFA (i.e. <8 carbons) could overcome these limitations. These issues however need further investigations. Second, at high concentrations, NEFAs can exhibit some toxic effects. Indeed, even if these compounds are physiologically present in the human blood (in the millimolar range) and are quickly metabolized by beta-oxidation, they exhibit a detergent activity onto cellular membranes and especially red blood cells leading to hemolysis [54–56]. Because of the high concentrations needed to induce the displacement, hemolysis can dramatically limit their use for a safe strategy. Docosahexaenoic acid (DHA, C22:6 (n-3)), a common polyunsaturated fatty acid (PUFA) belonging to the omega-3 family, have been successfully used to displace the binding of IS in an in-vitro setting. A continuous perfusion of DHA at 1 mM increased the removal of IS from 10% to 27% in an ex-vivo HD procedure [57]. However, large amount of PUFA or DHA could not be wise in CKD as oxidative stress is enhanced. Indeed, increase the formation of oxidized lipids can lead to cardiovascular complications [58] that are already a central issue in CKD patients (See Table 3). Furthermore, DHA is rather expensive and its extensive use could not be cost effective.

#### 5.2.2. Drugs: furosemide and ibuprofen

Serum albumin has been known for decades to be a major

carrier of hydrophobic drugs in plasma [59]. Some medicines can therefore be used as displacers to overcome the binding of PBUT. As substantial advantages, their easiness of use and their well-documented safety in numerous situations. Many drugs are carried by albumin on either site I or site II (See Table 3). Furosemide, a Henle-loop diuretic, is known to bind to Sudlow's site I of albumin. Its widespread use in nephrology and especially in HD renders its binding capacity interesting in a displacing strategy. Tao et al. reported that a 1 mM solution of furosemide can significantly increase the free fraction of HPA in a static dialysis device [60]. In the same study, furosemide also showed a significant, although more limited, effect on the displacement of IS and p-CS on Sudlow's site II [60]. Nevertheless, this dose of furosemide could be hard to reach in routine hemodialysis. Indeed, the range of circulating concentrations of furosemide is from 1 to 40 µg/mL (i.e. from 3 to 121 µM) [61–63]. In order to obtain a steady state concentration of 1 mM during HD session, massive doses of furosemide should be infused exposing patient with putative adverse effects such as ototoxicity [64].

Ibuprofen, a non-steroidal anti-inflammatory drug, is also tightly bound to plasma proteins and especially on Sudlow's site II of HSA [65]. Tao et al. reported that a concentration of 1 mM of ibuprofen can tremendously increase by a 3-fold ratio the free fraction of IS and p-CS in uremic plasma [60]. As furosemide, these concentrations could hardly be obtained in CKD patients as intravenous (IV) injection of high doses of ibuprofen (bolus of 200–800 mg) were associated with a maximal concentration of 117.12 µg/mL (i.e. 0.57 mM) in healthy patients [66]. Therefore, handling high doses of ibuprofen can be at risk for HD patients as it can trigger hypertension, infection and annihilate the residual renal function known to be precious for the removal of PBUT.

### 5.2.3. Tryptophan

Tryptophan is the precursor of IS in the gut where it is metabolized by the microbiota. As it shares structural similarities, tryptophan can bind on Sudlow's site II of albumin [67]. Tao et al. showed that a 1 mM concentration of tryptophan increased the free fraction of IS and p-CS by a 2-fold ratio [60]. Tryptophan has also been shown to compete with indole acetic acid for binding to HSA [68]. However, the injection of high doses of tryptophan in HD patients has to date not been tested since an aggravation of the uremic syndrome could be induced by the metabolism of the tryptophan.

## 5.3. Considerations and limitations of the strategy of displacement

### 5.3.1. Factors that could mitigate the efficacy of displacers

In-vitro experiments showed interesting results with displacers but the situation encountered in uremic patients is far much more complex involving the retention of multiple compounds competing for binding to the same sites on plasma proteins.

On one hand, the phenomenon of “cascade displacement” is difficult to evaluate. This phenomenon is explained by the intertwinement of displacements of such solutes. Then, solute from a site will itself displace another solute either on the same site of another particle of albumin or on another site of the same particle. Nishio et al. showed that in some patients, the displacement of NEFA by <sup>14</sup>C-Diazepam modified the allosteric conditions of binding on this site by a fixation of those NEFA on another site [69]. On the other hand, the competitive effect could be on the same site from many displaced solutes. Then with both allosteric and competitive effects, we could have unexpected increases in some concentration of solutes that we wanted to remove.

Second, because of the displacement, free toxins can rapidly bind on other sites as Sudlow site I for IS and p-CS, even if this site is not the predilection site of those toxins. Thus, a good strategy of displacement could be the use of mix of multiple displacers. Tao et al. showed that the use of both furosemide and ibuprofen at a 1 mM concentration each could significantly improve the removal of IS and indole-3 acetic acid by respectively a 2.9 and a 2.1-fold ratio [60] more than the control (phosphate-buffered saline). However, the infusion of multiple displacers can increase the risk of toxicity and the global effect is more unpredictable.

Third, the time of contact between the displacer and albumin is an important variable as in routine HD, IV administration is limited to the very beginning of the session until the end. Indeed, the exact kinetic of binding to the protein is unknown for most toxins. In Tao et al. study, the infusion of the displacer was delivered with an infusion pump upstream from the arterial port of the dialyzer. This setting allowed a 15-second time of contact between albumin and the displacers. According to their results, this could be sufficient for an effective displacement [60].

### 5.3.2. Safety of use of displacers

As previously discussed, displacers need to be safe and easy to handle. NEFAs are very effective but they can promote hemolysis in anemic HD patients [54–56]. High doses of furosemide can lead to ototoxicity [64] and the manipulation of high doses of ibuprofen is

**Table 3**  
Displacers used in the literature, efficiency, advantages and disadvantages.

Displacer	Advantages	Disadvantages	Tested PBUT	Effect on PBUT removal	References
Sodium octanoate (0.24 M)	Very efficient	Induce hemolysis	IS PCS	100% of displacement	[53]
DHA (1 mM)	Efficient Beneficial fatty acid	Hemolysis Risk of oxidation Costly	IS	40% of removal vs 10% with the vehicle	[57]
Tryptophan (1 mM)	Harmless	Could increase uremic symptoms Poor efficiency	IS IS + PCS HIPA	18.5% vs 10.2% with the vehicle 2-fold increase of the free fraction vs PBS No significant change	[57] [60] [60]
Furosemide (1 mM)	Widespread use in HD	Ototoxicity with massive doses Poor efficiency alone	IAA IS + PCS HIPA	1.4-fold more removal compared to PBS 1.3-fold increase of the free fraction vs PBS 1.5-fold increase of the free fraction vs PBS	[60] [60] [60]
Ibuprofen (1 mM)	Efficient displacer	Safety in HD patients	IS + PCS HIPA	3-fold increase in the removal vs PBS 1.3-fold increase of the free fraction vs PBS	[60] [60]
Furosemide + Ibuprofen (1 mM)	Very efficient association	Multiplication of potential side effects	IS IAA HIPA	2.9-fold increase in the removal vs PBS 2.1-fold increase in the removal vs PBS No significant change	[60] [60]

DHA, docosahexaenoic acid; IS, indoxyl sulfate; p-CS, p-cresyl sulfate; HIPA, hippuric acid; IAA, indole acetic acid; PBS, phosphate buffer saline.

at risk in HD patients. Tryptophan could be the harmless displacer but the effects of its chronic infusion has not been studied yet. Thus, the perfect displacer is not known yet (See Table 3) and further studies are needed to find harmless, efficient and cost effective displacers.

## 6. Conclusion

Displacers could be a valuable option to improve PBUT clearance in hemodialysis. Indeed, PBUT are widely bound to albumin and efficient displacers could increase the free fraction of those toxins. PBUT are mainly bound on albumin in the human plasma and especially onto Sudlow's sites I and II. For an efficient strategy of displacement, the selected compound should displace the binding on both sites and need to be safe for a chronic IV use in HD. Despite some promising candidates, none of them fulfilled all the characteristics of safety, efficiency and cost-effectiveness yet.

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## **1.2 Discovery of the potential candidates of displacers for binding site II on human serum albumin (HSA)**

Our last review about human serum albumin (HSA) and uremic toxins<sup>78</sup> introduced hemodialysis (HD), protein bound uremic toxins (PBUTs) and HSA, summarized and discussed a new strategy of PBUTs removal in CKD patients, which is chemical binding competitor of HSA. Based on this theory, we tried to find putative ligands for binding to the site II of HSA, on which the two main uremic toxins indoxyl sulfate (IS) and p-cresyl sulfate (p-CS) are prone to bind. According to the result of a virtual screening and a docking experiment, we were interested in N-phenyl glycine and some of its derivatives. Some derivatives were designed and synthesized from the structure of N-phenyl glycine. Then the binding affinity of all the candidates to HSA was tested by the displacement capacity of dansylsarcosine, a specific probe for site II, using spectrofluorimetry.

This article compared all the binding affinity of the candidates we got and synthesized, and also summarized the method to synthesize the derivatives. The candidates are: N-phenyl glycine, p139, p141, p142, p143, p144 (Name from P139 to P144 are referred to the pages of the laboratory notebook, Number: C75627, User: Dan YI), N-benzenesulfonyl glycine (BSG), N-phenyl-N-benzenesulfonyl glycine (PBSG), phenoxyacetic acid, p-cresol phosphate (p-CP), octanoic acid (C8), decanoic acid (C10).





## **Discovery of the potential candidates of displacers for binding site II on human serum albumin (HSA)**

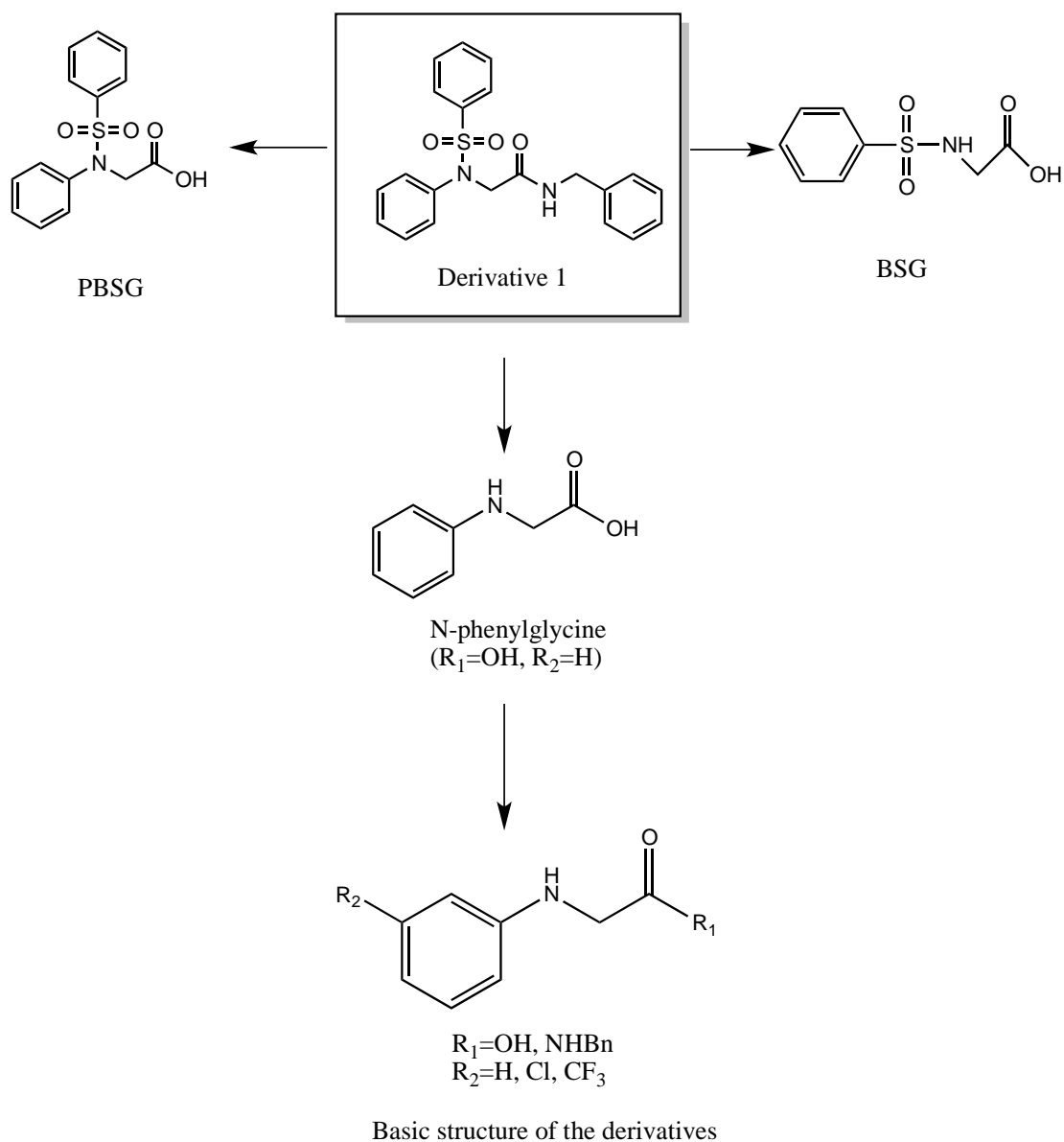
To discover putative human serum albumin (HSA) ligands for site II, we performed a **docking-based virtual screening** targeting with a subset chemical library of 12,055 compounds from Asinex.

After visual inspection of top ranked compounds, we were interested by the N-phenyl glycine (derivative 1). Indeed, this compound has a sulfonamide function such as N-dansyl-sarcosine. However, this compound is considered as very hydrophobic and exhibits a poor solubility in water, which could challenge its use in biological media. Based on this compound, we thus decide to study N-phenyl glycine derivatives structurally related to compound 1 namely N-phenyl-N-benzenesulfonyl glycine (PBSG), N-benzenesulfonyl glycine (BSG) and N-phenyl glycine (**Figure 23**).

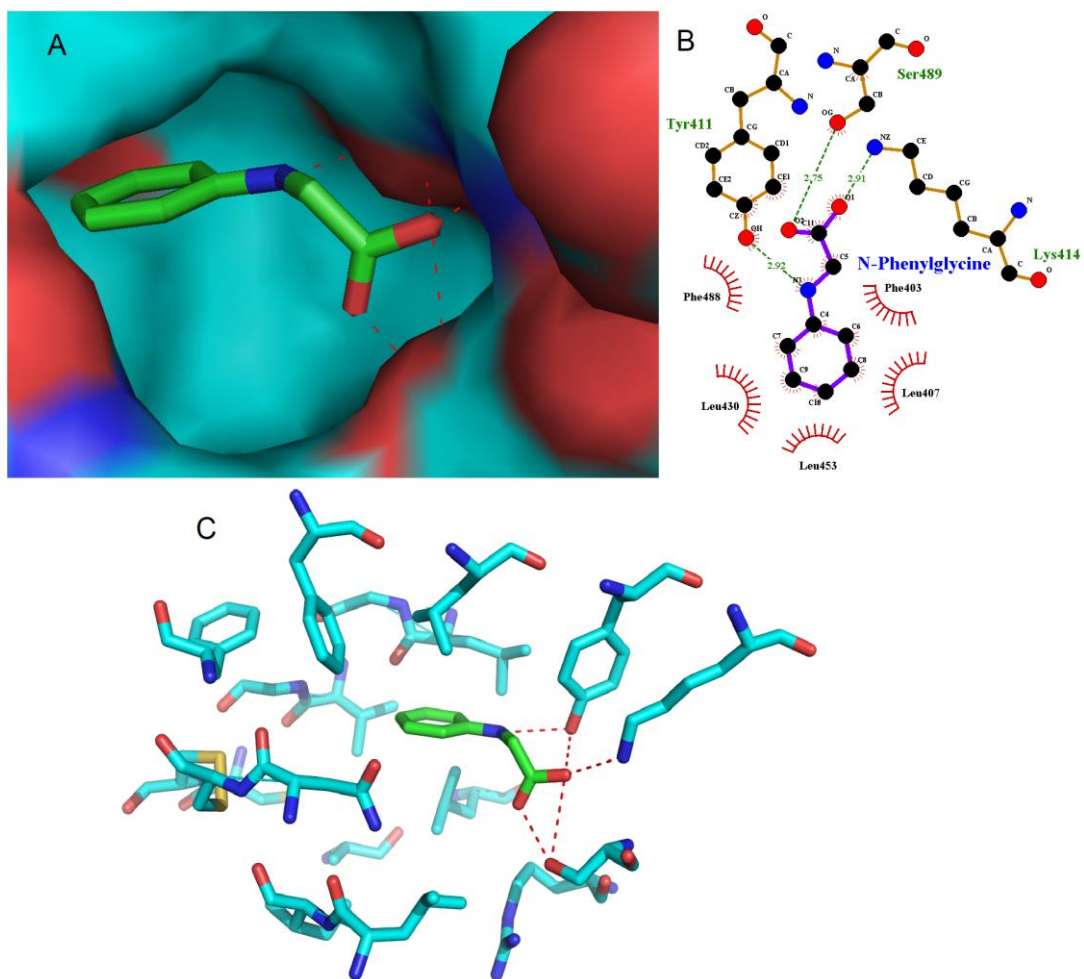
We then examined the binding mode of N-phenyl glycine via docking experiments targeting the site II of HSA. N-phenyl glycine fits very well with several hydrogen bonds with Tyr411, Ser489 and Lys414 (**Figure 24**). All heteroatoms of N-phenyl glycine are involved in hydrogen bonds and the phenyl group tightly interacts with hydrophobic residues Phe488, Phe403, Leu407, Leu430 and Leu453. The docking-based virtual screening was performed using a shape based algorithm with a subset chemical library of 12,055 compounds from Asinex using the complex HSA/N-dansyl sarcosine (pdb code 2XVQ). The binding mode of N-phenyl

glycine obtained as a result of docking experiment was generated with PyMol and the 2D-representation was generated using LigPlot+.

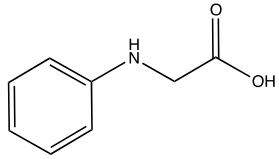
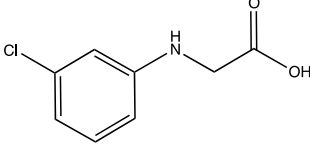
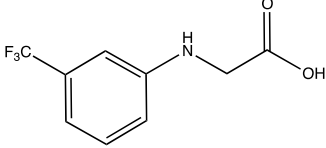
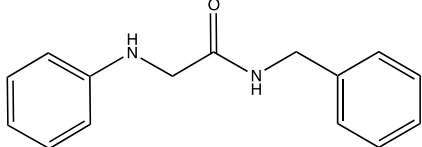
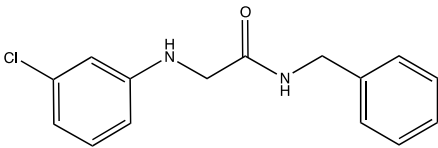
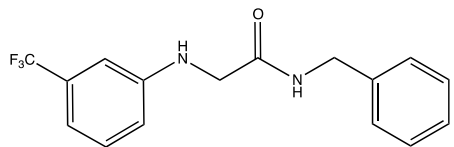
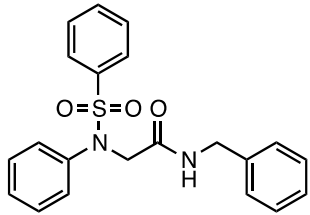
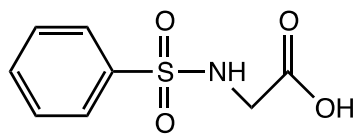
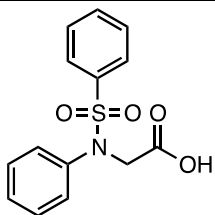
Due to the strong interactions between N-phenyl glycine and HSA from the docking experiment, we designed and synthesized a series of derivatives (**Figure 23**) according to the basic structure of N-phenyl glycine in order to test their binding affinity to site II on HSA. The structure determines the binding property. Because phenoxyacetic acid has a very similar structure with N-phenyl glycine (i.e. the nitrogen is replaced by the oxygen), and p-cresyl phosphate has a similar structure with p-cresyl sulfate, they were selected and expected to be good candidates binding to site II. Besides, we also tested two long-chain compounds: octanoic acid (C8) and decanoic acid (C10). **Table 9** shows the information and structures of all the compounds that we designed, selected, synthesized and have already been tested.

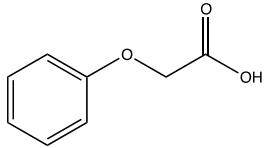
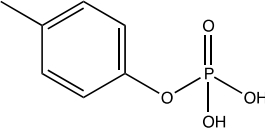
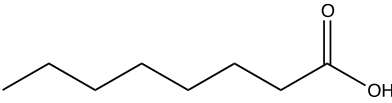
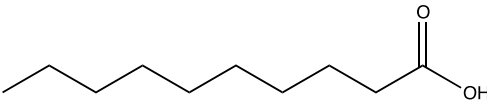


**Figure 23.** N-phenyl glycine derivatives structurally related to the compound named “Derivative 1”. Some other derivatives were designed from the basic structure of N-phenylglycine.



**Figure 24.** Proposed binding mode of N-phenyl glycine in the binding site II of HSA: interacting with N-phenyl glycine colored in green.

Name	Mw	R1	R2	Structure
N-phenyl glycine	151.16	OH	H	
P143	185.61	OH	Cl	
P144	219.16	OH	CF <sub>3</sub>	
P139	240.30	NHBn	H	
P140	274.75	NHBn	Cl	
P141	308.30	NHBn	CF <sub>3</sub>	
Derivative 1 (P142)	380.46	/	/	
N-benzenesulfonyl glycine (BSG)	215.23	/	/	
N-phenyl-N-benzenesulfonyl glycine (PBSG)	291.32	/	/	

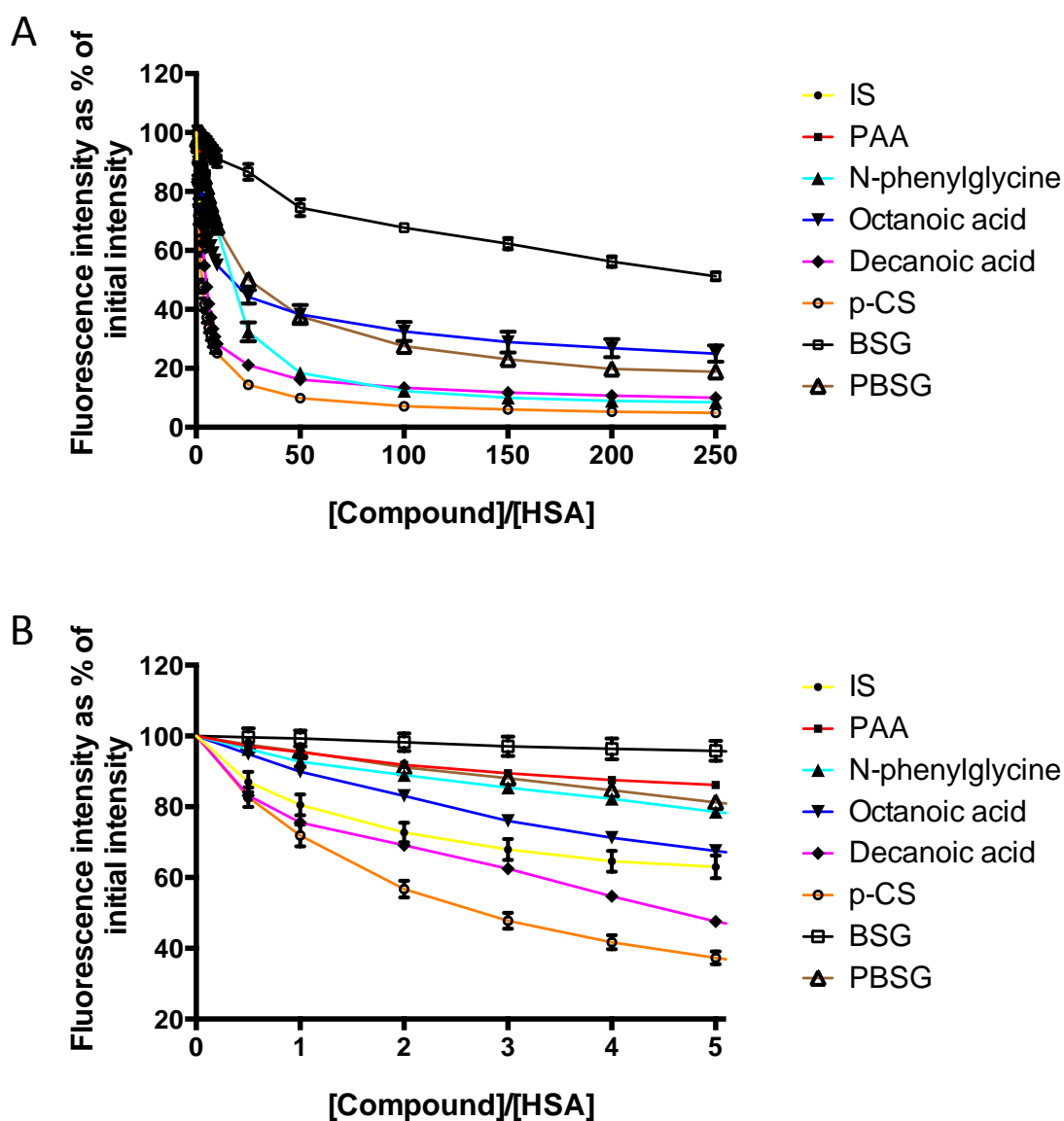
Phenoxyacetic acid (A7)	152.15	/	/	
p-Cresol phosphate (p-CP)	188.12	/	/	
Octanoic acid (C8)	144.21	/	/	
Decanoic acid (C10)	172.26	/	/	

**Table 9.** The list of molecules of which we tested the binding affinity to site II on HSA. Name from P139 to P144 are referred to the pages of the laboratory notebook (Number: C75627, User: Dan YI).

Dansylsarcosine is used as a specific probe for site II. The binding of racemic mixtures of dansylsarcosine to HSA is associated with an increase in the fluorescence quantum yield of these compounds. This property made dansylsarcosine to be routinely used as a specific probe to identify ligands to binding site II on HSA. The fluorescence yield of dansylsarcosine highly enhances on binding to HSA with an exciting light at the wavelength of 350nm. The binding of dansylsarcosine and the candidate molecules occurs in a competitive manner. The decreased intensity of fluorescence indicates the competitive displacement of dansylsarcosine from albumin. The results of displacement experiments of all our potential candidates for binding to site II are showed in **Figure 25**, **Figure 26** and **Figure 27**. Dansylsarcosine is used as site II probe with the excitaton/emission wavelength 350nm/475nm. With a high concentration, N-phenyl glycine, octanoic acid, decanoic acid and PBSG had a

high affinity binding to site II of HSA (**Figure 25A**), and had a higher affinity than PAA in low concentration (**Figure 25B**). Decanoate even had a higher binding affinity than IS, indicating to be a good candidate to displace IS binding on HSA. But none of them demonstrated a greater binding affinity than p-CS. Therefore, we continued to design and synthesis some other compounds according to the binding affinity to site II analyzed by the docking experiment, such as p139, p140, p141, p142, p143 and p144 (**Table 9**), and we also chose phenoxyacetic acid which shares a similar structure with N-phenyl glycine, one of the good ligands we had tested for site II (**Figure 25**), and p-cresyl phosphate which just has one atom difference with p-cresyl sulfate. However, even if with very similar structures with good ligands, neither phenoxyacetic acid nor p-cresyl phosphate exhibited a high affinity binding to site II (**Figure 26**). Because of the low solubility of some new synthesized compounds, we added 1% (v/v) dimethyl sulfoxide (DMSO) to prepare the working solution and stopped the test once the precipitation occurred. We found that compounds p143 and p144 could produce fluorescence themselves at the excitation wavelength 350nm, leading to a increasing of fluorescence in high concentrations (**Figure 26A**). To adjust the decreased fluorescence by the displacement of dansylsarcosine, we tested the fluorescence of p143 and p144 alone in PBS. **Figure 27** shows the data adjusted by the fluorescence produced by the compounds alone. Compounds p143 and p144 could not reach to the displacement capacity of N-phenyl glycine. So far, N-phenyl glycine is the best ligand that we found.



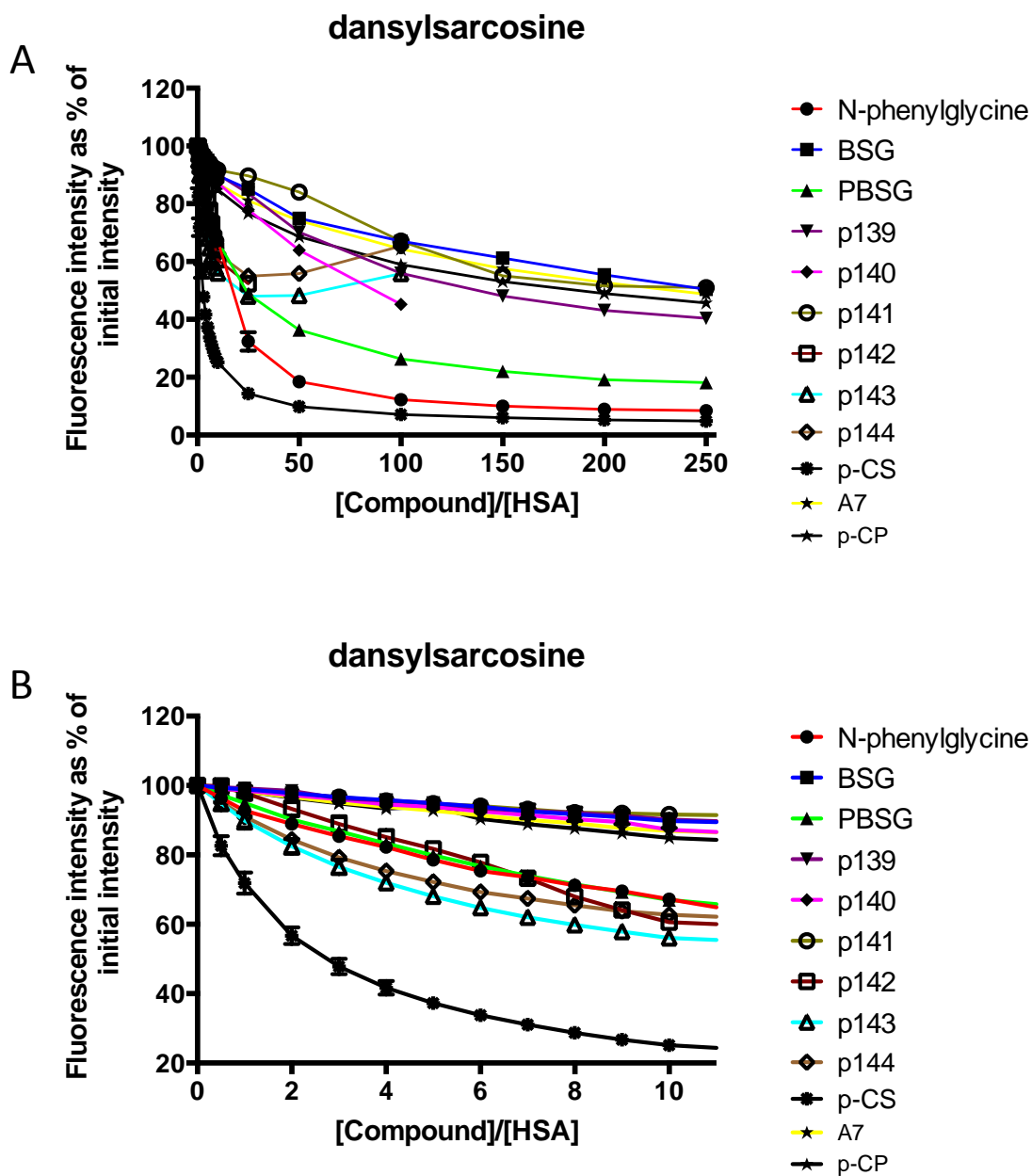


**Figure 25.** Displacement capacity of IS, PAA, N-phenylglycine, octanoic acid, decanoic acid, p-CS, BSG and PBSG for dansylsarcosine on binding site II of HSA.

A: [compound]/[HSA] concentration ratio from 0 to 250.

B: [compound]/[HSA] concentration ratio from 0 to 10.

**Abbreviations:** IS, indoxyl sulfate; PAA, phenylacetic acid; p-CS, p-cresyl sulfate; BSG, N-benzenesulfonyl glycine; PBSG, N-phenyl-N-benzenesulfonyl glycine.



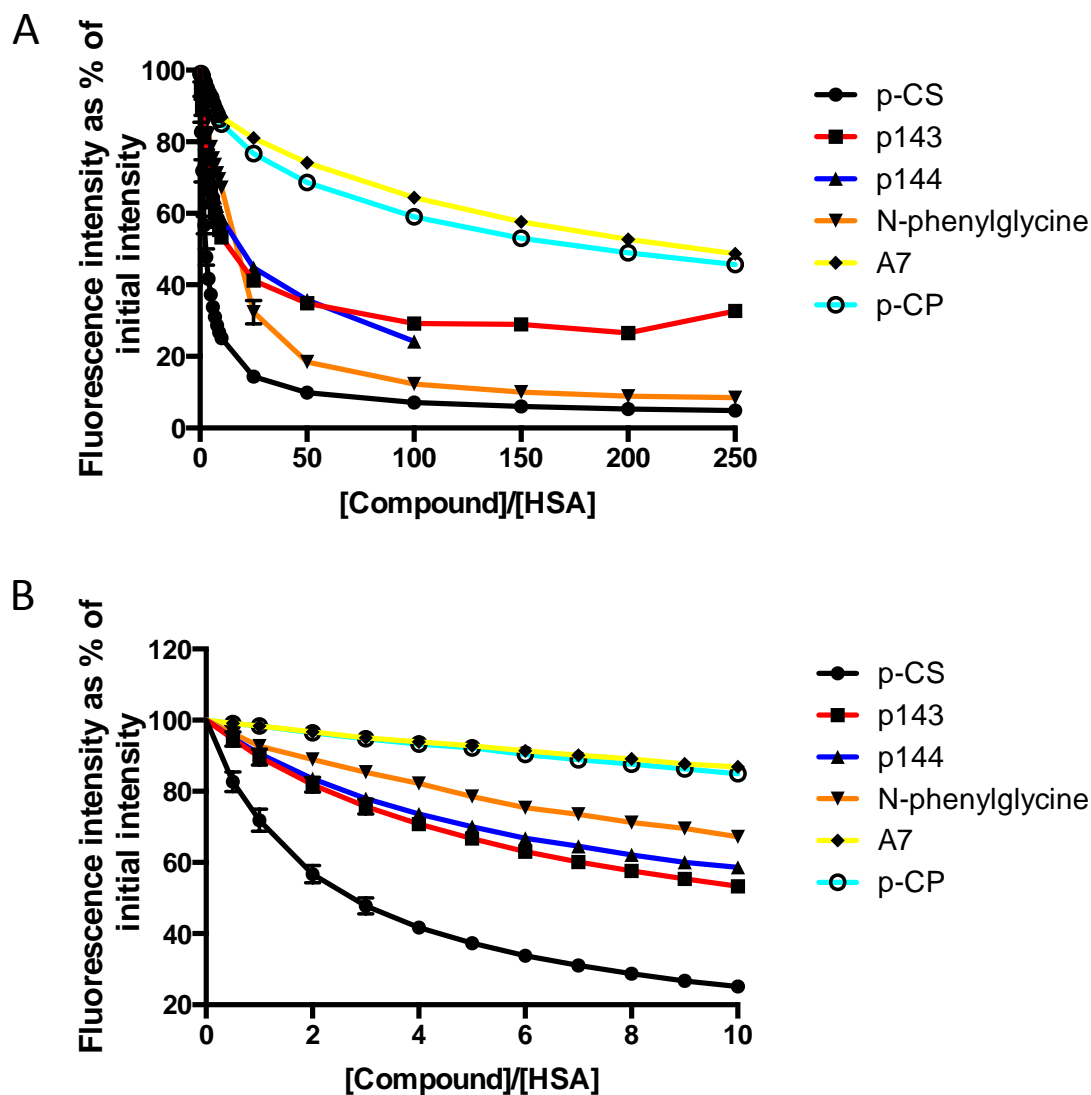
**Figure 26.** Displacement capacity of N-phenylglycine, p139, p140, p141, p142, p143, p144, phenoxyacetic acid, p-CS, p-CP, BSG and PBSG to dansylsarcosine on binding site II of HSA.

A: [compound]/[HSA] concentration ratio from 0 to 250.

B: [compound]/[HSA] concentration ratio from 0 to 10.

**Abbreviations:** p-CS, p-cresyl sulfate; BSG, N-benzenesulfonyl glycine; PBSG,

N-phenyl-N-benzenesulfonyl glycine; A7, phenoxyacetic acid; A7, phenoxyacetic acid; p139 to p144 are compounds referred to the pages of the laboratory notebook (Number: C75627, User: Dan YI) (Table 9).



**Figure 27.** Displacement capacity of N-phenylglycine, p143, p144, phenoxyacetic acid, p-CS, p-CP BSG and PBSG to dansylsarcosine on binding site II of HSA. Data of compounds p143 and p144 were adjusted by the fluorescence produced by themselves.

A: [compound]/[HSA] concentration ratio from 0 to 250.

B: [compound]/[HSA] concentration ratio from 0 to 10.

**Abbreviations:** p-CS, p-cresyl sulfate; BSG, N-benzenesulfonyl glycine; PBSG, N-phenyl-N-benzenesulfonyl glycine; A7, phenoxyacetic acid; p-CP, p-cresyl phosphate; p143 and p144 are compounds referred to the pages of the laboratory notebook (Number: C75627, User: Dan YI) (**Table 9**).

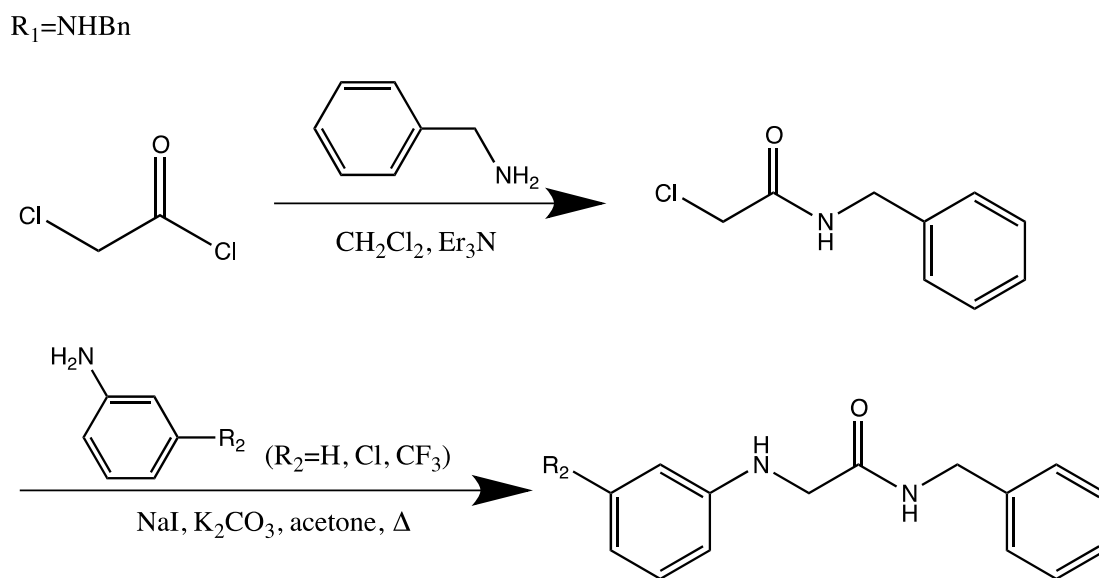
### Synthesis

N-phenyl-N-benzenesulfonyl glycine. A solution of N-phenylbenzenesulfonamide (3g, 12.86 mmol), tert-butyl 2-bromoacetate (3.763 g, 19.29 mmol) and  $K_2CO_3$  (3.555 g, 25.72 mmol) in acetone (30 mL) was refluxed for 3 hours. The solution was filtered and the filtrate was evaporated. The corresponding ester intermediate was purified using column chromatography pentane/diethyl ether: 7/3 to give a white solid (2.753 g, 80 %).  $^1H$  NMR (300 MHz,  $CDCl_3$ ): 1.40 (s, 9H); 4.32 (s, 2H); 7.19-7.22 (m, 2H); 7.29-7.32 (m, 3H); 7.44-7.49 (m, 2H); 7.55-7.61 (m, 1H); 7.67-7.70 (m, 2H). The tert-butyl ester (1.05 g, 3.94 mmol) was deprotected for 24 hrs using a mixture TFA/ $CH_2Cl_2$ : 4/6 (10 mL). After evaporation, a white solid was recovered in quantitative yield.  $^1H$  NMR (300 MHz,  $CDCl_3$ ): 4.45 (s, 2H); 7.16-7.19 (m, 2H); 7.28-7.32 (m, 3H); 7.42-7.48 (m, 2H); 7.55-7.60 (m, 1H); 7.64-7.67 (m, 2H).

N-benzenesulfonyl glycine. A solution of glycine (1 g, 13.32 mmol),  $NaHCO_3$  (2.80 g, 33.30 mmol) and benzenesulfonyl chloride (2.34 g, 13.32 mmol) in water (10 mL) was stirred for 3 hours at 80°C. After cooling at RT, the pH was adjusted at pH 1

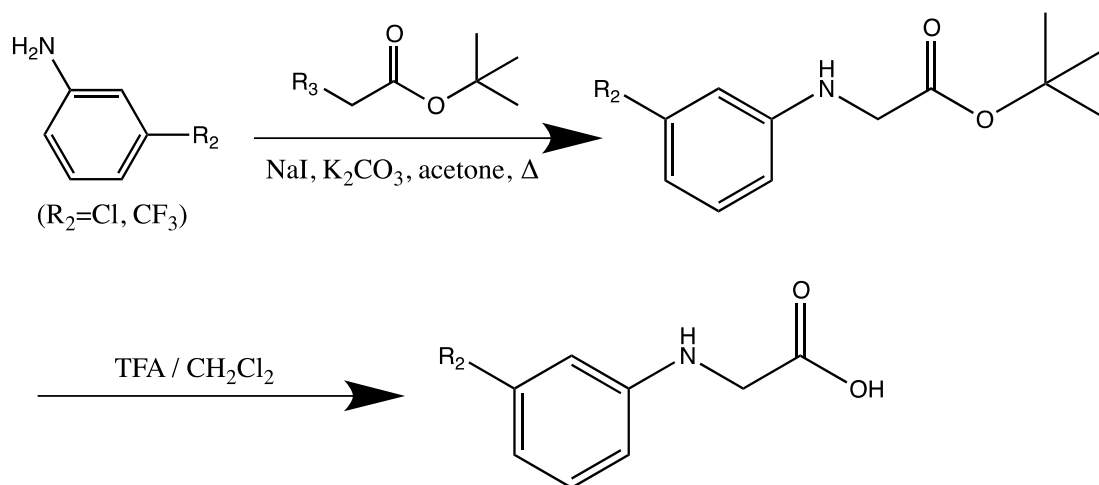
with HCl 37 % and the solution kept at 4°C until a white solid appears. The solid was recovered by filtration (1.76 g, 62 %). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 3.58 (d, J = 6 Hz, 2H); 7.55-7.66 (m, 3H), 7.79-7.81 (m, 2H), 8.05 (t, J = 6 Hz, 1H).

N-phenyl glycine derivatives of p139, p140 and p141 were synthesized according to the scheme in **Figure 28**. N-phenyl glycine, p143 and p144 were synthesized according to the scheme in **Figure 29**. Derivative 1 (p142) was synthesized by the scheme in **Figure 30**.

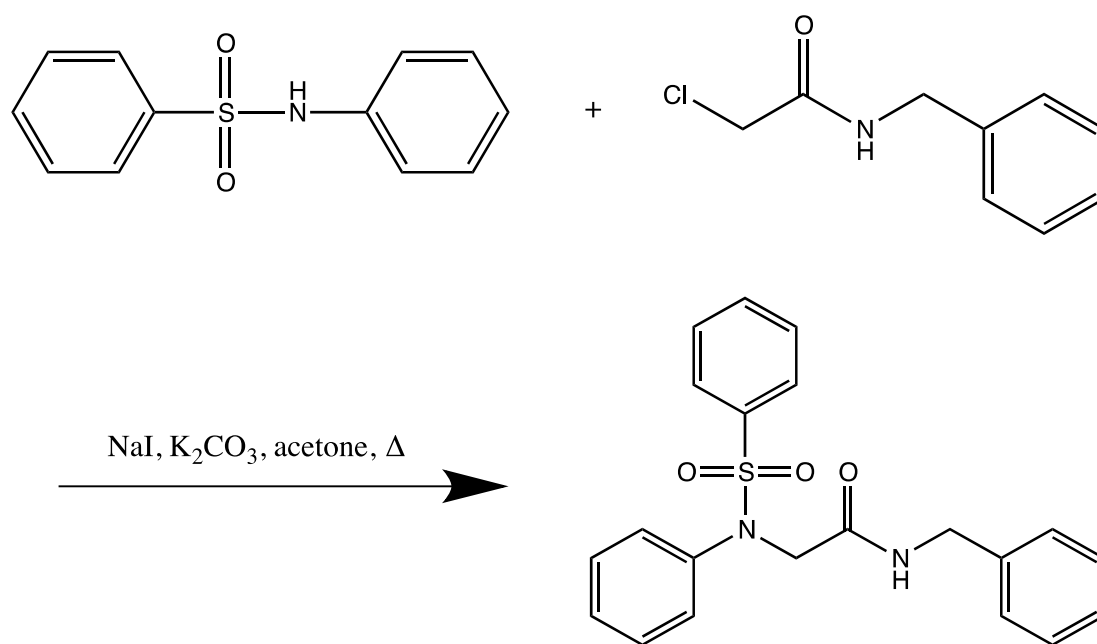


**Figure 28.** Scheme to synthesize compounds p139, p140 and p141.

$R_1=OH$



**Figure 29.** Scheme to synthesize compounds N-phenyl glycine, p143 and p144.



**Figure 30.** Scheme to synthesize compound derivative 1 (p142).



## 2 Determination of the binding properties of uremic toxins

### 2.1 Phenylacetic acid (PAA)

*Determination of the binding properties of the uremic toxin phenylacetic acid to Human Serum Albumin*

*Juliana F. Saldanha\*, Dan Yi\* et al, Biochimie, 2016; 125: 53-58.*

*\*These authors contributed equally to this work.*

Protein bound compounds, such as p-cresyl sulfate (p-CS) and indoxyl sulfate (IS), are characterized by their tight binding to plasma proteins, so that these hydrophobic uremic toxins are poorly cleared using conventional dialysis and thus strongly accumulate in end stage renal disease patients. Among variable protein bound uremic toxins, phenylacetic acid (PAA) is a weak aromatic acid and was first identified as a protein bound uremic solute in 2009.<sup>105</sup> PAA is a degradation product of phenylalanine metabolism. In vivo studies showed that roughly 30% of PAA is protein bound in CKD patients. Some studies reported that PAA may contribute significantly to the progression of CKD and to the increased risk of cardiovascular disease observed in CKD patients, and PAA concentration in plasma increased more than 300 times of end stage renal disease (ESRD) patients compared with healthy people.

The protein binding of uremic toxins is a critical issue in CKD patients. Since PAA most likely has a strong impact on the pathogenesis of cardiovascular disease and a pro-inflammatory action in CKD.<sup>106</sup> The importance of removal of this uremic



toxin in patients is imperative. Total plasma PAA level consists of a protein bound fraction and an unbound fraction. The unbound fraction is biologically active and to be removed using dialysis. A better understanding of the interactions of phenylacetic acid with HSA and plasma protein is mandatory to develop new dialysis strategies aimed at the removal of PAA. However, no data is available in the literature regarding the characteristics of the binding of PAA to HSA. Therefore, the purpose of this study was therefore to investigate in vitro the interaction between PAA and human serum albumin (HSA) at physiological and pathological concentrations.

We elucidated some questions and try to help future clinical strategies of uremic toxins removal. To our knowledge, this is the first determination of binding constant of PAA to HSA. This article was designed to investigate the interactions between PAA and human serum albumin. We characterized the binding PAA percentage in vitro and detected binding site of PAA to HSA. About 26% PAA is binding to HSA (**Table 10**). The binding parameters of PAA to HSA are shown in **Table 11**. Single-binding site model is the best fitting model, indicating that there is only one single high-affinity binding site for PAA with a binding constant ( $K_i$ ) of  $3.4 \times 10^4 \text{M}^{-1}$ . Although PAA neither bound to site I nor site II on HSA, we also explored its competition with two common uremic toxins p-cresyl sulfate and indoxyl sulfate for HSA binding. There is no competition between these two uremic toxins and PAA for binding to HSA.

	Bound, %	Free, %	r
Human Serum Albumin	25.8 ± 1.7	74.2 ± 1.7	1.61 ± 0.08
Bovine Serum Albumin	20.6 ± 3.2	79.4 ± 3.2	1.36 ± 0.22
<i>P-value</i>	0.182	0.182	0.324

**Table 10.** Protein binding of PAA as determined by ultrafiltration at pH 7.4 and 37°C. Human serumalbumin (500 µM) or bovine serumalbumin (500 µM) were incubated for 1 h with PAA at the concentration encountered in end stage renal disease patients (450 mg/L i.e. 3.3 mM). Data are the mean ± SEM for 6 independent experiments. r, number of mole of PAA bound per mole of HSA.

Model	N <sub>i</sub>	K <sub>i</sub> , M <sup>-1</sup>	χ <sup>2</sup>	AIC
Single binding site	1.875	3.38×10 <sup>4</sup>	0.107	-226.21
Two binding sites	1.831	3.86×10 <sup>4</sup>	0.103	-197.84
	0.076	3.66×10 <sup>5</sup>		

**Table 11.** Binding parameters of PAA to HSA as determined by ultrafiltration at pH 7.4 and 37°C. N<sub>i</sub>, number of binding site; K<sub>i</sub>, dissociation constant; χ<sup>2</sup>, residues; AIC, Akaike information criteria. Note that from AIC analysis, one site model is the best fit to model the experimental data since it exhibited the lowest AIC score.





## Research paper

# Determination of the binding properties of the uremic toxin phenylacetic acid to human serum albumin



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## ABSTRACT

Uremic toxins are compounds normally excreted in urine that accumulate in patients with chronic kidney disease as a result of decreased renal clearance. Phenylacetic acid (PAA) has been identified as a new protein bound uremic toxin. The purpose of this study was to investigate *in vitro* the interaction between PAA and human serum albumin (HSA) at physiological and pathological concentrations. We used ultrafiltration to show that there is a single high-affinity binding site for PAA on HSA, with a binding constant on the order of  $3.4 \times 10^4 \text{ M}^{-1}$  and a maximal stoichiometry of 1.61 mol per mole. The PAA, at the concentration reported in end-stage renal patients, was 26% bound to albumin. Fluorescent probe competition experiments demonstrated that PAA did not bind to Sudlow's site I (in subdomain IIA) and only weakly bind to Sudlow's site II (in subdomain IIIA). The PAA showed no competition with other protein-bound uremic toxins such as *p*-cresyl-sulfate or indoxyl sulfate for binding to serum albumin. Our results provide evidence that human serum albumin can act as carrier protein for phenylacetic acid.

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## 1. Introduction

The prevalence of chronic kidney disease (CKD) has reached epidemic proportions and today about 10% of the population shows signs of kidney dysfunction [1]. In CKD, the urinary excretion of many metabolites decreases and causes the accumulation in the body fluids of many deleterious compounds often referred to as uremic toxins [2,3] that contribute to pathophysiology of the

uremic syndrome [4], associated with the cardiovascular morbidity and mortality in this population [5–8].

Among the uremic toxins, the protein-bound compounds, like *p*-cresyl sulfate (*p*-CS) and indoxyl sulfate (IS), are characterized by their difficult removal by dialysis [9] due to their tight binding to plasma proteins, especially to human serum albumin (HSA) [10]. Human serum albumin is the most abundant serum protein, which carries numerous endogenous compounds as well as many xenobiotics and drugs [11]. HSA binding is therefore of great interest as its impacts the drug pharmacokinetic properties [12]. Most aromatic and heterocyclic ligands bind within two hydrophobic pockets in subdomains IIA and IIIA, referred to as Sudlow's site I (known as the warfarin binding site) and site II (known as the benzodiazepine and indole binding site) [13]. Several drug binding sites have been localized in subdomains IB, IIIA, IIIB but also in the subdomain interfaces. Regarding the uremic toxins, phenylacetic acid (PAA) is a weak aromatic acid with the molecular formula  $\text{C}_8\text{H}_8\text{O}_2$ , structured as shown in Fig. 1, and with a molecular weight of 136.15 Da [14]. It gained attention in 2009 as a newly identified

**Abbreviations:** BSA, bovine serum albumin; CKD, chronic kidney disease; ERK, Extracellular signal-regulated kinases; ESRD, end-stage renal disease; HSA, human serum albumin; HD, hemodialysis; JNK, c-Jun N-terminal kinases; IL-1 $\beta$ , interleukine 1 $\beta$ ; IS, indoxyl-sulfate; PAA, phenylacetic acid; *p*CS, *p*-cresyl sulfate; CMPF, 3-Carboxy-4-methyl-5-propyl-2-furanpropanoic acid; ROS, reactive oxygen species; PBS, phosphate buffered saline; CVD, cardiovascular diseases; iNOS, inducible nitric oxide synthase; NO, nitric oxide.

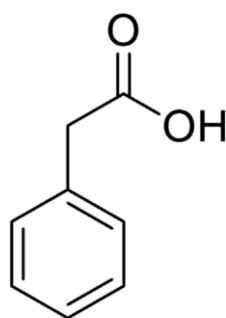
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## 2-phenylacetic acid

Fig. 1. The molecular structure of phenylacetic acid.

uremic toxin belonging to the group of protein-bound uremic solutes [15]. PAA, originally identified by Jankowski et al. [16], is a degradation product of phenylalanine metabolism, and was shown to be increased >300-fold in the plasma of end stage renal disease (ESRD) patients compared to healthy people [17]. From *in vivo* studies, it was initially reported that roughly 30% of PAA is protein-bound in CKD patient plasma [17].

PAA exerts many deleterious biological activities and in particular impairs inducible NO synthase (iNOS) regulation in many cell types. In mononuclear leukocytes, PAA dose dependently inhibits LPS-induced iNOS expression [17]. In murine macrophage cells line RAW 264.7, PAA inhibits iNOS mRNA and protein expression, nitrite formation and activation of the mitogen-activated protein kinases ERK1/2 and JNK [18], and this inhibition might affect immunoregulatory processes in CKD patients [15]. Also, PAA demonstrated an ability to increase reactive oxygen species (ROS) production, through the enhancement of the IL-1 $\beta$ -mediated induction of iNOS expression in vascular smooth cells [19], suggesting that this uremic toxin may contribute to oxidative stress in CKD patients [15]. In addition, PAA is suggested to play a role in the pathogenesis of CKD-associated bone disease by its inhibitory effect on osteoblast function [20]. Jankowski et al. further observed that PAA is sufficiently lipophilic to penetrate the blood-brain barrier and to accumulate in cerebral tissue, inhibiting Ca<sup>2+</sup> ATPase, which could be of importance in renal failure for cerebral toxicity [16].

The protein binding of uremic toxins is a critical issue in CKD patients. However, to date, no data is available in the literature regarding the characteristics of the binding of PAA to HSA. Since PAA has most likely has a strong impact on the pathogenesis of cardiovascular disease and inflammatory system in CKD, the importance of removal of this uremic toxin in patients is imperative [14]. Therefore, to elucidate some questions and try to help future clinical strategies of uremic toxins removal, the aims of this study were: (1) to characterize the binding PAA percentage to HSA *in vitro*; (2) to explore the binding site of PAA on HSA; and, (3) to evaluate its possible competition with two common uremic toxins *p*-cresyl sulfate and indoxyl-sulfate for HSA binding.

## 2. Material and methods

### 2.1. Chemicals

Phenylacetic acid (reference P16621), warfarin (reference A2250), Bovine Serum Albumin (BSA, reference A2153) and Human Serum Albumin (HSA, reference A9511) were purchased from Sigma Aldrich, (Saint Quentin Fallavier, France). We chose not to

use defatted HSA, to mimic normal physiological conditions, where 0.1–2 fatty acid molecules are bound to albumin [21,22]. Dansyl-sarcosine was synthesized according to the method described by Parameswaran & Lorand [23]. Para cresyl-sulfate (*p*-CS) was synthesized according to the method described by Feigenbaum & Neuberger [24]. All solvents were purchased from Carlo-Erba reagents (Val-de-Reuil, France) and were of HPLC grade. Unless otherwise stated, all chemicals were obtained from Sigma Aldrich.

### 2.2. Determination of *in vitro* HSA PAA binding

A quantitative analysis of potential interaction between PAA bound to HSA was performed by ultrafiltration according to the guidelines of Zhang et al. (2012) [25]. The ultrafiltration was carried out using an ultrafiltration system (Centrisart<sup>®</sup>, Sartorius Stedim Biotech GmbH, Germany) of molecular weight cut-off (MWCO) 10,000 Da. HSA (500  $\mu$ M) and PAA (1–450  $\mu$ g/ml i.e. 7.3–3300  $\mu$ M) were dissolved in PBS, the pH was adjusted to 7.30 with sodium hydroxide (5 M), and the solution was incubated at 37 °C for 1 h. One ml of each sample was ultrafiltered at room temperature and 1000 rpm for 5 min, then 3000 rpm for 15 min. Free PAA concentration was measured by HPLC coupled to UV detection as described below. The bound PAA concentration was calculated using the following equations:

$$NSB = \frac{C_D - C_F}{C_D} \quad (1)$$

$$F_U = \frac{C_{PF}}{(1 - NSB) \times C_{PD}} \quad (2)$$

$$Bound(\%) = (1 - F_U) \times 100 \quad (3)$$

We used Eqs. (1)–(3) to determine the NSB and % protein binding. NSB is non-specific binding;  $C_D$  is the concentration of PAA in PBS;  $C_F$  is the concentration of PAA in filtrate after ultrafiltration of PAA in PBS;  $C_{PD}$  is the concentration of PAA in HSA solution;  $C_{PF}$  is the concentration of drug in filtrate after ultrafiltration of drug in HSA solution;  $F_U$  is the unbound i.e. the free fraction of drug.

The binding data were analyzed according to the two following equations assuming one or two binding site:

$$r = \sum_{i=1}^n \frac{n_i C_f}{K_i + C_f}$$

where  $r$  is the number of moles of bound drug per mole of protein,  $n_i$  is the number of binding sites per protein molecule,  $K_i$  is the binding constant in the  $i$ th binding class and  $C_f$  is the concentration of the free fraction of PAA. Non-linear regression was used to predict binding of PAA to HSA using GraphPad Prism 4.5 for Mac OS X (GraphPad Softwares, San Diego, USA). The number of moles of bound drug per mole of protein ( $r$ ) was calculated according to the following equation:

$$r = \frac{Cb}{[HSA]}$$

where  $C_b$  is the concentration of PAA bound to albumin.

### 2.3. HPLC assay of phenylacetic acid

The HPLC system consisted of an Agilent technologies LC 1200 series system coupled to an UV-visible diode array detector. A RP-C18 Spherisorb ODS2 column (5  $\mu$ m, 4.6  $\times$  250 mm, Grace Discovery Sciences, Epernon, France) was used as the stationary phase.

The mobile phase consisted of 50% (v/v) acetonitrile and 50% (v/v) ultrapure water acidified (pH 3.0) with H<sub>3</sub>PO<sub>4</sub> 12 N. The flow rate was 1.5 ml/min. PAA was detected at 210 nm. Under these conditions, the retention time of PAA was 2.3 min.

#### 2.4. Fluorescent probe displacement

Fluorescence was measured with a Jasco FP-8300iRM Spectrofluorometer (Jasco, Tokyo, Japan). The excitation and emission wavelengths were set at 320/390 nm and 350/475 nm for warfarin and dansylsarcosine, respectively. HSA and ligands were dissolved in PBS (pH 7.4). Within the limits of sensitivity, the excitation wavelengths were chosen to ensure the least possible absorption of incidental light. Emission wavelengths were chosen to maximize fluorescence of the HSA-bound probe, with negligible fluorescence from the buffer (see typical spectra in [Supplementary Fig. 1](#)). The concentration of HSA used in the fluorescence experiments was 20 μM. The probe/HSA ratio was kept at 1:10, respectively, to minimize the nonspecific binding of probes. The percentage of displacement of the probe was determined according to the method of Sudlow et al. [26]. The percentage of binding was calculated as follows:

$$\frac{(F_1 \times F_2)}{F_1} \times 100$$

where F<sub>1</sub> and F<sub>2</sub> represent the fluorescence of the probe plus HSA without or with PAA, respectively.

#### 2.5. Competition experiments: interaction of p-CS and IS with HSA binding of PAA

To further investigate whether competitive interaction with PAA occurs under the CKD condition, the binding experiments of PAA with HSA were performed by ultrafiltration in the presence of indoxyl-sulfate (IS) or p-cresyl-sulfate (p-CS) at a physiologically achievable concentrations [3,27]. The concentrations of HSA and PAA were set at 500 μM and 200 μM, respectively. Displacer concentrations of IS or p-CS in the range 250–1000 μM were used.

#### 2.6. Statistical analysis

Statistical analyses were performed by using one-way analysis of variance. A probability value of P < 0.05 was considered to be significant. GraphPad Prism software version 6.0 (Graphpad software, La Jolla, CA) was used to perform statistical analyses.

#### 2.7. Non-linear fitting of binding data

We used non-linear fitting techniques for the dose response estimation of PAA versus HSA. The whole dataset comprises of PAA measured  $p_i$  for a given dose of HSA  $x_i$ . For a given function  $F$  and a set of parameter  $\theta$ , the error  $\chi^2 = \sum (p_i - F(x_i, \theta))^2$  must be minimized. We used a customized MATLAB script to minimize this quantity using Nelder-Mead simplex algorithm (function *Fminsearch*). The goodness of fit was then evaluated using Akaike Information Criterion (AIC) [28] using the following formula:  $AIC = 2n + N \log(\chi^2/N)$  where  $n$  is the number of parameters in  $\theta$  and  $N$  the total number of data points (see [Online Appendix](#)).

### 3. Results

#### 3.1. Determination of binding parameters of PAA for HSA

The binding parameters of PAA for HSA were determined using

**Table 1**  
Protein binding of PAA as determined by ultrafiltration at pH 7.4 and 37 °C.

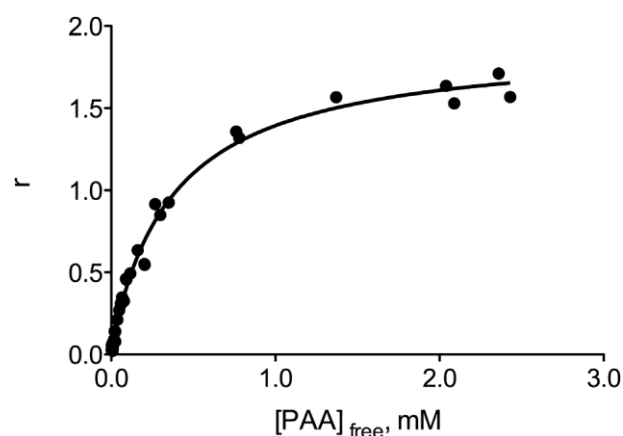
	Bound, %	Free, %	r
Human serum albumin	25.8 ± 1.7	74.2 ± 1.7	1.61 ± 0.08
Bovine serum albumin	20.6 ± 3.2	79.4 ± 3.2	1.36 ± 0.22
P-value	0.182	0.182	0.324

Human serum albumin (500 μM) or bovine serum albumin (500 μM) were incubated for 1 h with PAA at the concentration encountered in end stage renal disease patients (450 mg/L i.e. 3.3 mM). Data are the mean ± SEM for 6 independent experiments.

an ultrafiltration method ([Table 1](#)). At the concentration encountered in ESRD patient (roughly 3.3 mM), 26% of PAA is bound to HSA. Under these conditions, 1 mol of HSA can bind 1.6 mol of PAA ([Fig. 2](#)). The individual binding parameters, the Ni and Ki values, obtained by a non-linear Nelder-Mead method, are summarized in [Table 2](#). The Akaike information criterion (AIC) was used to measure the relative quality of the different statistical models (single versus two binding sites) for our data set. Akaike Information Criteria (see [Table 2](#)) was calculated as follow:  $AIC = 2k + n \times \ln(\chi^2/n)$  where  $k$  is the number of variables,  $\chi^2$  the residues et  $n$  the number of data. Given a set of candidate models for the data, the preferred model is the one that minimizes AIC value. In all models, the fitting procedure reached convergence and yielded appropriate values. The AIC analysis showed that there was only one single high-affinity binding site for PAA with a binding constant (Ki) of  $3.4 \times 10^4 \text{ M}^{-1}$ . We performed further experiments with BSA. At the concentration encountered in ESRD patients (3.3 mM), 21% of PAA was bound to BSA. Under these conditions, 1 mol of HSA can bind 1.4 mol of PAA ([Table 1](#)). No significant difference was found between PAA-HSA binding and PAA-BSA binding (P = 0.182).

#### 3.2. Identification of the binding site of PAA using a displacement fluorescence method

High-resolution crystal structures of HSA complexed with various molecules have demonstrated that there are two main binding sites named site I (in sub domain IIA) and site II (in sub-domain IIIA) [13]. We performed site marker displacement experiments using representative fluorescent probes, warfarin (a commonly used site I probe) and dansylsarcosine (a site II probe), to



**Fig. 2. Human serum albumin binding curve of PAA.** Human serum albumin (500 μM) was incubated for 1 h at 37 °C pH 7.4 with different concentrations of PAA ranging from 0.7 μM to 3.3 mM. Free fraction was separated using ultrafiltration and PAA was assayed in HPLC-Vis. r, number of mole of PAA bound per mole of HSA. The binding was modeled by non-linear regression methods. This curve summarizes the results of 39 independent experiments ( $r^2 = 0.991$ ).

**Table 2**  
HSA binding parameters of PAA as determined by ultrafiltration at pH 7.4 and 37 °C.

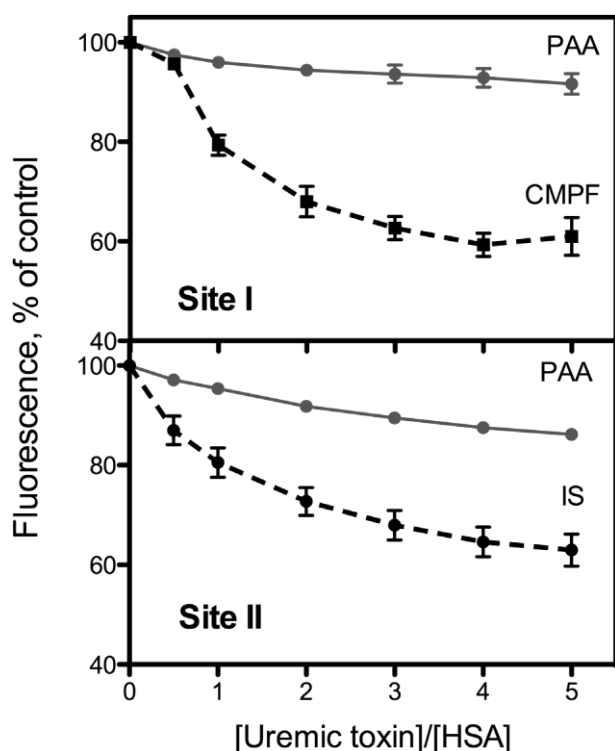
Model	N <sub>i</sub>	K <sub>i</sub> , M <sup>-1</sup>	χ <sup>2</sup>	AIC
Single binding site	1.875	3.38 × 10 <sup>4</sup>	0.107	-226.21
Two binding sites	1.831	3.86 × 10 <sup>4</sup>	0.103	-197.84
	0.076	3.66 × 10 <sup>5</sup>		

N<sub>i</sub>, number of binding site, K<sub>i</sub>, dissociation constant for site i, χ<sup>2</sup>, residues, AIC, Akaike information criteria. Note that from AIC analysis, one site model is the best fit to model the experimental data since it exhibited the lowest AIC score.

identify the binding site of PAA on HSA, accordingly to the method proposed by Sudlow et al. [26]. The results were compared with those obtained for 2 uremic toxins, 3-Carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) and IS, reported to bind to sites I and II, respectively [29]. As shown in Fig. 3A, warfarin was significantly displaced by CMPF (-60%, P < 0.005) while PAA only induced a modest decrease in the fluorescence of the warfarin-HSA complex (-8%, P = 0.414). The fluorescence of dansylsarcosine was slightly decreased in the presence of PAA (-13%, P < 0.013) while IS induced a sharp decrease in fluorescence (-50%, P < 0.005) (Fig. 3B). These data suggested that the main binding site of PAA on HSA is neither Sudlow's site I nor site II.

### 3.3. Interaction of PAA and IS and p-CS in HSA binding

It was reported previously that the serum concentrations of PAA was increased to 3490 ± 330 μM in patients with CKD, or 300-fold



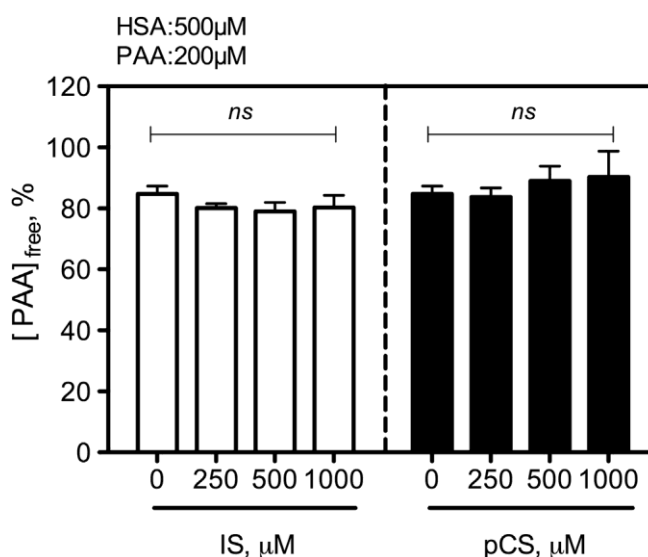
**Fig. 3. Identification of the binding site of phenylacetic acid to human serum albumin.** Effect of uremic toxins on the fluorescence intensity of warfarin (a site I specific probe), and dansylsarcosine (a site II specific probe) bound to HSA. The following concentrations were used: HSA, 20 μM warfarin and dansylsarcosine, 2 μM, and PAA 10–100 μM. The excitation and emission wavelengths were set at 320/390 nm and 350/475 nm for warfarin and dansylsarcosine, respectively. Indoxyl-sulfate (IS) and 3-Carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) were used as positive controls. Values are expressed as the mean ± S.D. (n = 5).

higher than those of healthy people [14]. Vanholder et al. reported that serum concentrations of p-CS and IS were increased to the range of 100–500 μM and 100–1000 μM in patients with CKD stage 4–5, respectively [4]. To investigate whether competitive interaction of PAA and IS and p-CS could occur, the binding experiments of PAA with HSA in the presence of IS and p-CS were performed by ultrafiltration at a highest physiologically achievable concentrations. The concentrations of PAA and HSA were set at 200 and 500 μM, respectively. Concentrations of IS and p-CS, used as displacers, were 200, 500 and 1000 μM. Fig. 4 shows that there was no changes in free percentage of PAA to HSA in the presence of IS (Fig. 4A) or p-CS (Fig. 4B). In good agreement with the results of fluorescent probe displacement, our data suggest that PAA did not interfere with the binding of IS and p-CS to site II of HSA.

## 4. Discussion

Protein-bound uremic toxins, such as PAA, may contribute significantly to the progression of CKD and to the increased risk of cardiovascular disease observed in CKD patients. Indeed, based on their tight binding to plasma proteins, these hydrophobic uremic toxins are poorly cleared using conventional dialysis and thus strongly accumulate in end stage renal disease patients. Jankowski et al. (2003) reported that PAA accumulated in end-stage CKD patients, inhibiting iNOS expression and, consequently, NO production, which may contribute to accelerated atherosclerosis and hence to increased CVD morbidity in these patients [17]. Total plasma PAA level consists of a protein-bound fraction and an unbound biologically active fraction, the latter being the only one to be removed using dialysis. Protein-bound uremic toxins are therefore recognized as important targets of therapeutic removal. Phenylacetic acid protein binding has received little attention in the scientific literature and, to our knowledge, this is the first determination of its binding constant for HSA. The present study was therefore designed to investigate the interactions between PAA and human serum albumin.

The present data show that the binding constant of the high



**Fig. 4. Interactions between phenylacetic acid (PAA), indoxylsulfate (IS) and p-cresyl-sulfate (p-CS) for binding to human serum albumin.** Competitive HSA binding of PAA, PCS and IS at pH 7.4 and 25 °C. The binding of PAA (200 μM) to HSA (500 μM) was measured in the presence of 200, 500 or 1000 μM IS or p-CS. Values are expressed as the mean ± S.D. (n = 3). No difference was found to be significant at the P < 0.05 level.

affinity site on HSA for PAA was  $3.4 \times 10^4 \text{ M}^{-1}$  using an ultrafiltration method, which allows direct measurement of the free concentration of ligand. The bound percentage of PAA to HSA was found to be ~30%, indicating that PAA binding to HSA was weak to moderate and this is in good agreement with the clinical data. We used in the present *in vitro* study PAA concentrations ranging from 7.5  $\mu\text{M}$  to 3.3 mM to estimate the binding constant. The highest concentration was chosen to represent the concentrations observed in patients with CKD [3,17]. We estimated the binding percentage of PAA, under conditions that represent CKD stage 4–5, to be ~30%, which corresponds to the bound percentage found in the serum of patients with CKD. Indeed, in ESRD patients, total and free plasma concentrations of PAA were reported to be 3.49 and 2.50 mM, respectively corresponding to the binding percentage of 29% [17]. Ito et al. (2012) measured PAA in plasma from HD patients by liquid chromatography/electrospray ionization–mass spectrometry/mass spectrometry (LC/ESI-MS/MS) and found concentrations of 0.1 and 0.5 mg/L (i.e. 0.7 and 3.5  $\mu\text{M}$ ) in control and HD patients, respectively [8]. They calculated the reduction rate of PAA during HD to be 35% and protein binding to be 60.5%. The discrepancies between these concentrations (0.5 mg/L versus 475 mg/L or 950-fold) cannot be explained solely by the difference of sensitivity between the methods used to assay PAA (i.e. LC-MS/MS versus  $^1\text{H}$ -RMN) and will deserve further examination. In 2013, Brettschneider et al. reported this concentration to be 1.08 mM in HD patients [30]. However, in a recent *in vitro* study with human plasma spiked with PAA, Böhringer et al. [31] reported the protein bound fraction of PAA to be 59% [31]. Taken together, these data strongly suggest that PAA could bind to proteins other than HSA in human plasma. Meijers et al. pinpointed that CKD patients commonly exhibited a “dysfunctional” albumin regarding the binding of uremic toxins [32]. Recently Rueth et al. [33] demonstrated that some post-translational modifications of albumin could account for this decrease in binding of uremic toxins. Many post-translational modifications are known to occur under uremic conditions such as glycation, oxidation, carbamylation or guanidylation. Albumin isolated from plasma of patients with CKD is specifically post-translationally modified by guanidinylation of its lysine residues [33]. Furthermore, *in vitro* guanidinylation of native serum albumin (isolated from healthy subjects) results in a decreased binding capacity of binding for indoxyl-sulfate. Under uremic conditions, post-translational modifications of HSA could alter its binding affinity for PAA.

Protein-bound uremic retention solutes like phenylacetic acid are usually characterized by their hydrophobic structure, e.g. an aromatic phenyl groups in the case of PAA. Our results from fluorescence probe displacement experiments using warfarin and dansylsarcosine, as prototypical site I and II marker probes respectively [26], suggest that the high-affinity binding site of PAA is neither of these sites. In good agreement, no competition was found between PAA and *p*-CS or IS which are known to bind to site II of HSA. Site I (subdomain IIA) generally binds lipophilic drugs with a centrally located negative charge or an electronegative feature while site II (subdomain IIIA) binds lipophilic drugs with a peripheral negative charge or an electronegative feature. At physiological pH, PAA (pKa 4.31) is mostly in a deprotonated form and therefore exhibits a peripheral negative charge. According to these general rules, PAA should therefore be more prone to bind to site II of albumin due to its peripherally located negative group (carboxylate group). We however only noticed a weak binding to site II and no evidence of binding to site I. Notably, the affinity of PAA for site II was lower than that of *p*-CS or IS. The effect of PAA on HSA fluorescence intensity is shown in [Supplementary Fig. 2](#). HSA exhibited a fluorescence emission with a peak at 350 nm, mainly due to the only tryptophan residue (Trp-214) located on subdomain IIA (Sudlow's

site I). Indeed, HSA is a class B protein and its fluorescence emission comes solely from Trp-214 residue. Addition of PAA to HSA did not significantly trigger a quenching of the fluorescence indicating that the microenvironment around the Trp-214 residue was not altered; thus, in good agreement with competition experiment, the PAA binding on HSA site should not involve Trp residue located in Sudlow's site I.

The European Uremic Toxin Workgroup (EUTox) published guidelines for handling uremic toxins in *in vitro* studies aimed at deciphering the biological effects of uremic toxins [27]. These guidelines recommend adding human serum albumin at the average uremic concentration of 35 g/l to any test system. Indeed if a substantial fraction of the uremic toxin is protein bound, only the free fraction is assumed to be responsible for biological effects. As bovine serum albumin (BSA) exhibited a very similar binding capacity for PAA ([Table 1](#)), we suggest that it could be used as a surrogate of HSA (far less expensive and easier to provide) for *in vitro* studies investigating biological effects of PAA.

Protein-bound uremic toxins strongly contribute to the progression of cardiovascular diseases (CVD) in CKD patients but are unfortunately poorly cleared by most of the conventional dialysis therapies due to their tight protein binding [17,34,35]. Recently, Itoh et al. [10] described the potential of removing some uremic toxins by HD. In this study, they categorized the uremic toxins as easily or difficultly removable (more and less than 35% binding, respectively). Accordingly, PAA is considered as a uremic toxin with a difficult removal by HD (35%), which reaffirms the relevance of understanding its interaction with plasma proteins. Two recent studies demonstrated the interest of developing new strategies to dissociate the uremic toxin/protein complexes. Böhringer et al. [31] proposed a new dialysis strategy where the protein-bound uremic retention solutes were removed more efficiently using high ionic strength to disrupt the electrostatic interaction of PAA with proteins and hence increase the free fraction. The protein-bound fraction of phenylacetic acid, was then significantly decreased from 59 to 36%. In these conditions, the clearance during the *in vitro* dialysis was increased by 13%. Brettschneider et al. [30] used hydrophobic and cationic adsorbents to more effectively remove protein-bound, hydrophobic uremic toxins using a *Fractionated Plasma separation, Adsorption, and Dialysis (FPAD)* system. In a small group of 5 HD patients, elimination of PAA was increased by 130% compared to conventional high-flux hemodialysis. Such studies could likely represent the first step for establishing novel therapeutic approaches to remove protein-bound retention solutes such as PAA.

## 5. Conclusion

In conclusion, our study provides important quantitative data on the binding affinity of phenylacetic acid to human serum albumin, a major drug carrier protein. A better understanding of the interactions of phenylacetic acid with HSA and plasma protein is mandatory to develop new dialysis strategies aimed at removing this compound. Indeed, current HD methods do not efficiently remove protein-bound uremic toxins such as phenylacetic acid because of their strong interaction with plasma proteins. Thus novel dialysis methods should be developed to more efficiently remove these protein-bound uremic toxins in order to prevent uremic complications.

## Author contributions

JFS, DY, MBSP and COS researched data. SC synthesized the fluorescent probe dansylsarcosine and *p*-CS. HAS performed the mathematical analysis. JFS and COS wrote the manuscript. DM and



DF reviewed and edited the manuscript.

### Author disclosure

The authors declare no conflicts of interests.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biochi.2016.03.002>.

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## 2.2 p-cresyl glucuronide (p-CG)

*Determination of the binding properties of p-cresyl glucuronide to human serum albumin*

**Dan Yi** *et al*, *Biochimie* 2018; 150:1-7.

Chronic kidney disease is characterized by the progressive accumulation of retention solutes, some of which are issued from the metabolism of dietary proteins. p-Cresol is the main product of tyrosine biotransformation by anaerobic intestinal bacteria and it generates p-cresyl sulfate (p-CS) by sulfatation and p-cresyl glucuronide (p-CG) by glucuronidation in the liver. Both of these p-cresol derivatives are protein bound uremic solutes, which are poorly removed common dialysis techniques. However, the percentage of protein binding varies drastically between these two conjugates. p-CS is found to be approximately 90% protein bound, while p-CG is only 10% approximately. Due to this significant variation, their absolute concentrations are very similar, and p-CG could present similar impact in outcome studies in comparison to its counterpart. p-CS is the main metabolite of p-cresol. Experimental studies have demonstrated that p-CS can exert deleterious effects, such as induction of oxidative stress and insulin resistance<sup>81</sup>. In contrast to p-CS, p-CG exhibited no effect on insulin sensitivity and no synergistic inhibiting effect in combination with p-CS.<sup>107</sup>

The deleterious effects of p-CS have been extensively studied, while very limited publications in literature were dedicated to p-CG. In human, p-cresol is predominantly

subjected to sulfation. However, the contribution of glucuronidation is greater among patients with lower glomerular filtration rate (eGFR). Recent study showed that free p-CG concentrations were associated with mortality, independently of well-known predictors of survival, such as age, vascular calcification, anemia, inflammation and eGFR in predialysis patients.<sup>108</sup>

As a protein bound uremic toxins, although the binding proportion of p-CG is not as high as p-CS, p-CG is also poorly removed by hemodialysis. To investigate the binding properties of p-CG to HSA will contribute to the removal pattern and deputation of p-CG and finally contribute to the clinical therapy of CKD. However, very few data are available in the literature regarding the characteristics of the binding of p-CG to HSA. This article characterized the binding p-CG percentage to HSA in vitro. Mainly 79% of p-CG circulates under the unbound form in plasma (**Table 12**). Binding constants are shown in **Table 13**. Single-binding site model fits best to the binding properties of p-CG to HSA with a dissociation constant  $K_1=2.77 \times 10^3 \text{ M}^{-1}$ . Binding sites of p-CG on HSA is neither Sudlow's site I nor site II. We evaluated its competition with its counterpart p-cresyl sulfate for HSA binding. Due to its weak binding affinity, p-CG cannot compete with p-CS for binding to HSA.

	Bound, %	Free, %
Human serum albumin	22.98 ± 2.15	77.02 ± 2.15
Control plasma	28.22 ± 3.26	71.78 ± 0.33

**Table 12.** Protein binding of p-cresyl glucuronide (p-CG) as determined by ultrafiltration at pH 7.4 and 37°C. Data are expressed as mean ± SD or as median (interquartile range).

Parameters	CI <sub>95</sub>
$K_1, \times 10^3 \text{ M}^{-1}$	2.77 ± 0.38 (2.01-3.54)
$n_1$	3.80 ± 0.17 (3.46-4.14)
<b>Goodness of fit</b>	
Degree of freedom	28
$r^2$	0.9839

**Table 13.** Characteristics of the binding of p-cresyl glucuronide (p-CG) to HSA by single-binding site model. Data are expressed as mean±SD.

**Abbreviations:**  $n_1$ , number of binding site;  $K_1$ , dissociation constant; CI<sub>95</sub>, confidence interval.

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## Research paper

Determination of the binding properties of *p*-cresyl glucuronide to human serum albumin

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## ABSTRACT

*p*-Cresyl glucuronide (*p*-CG) is a by-product of tyrosine metabolism that accumulates in patients with end-stage renal disease. *p*-CG binding to human serum albumin in physiological conditions (37 °C, pH 7.40) was studied by ultrafiltration (MWCO 10 kDa) and data were analyzed assuming one binding site. The estimated value of the association constant was  $2.77 \times 10^3 \text{ M}^{-1}$  and a maximal stoichiometry of 3.80 mol per mole. At a concentration relevant for end-stage renal patients, *p*-CG was 23% bound to albumin. Competition experiments, using fluorescent probes, demonstrated that *p*-CG did not bind to Sudlow's site I or site II. The *p*-CG did not interfere with the binding of *p*-cresyl-sulfate or indoxyl sulfate to serum albumin.

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## 1. Introduction

Chronic kidney disease (CKD) is characterized by the progressive and irreversible loss of renal function. Many solutes normally excreted in urine by the kidneys accumulate in the body fluids of patients with CKD. These retention solutes may interact with biological functions and exert major toxic effects; for this reason, they are often referred to as uremic toxins [1,2]. Of note, protein-bound uremic toxins are especially relevant, due to their poor removal by commonly used dialysis techniques and high toxicity [3,4]. *p*-Cresol is the main product of tyrosine biotransformation by anaerobic intestinal bacteria and is transformed into both *p*-cresyl sulfate (*p*-CS) and *p*-cresyl glucuronide (*p*-CG) in the liver by sulfatation and

glucuronidation, respectively (Fig. 1). *p*-CS, the most preponderant metabolite of *p*-cresol in human, is frequently associated with increased mortality rate and risk of cardiovascular diseases among uremic patients [5–8]. Experimental studies demonstrated that *p*-CS can exert deleterious effects both *in vitro* and *in vivo*, such as induction of oxidative stress [9], endothelial damage [10,11], proximal tubular injury [12] and insulin resistance [13]. While *p*-CS has been extensively studied, only limited publications in literature were dedicated to *p*-CG behavior. This later metabolite is produced at markedly lower concentrations in human and further investigations are needed in order to better elucidate its role in uremia. Total and free *p*-CG concentrations are independently associated with mortality in predialysis patients [14]. Additionally, *p*-CG was found to act synergistically with *p*-CS increasing the oxidative burst in leucocytes and contributed to the pro-inflammatory effect [15]. Other authors also demonstrated that *p*-CG could exert pro-inflammatory effects that contributed to vascular damage by stimulating crosstalk between leukocytes and vessel wall, when acted synergistically with *p*-CS [16]. Both *p*-cresol derivatives are bound to plasmatic proteins in biological systems; however, the percentage of protein binding varies drastically between the two conjugates. *p*-CS is found to be approximately 90%

**Abbreviations:** CKD, chronic kidney disease; CMPF, 3-Carboxy-4-methyl-5-propyl-2-furanpropanoic acid; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; HPLC, high pressure liquid chromatography; HSA, human serum albumin; NSB, non specific binding; IS, indoxyl-sulfate; PBS, phosphate buffered saline; *p*-CG, *p*-Cresyl glucuronide; *p*CS, *p*-cresyl sulfate.

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### 3 Indoxyl sulfate

#### 3.1 The uremic toxin indoxyl sulfate exacerbates reactive oxygen species production and inflammation in 3T3-L1 adipose cells

*Milena B. Stockler-Pinto, Juliana F. Saldanha, Dan Yi et al, Free Radical Research, 2016; 50(3): 337-344.*

Inflammation and oxidative stress are common features of patients with chronic kidney disease (CKD) and many uremic toxins retained in these patients could be involved in these processes. Uremic toxins are solutes that accumulate in body fluids of CKD patients, which result in a decrease in their renal clearances. Among the variable uremic toxins, protein bound uremic molecules are the most difficult to remove by conventional hemodialysis because of their interaction with plasma protein. Indoxyl sulfate (IS), the prototype of PBUTs, and has recently attracted great attention due to its major cardiovascular toxicity, so that can accumulate to high concentration in CKD patients and causes renal injury. Indoxyl sulfate is synthesized in the liver from indoles, which are produced by the intestinal bacteria as a metabolite of tryptophan derived from dietary proteins. Normally, indoxyl sulfate is excreted into urine. In CKD patients, because of the reduced function of kidneys, indoxyl sulfate accumulate in the blood.

Many studies have reported the relationship between uremic toxin and some pathological conditions related to cardiovascular mortality and progression of CKD such as inflammation and oxidative stress. White adipose tissue (WAT) was recently



recognized as an important source of inflammation and oxidative stress. WAT is active metabolic organ that secretes important bioactive compounds, some of which stimulating oxidative stress and inflammation. Therefore, understanding the pathophysiological impact of IS on white adipose tissue might be an important step toward development of new intervention to modulate inflammation in patients with CKD. However, the impact of IS on adipose tissue remains unclear. Thus, we examined the effect of indoxyl sulfate on adipocytes oxidative stress production.

This article investigated whether IS, at clinically relevant concentrations, may have effects on oxidative stress production and inflammation in adipose cells. We used 3T3-L1 adipose cells, and incubated them with indoxyl sulfate to mimic the conditions encountered in uremic patients to examine the effect of indoxyl sulfate on adipocytes. This article identified IS as a new player for adipocyte dysfunction. The increased level of IS triggered both oxidative stress and inflammation. Indoxyl sulfate induced oxidative stress seemed to play a key role in the perturbation of adipocyte biology.



## The uremic toxin indoxyl sulfate exacerbates reactive oxygen species production and inflammation in 3T3-L1 adipose cells

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ORIGINAL ARTICLE

## The uremic toxin indoxyl sulfate exacerbates reactive oxygen species production and inflammation in 3T3-L1 adipose cells

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### ABSTRACT

Inflammation and oxidative stress are common features of patients with chronic kidney disease (CKD) and many uremic solutes retained in these patients could be involved in these processes, among which protein-bound solutes such as indoxyl sulfate (IS). White adipose tissue recently gained attention as an important source of inflammation and oxidative stress. To examine the effect of IS on adipocytes, 3T3-L1 adipose cells were incubated with IS to mimic the conditions encountered in uremic patients. Incubation of adipose cells with IS increased reactive oxygen species production generated mainly through activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase since it was prevented by the NADPH oxidase inhibitor apocynin. Exposure to IS furthermore exacerbated the secretion of tumor necrosis factor- $\alpha$  and interleukin-6 by adipose cells. This inflammatory response was prevented by NADPH oxidase inhibition pinpointing the pivotal role of intracellular oxidative stress. IS induces adipocyte perturbation and promotes inflammatory state mainly through induction of oxidative stress. IS, a uremic toxin, accumulates in CKD patients could, therefore, be an important mediator of adipocyte dysfunction in these patients.

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### Introduction

Uremic toxins are solutes that accumulate in body fluids of patients with chronic kidney disease (CKD) as a result of a decrease in their renal clearances. Uremic toxins were classified by the “European Uremic Toxin Work Group” (EUTox) [1] into three groups mainly based on their behavior during dialysis procedure [2]: the low molecular weight water-soluble molecules (such as urea), the middle molecules (such as  $\beta_2$ -microglobuline), and the protein-bound molecules, which are poorly removed by conventional hemodialysis treatment and thus accumulates in these patients. Amongst protein-bound solutes, indoxyl sulfate (IS) has recently attracted great attention because of its major cardiovascular toxicity [3,4]. IS is synthesized in the liver from indoles, which are produced by the intestinal bacteria as a metabolite of tryptophan derived from dietary proteins [3,5].

Many studies have reported a relationship between uremic toxins like IS and some pathological conditions related to cardiovascular mortality and progression of CKD such as inflammation and oxidative stress. Moreover, incubation of cells with uremic plasma was shown to

promote synthesis of inflammatory mediators as cytokines and monocyte chemoattractant protein-1 [6]. The mechanism by which IS induces toxicity has recently been clarified. The intracellular accumulation of IS via organic anion transporters (OAT) and the subsequent increased production of reactive oxygen species (ROS) seems to play a key role in the toxicity of IS [7,8]. IS was shown to induce oxidative stress in various cells types, including vascular endothelial cells, vascular smooth muscle cells, renal tubular cells, monocytes, and macrophages [9–12]. According to Gelasco and Raymond [13], this ROS production likely involves nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Masai et al. [14] demonstrated that IS increases NADPH-oxidase-derived ROS, which, in turn, activates the mitogen-activated protein kinase (MAPK)/nuclear factor kappa-b (NF- $\kappa$ B) pathway in human umbilical vein endothelial (HUVEC) cells. Shimizu et al. [12], showed that IS, through an increase in ROS production, upregulated mRNA and protein expression of intercellular adhesion molecule-1 (ICAM-1) in human kidney-2 (HK-2) cells.

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### **3.2 Indoxyl sulfate, aryl hydrocarbon receptor (AhR) activation and metabolic risk in chronic kidney disease (CKD)**

Chronic kidney disease (CKD) is associated with an increased risk of cardio-vascular mortality and morbidity and is also frequently associated with peripheral insulin resistance (IR), which is also a major cardiovascular risk factor.<sup>109</sup> CKD leads to the progressive retention uremic toxins, such as indoxyl sulfate (IS) and p-cresyl sulfate (p-CS). Healthy individuals can excrete or metabolize uremic toxins by the kidneys. However, in CKD patients, because of the impaired function of kidneys, uremic toxins accumulate in the blood.

Indoxyl sulfate (IS) is one of the main uremic toxins in CKD patients, poorly removed by hemodialysis (HD), so that accumulates in CKD patients. IS is derived from tryptophane metabolism by intestinal microbes, but little is known about its adverse effects on metabolism. Our previous study shows that p-CS, another major uremic toxin, promotes insulin resistance in CKD. So, our working hypothesis was that IS could also impair the insulin signaling pathways and induces IR. IS was recently identified as an endogenous agonist for aryl hydrocarbon receptor (AhR). The aim of this project is therefore to investigate whether IS, through activation of AhR could contribute to the development of insulin resistance and other metabolic disorders associated with CKD.

We performed both *in vivo* and *in vitro* experiments. In the *in vitro* experiment, we demonstrated that IS at CKD-relevant concentration triggered insulin resistance,

through a direct activation of AhR. C2C12 myotubes pretreated with AhR inhibitor ANF or probenecid prevented the development of insulin resistance. However, we got different findings in the in vivo experiment. During the in vivo experiments, we developed and validated a new and non-surgical model of CKD, which is adenine rich diet. Chronic administration of IS were performed in this new model. We were able to experimentally increase plasma IS concentration in mice to mimic levels encountered in CKD. However, in vivo, IS failed to trigger insulin resistance. This essay shows our findings of the effect of IS in vivo and in vitro, the process to perform adenine diet model and the biometric data of our new model of CKD.

## **Indoxyl-sulfate, aryl hydrocarbon receptor (AhR) activation and cardio-metabolic risk in chronic kidney disease**

### **Conferences:**

- The 24th meeting of G2L2 association (Geneve, Grenoble, Lyon, Lausanne), May 19, 2017, Lyon, France
- 20ème Journée Scientifique de l'EDISS, le 15 Octobre 2015, Lyon, France

### **Abstract**

#### **Background:**

Chronic kidney disease (CKD) is associated with an increased risk of cardio-vascular mortality and morbidity and is also frequently associated with peripheral insulin resistance which is also a major cardiovascular risk factor. The uremic syndrome is attributed to the progressive retention of numerous compounds, which in healthy individuals are normally excreted or metabolized by the kidneys. Indoxyl sulfate (IS), one of these compounds, is poorly removed by hemodialysis (HD) and accumulates in CKD patients. IS is derived from tryptophane metabolism by intestinal microbiota, but little is known about its adverse effects on metabolism. Recent studies identified IS as an endogenous ligand for aryl hydrocarbon receptor (AhR). The aim of the present project is therefore to investigate whether IS, through activation of AhR could contribute to the development of insulin resistance and other metabolic disorders associated with CKD.

## **Methods:**

**In vitro study:** C2C12 myotubes were incubated with 250 $\mu$ M of IS (a concentration chosen to mimic that encountered in CKD patients) for 30 min and stimulated with 100 nM insulin for 20 min. In order to detect IS intra- or extra-cellular effects, C2C12 myotubes were preincubated with probenecid (1mM, 1h), a potent inhibitor of organic anion transporter-3 (OAT-3), prior to incubation with IS and stimulation with insulin. Alpha-naphthoflavone (ANF), a potent inhibitor of AhR was used to prevent AhR activation in muscle cells. C2C12 myotubes were incubated with ANF (1 mM, 1h), prior to incubation with IS and stimulation with insulin. The insulin signaling pathway was explored by Western Blotting through the phosphorylation of Protein kinase B (PKB/Akt).

**In vivo study:** To induce chronic kidney disease, mice were fed for 4 weeks with a diet containing adenine (0.25% w/w). HPLC-Fluorescence assay was used to determine the plasma concentration of IS. The disposition of IS was explored after an intraperitoneal injection of IS (100mg/kg).

## **Results:**

Exposition of C2C12 myotubes to IS at CKD-relevant concentration triggered insulin resistance, through a direct activation of AhR. Pretreatment of C2C12 myotubes with the AhR inhibitor ANF or probenecid prevented the development of insulin resistance. We developed and validated a non-surgical model of CKD in rodents and demonstrated that IS accumulates in CKD mice as observed in human. We further demonstrated that plasma IS concentration can be experimentally raised in



mice but does not promote insulin resistance in vivo.

### **Conclusion:**

IS, through activation of AhR, disrupts insulin signaling pathways and lead to the development of insulin resistance associated with CKD. IS could be a very important uremic retention solutes that could contribute to insulin resistance in some conditions.

**Keywords:** Chronic kidney disease (CKD), Insulin resistance (IR), Indoxyl sulfate (IS), aryl hydrocarbon receptor (AhR).

### **Introduction**

Chronic kidney disease (CKD) is associated with an increased risk of cardio-vascular mortality and morbidity.<sup>110,111</sup> This increase in disease burden is not solely explained by traditional risks factor like, tobacco, hypertension, age, etc. The uremic syndrome is attributed to the progressive retention of numerous compounds, which in healthy individuals are normally excreted or metabolized by the kidneys. These uremic retention molecules (URMs) can originate from endogenous metabolism, microbial metabolism or exogenous intake. Indoxyl sulfate (IS) and p-cresyl sulfate (p-CS), are protein-bound uremic toxins, that increase significantly during CKD. These URMs are generated in part in the gastrointestinal tract and are poorly removed by conventional dialysis. It was demonstrated that some of these uremic solutes have an impact on the survival of CKD patients.<sup>112</sup> Indoxyl sulfate, a protein bound uremic solutes is associated with mortality and cardiovascular events. IS is derived from tryptophane metabolism by intestinal microbes and evidence

suggest that besides the passive accumulation of bacterial uremic toxins, increased colonic generation or absorption may also explain the high level of these uremic toxins in CKD.

Chronic kidney disease is frequently associated with peripheral insulin resistance which is a major cardiovascular risk factor<sup>27</sup>. Fasting hyperglycemia, abnormal glucose tolerance and hyperinsulinemia are hallmarks of insulin resistance and common features in patients with CKD. Insulin resistance was unambiguously evidenced in CKD patients using hyperinsulinemic euglycemic clamp testing<sup>113</sup>. However, to the best of our knowledge, little is known about why CKD patients often develop insulin resistance. Insulin mainly decreases blood glucose through its action on 3 main target tissues: skeletal muscle and white adipose cells, where it stimulates glucose uptake, and liver where it inhibits endogenous glucose production (i.e. glycogenolysis and neoglucogenesis). At the cellular level, in the presence of insulin, the insulin receptor autophosphorylates, recruits and phosphorylates IRS 1/2 proteins, and in turn activates the phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway<sup>114</sup>, which is responsible for most of the metabolic action of insulin. The insulin-induced glucose transport is significantly reduced in muscles from uremic patients<sup>115</sup>. In skeletal muscle of human<sup>115</sup> or rats<sup>116</sup> with CKD the insulin receptor number, affinity, or tyrosine kinase activity are however normal suggesting that insulin resistance observed in uremic patients results from post-receptor defects in the insulin signaling pathway. In animal models of CKD, PI3K activity is reduced<sup>117</sup>. Thomas and al noticed a decrease in tyrosine-phosphorylation of IRS-1<sup>118</sup> in muscles

of uremic mice. Many mechanisms could be involved since downstream dysfunction of the IRS/PI3K/Akt pathway is associated with chronic inflammation, metabolic acidosis, vitamin D and parathyroid hormone status, uremic toxins, or adipokines<sup>119</sup>. Interestingly, McCaleb et al. demonstrated that isolated rat adipocytes exhibited a blunted insulin stimulated glucose uptake when incubated with serum from CKD patients but not with serum from obese or type 2 diabetes patients<sup>120,121</sup>. This observation suggested that one or several unknown circulating molecules unique to uremia could induce insulin resistance and impairs downstream function of the IRS/PI3K/Akt pathway. Although numerous fractionation studies attempted to decipher the precise molecular nature of this factor, these studies have so far only yielded inconclusive data.

Excessive retention of uremic toxins, normally removed by the kidneys and excreted into the urine, may play a key role. Two of these compounds are p-cresyl sulfate and indoxyl-sulfate, 2 major uremic toxins produced by intestinal bacteria from fermentation of dietary proteins. Due to their strong binding to plasma proteins, both p-CS and IS are poorly removed by common dialysis techniques and therefore accumulates in end-stage renal disease (ESRD) patients. We determined whether the accumulation of p-CS could contribute to CKD-associated insulin resistance and dysmetabolism. We recently addressed this issue<sup>81</sup> and found, as a proof of concept, that chronic administration of p-CS in mice with normal renal function, triggered insulin resistance, a loss of fat mass, and ectopic lipid redistribution in muscles and liver, mimicking the metabolic disturbances associated with CKD. We also found that

mice treated with p-CS exhibited a disruption of the insulin signaling pathway in skeletal muscle related to activation of some kinases (Extracellular signal-regulated kinases 1/2 or ERK1/2). These data were further confirmed by in vitro studies in which exposure of C2C12 myotubes to p-CS (at concentrations chosen to mimic those observed in ESRD patients) causes direct activation of kinases ERK1/2 and inhibitory serine phosphorylation of insulin receptor substrate-1 (IRS-1). Prebiotic are non-digestible fibers that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. Treatment of mice with CKD with a prebiotic (arabino-xylo-oligosaccharide, Optiflor ®, Witaxos) which reduces the intestinal production of p-CS and decreases blood levels of this toxin prevented insulin resistance and lipid abnormalities associated with renal failure. Taken together, these results suggest that the protein bound uremic toxins could contribute to insulin resistance and their elimination could help to improve the health of patients with chronic kidney disease.

Indoxyl sulfate is an uremic toxin that causes renal injury, but little is known about its adverse effects on metabolism. The aryl hydrocarbon receptor is a ligand-activated transcriptional factor that mediates adaptive and toxic responses in cells. AhR is now recognized as being the receptor mediating the effects of some persistent organic pollutants such as dioxins. Recent studies identified IS as an endogenous agonist for AhR, as well as some other tryptophan metabolites<sup>122</sup>. Indoxyl sulfate is associated with cardiovascular mortality in CKD patients<sup>123</sup>. Indoxyl sulfate

was unambiguously shown to promote a vascular procoagulant phenotype that could contribute to the increased cardiovascular risk in CKD patients.<sup>124-126</sup>

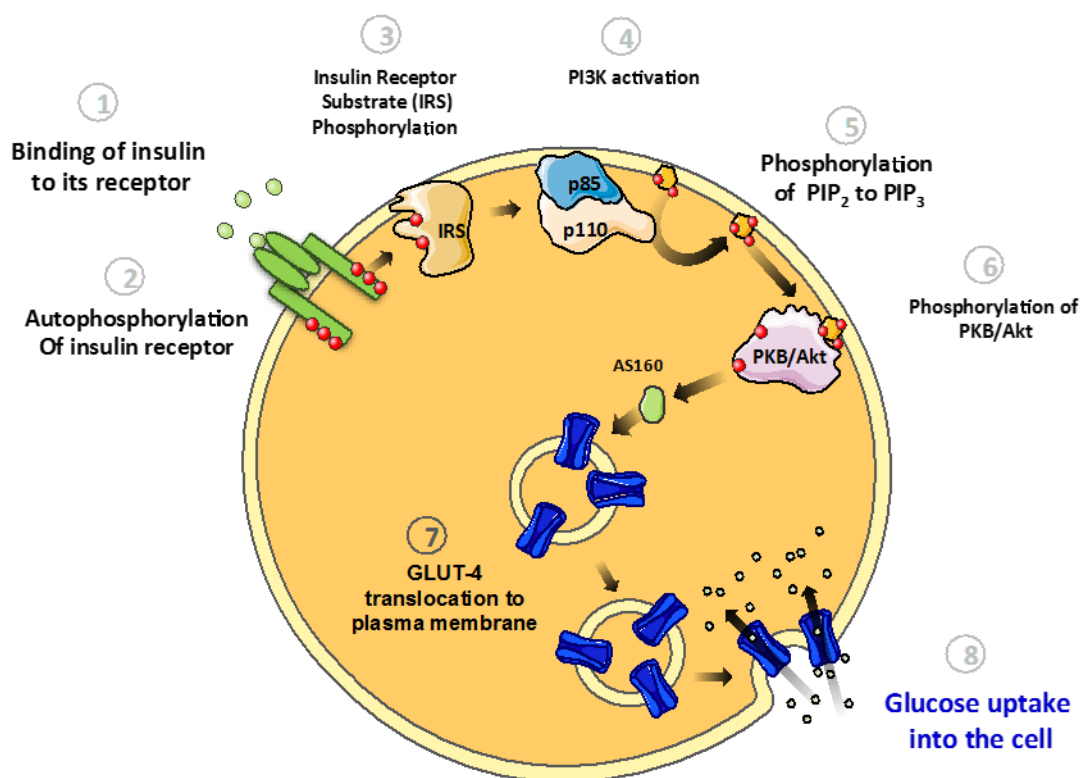
Numerous man-made pollutants activate the AhR and are recognized as risk factors for type 2 diabetes<sup>127</sup>. Indeed, AhR signaling also affects molecular clock genes to influence glucose metabolism. However, to the best of our knowledge there is no data in the literature regarding the role of IS for metabolic disturbances associated with CKD. The aim of the present project was therefore to investigate whether IS, through activation of AhR, with subsequent inflammatory responses could contribute to the development of insulin resistance and other metabolic disorders associated with CKD. In a preliminary data, we have shown that exposing C2C12 muscle cells to IS leads to altered insulin signaling (through a very different mechanism than p-CS) involving AhR also known to mediate the cellular response to some pollutants, such as dioxins. We demonstrated as a proof of concept that the use of the AhR inhibitor alpha-naphthoflavone (ANF) thoroughly prevented IS-induced insulin resistance in muscle cells. These results suggest that in CKD, accumulation of IS may act like **an endogenous pollutant** resulting in increased cellular stress and promoting insulin resistance.

## **Method**

### **In vitro experiments**

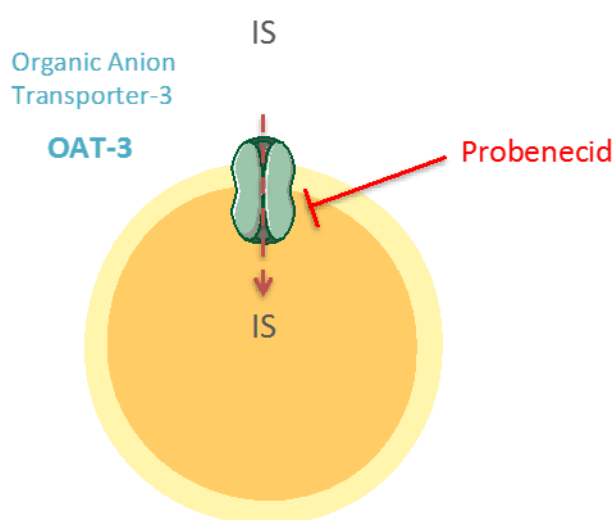
All in vitro experiments were performed according to the standard approach and the recommendations for handling uremic retention solutes published by the European

Uremic Toxin Work Group (EUTox, <http://www.uremic-toxins.org>). To evaluate the mechanisms responsible for the inhibition of glucose uptake, we analyzed the insulin signaling pathways in the absence or presence of IS. C2C12 myotubes were incubated with 250 $\mu$ M of IS, which is the concentration found in CKD patients as reported by the EUTox, to mimic encountered IS in CKD patients, for 30 min and then stimulated by 100 nM insulin for 20 min. **Figure 31** shows the insulin signaling PI3K / Akt pathway. We explored the insulin pathway by western blotting using the phosphorylation of protein kinase B (PKB / Akt) as showed in the sixth step of **Figure 31**.



**Figure 31.** Insulin signaling: PI3K / Akt pathway in muscle and adipose cells.

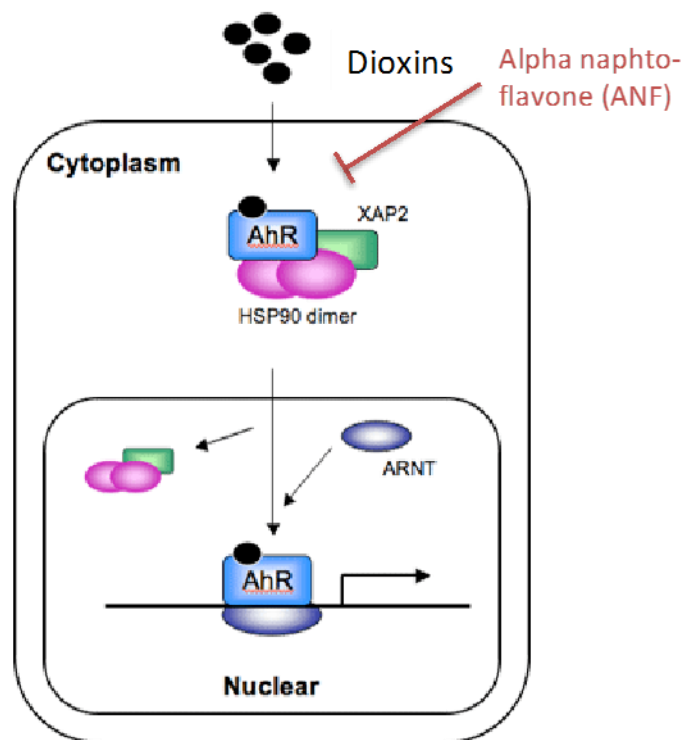
Indoxyl sulfate is thought to enter the cell via organic anion transporters (OAT), especially OAT-3 (SLC22A8). OAT3 are abundantly expressed on the basolateral membrane of proximal tubule cells, as well as several other epithelial tissues, and is responsible for the renal uptake and excretion of a wide variety of drugs, exogenous toxins, nutrients, and endogenous metabolites, including uremic toxins, such as indoxyl sulfate, p-cresyl sulfate, CMPF and TMAO.<sup>128, 129</sup> IS is transferred into the cells by organic anion transporter. In order to determine if IS effects inside or outside the cells, we used a potent inhibitor of OAT-3, probenecid, which can prevent OAT-3 from transferring IS into the cells (**Figure 32**). C2C12 myotubes were pre-incubated with 1mM probenecid for 1h, prior to incubation with 250 $\mu$ M IS for 30 min and stimulation with 100nM insulin for 20 min. The insulin signaling pathway was explored by western blotting using the phosphorylation of protein kinase B (PKB/Akt).



**Figure 32.** Probenecid prevents organic anion transporter 3 (OAT3) from transferring IS into the cells.

Recent study shows IS, as well as other tryptophan metabolites, to be the first identified relatively high effect endogenous agonist for AhR and plays an important role in CKD progression.<sup>83</sup> AhR is a ligand-activated transcriptional factor mediating the effects of some persistent organic pollutants such as dioxins. Normally, AhR is inactive, and bound to several co-chaperones. Upon ligand binding to chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the chaperones dissociate resulting in AhR translocation into the nucleus and dimerization with AhR nuclear translocator (ARNT), leading to changes in gene transcription. IS was identified as an endogenous agonist for AhR. Aimed to study if indoxyl sulfate induces insulin resistance through activation of AhR, alpha-naphthoflavone (ANF), a potent inhibitor of AhR was used to prevent AhR activation in muscle cells (**Figure 33**). C2C12 myotubes were incubated with 1mM alpha-naphthoflavone for 1 h, prior to incubation with 250  $\mu$ M IS for 30 min and stimulation with 100nM insulin for 20 min. The insulin signaling pathway was explored by western blotting using the phosphorylation of protein kinase B (PKB/Akt).





**Figure 33.** Alpha-naphthoflavone (ANF) prevents aryl hydrocarbon receptor (AhR) activation in cells.

### **In vivo study**

#### **Animal experiments**

Animal experiments were performed under the authorization n°69-266-0501 (INSA-Lyon, DDPP-DSV), according to the guidelines laid down by European Union Council Directive 2010/63UE. CD1 Swiss and C57BL/6J mice were purchased from Janvier SA (Le Genest-Saint-Isle, France) and housed in an air-conditioned room with a controlled environment of  $21^{\circ}\text{C}\pm 0.5^{\circ}$  and 60%–70% humidity, under a 12-hour light/dark cycle (light on from 07:00 to 19:00) with free access to food and water in the cages.

Moderate CKD was induced by two methods. 5/6 subtotal nephrectomy is the

common method to induce CKD in rodents. Mice were operated with 5/6 subtotal nephrectomy performed in two steps. First week, unilateral nephrectomy was performed. Then mice were performed with electrocoagulation of 2/3 of the remnant kidney the second week. As 5/6 nephrectomy is a surgical model, sham operated mice were performed as control. Meanwhile, we gave different diet, adenine diet and normal diet to the mice for 4 weeks to develop a new model of CKD in mice. Mice were fed for 4 weeks with a diet containing 0.25% (w/w) adenine. This model is a non-surgical model of CKD in rodents.

We performed a chronic administration of indoxyl sulfate on mice to detect the effect of IS. Mice were randomly assigned to receive twice daily intraperitoneal injections of IS (100 mg/kg) or with 0.65% (w/v) IS in drinking water.

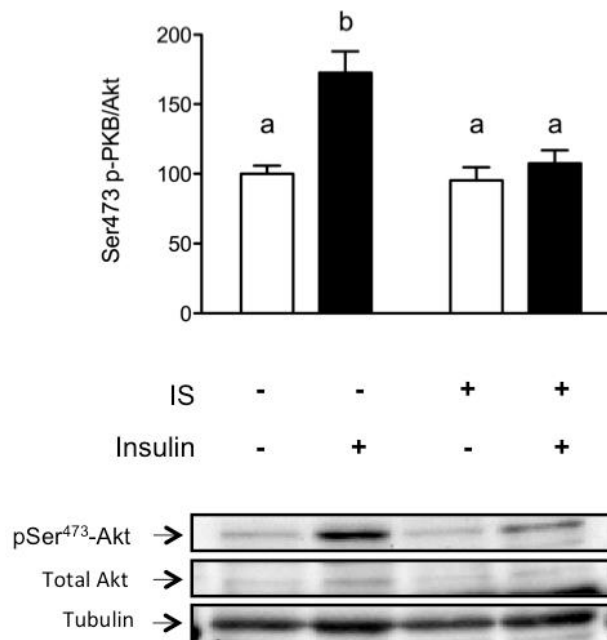
### **Pharmacokinetic studies**

The metabolic impacts were studied with intraperitoneal glucose and insulin tolerance tests. Biometric data (body weight, Lee adiposity index, fat accretion), metabolic (total cholesterol, triglycerides, glucose) were measured using standard methods. HPLC-Fluorescence assay was used to determine the plasma concentration of IS (Pr. Stéphane Burtey, Vascular Research Center, Marseille). The disposition of IS was explored after an intraperitoneal injection of IS (100mg/kg). Blood was removed serially to perform IS assay. HPLC-Fluorescence assay was used to determine the plasma concentration of IS.

## Results

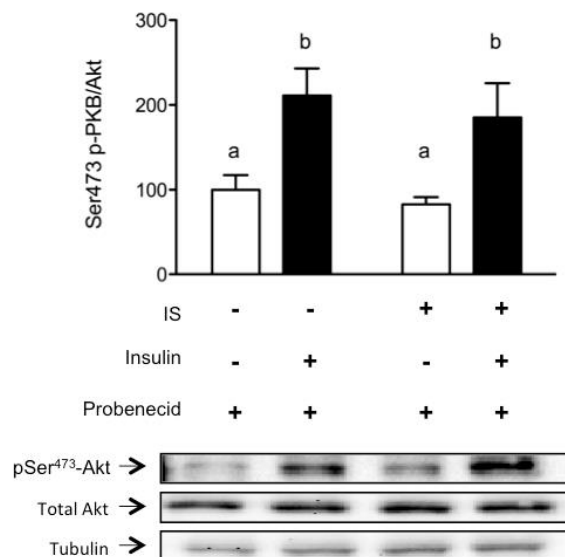
### In vitro experiments

Insulin signaling pathways were analyzed in the absence or presence of IS. Activation of the downstream kinase PKB/Akt was estimated through the insulin-induced phosphorylation of serine 473, which was significantly increased after a treatment by 100nM insulin for 20 minutes, but this effect was abolished with a preincubation with 250  $\mu$ M IS for 30 min. (**Figure 34**). This proved that IS disrupts insulin signaling pathways in C2C12 myotubes.



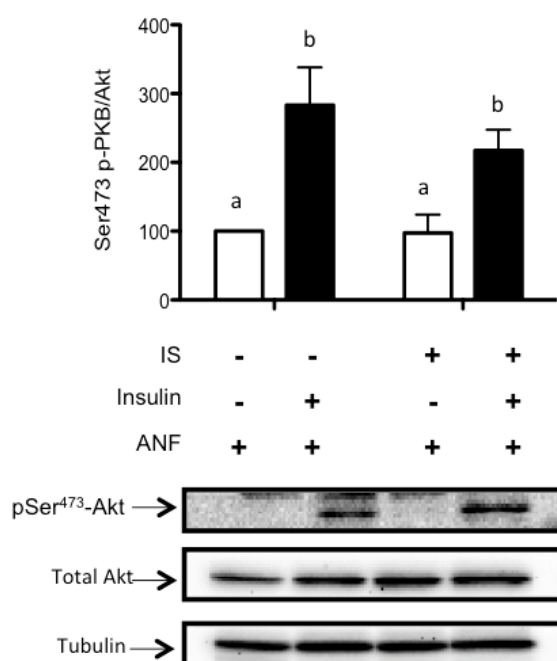
**Figure 34.** IS disrupts insulin signaling pathways in C2C12 myotubes. C2C12 myotubes were incubated with IS (250  $\mu$ M) for 30 min and stimulated by 100 nM insulin for 20 min. Insulin signaling pathway was explored by Western Blotting. Effect of IS on serine 473 phosphorylation of PKB/Akt Data are mean  $\pm$  SEM for n=7-8. Different letters indicate a significant difference at p <0.05.

IS is thought to enter the cell through organic anion transporters (OAT), especially OAT3. C2C12 muscle cells were pretreated with probenecid (1 mM, 1 hour), a potent inhibitor of OAT family, before incubation with 250 $\mu$ M IS for 30 min. Pretreatment with probenecid prevented IS-induced disruption of insulin signaling pathways, as evidenced by the measurement of insulin-induced PKB/Akt phosphorylation (**Figure 35**).



**Figure 35.** Probenecid prevents IS-induced disruption of insulin signaling pathways in C2C12 myotubes. C2C12 myotubes were incubated with probenecid (1 mM, 1 h), an inhibitor of OAT-3, prior to incubation with IS (250  $\mu$ M, 30 min) and stimulation by insulin (100 nM, 20 min). Effect of IS on serine 473 phosphorylation of PKB/Akt. N=4~5. Different letters indicate a significant difference at  $p < 0.05$ .

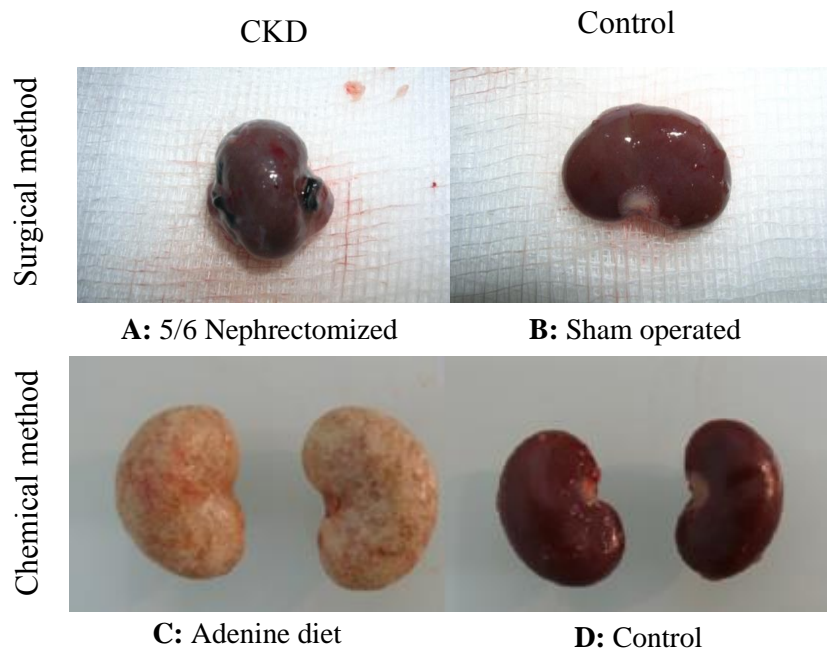
Recent studies identified IS as an endogenous agonist for AhR, which is a ligand-activated transcriptional factor that mediates adaptive and toxic responses in cells. We pretreated C2C12 myotubes with alpha naphtho-flavone (ANF, 1 mM, 1 hour) prior to the incubation with 250 $\mu$ M IS. Pretreatment with ANF prevented IS-induced disruption of insulin signaling pathways in C2C12 muscle cells, as a result of the measurement of insulin-induced PKB/Akt phosphorylation (**Figure 36**).



**Figure 36.** Alpha naphtho-flavone (ANF) prevents IS-induced disruption of insulin signaling pathways in C2C12 myotubes. C2C12 myotubes were incubated with alpha naphtho-flavone (1 mM, 1 h), a potent inhibitor of AHR, prior to incubation with IS (250  $\mu$ M, 30 min) and stimulation by insulin (100 nM, 20 min). Effect of IS on serine 473 phosphorylation of PKB/Akt. N=4~5.

### **In vivo experiments**

5/6 nephrectomy is the most frequently used experimental animal model to induce chronic renal disease. However, as it is a surgical method with two steps of operation, in our hand roughly 20% of mortality occurs during the surgical procedure. Therefore, we developed and validated a new model of CKD in mice, using an adenine diet. Chemical nephrectomy was induced by adenine rich diet. This method is no surgical, easily performed and quickly achieved. Mice were fed with 0.25% (w/w) adenine diet for 4 weeks. **Figure 37** shows the kidneys of different CKD animal models. Kidneys of adenine diet mice (**Figure 37C**) have an apparent difference of color and pathological features from the control mice (**Figure 37D**) that were fed with normal food.



**Figure 37.** Implementation of a new model of CKD in mice. **A, B, C** and **D** are kidneys from different CKD animal models on mice.

**A:** Kidney of 5/6 nephrectomized mice. Mice were operated with 5/6 subtotal nephrectomy performed in two steps. Week 1: Unilateral nephrectomy; Week 2: Electrocoagulation of 2/3 of the remnant kidney.

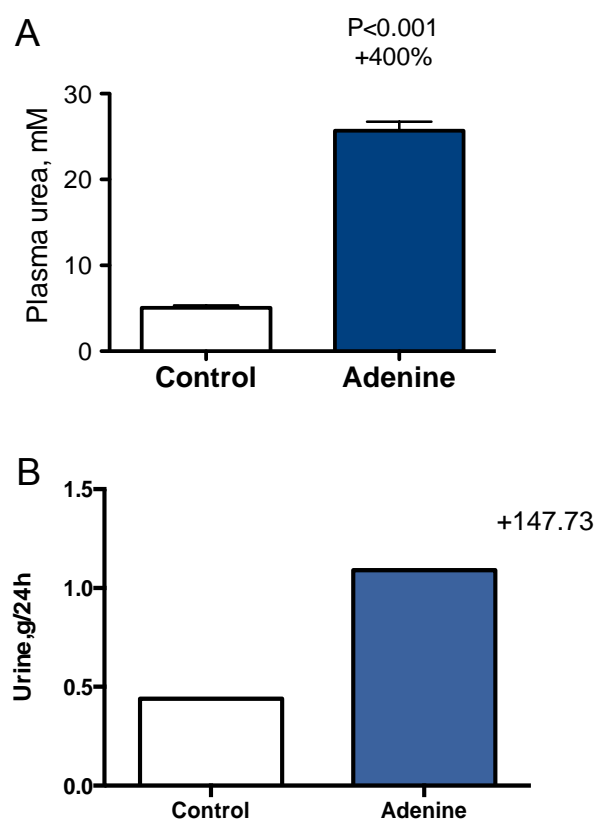
**B:** Kidney of sham operated mice.

**C:** Kidneys of mice fed an adenine rich diet. Chemical nephrectomy was induced by Adenine rich diet. Mice were fed with 0.25% (w/w) adenine diet for 4 weeks. Adenine rich diet induced chemical nephrectomy which is a non-surgical model of CKD in rodents.

**D:** Kidneys of control mice.

The concentration of plasma urea and diuresis are two important indexes to refer to of clinical diagnosis on CKD. Thus, we checked the plasma urea level and amount of urine excreted by the adenine diet model of CKD mice. Plasma urea (**Figure 38A**) and diuresis (**Figure 38B**) were both found a significant increase in adenine diet mice compared with the control group fed with normal food. These results evidence

adenine diet induced increased plasma urea and diuresis in mice.



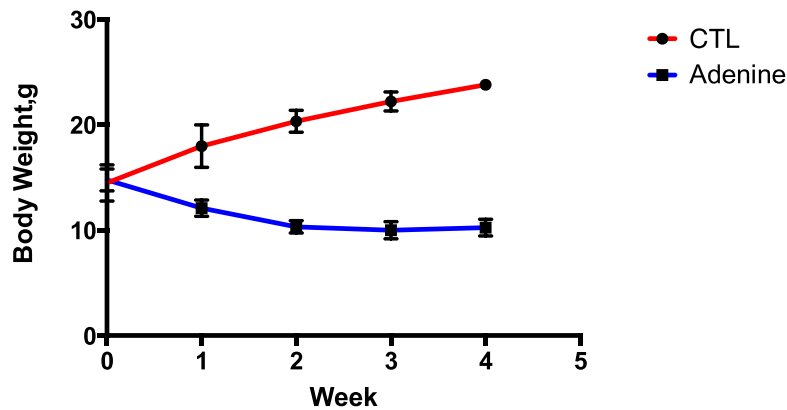
**Figure 38.** Significant differences in indexes of renal function in adenine induced kidney failure in mice.

**A:** Plasma urea level significantly increased in adenine diet mice.

**B:** Diuresis significantly increased in adenine diet mice (i.e. polyuria). Urine were collected every 24h for 2 days.

Adenin diet is an easy and efficient method to induce CKD in mice. Oral intake of adenine is simple and can induce CKD rapidly, but it is a very different method from 5/6 nephrectomy. We found this method caused a substantial weight loss in mice (**Figure 39**), which was due to a very significant decrease in food intake. This point should therefore be taken into account when performing adenine diet CKD model.





**Figure 39.** Adenine diet blunted body weight gain in mice.

Biometric data of control and adenine diet mice are summarized in **Table 14**.

Body weight gain was significantly reduced in mice fed an adenine diet and most organ weights were significantly decreased. Adenine mice exhibited a striking decrease in white adipose tissue accretion (-49%,  $P < 0.001$ ). Adenine diet mice, however exhibited an increased plasma cholesterol level (+21%,  $P < 0.05$ ) compared to control mice.

Variable	Control Mice	CKD Mice	Change(%)	If Significant
Mice(n)	10	10	-	-
Body weight(g)	25±0.4	15±0.5	-39.79	****
Body length(cm)	9.3±0.1	7.8±0.1	-15.63	****
Lee index(×100)	31.5±0.3	31.5±0.3	0.05	No
Liver weight(mg)	1,361±67.0	771±31.7	-43.35	****
Heart weight(mg)	125±4.5	94±3.1	-24.80	****
Kidney weight(mg)	281±10.3	119±3.8	-57.65	****
Gastrocnemius weight(mg)	126±3.7	77±3.3	-38.89	****
eWAT weight(mg)	318±27.8	117±14.1	-63.21	****
rWAT weight(mg)	63±9.0	34±6.9	-46.03	*
scWAT weight(mg)	149±13.1	121±14.3	-18.79	No
Total WAT weight(mg)	530±47.8	272±32.5	-48.68	***

**Table 14.** Biometric data. Data are expressed as mean ± SEM. Lee index was calculated as the cubic square of body weight divided by naso-anal length. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\* $P < 0.005$ , \*\*\*\* $P < 0.001$ , Significant difference between control and adenine mice.

**Abbreviations:** WAT, white adipose tissue; eWAT, epididimal WAT; rWAT, retroperitoneal WAT; scWAT, subcutaneous WAT.

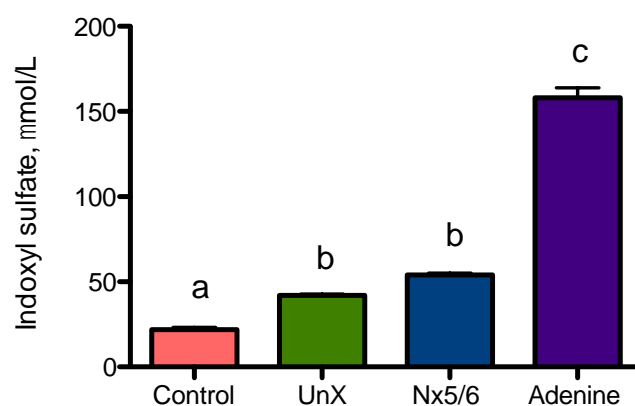
Variable	Control Mice	CKD Mice	Change(%)	If Significant
Mice(n)	10	9	-	-
Diuresis(ml/24hr)	0.44	1.09	147.73	-
Water intake(ml/24hr)	3.07	6.50	111.73	-
Proteinuria(mg/24hr)	128.15	53.18	-58.50	-
Triacylglycerols(mg/dl)	1.00±0.09	0.31±0.06	-68.54	****
Total cholesterol(mg/dl)	1.26±0.11	1.61±0.10	28.29	*
Fasting glucose(mg/dl)	93.60±4.49	105.10±6.82	12.29	No

**Table 15.** Metabolic parameters. Data are expressed as mean ± SEM.

**Table 15** shows the biochemical data of adenine diet induced CKD mice. Diuresis and water intake significantly increased in CKD mice. However, proteinuria was reduced in adenine diet mice due to the dilution of urines. Triacylglycerols and total cholesterol had a higher level in CKD mice, while fasting plasma glucose was within the range of control mice.

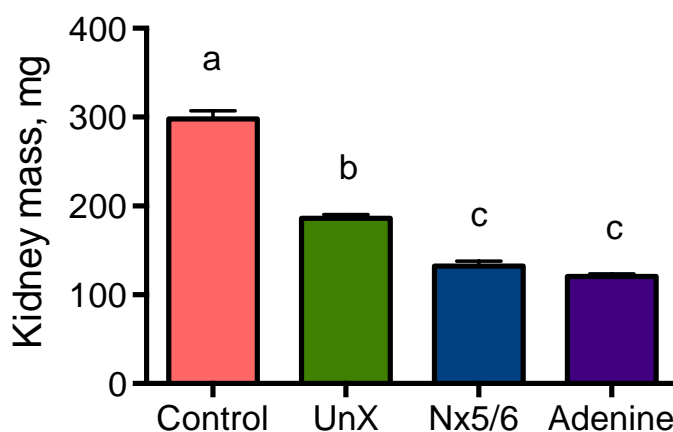
The accumulation of indoxyl sulfate is observed in CKD patients because of the reduced function of kidneys. For the purpose of detecting if IS also accumulates in CKD models, we used HPLC-Fluorescence assay to determine the plasma concentration of IS in different animal models. Unilaterally nephrectomized mice and 5/6 nephrectomized mice exhibited a significant increased level of IS. Compared with the nephrectomized mice, the plasma level of IS in mice fed an adenine diet significantly increased (**Figure 40**). Adenine diet induced the highest amount of IS accumulation.

Kidney weight is a gross index of renal failure in the different experimental models. We measured and calculated the kidney mass for each model as one kidney. 5/6 nephrectomy and adenine diet both significantly reduced kidney mass in mice compared with control and uninephrectomized mice (**Figure 41**).



**Figure 40.** HPLC-Fluorescence assay of IS in different CKD animal models.

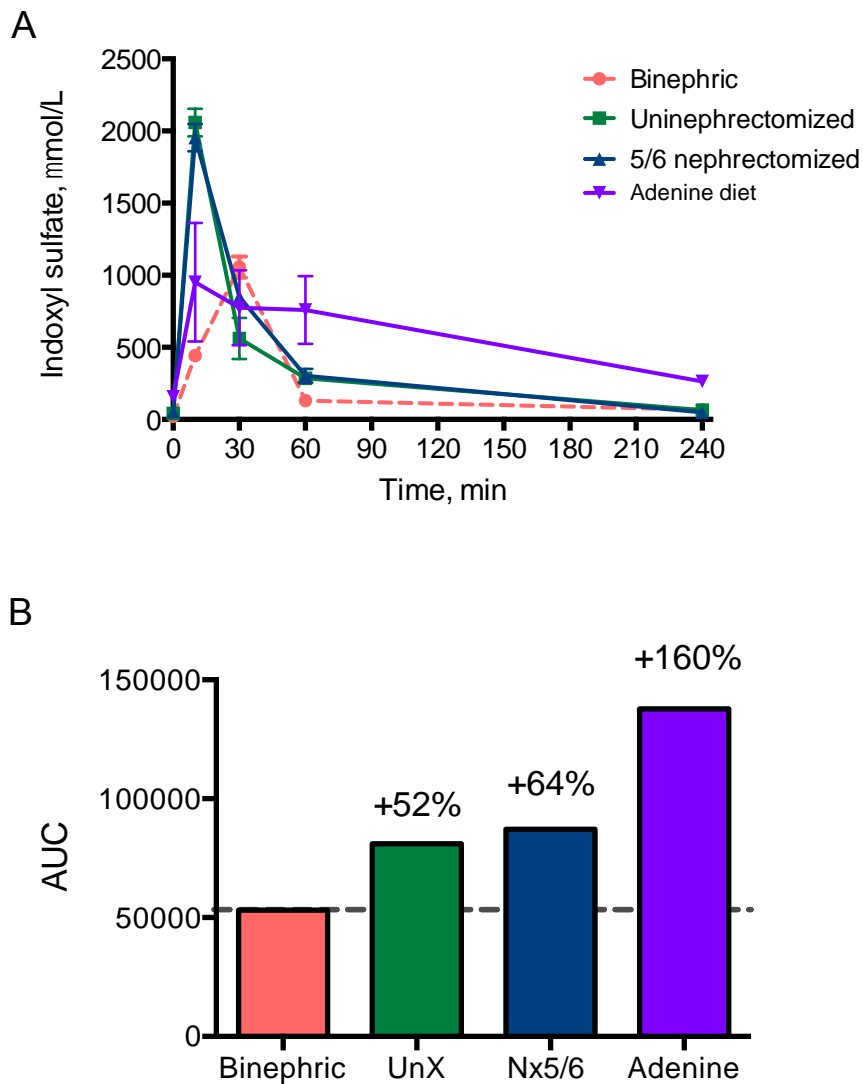
Control: Binephric mice, n=15; UnX: Uninephrectomized mice, n=15; Nx 5/6: 5/6 nephrectomized mice, n=15; Adenine: Adenine fed mice, n=15.



**Figure 41.** Kidney weights in the different experimental models of renal failure.

Control: Binephric mice, n=15; UnX: Uninephrectomized mice, n=15; Nx 5/6: 5/6 nephrectomized mice, n=15; Adenine: Adenine fed mice, n=15.

CKD does not happen overnight. Clinically, based on the level of renal function, CKD is divided into 5 stages. Kidney function decreases from stage 1 to stage 5, where the accumulation of uremic toxins is increasing. In order to study if IS accumulation can experimentally increased in CKD animal models as observed in CKD patients, the disposition of IS was explored after an intraperitoneal injection of IS (100mg/kg). Blood was removed serially to perform IS assay and HPLC-Fluorescence assay was used to determine the plasma concentration of IS. We found that IS concentration was transiently increased but does not accumulate in the plasma (**Figure 42A**). In the first 30 min after injection, IS concentration in uninephrectomized and 5/6 nephrectomized mice was transiently increased but rapidly decreased in 60 min and returned to the baseline after 4h. For the control group, IS concentration slowly increased in the first 30 min after injection, but quickly reduced to the baseline at 60 min. Adenine diet changed the kinetic of the metabolism of IS. IS concentration in adenine diet mice did not reach as high as in 5/6 nephrectomized mice, but decreased slower to reach to the baseline. IS stayed for a longer time in adenine diet induced CKD mice. **Figure 42B** shows the area under the curve (AUC) of integral by IS concentration. Nephrectomy and adenine diet changed the kinetic of IS. CKD increased the mean residence time of IS. Adenine diet method has the longest residence time of IS, so caused highest IS accumulation. IS concentration can be experimentally increased indoxyl sulfate concentration in mice although this accumulation is only transient.



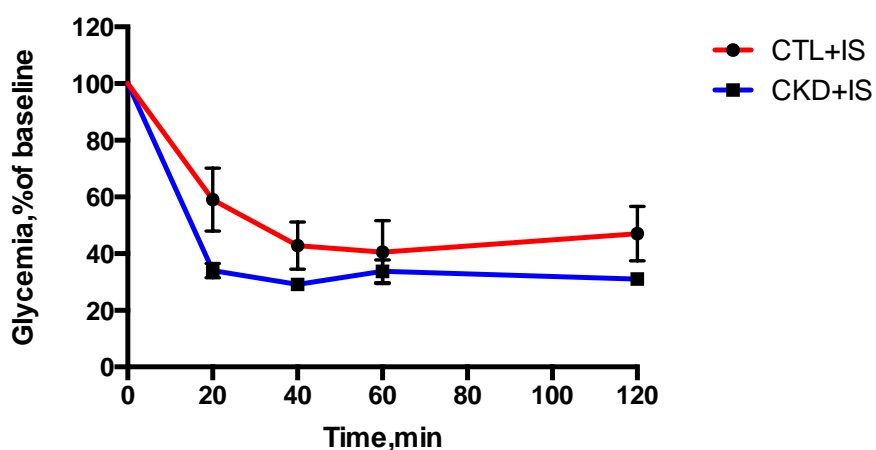
**Figure 42.** Plasma kinetics of IS in mice injected with indoxyl sulfate (100 mg/kg i.p.).

A: Pharmacokinetics of IS in different animal models of kidney failure after intraperitoneal injection of IS.

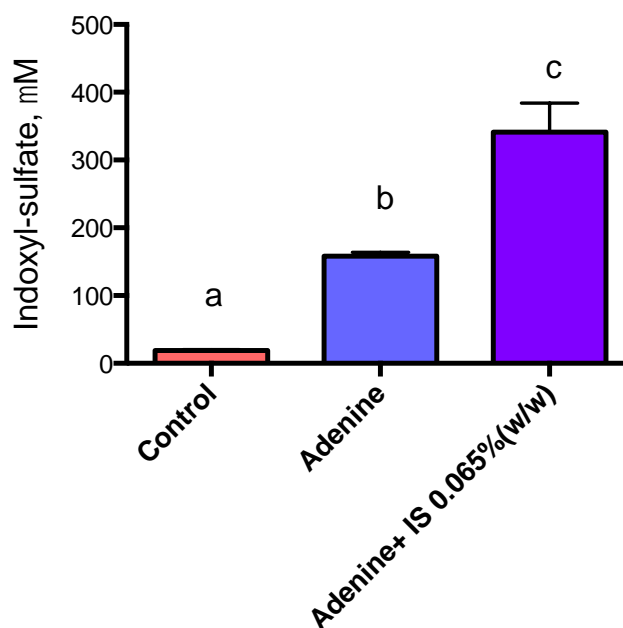
B: Area under the curve of IS concentration in different animal models of kidney failure after intraperitoneal injection of IS.

To detect the effect of a chronic administration of indoxyl sulfate on mice, we tried two methods: daily intraperitoneal injections and oral intake of IS in drinking water. However, we did not find insulin resistance in vivo study. For the insulin

tolerance test, control and adenine diet mice with chronic administration of IS (0.65%, w/w) for 17 days. The insulin tolerance test (insulin, 0.50 IU/kg body weight) was performed after an overnight fast. Blood glucose values were determined using an automatic glucose monitor (Accu-Check Performa, Roche, Meylan, France), from a drop of blood sampled from the terminal portion of the tail. No difference in fasting glucose was found with administration by IS in vivo study (**Figure 43**). IS did not induce the development of insulin resistance in mice, showing a very different characteristics from p-cresyl sulfate that contributes to insulin resistance<sup>81</sup>. Thus, we paused the study of in vivo experiments of IS on adenine diet mice. **Figure 44** shows IS concentration significantly increased in adenine diet mice with a chronic administration of IS (0.65%, w/w) in drinking water. Daily intake of IS has a harmful impact on the kidneys of adenine induced CKD mice.



**Figure 43.** Insulin tolerance test of control and CKD mice with a chronic administration of IS (0.65%, w/w) in drinking water. Data are mean  $\pm$  SEM for n=5 mice.



**Figure 44.** IS concentration increased in adenine diet mice with a chronic administration of IS (0.65%, w/w) in drinking water.

## Conclusion

IS, through activation of AhR, disrupts insulin signaling pathways and lead to the development of insulin resistance associated with CKD. IS appears to be a very important uremic retention solutes that could contribute to insulin resistance in patients with CKD. Adenine rich diet is an easy efficient method to induce CKD in mice. IS can be experimentally raised in mice with kidney failure to mimic the situation encountered in ESRD patients. Unfortunately we failed to find any difference in metabolic parameters or in insulin sensitivity in CKD mice supplemented with IS.





#### 4 Zinc-alpha2-glycoprotein as an uremic toxin

*Serum levels of the adipokine Zinc-Alpha2-Glycoprotein (ZAG) predict mortality in hemodialysis patients*

Anaïs Bouchara\*, **Dan Yi**\* et al, *Kidney International*, 2018; in press.

*\*These authors contributed equally to this work.*

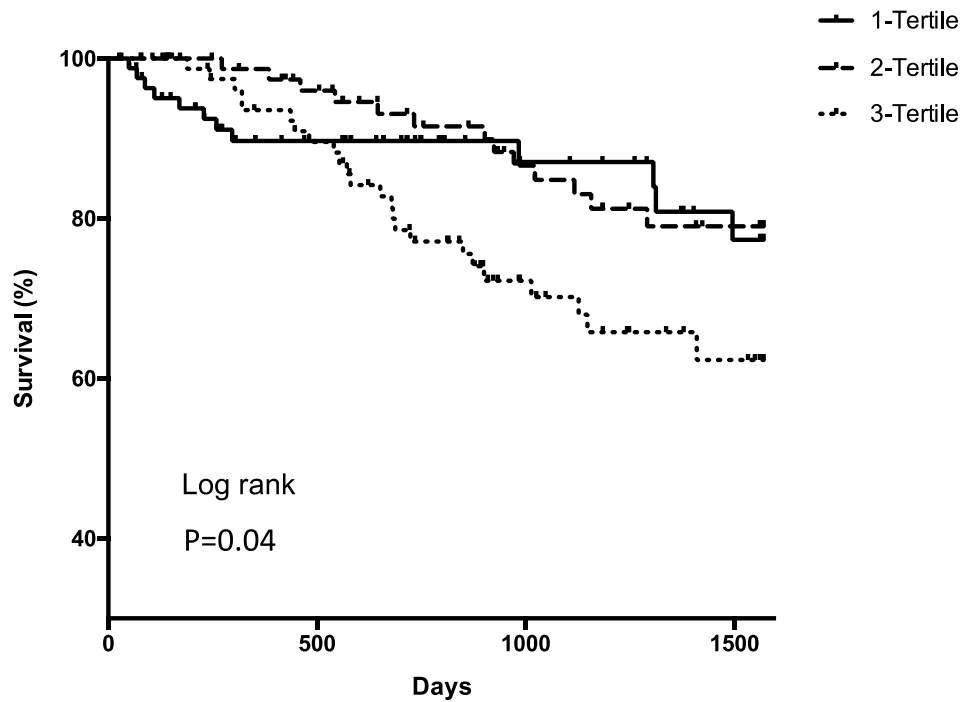
Chronic kidney disease (CKD), especially end stage renal disease (ESRD) is a risk factor for cardiovascular (CV) diseases. CV is the most common cause of death due to more than half of patients with hemodialysis (HD) treatment die of CV complications. It is hard to explain the increased risk for CV caused morbidity and mortality in ESRD with classic traditional risk factors, such as age, diabetes, dyslipidemia and hypertension, which have prompted the search for specific CKD biomarkers for CV diseases.

Uremic toxins predicted cardiovascular and global mortality in CKD. Zinc-alpha2-glycoprotein (ZAG), a 43-kDa soluble glycoprotein, is a middle molecule uremic toxin. ZAG was initially identified more than 50 years ago, but its actual biological function still remains poorly understood. Recently, ZAG was described as a new adipokine secreted by adipose and epithelial cells of multiple organs, which involved in cachexia due to its potent lipolytic effects. It is reported that ZAG circulates in high concentration in CKD and ESRD patients as many other adipokines, such as adiponectin, leptin or retinol binding protein-4 (RBP-4). Plasma ZAG concentration was increased in chronic kidney disease (CKD) and patients on

hemodialysis (HD) treatment.<sup>96,97</sup> High ZAG serum concentration in this population probably results from a decreased glomerular filtration, a blunted renal degradation and overproduction by adipocytes stimulated by the uremic environment.

However, the impact of ZAG accumulation on mortality and cardiovascular risks has never been studied. Therefore, we investigated whether ZAG, a potent cachectic factor and lipid mobilizing factor and increasing during CKD, predicts clinical outcomes in patients in hemodialysis patients. For this article, we followed 252 patients undergoing maintenance hemodialysis at least 3 months, without progressive cancer, and quantified serum ZAG by enzyme immunoassay. Mortality and cardiovascular events have been registered during 4 years. We stratified patients into three tertiles and examined the relationship between ZAG level and mortality in time-to-event analyses. **Figure 45** shows there are significantly more deaths among patients with high serum ZAG concentration in the third tertile (tertiles 1-3: 12, 13 and 24 events, respectively; log rank  $P=0.04$ ).

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	Number of patients at risk			
	0 day	500 days	1000 days	1500 days
Tertile 1	84	60	33	23
Tertile 2	84	70	50	35
Tertile 3	84	68	36	18

**Figure 45.** Kaplan–Meier estimates of cumulative survival of all patients according to plasma Zinc-Alpha2 Glycoprotein (ZAG) level at inclusion. Tertile cut-off (ZAG,  $\mu\text{g/ml}$ ): T1,  $< 17.9$ ; T2,  $[17.9 - 48.0]$ ; T3,  $> 49.0$ . Numbers of events were 12, 13, and 24 for tertile 1 to 3, respectively. The number of patients indicated in the tables is the number of patients still followed at the indicated time.



## **Serum levels of the adipokine Zinc-Alpha2-Glycoprotein (ZAG) predict mortality in hemodialysis patients**

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**Running title:** ZAG and mortality in hemodialysis patients.

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**Word count:** 4623 words

**References:** 64

**Figures:** 6

**Tables:** 9

## **Abstract (240 words)**

Wasting predicted cardiovascular and global mortality in CKD. We investigated whether Zinc-alpha2-glycoprotein (ZAG) a potent cachectic and lipid mobilizing factor, increased during CKD, predicts clinical outcomes in patients in hemodialysis patients. Therefore, we prospectively followed 252 patients undergoing maintenance hemodialysis and quantified serum ZAG. During the mean study period of 954 days, 49 patients died and 62 experienced a major cardiovascular event. Serum ZAG concentration was inversely correlated with serum albumin ( $P<0.01$ ), creatinine ( $P=0.01$ ) and triglycerides ( $P=0.04$ ). By contrast, it was positively correlated with age ( $P<0.01$ ). Kaplan-Meier analysis revealed a significant correlation between serum ZAG concentration and overall mortality (log rank,  $P=0.04$ ) and cardiovascular events (log rank,  $P<0.01$ ). Multivariable Cox regression analysis demonstrated that serum ZAG concentration was a significant predictor of mortality and cardiovascular events after adjustment for demographic factors (age, sex, dialysis vintage), metabolic parameters (serum albumin, prealbumin, triglycerides, cholesterol, normalized protein catabolic rate, body mass index) and cardiovascular risks (diabetes, dyslipidemia, history of cardiovascular diseases, tobacco, diuretic medication (as a proxy of residual renal function)). By contrast with patients having a cancer, serum ZAG accumulation does not correlate with protein energy wasting. Finally, in our cohort, ZAG did not seem to be involved in metabolic disturbances (type 2 diabetes, dyslipidemia). In conclusion, serum ZAG may be a strong and independent predictor of mortality and cardiovascular events in patients in the end-stage renal disease. Further studies are necessary to confirm this association and to elucidate the underlying mechanisms.

**Key words:** Zinc-alpha2-glycoprotein, cardiovascular disease, end stage renal disease, wasting, mortality

## Introduction

Chronic kidney disease (CKD) and especially end-stage renal disease (ESRD) are major risk factors for cardiovascular (CV) diseases <sup>1</sup>. In fact, CV mortality is the most common cause of death in CKD patients <sup>2</sup> and more than 50% of patients on hemodialysis (HD) treatment die from CV complications. The traditional risk factors do not fully explain the CV morbidity and mortality in ESRD patients. There is therefore a need for specific biomarkers of CV disease in CKD patients. Indeed, a 10- to 20-fold increased risk of CV death still exists in this population after adjustment for classical CV risk factors <sup>3</sup>.

White adipose tissue (WAT) is recognized as an endocrine organ since it secretes a large variety of biologically active compounds referred to as adipokines. Zinc-alpha2-glycoprotein (ZAG), a recently described adipokine, is a 43-kDa soluble glycoprotein secreted by adipocytes but also by epithelial cells from multiple organs. ZAG circulates in high concentration in CKD and ESRD patients <sup>4,5</sup>. High ZAG serum concentration in this population is thought to result from a decreased glomerular filtration and/or a blunted renal degradation but also from overproduction by adipocytes stimulated by the uremic environment <sup>4,6</sup>. ZAG was initially identified more than 50 years ago, but actually its biological functions still remains poorly understood. Various roles of ZAG were issued from structure/activity studies available in literatures <sup>7</sup>.

The discovery that ZAG shares sequence identity with a long sought, lipid mobilizing factor, first involved ZAG in lipid metabolism <sup>8,9</sup>. Subsequently, several studies supported the idea that ZAG was involved in the regulation of body weight and could contribute to cachexia associated with cancer <sup>10</sup>. Indeed, experimental data demonstrated that ZAG induces lipolysis on adipose cells through the stimulation of adipocyte adenylyl cyclase <sup>11</sup>, and

through stimulation of beta<sub>3</sub>-adrenoceptors<sup>12</sup>. Furthermore, rodents administered with exogenous ZAG exhibited increased fat mobilization<sup>13</sup>, whereas genetic deletion of ZAG was associated with an increased fat deposition<sup>14</sup>. ZAG could also increase resting energy expenditure by activating the uncoupling proteins in brown adipose tissue and muscle, increasing thermogenesis<sup>15</sup>. Therefore, a role for ZAG in maintaining appropriate fat mass and insulin sensitivity has been proposed. However, the correlation between serum ZAG concentrations, fat mass and insulin resistance in human with normal renal function remain controversial<sup>16-24</sup>.

The same conflicting results were reported in CKD patients. No correlation was found between plasmatic ZAG levels and protein energy wasting (PEW), fat mass or metabolic parameters in CKD<sup>4</sup> and HD patients<sup>5,25-27</sup>. In contrast, some others studies observed that ZAG was inversely correlated with insulin resistance markers such as fasting insulin and HOMA in patients with GFR > 50 ml/min<sup>5</sup> or body fat mass in HD patients<sup>28</sup>. One explanation for these discordant results could be that ZAG mainly acts as a paracrine/autocrine regulator of human adipose cells and that there is no straightforward relationship between ZAG plasma concentration and expression in WAT as observed in obesities<sup>17</sup> and patient with cancer<sup>29,30</sup>.

Until now the role of ZAG in CV complications have never been thoroughly explored. The central role of ZAG in lipid mobilization and maybe insulin-resistance suggest that ZAG could participate to dyslipidemia and diabetes recognized as major CV risk factors. As many adipokines, ZAG could promote endothelial dysfunction and impair the structural integrity of the CV system<sup>31</sup>. Indeed ZAG was shown to induce an increase in vascular tone through the activation of RhoA kinases and contribute to elevated blood pressure<sup>32</sup>. However, the association between hypertension and ZAG was not confirmed in other studies<sup>33</sup>. ZAG is significantly increased in clinical situations of endothelial dysfunction such as preeclampsia



which significantly increased future risk for CV diseases<sup>34</sup> and in heart failure patients<sup>35</sup>. However, physiological significance of ZAG upregulation in this situation remains unclear. At the opposite, ZAG has ~~also~~ been suggested as a CV protective factor in HD patients because ZAG was inversely associated with proatherogenic factors and oxidative stress<sup>28</sup>. ZAG is down regulated by pro-inflammatory cytokine and high ZAG level could indicate a low inflammatory state<sup>36</sup>.

The functional effect of elevated plasmatic ZAG levels in humans and particularly in those with renal disease remains unclear and controversial. Because, we thought that ZAG could play a role in metabolism complications and CV events, we measured the serum concentrations of ZAG in a large cohort of HD patients, we sought its functional correlates, and tested its predictive power for mortality and CV events.

## Results

### Systemic Levels of ZAG are Associated with CV events in HD Patients

Between March 2012 and December 2014, 266 prevalent patients under HD for more than 3 months were included. Measurements of serum ZAG were available in a total of 252 patients at baseline and in 145 patients (57% of the initial population) one year later. Baseline patients demographic and clinical characteristics, and laboratory measurements are presented in **Table 1**. The mean duration of follow-up was  $954 \pm 514$  days. The median age was 64 (17-88) years old. 94 patients were females (37%), with a median BMI of  $25.4 \text{ kg/m}^2$  (min-max,  $16.2\text{-}66.7 \text{ kg/m}^2$ )  $\text{kg/m}^2$  and median body fat mass of 38.0% (min-max,  $17.6\text{-}86.6\%$ ). 81 patients had diabetes (32%) and 97 were smokers (38%). The mean pre-dialysis systolic blood pressure (BP) was  $130 \pm 22$  mmHg and the median serum total cholesterol was

4.1 mmol/L (min-max, 1.5-7.5 mmol/L). Median serum albumin and pre-albumin were respectively 39.1 g/L (min-max, 22.7-45.6 g/L) and 330 mg/L (min-max, 40-770 mg/L). Median nPCR was 1.10 g/kg/day (min-max, 0.44-2.37 g/kg/day). Higher ZAG was significantly associated with an unfavorable CV risk profile, including higher age ( $P<0.01$ ) and history of dyslipidemia ( $P<0.01$ ); lower albumin ( $P=0.01$ ), and high triglycerides ( $P<0.01$ ) (**Table 1**). The analyzed population made by the 252 patients, was not different from the national population, as described in two previous French cohorts<sup>37,38</sup>. In our overall HD study cohort, the median log-transformed ZAG level was 1.42  $\mu\text{g/ml}$  (min-max, 0.53-2.82  $\mu\text{g/ml}$ ). ZAG concentration is stable over the time with no significant difference between initial ZAG concentrations and one year later (**Supplementary Figure 1**). ZAG was significantly higher in patients who presented CV events during the follow-up period ( $n=62$ ; median, log-transformed ZAG: 1.67  $\mu\text{g/ml}$  (min-max, 0.53-2.75 $\mu\text{g/ml}$ ) than among patients who presented no CV events ( $n=190$ ; median, log-transformed ZAG: 1.37  $\mu\text{g/ml}$  (min-max 0.55-2.82  $\mu\text{g/ml}$ ) ( $P<0.01$ ) (**Supplementary Table 1**). The concentration of ZAG was not significantly different between patients without or with RRF (log-transformed ZAG: 1.27  $\mu\text{g/ml}$  (min-max, 0.53-2.82) vs 1.48  $\mu\text{g/ml}$  (min-max, 0.55-2.70),  $p=0.06$ , respectively) or without/with use of diuretic (log-transformed ZAG: 1.42  $\mu\text{g/ml}$  (min-max, 0.67-2.82) vs 1.43  $\mu\text{g/ml}$  (min-max, 0.53-2.75),  $p=0.34$ ). The use of diuretic and the presence of RRF are not different between tertiles (**Table 1**).

Correlation studies on relevant clinical characteristics and laboratory measurements in the serum of these patients are shown in **Table 2**. Serum ZAG concentration was inversely correlated with serum albumin ( $P<0.01$ ), creatinine ( $P<0.01$ ) and triglycerides ( $P=0.04$ ). In contrast, serum ZAG concentration was positively correlated with age ( $P<0.01$ ). Serum ZAG was independent of serum CRP, parathyroid hormone, LDL cholesterol and glycated hemoglobin. While ZAG is described as a cachectic agent, ZAG concentration was not

associated with PEW ( $P=0.32$ ) (see **Supplementary Figure 2 and Supplementary Table 2**). Moreover, we failed to observe any significant correlation between systemic concentrations of ZAG and BMI, ~~body fat mass~~, prealbuminemia and nPCR (**Table 2**). We failed to observe a significant correlation between ZAG levels and body fat mass estimated by anthropometric formula (**Table 2**) or measured by BIS ( $r= -0.08$ ; 95% confidence interval [95% CI],  $-0,31$  to  $0,16$ ,  $p=0.50$ ), (*data not shown*).

### **Systemic Levels of ZAG Have Prognostic Utility Among HD Patients**

We stratified patients into three tertiles and examined the relationship between ZAG concentration and death or CV events in time-to-event analyses. At follow-up, we observed a total of 49 deaths (**Supplemental Table 3**), with significantly more deaths among patients with serum ZAG in the third tertile (tertiles 1-3: 12, 13 and 24 events, respectively; log rank  $P=0.04$ ) (**Figure 1**). We further observed a relationship between ZAG concentration 1 year after inclusion and all-cause mortality (log rank  $P= 0.05$ ) (**Supplementary Figure 3**). In univariable Cox proportional hazard analysis with ZAG as a continuous variable, serum ZAG was also significantly associated with overall mortality (hazard ratio [HR], 1.222; 95% CI, 1.110 to 1.345;  $P<0.01$ ) (**Table 3**). This association remained significant after adjustment for age, sex and dialysis vintage (model 1, HR, 1.194; 95% CI, 1.081 to 1.320;  $P<0.01$ ), for metabolic and nutritional parameters (model 2, HR, 1.173; 95% CI, 1.012 to 1.359;  $P=0.03$ ) or CV risk factors (model 3, HR, 1.277; 95% CI, 1.103 to 1.407;  $P<0.01$ ) (**Table 3**).

We further explored the relationship between serum ZAG and CV diseases. We have observed 62 CV events during follow-up (**Supplemental Table 4**). The number of events was significantly higher among patients with ZAG in higher tertiles of serum ZAG (tertiles 1-3: 12, 20 and 30 events, respectively; log rank  $P<0.01$ ) (**Figure 2**). Serum ZAG was also significantly associated with CV events in univariable Cox proportional hazard analysis with ZAG as a

continuous variable (HR, 1.190; 95% CI, 1.091 to 1.298;  $P < 0.01$ ) (**Table 3**). In addition, the relationship between serum ZAG and CV events during follow-up remained significant after several multivariable adjustments (**Table 3**). Of note, the association remained significant even after adjustment for age, sex and dialysis vintage (model 1, HR, 1.162; 95% CI, 1.061 to 1.272;  $P < 0.01$ ), metabolic and nutritional parameters (Model 2, HR, 1.144; 95% CI, 1.002 to 1.281;  $P = 0.02$ ) or CV risk (Model 3, HR, 1.158; 95% CI, 1.050 to 1.277;  $P < 0.01$ ). We did not find any association between ZAG levels and death by cancer, infection or other cause of mortality (**Supplementary Table 5**).

**Supplementary Table 6** shows the AUC of the ROC curves of ZAG and other traditional CV risk factor at the time of inclusion as predictors of CV events-related and all-cause mortality. ZAG had a significant diagnostic value to predict all-cause mortality and CV events since AUC were 0.61 (95% CI, 0.52 to 0.71);  $P = 0.012$  and 0.63 (95% CI, 0.55 to 0.72) respectively, and were significantly greater than the threshold of diagnostic indifference (50%). ZAG had the highest predictive value compared to the other variables for all-cause mortality and CV events, except for Charlson score, age, CRP and NT-pro BNP. A negligible improvement was seen with the addition of ZAG with Charlson score. When we considered the prognostic accuracy of ZAG for all cause of mortality, there was no significant difference between the AUCs of the ZAG and established risk factors excepted for Charlson score and age. ROC curves are shown in **Supplementary Figure 4**.

## Discussion

This study showed for the first time that high ZAG level can predict all-cause, and specifically, CV mortality in HD patients. This effect was independent of age, gender, RRF, metabolic and nutritional parameters and traditional CV risk factors. ZAG appeared as a robust biomarker,

stable over the time, and not influenced by RRF. When we considered model discrimination using ROC curve, ZAG appears as an interesting biomarker to predict mortality in HD patients. However, it did not perform as well as composite clinical index such as Charlson score. We confirm the absence of predictive capacity of systemic levels of ZAG in terms of estimated fat mass or diagnostic of PEW in our cohort.

Over the last ten years, evidences have accumulated that WAT produces many molecules that are metabolically active and that interfere with several organ systems. Overproduction of TNF- $\alpha$  or plasma activator inhibitor-1 (PAI-1) by adipose cells is involved in insulin resistance in obesity or vascular thrombosis, respectively <sup>39,40</sup>. The exploration of the relationship between adipokine levels and morbidity/mortality in patients with CKD has yielded some potential candidate biomarkers <sup>41,42</sup>. Given this complexity, it seems likely that one adipokine such as leptin is not the only piece to the CV events mediator puzzle in HD. A more detailed characterization of the role of adipokines in HD patients may leave us with the opportunity to tailor specific treatment strategies and to modify this risk factor accurately. Several factors could account for the relationship between ZAG concentration and an increased CV risk in HD patients with CKD; however, it must be acknowledged that our study, although hypothesis generating, does not directly address any of them.

First, given the role of ZAG in lipolytic pathways on WAT, we could suppose that its accumulation may reflect or mediate the PEW and malnutrition that characterize this disease state <sup>43</sup>. In this study, a negative correlation was found between ZAG concentration and plasma albumin ( $r=-0.17$ ,  $P<0.01$ ) or triglycerides ( $r=-0.15$   $p=0.04$ ). These correlations are weak (less than 0.20) and we were not able to observe a significant correlation between ZAG concentration and body fat mass as previously reported in HD patients <sup>25-27</sup>. The inverse relationship of ZAG gene expression and its protein level in WAT with BMI and body fat percentage were confirmed in human with normal renal function <sup>17,22,44</sup> or in cancer-

associated cachexia<sup>29,30</sup>. Several studies have reported the same correlation for ZAG circulating levels<sup>19,22,23</sup>; however, there are also some contradictory results that highlighted that ZAG released from abdominal WAT did not significantly contribute to the circulating levels<sup>17,24,29,30</sup>. In our previous work, we observed an increased ZAG protein content in WAT in rodent CKD models and non-dialysed-CKD patients<sup>6</sup>. In rodent CKD model, we observed a correlation between ZAG protein content in WAT and WAT weight but unfortunately serum ZAG concentration was not explored. In CKD patients, we observed an increased plasma ZAG concentration and ZAG expression in WAT but we lacked information about percentage of fat mass in these patients. In this cohort, we estimated the fat mass with BIS and anthropometric formula. Until now, the most appropriated method to estimate body composition and fat mass in HD patients is still under debate. BIS appears to be a robust tool for measuring and monitoring total-body fat in this population<sup>45-48</sup> even if there are some conflicting data<sup>49</sup>. The Durenberg formula provided similar estimates obtained by BIS (*data not shown*) in our cohort. Even Dual-energy X-ray absorptiometry (DEXA) analyses, often considered as a gold standard to measure fat mass, was not used in the present study, we can conclude with a good confidence that ZAG circulating levels is not correlated with WAT weight in HD patients. In good agreement, we failed to find any significant increase of plasma ZAG in HD patients with PEW in line with previous report in non-dialysed<sup>4</sup>, dialysed<sup>25</sup> or heart failure patients<sup>35</sup>. ZAG could, however, contribute to metabolic disturbances since some studies reported that ZAG serum levels negatively correlate with homeostasis model assessment of insulin resistance in patient with GFR > 50 ml/min/1.73m<sup>2</sup><sup>5</sup> or with adiposity estimated with sum of skinfolds in HD patients using<sup>28</sup>. Other studies reported that ZAG serum levels correlate with serum levels of metabolic parameters such as cholesterol, insulin resistance indices in healthy subjects<sup>24,50</sup>. The low proportion of patients with PEW criteria in our cohort (9.9%) may explain the absence of a significant difference of

serum ZAG between PEW and no PEW patients. All this data suggests that the lipolytic action of ZAG is mainly mediated by its local expression in adipose tissue (autocrine/paracrine action) although we cannot exclude a minor endocrine action and could explain the absence of correlation between ZAG serum and nutritional and metabolic parameters. Further analyses of adipose tissue expression of ZAG in HD will be needed to determine the role of ZAG in PEW. It should also be considered that ZAG can originate from other non-adipose tissue secreting sources which might also play a role. Furthermore, turnover rate of ZAG in plasma remains to date largely unknown. The impact of RRF seems negligible in our cohort but we previously observed that serum ZAG concentration increased during HD independently of hemoconcentration, implying that additional ZAG is secreted during HD without clear explanation<sup>6</sup>.

The receptors and ligands of ZAG still remains to be identified. Some biochemical evidences suggest that ZAG induce lipolysis through interaction with a  $\beta_3$ -adrenoreceptor and increased intracellular cAMP levels. The nature of ligand may be a fatty acid<sup>51,52</sup>. It is possible that the particular fatty acid bound to ZAG may determine its biological effect depending of nature of tissue. For example it may also be pertinent that eicosapentaenoic acid in conjunction with ZAG induces lipolysis in cultured adipocytes, whereas docosahexaenoic acid does not exhibit such activity<sup>53</sup>. It may therefore be possible to manipulate the biological effects of endogenous ZAG in clinical situations or local environment and this might explain pleiotropic effects of ZAG. The multiple sources and different regulatory mechanisms, that lead to plasma circulating ZAG protein would question the usefulness of circulating levels of this protein as an index of its lipolytic activity and further study will be needed to answer this question.

Second, some studies have suggested that ZAG could have direct effect on the CV system. A positive association was observed with ZAG levels and heart disease, preeclampsia and hypertension<sup>32,34,35</sup> even if it remain controversial<sup>28,33</sup>. ZAG was identified as a potential candidates involved in the pathogenesis of proliferative diabetic retinopathy<sup>54</sup> and as a biomarker for glomerular disease<sup>55</sup> who are recognized as CV risk factors. However, in our cohort, we did not find any relationship between ZAG and a common heart failure marker, NT pro-BNP. ZAG levels were not significantly different between patients with and without CV history (log-transformed ZAG: 1.51 µg/ml (min-max, 0.53-2.75) vs 1,33 (0,50-2.82); p= 0.15). However, NT pro-BNP is not validated as a biomarker to manage heart failure in HD and cardiac function was not estimated in the present study (using for instance echocardiography). ZAG concentrations were not different between diabetes state or etiology of renal disease. The reason for inconsistency between these studies might depend on the pathophysiological states and need further studies.

Third, ZAG has been detected in cancer. Therefore, the observed relationship between ZAG and mortality may reflect the presence of cancer not detected at the time of inclusion. Indeed, ZAG was described to be increased in colon cancer and permitted a more accurate diagnostic of cancer in combination with traditional markers, such as ACE or CA 19.9<sup>56</sup>. Similar results were found for prostate cancer: where serum ZAG concentration was higher in men with prostate cancer than men with nonmalignant prostate disease or healthy men<sup>57,58</sup>. In our cohort, we did not find any association between ZAG and cancer mortality. However, patients who developed cancer (n=20) during the follow-up period had significantly higher serum ZAG concentration ( $85.9 \pm 85.2$  µg/ml versus  $64.3 \pm 51.4$  µg/ml; P=0.03). Thus, we can suggest that ZAG could be a predictor of cancer in HD patients. But



also, the appearance of cancer and associated inflammatory state may contribute to CV complications<sup>59</sup>.

Fourth, ZAG accumulation could be associated with an inflammatory state. Chronic low-grade inflammation is common in HD patients and contributes to increased CV mortality. There are some experimental evidence suggesting that inflammatory cytokines decrease ZAG expression<sup>22,36</sup> but no always confirmed as in our previous study<sup>6</sup>. Therefore, we failed to observe any correlation between CRP and ZAG as previously reported<sup>5,25</sup>. However, we didn't measure other inflammatory biomarkers like TNF-alfa or IL-6 to confirm this result. Further study will be necessary to better understand to exact role of inflammation in the regulation of ZAG expression. Due to high degree of structural similarities to MHC-I, ZAG could have a plausible role in immunity is through its capacity for binding and presenting lipidic ligands to T cells<sup>7</sup>. Although the role of ZAG in immunoregulation cannot be denied, the immune system does not seem to be significantly altered in the absence of ZAG<sup>7,14</sup>. It has been shown that the RNase activity of ZAG has similarities to other RNases such as human pancreatic RNase<sup>60</sup>. The RNase activity may be important for the physiologic function of ZAG like immunoregulation, and antitumor activity.

The strengths of this prospective study include the large number of patients and the long-term follow-up. However, this study has several limits. Foremost, the patients studied in this cohort are from the same hemodialysis center from France, and the applicability of the study findings across nationalities remains unclear. Because all our patients were Caucasians our findings do not apply to black patients. The main limitation is represented by the potential misclassification of causes of death or CV events; however, this should not influence the relationship between ZAG and all-cause mortality.

In conclusion, the present study demonstrates for the first time the potential clinical utility of ZAG levels for risk-stratifying HD patients. Routine use of this biomarker does not seem feasible in relation to its costs. However, it could provide a more comprehensive assessment of the CV risk in clinical trials, and its molecular size (43 kDa) makes it a reasonable surrogate marker for elimination studies using medium cut-off and high cut-off membranes<sup>61</sup>.

Although increased serum ZAG remained associated with fatal events after adjustment for multiple confounders, this study leaves the question unanswered of whether increased serum ZAG is related causally to mortality in HD patients or simply indicates risk as an innocent bystander. Largest prospective and mechanistic studies are needed to address the question of functional involvement

## **Materials and Methods**

### **Ethic statement**

The study protocol was approved by the local ethics committees (DC-2009-1066) and conducted in accordance with its ethical standards and the principles of the second Declaration of Helsinki. All subjects involved in the research signed written informed consent forms prior to enrolment.

### **Study Design and Population**

Prevalent HD patients 18 years of age or older followed at a single HD center at AURAL in Lyon, France, were invited to participate in this observational, prospective, cohort study. All patients were recruited between March 1<sup>st</sup>, 2012 and December 31<sup>st</sup>, 2014. Inclusion criteria were regular 4-h HD sessions, three times a week, for at least 3 months. All patients received uniform HD treatments *via* high-flux membranes with reuse and standard water purification

and processing techniques. Exclusion criteria were: current hospitalization, pregnancy, active or invasive malignancy. Patients with local non-melanoma skin cancers, in situ cancer or cancer history (over 5 years old) were included. Demographic factors, relevant medical history, renal residual function (RRF) defined as zero-RRF in patients with 24 h-residual urine volume <100 ml, Charlson comorbidity index <sup>62</sup> and any concomitant medication, were ascertained at the time of the inclusion by review of medical records and patient interviews. For descriptive purposes, patients who reported current or past use of insulin and/or oral hypoglycemic drugs were considered to have diabetes. Previous CV diseases was defined as a history of any of the following events: myocardial infarction, stroke, heart failure, angina pectoris, or surgical procedures for angina or coronary/peripheral artery disease (including percutaneous transluminal angioplasty). Dialysis vintage was defined as the time period between the date of study entry and the date of HD initiation.

### **Laboratory measurements**

All laboratory data were measured at the baseline visit, in a fasting state, during weekday HD treatments and one year later. Serum samples were also collected at the time of the baseline and one year later and stored at -20°C for future use. Biochemical parameters measurements were made using standard methods in the routine clinical laboratory. Albumin was measured by immunonephelometry. Creatinine assay was performed by enzymatic method (Roche, Meylan, France), with calibrators assigned by an isotope-dilution mass spectrometry. Serum ZAG concentrations were determined with a commercially available ZAG Enzyme Immunoassay (RayBiotech Inc, Tebu-Bio, Le-Perray-en-Yvelines, France) according to the manufacturer's instructions. All immunoassays were from the same batch. The detection limit was 21 pg/ml and intra and inter-assay coefficient were 4.1% and 11.3%, respectively (in line with manufacturer's recommendations: intra and inter-assay

coefficients <10% and <15%, respectively). All measurements were performed at least in duplicate

### **Follow-up study**

During the study period, clinical events, including overall mortality and CV events, were recorded by considering all patients included at least 18 months before the end of the study date (July 1<sup>st</sup>, 2016). Each medical chart was reviewed and the cause of death was assigned by a physician on the basis of all the available clinical information.

The primary outcome of interest was all-cause mortality. Secondary analysis was performed for CV events. CV events were defined as CV death or any adverse event directly related to a CV system dysfunction (stroke, angina pectoris/myocardial infarction, congestive cardiac failure, new-onset arrhythmia or peripheral ischemia) or surgical procedures for angina or coronary/peripheral arterial disease. CV death was diagnosed when death was attributed to myocardial infarction, cardiogenic shock, peripheral ischemia (including mesenteric ischemia) or stroke. Death occurring outside the hospital for which no other cause was specified was regarded as sudden cardiac death and included in the definition of CV death.

### **Nutritional status**

The body mass index (BMI) was defined as the post-dialysis weight (in kg) divided by height-squared (in m<sup>2</sup>). Body fat mass was calculated according to Deurenberg et al <sup>63</sup> using the following formula:  $\text{Body fat} = (1.20 \times \text{BMI}) + (0.23 \times \text{Age}) - (10.8 \times \text{Gender})$  where male gender = 1, female gender = 0. CKD patients were considered having PEW if they presented at least 3 out of 4 of the following criteria, BMI < 23 kg/m<sup>2</sup>, Albumin < 38g/L, Pre-albumin < 300mg/L or normalized protein catabolic rate (nPCR) < 0.8 g/kg.24 h according to Fouque et al (2008) <sup>43</sup>.

In our cohort, body composition was assessed by whole-body bioimpedance spectroscopy (BIS; Fresenius Medical Care) at baseline in a sub-population of 71 patients. In order to control for potential variability and the effect of overhydration, all BIS analyses were performed before a mid-week dialysis session. The BIS monitor uses a whole spectrum of low and high frequencies from 5 to 1000 kHz.

### **Statistical analysis**

Data were analyzed using GraphPad Prism 6.0 (GraphPad softwares, La Jolla, USA) and Statview 5.0 (Abacus concept, Berkeley, USA) softwares. The data are expressed as mean  $\pm$  standard deviation (SD) or as median (range i.e. minimum-maximum) when variables were not normally distributed. Distributions were tested for normality using d'Agostino-Pearson test. ZAG was classified as non-normally distributed ( $P < 0.01$ ) and was log-transformed before analysis. Differences between groups were assessed by Kruskal & Wallis test followed, when appropriated, by Dunn tests. Simple comparisons were made using Mann & Whitney U test. A paired Wilcoxon test was used to compare ZAG at baseline and one year later. Chi-squared tests were used to compare categorical measures. Univariable analysis was performed using the Spearman rank correlation method. The diagnostic performance of ZAG compare to traditional risk factors for mortality was assessed by analysis of the receiver operating characteristic (ROC) curve. We compared areas under the ROC curves (AUC) using nonparametric test according to the approach proposed by DeLong et al<sup>64</sup>. HD patients were stratified into tertiles according to their concentration of log-transformed ZAG (lowest tertile [n=84]: median, 1.10  $\mu\text{g/ml}$  [min-max, 0.53–1.25  $\mu\text{g/ml}$ ]; middle tertile [n=84]: median, 1.42  $\mu\text{g/ml}$  [min-max, 1.25–1.68  $\mu\text{g/ml}$ ]; highest tertile [n=84]: median, 2.03  $\mu\text{g/ml}$  [min-max, 1.690–2.82  $\mu\text{g/ml}$ ]) for survival analysis.

We estimated the association between serum ZAG and all-cause mortality, CV events using Kaplan-Meier plots with the log rank test to compare differences between tertiles of serum ZAG.

Hazard ratios for death or CV events were generated using Cox proportional hazards modeling with ZAG as a continuous variable with three incremental levels of covariate adjustments:

- (1) Adjusted for demographic characteristics (Model 1): age, sex, and dialysis vintage
- (2) Adjusted for metabolic and nutritional parameters (Model 2): age, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, albumin, prealbumin, nPCR and BMI.
- (3) Adjusted for CV risk factors (Model 3): age, tobacco, diabetes, dyslipidemia, prior CV disease, diuretic medication (as a proxy of RRF).

For overall mortality and CV events, data were censored at renal transplantation, loss to follow-up, or the end of the study observation period. A  $P < 0.05$  was considered as statistically significant in all analysis.

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## **Author contributions**

AB performed the study, researched data, analyzed the results, and reviewed the manuscript. YD, MP, SG, JCS, ML, WA, SP, DF researched data and analyzed the results. CS and LK conceived the study, analyzed the results, and wrote the manuscript. LK is the guarantor of this work and, as such, take full responsibility for it.

## Disclosure:

The authors have no conflict of interest to disclose.

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## Figure legends

### **Figure 1. Kaplan–Meier estimates of cumulative survival of all patients according to plasma Zinc-Alpha2- Glycoprotein (ZAG) level at inclusion.**

Tertile cut-off (log-transformed ZAG,  $\mu\text{g/ml}$ ): T1,  $< 1.25$  ; T2,  $[1.25 - 1.68.]$ ; T3,  $> 1.69$ .

Number of events were 12, 13, and 24 for tertile 1 to 3, respectively. The number of patients indicated in the tables is the number of patients still followed at the indicated time.

### **Figure 2. Kaplan–Meier estimates of time to first cardiovascular event according to plasma Zinc-Alpha2- Glycoprotein (ZAG) level at inclusion.**

Tertile cut-off (log-transformed ZAG,  $\mu\text{g/ml}$ ): T1,  $< 1.25$  ; T2,  $[1.25 - 1.68.]$ ; T3,  $> 1.69$ .

Tertiles of serum ZAG. Number of events were 12, 20, and 30 for tertile 1 to 3, respectively.

The number of patients indicated in the tables is the number of patients at risk at the indicated time.

### **Supplementary Figure 1. Plasma ZAG concentration in hemodialysis patients at baseline and one year later.**

Data are expressed as median (interquartile rang). Note that no difference was found to be significant at the  $P < 0.05$  level. Abbreviations: ZAG, zinc alpha-2 glycoprotein, Ln-transformed: log-transformed.

### **Supplementary Figure 2. Plasma ZAG concentration in hemodialysis patients with a diagnostic of protein-energy wasting.**

Data are expressed as median (interquartile rang). Note that no difference was found to be significant at the  $P < 0.05$  level. **Abbreviation:** PEW, protein energy wasting, Ln-transformed: log-transformed, ZAG, zinc alpha-2 glycoprotein

### **Supplementary Figure 3: Kaplan–Meier estimates of cumulative survival of all patients according to plasma Zinc-Alpha2- Glycoprotein (ZAG) concentration measured after one year of inclusion.**

### **Supplementary Figure 4. Receiver operator characteristic (ROC)**

(A-C) ROC curve for all-cause mortality for ZAG, different traditional risk factors and the addition of ZAG with Charlson score. (D-E) ROC curves for cardiovascular events for ZAG, different traditional risk factors and the addition of ZAG with Charlson score

Abbreviations: ZAG, zinc alpha-2 glycoprotein

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## Tables

**Table 1. Baseline Characteristics According to ZAG Tertiles in Maintenance Hemodialysis Patients**

	Tertile 1	Tertile 2	Tertile 3	Overall population	P-value
No. of patients	84	84	84	252	
<b>Clinical Characteristics</b>					
Age (years)	59 (17-86)	65 (21-87)	68 (27-88)	64 (17-88)	<b>&lt;0.01</b>
Sex (% female)	38	45	29	37	0.13
BMI (kg/m <sup>2</sup> )	25.6 (17.9-40.7)	24.6(16.2-66.7)	25.7(16.4-49.3)	25.4 (16.2-66.7)	0.31
Body fat masse (%)	37.2 (17.7-61.9)	37.8 (19.5-86.6)	39.2 (20.4-74.4)	38.0 (17.6-86.6)	0.38
PEW (%)	9.5	8.3	11.9	9.9	0.73
Dialysis vintage (y)	2.3 (0.3-21.9)	3.4 (0.2-20.3)	1.6 (0.2-17.2)	2,2 (0.2-21.9)	<b>&lt;0.01</b>
nPCR (g/kg/day)	1.14 (0.44-2.37)	1.10 (0.58-1.82)	1.07 (0.53-2.30)	1.10 (0.44-2.37)	0.66
Dialysis dose (Kt/V)	1.6 (0.9-2.6)	1.7 (1.1-2.7)	1.6 (0.6-2.6)	1.7 (0.6-2.7)	0.17
Predialysis SBP (mmHg)	129 ± 21	131 ± 22	131 ± 22	130 ± 22	0.53
RRF (%)	45	61	74	60	0.06
Diabetes (%)	29	26	42	32	0.07
AVF/AVG (%)	7	7	10	8	0.80
Smoking (%)	39	33	43	38	0.47
CV history (%)	29	33	42	34	0.19
Hypertension (%)	85	89	95	89	0.07
Dyslipidemia (%)	46	56	69	57	<b>0.01</b>
Cancer history (%)	14	10	15	13	0.48
<b>Causes of nephropathy</b>					
Diabetes (n)	21	19	27	67	0.35
Vascular (n)	17	12	18	47	0.22
Glomerulopathy (n)	17	22	17	56	0.56
ADPKD (n)	6	7	8	21	0.86
Interstitial nephritis (n)	5	4	4	13	0.92
Urology (n)	9	12	3	24	0.06
Unknown (n)	9	8	7	24	0.87
<b>Laboratory Tests</b>					
ZAG (µg/ml)	12.6 (3.4-17.9)	26.4 (17.9-48.0)	106.2 (49.0-667.5)	26.4 (3.4-667.5)	
Ln-transformed ZAG levels	1.10 (0.53-1.25)	1.42 (1.25-1.68)	2.03 (1.69-2.82)	1.42 (0.53-2.82)	
levels levels					
Serum albumin (g/dL)	40.0 (22.7-45.5)	39.6 (25.5-45.6)	37.7 (27.5-45.5)	39.1 (22.7-45.6)	<b>0.01</b>
Serum prealbumin (mg/L)	360 (40-650)	330 (120-770)	310 (120-590)	330 (40.0-770)	0.18
Creatinine (µ mol/L)	830 (136-1526)	736 (136-1406)	687 (280-1872)	747 (136-1872)	0.07
Urea (mmol/L)	21.4 (6.1-39.0)	20.3 (5.2-32.8)	19.8 (7.5-46.0)	20.3 (5.2-46.0)	0.68
Bicarbonate (mmol/L)	20.9 ± 2.4	21.3 ± 2.9	21.5 ± 3.6	21.2 ± 3,0	0.43
Calcium (mmol/L)	2.2 (1.6-2.6)	2.3 (1.2-2.7)	2.3 (0.8-2.8)	2.2 (0.8-2.8)	0.27
Phosphorus (mmol/L)	1.4 (0.7-2.7)	1.4 (0.5-3.0)	1.5 (0.3-3.3)	1.4 (0.3-3.3)	0.85
PTH (pmol/L)	26,2 (0.2-149)	27.9 (0.6-207)	22.6 (1.3-144)	26.0 (0.2-207)	0.72
25-OH VitamineD3 (nmol/L)	81.9 (29.0-143)	78.6 (22.0-177)	80.9 (6.0-190)	80.3 (6.0-190)	0.78
Hemoglobin (g/dL)	11.3 ± 1.3	11.2 ± 1.0	11.5 ± 1.5	11.3 ± 1.3	0.36
Platelet count (×10 <sup>9</sup> /L)	211 (84-463)	220 (103-404)	219 (95-475)	218 (84-475)	0.45
WBC count (×10 <sup>9</sup> /L)	6.1 (2.0-12.2)	5.9 (3.6-11.7)	6.3 (1.5-14.4)	6.1 (1.5-14.4)	0.64
Ferritin (µ g/L)	343 (0.2-1686)	501 (12.8-3075)	396 (20.0-2867)	387 (0.2-3075)	0.06
Glucose (mmol/L)	6,0 (3.3-33.0)	1.0 (3.3-33.0)	6.0 (3.3-38.5)	6.0 (3.3-38.5)	0.80
Hemoglobin A1c (%)	5.6 (3.9-11.0)	5.6 (3.4-9.3)	5.7 (4.2-9.4)	5.6 (3.4-11.0)	0.18
Total cholesterol (mmol/L)	4.4 (1.5-7.5)	4.1 (2.3-6.7)	4.1 (1.8-6.5)	4.1 (1.5-7.5)	0.46
HDL cholesterol (mmol/L)	1.0 (0.5-1.8)	1.0 (0.5-2.6)	1.0 (0.5-2.1)	1.0 (0.5-2.6)	0.84
LDL cholesterol (mmol/l/l)	2.3 (0.8-9.6)	2.3 (1.0-9.8)	2.3 (0.8-4.6)	2.3 (0.8-9.8)	0.93
Triglycerides (mmol/L)	2.3 (0.7-6.9)	1.5 (0.6-6.8)	1.6 (0.4-4.5)	1.7 (0.4-6.9)	<b>&lt;0.01</b>
CRP (mg/L)	5.4 (0.6-72.0)	4.5 (0.2-61.0)	5.2 (0.4-104.0)	5.0 (0.2-104)	0.43
NT-ProBNP (ng/L)	1883 (18.4-70000)	1506 (29.2-47369)	2600 (74.0-54028)	1883 (18.4-70000)	0.26
<b>Treatment</b>					
Anti-diabetic therapy (%)	24	23	32	26	0.31
Lipid-lowering therapy (%)	46	54	57	52	0.36
RAA % (%)	36	46	52	45	0.09
Beta blocking agents (%)	45	37	57	46	<b>0.03</b>
AAP (%)	50	55	56	53	0.71
Diuretic (%)	42	46	54	47	0.29

**Abbreviations:** AVF, arteriovenous fistula; AVG, arteriovenous graft; BMI, body mass index; SBP, systolic blood pressure; PTH, parathyroid hormone; CRP, C-reactive protein; BMI, body mass index; PEW, protein-energy wasting; ADPKD, Autosomal dominant polycystic kidney disease; CV, cardiovascular; RAA, Renin-angiotensin-aldosterone system antagonists; WBC, white blood cell; nPCR, normalized protein catabolic rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AAP, Antiplatelet agents; NT-proBNP, N-terminal pro-brain natriuretic peptide, ZAG: Zinc-Alpha2-Glycoprotein, Ln-transformed: log-transformed, RRF: Residual renal function.

Data are reported as mean  $\pm$  SD if the variable distribution is Gaussian, or as median (minimum-maximum) otherwise. Differences between tertiles were tested using Kruskal-Wallis or chi-squared test as appropriate.



**Table 2. Unadjusted Spearman Correlation Coefficients (r) of ZAG and Other Relevant Covariates in Hemodialysis Patients.**

Variable	r	95% CI
Dialysis vintage (y)	-0.08	-0,21 to 0,046
Age (y)	0.19	<b>0,067 to 0,31</b>
Body fat mass (%)	0.08	-0,053 to 0,21
Predialysis systolic BP	-0.01	-0,15 to 0,13
Predialysis diastolic BP	-0.11	-0,24 to 0,030
Hemoglobin (g/dL)	0.08	-0,052 to 0,20
WBC count ( $\times 10^9/L$ )	0.05	-0,082 to 0,17
Platelets ( $\times 10^9/L$ )	-0.02	-0,14 to 0,11
Ferritin ( $\mu g/L$ )	0.08	-0,056 to 0,21
Urea (mmol/L)	-0.03	-0,16 to 0,095
Creatinine ( $\mu mol/L$ )	-0.16	<b>-0,28 to -0,033</b>
Bicarbonate (mmol/L)	0.08	-0,052 to 0,20
Dialysis dose (Kt/V)	-0.02	-0,15 to 0,11
Calcium (mmol/L)	0.09	-0,037 to 0,22
Phosphorus (mmol/L)	-0.01	-0,13 to 0,12
PTH (pmol/L)	-0.07	-0,20 to 0,062
25-OH Vitamin D3 (nmol/L)	0.02	-0,12 to 0,15
CRP (mg/L)	-0.01	-0,14 to 0,12
Glucose (mmol/L)	-0.03	-0,16 to 0,095
Hemoglobin A1c (%)	0.10	-0,055 to 0,25
Total cholesterol (mmol/L)	-0.03	-0,17 to 0,12
LDL cholesterol (mmol/L)	0.02	-0,14 to 0,18
HDL cholesterol (mmol/L)	-0.01	-0,14 to 0,16
Triglycerides (mmol/L)	-0.15	<b>-0,30 to -0,0024</b>
Serum albumin (g/dL)	-0.17	<b>-0,29 to -0,040</b>
Serum prealbumin (mg/L)	-0.08	-0,21 to 0,049
nPCR (g/kg/day)	-0.04	-0,17 to 0,096
BMI (kg/m <sup>2</sup> )	-0.01	-0,14 to 0,12
NT-ProBNP (ng/L)	0.06	-0,069 to 0,20

**Abbreviations:** BMI, body mass index; BP, blood pressure; PTH, parathyroid hormone; CRP, C-reactive protein, BMI, body mass index, WBC, white blood cell; nPCR, normalized protein catabolic rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NT-proBNP: N-terminal pro-brain natriuretic peptide; CI : Confidence Interval.

**Table 3. Multivariable Cox Regression Analysis of Risk Factors at Baseline for All-Cause Mortality and cardiovascular Events**

Event	HR (95%CI)	P value
<b>Overall mortality</b>		
Unadjusted	1.222 (1.110-1.345)	<0.01
Model 1 <sup>a</sup>	1.194 (1.081-1.320)	<0.01
Model 2 <sup>b</sup>	1.173 (1.012-1.359)	0.03
Model 3 <sup>c</sup>	1.277 (1.103-1.407)	<0.01
<b>Cardiovascular events</b>		
Unadjusted	1.190 (1.091-1.298)	<0.01
Model 1 <sup>a</sup>	1.162 (1.061-1.272)	<0.01
Model 2 <sup>b</sup>	1.144 (1.002-1.281)	0.02
Model 3 <sup>c</sup>	1.158 (1.050-1.277)	<0.01

**Abbreviations:** HR, hazard ratio; 95% CI, 95% confidence interval.

<sup>a</sup>Model 1 was adjusted for demographic characteristics (age, sex, and dialysis vintage).

<sup>b</sup>Model 2 was adjusted for metabolic and nutritional parameters (age, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, albumin, prealbumin, nPCR and BMI).

<sup>c</sup>Model 3 was adjusted for traditional cardiovascular risk factors (age, tobacco, presence of diabetes, dyslipidemia, history of cardiovascular disease and diuretic medication).

## Supplemental Tables

**Supplemental Table 1. Characteristics of HD patients with cardiovascular events (CV events), n = 62 versus patients without CV events (No CV events ), n = 190.**

Clinical Characteristics	CV events, n=62	No CV events, n=190	P-value
Age (years)	73 (42-88)	60 (17-88)	<b>&lt;0.01</b>
Sex (% female)	19	43	<b>&lt;0.01</b>
BMI (kg/m <sup>2</sup> )	25.8 (16.4-44.2)	25.1 (16.2-66.7)	0.23
Body fat masse (%)	37.8 (23.1-70.0)	38.2 (17.7-86.6)	0.31
PEW (%)	13	9	0.37
Dialysis vintage (y)	2.9 (0.2-20.3)	2.0 (0.2-21.9)	0.25
nPCR (g/kg/day)	1.10 (0.64-1.74)	1.09 (0.44-2.37)	0.44
Dialysis dose (Kt/V)	1.7 (0.9-2.4)	1.7 (0.6-2.7)	0.40
Predialysis systolic BP (mmHg)	132 ± 22	130 ± 21	0.44
CV history (%)	73	22	<b>&lt;0.01</b>
Diabetes (%)	42	29	0.06
AVF/AVG (%)	10	7	0.56
Smoking (%)	55	33	<b>&lt;0.01</b>
Hypertension (%)	100	86	<b>&lt;0.01</b>
Dyslipidemia (%)	79	50	<b>&lt;0.01</b>
Cancer history (%)	15	13	0.70
<b>Causes of nephropathy</b>			
Diabetes (n)	19	48	0.40
Vascular (n)	17	30	<b>0.04</b>
Glomerulopathy (n)	13	43	0.78
ADPKD (n)	2	19	0.09
Interstitial nephritis (n)	2	11	0.43
Urology (n)	3	21	0.15
Unknown (n)	6	18	0.96
<b>Laboratory Tests</b>			
ZAG (µg/ml)	47.0 (3.4-559.0)	23.3 (3.6-667.5)	<b>&lt;0.01</b>
Ln-transformed ZAG levels	1.67 (0.53-2.75)	1.37 (0.55-2.82)	<b>&lt;0.01</b>
Serum albumin (g/dL)	38.2 (29.1-45.6)	39.5 (22.7-45.5)	0.09
Serum prealbumin (mg/L)	305 (120-650)	330 (40-770)	<b>0.03</b>
Creatinine (µmol/L)	715 (360-1149)	768 (136-1872)	0.15
Urea (mmol/L)	20.1 (10.5-33.0)	20.4 (5.2-46.0)	0.42
Bicarbonate (mmol/L)	21.6 ± 2.8	21.1 ± 3.1	0.28
Calcium (mmol/L)	2.3 (1.8-2.6)	2.2 (0.8-2.8)	0.07
Phosphorus (mmol/L)	1.5 (0.5-3.0)	1.4 (0.3-3.3)	0.47
PTH (pmol/L)	23.4 (0.6-144)	27.1 (0.2-207)	0.33
25-OH Vitamine D3 (nmol/L)	80.8 (6.0-155)	79.9 (10.5-190)	0.45
Hemoglobin (g/dL)	11.5 ± 1.4	11.3 ± 1.3	0.20
Platelet count (×10 <sup>9</sup> /L)	216 (105-475)	221 (84-462)	0.30
WBC count (×10 <sup>9</sup> /L)	6.4 (3.8-14.4)	5.9 (1.5-12.2)	<b>0.02</b>
Ferritin (µg/L)	424 (20-2867)	378 (0.2-3075)	0.36
Glucose (mmol/L)	6.0 (3.3-38.5)	1.0 (3.3-33)	<b>0.01</b>
Hemoglobin A1c (%)	5.8 (4.8-9.3)	5.6 (3.4-11.0)	<b>0.05</b>
Total cholesterol (mmol/L)	4.1 (2.3-6.7)	4.1 (1.5-7.5)	0.24
HDL cholesterol (mmol/L)	0.8 (0.5-2.0)	1.0 (0.5-2.6)	0.09
LDL cholesterol (mmol/L)	2.3 (0.8-3.9)	2.3 (0.8-9.8)	0.32
Triglycerides (mmol/L)	1.8 (0.6-6.8)	1.7 (0.4-6.9)	0.43
CRP (mg/L)	7.7 (0.4-104)	4.1 (0.2-72.0)	<b>&lt;0.01</b>
NT-ProBNP (ng/L)	3932 (498-54028)	1666 (18-70000)	<b>&lt;0.01</b>
<b>Treatment</b>			
Anti-diabetic therapy (%)	34	24	0.11
Lipid-lowering therapy (%)	73	46	<b>&lt;0.01</b>
RAA %, (%)	52	43	0.21
Beta blocking agents, (%)	60	42	<b>0.02</b>
AAP (%)	81	45	<b>&lt;0.01</b>

**Abbreviations:** AVF, arteriovenous fistula; AVG, arteriovenous graft; BMI, body mass index; SBP, systolic blood pressure; PTH, parathyroid hormone; CRP, C-reactive protein; BMI, body mass index; PEW, protein-energy wasting; ADPKD, Autosomal dominant polycystic kidney disease; CV, cardiovascular; RAA, Renin-angiotensin-aldosterone system antagonists; WBC, white blood cell; nPCR, normalized protein catabolic rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AAP, Antiplatelet agents; NT-proBNP, N-terminal pro-brain natriuretic peptide, ZAG: Zinc-Alpha2-Glycoprotein, Ln-transformed: log-transformed.

Data are reported as mean  $\pm$  SD if the variable distribution is Gaussian or as median (minimum, maximum) otherwise. Differences between tertiles were tested using Kruskal–Wallis or chi-squared test as appropriate.

**Supplemental Table 2. Characteristics of HD patients with PEW (PEW), n= 25 versus patient without PEW (No PEW), n = 227**

<b>Clinical Characteristics</b>	<b>PEW, n=25</b>	<b>No PEW, n=227</b>	<b>P-value</b>
Age (years)	71 (29-88)	64 (17-87)	0.35
Sex (% female)	28	38	0.31
BMI (kg/m <sup>2</sup> )	22.5 (16.7-33.6)	25.7 (16.2-66.7)	<b>&lt;0.01</b>
Body fat masse (%)	35.4 (19.3-56.3)	38.2 (17.7-86.6)	0.06
Dialysis vintage (y)	2.4 (0.2-20.3)	2.2 (0.2-21.9)	0.31
nPCR (g/kg/day)	0.78 (0.46-1.59)	1.10 (0.44-2.37)	<b>&lt;0.01</b>
Dialysis dose (Kt/V)	1.7 (0.9-2.2)	1.7 (0.6-2.7)	0.98
Predialysis systolic BP (mmHg)	126 ± 16	131 ± 22	0.36
CV history (%)	36	34	0.87
Diabetes (%)	24	33	0.43
AVF/AVG(%)	16	7	0.12
Smoking (%)	52	37	0.07
HTA (%)	92	89	0.69
Dyslipidemia (%)	56	57	0.90
Cancer history (%)	20	12	0.28
<b>Causes of nephropathy</b>			
Diabetes (n)	5	62	0.19
Vascular (n)	6	41	0.80
Glomerulopathy (n)	6	50	0.78
ADPKD (n)	1	20	0.30
Interstitial nephritis (n)	2	11	0.67
Urology (n)	2	22	0.58
Unknown (n)	3	21	0.90
<b>Laboratory Tests</b>			
ZAG( μ g/ml)	30.4 (3.4-331.0)	26.2 (3.6-667.5)	0.32
Ln-transformed ZAG levels	1.48 (0.53-2.52)	1.42 (0.55-2.82)	<b>0.32</b>
Serum albumin (g/dL)	35.4 (28.5-37.9)	39.7 (22.7-45.6)	<b>&lt;0.01</b>
Serum prealbumin (mg/L)	260 (120-290)	340 (40-770)	<b>&lt;0.01</b>
Creatinine ( μ mol/L)	561 (360-1125)	762 (136-1872)	<b>0.01</b>
Urea (mmol/L)	13.8 (7.5-25.0)	20.5 (5.2-46.0)	<b>&lt;0.01</b>
Bicarbonate (mmol/L)	22.9 ± 3.1	21.0 ± 2.9	<b>&lt;0.01</b>
Calcium (mmol/L)	2.2 (0.8-2.5)	2.2 (1.2-2.8)	0.03
Phosphorus (mmol/L)	1.2 (0.5-2.7)	1.5 (0.3-3.3)	0.09
PTH (pmol/L)	11.2 (0.2-96.4)	27.6 (0.7-207)	0.15
25-OH Vitamine D3 (nmol/L)	80.9 (43.9-177)	79.9 (6.0-190)	0.25
Hemoglobin (g/dL)	11.3 ± 1.6	11.3 ± 1.3	0.96
Platelet count (×10 <sup>9</sup> /L)	235 (99-404)	218 (84-475)	0.41
WBC count (×10 <sup>9</sup> /L)	6.2 (2.3-14.4)	6.1 (1.5-12.2)	0.16
Ferritin (μg/L)	363 (15-991)	387 (0.2-3075)	0.21
Glucose (mmol/L)	5.5 (3.3-11)	6.0 (3.3-38.5)	0.15
Hemoglobin A1c (%)	5.5 (3.4-7.7)	5.6 (3.9-11.0)	0.24
Total cholesterol (mmol/L )	4.6 (2.8-5.9)	4.1 (1.5-7.5)	0.31
HDL cholesterol (mmol/L)	1.0 (0.5-1.5)	1.0 (0.5-2,6)	0.85
LDL cholesterol (mmol/L )	2.8 (1.8-3.9)	2,3 (0.8-9.8)	0.42
Triglycerides (mmol/L )	1.5 (0.6-3.6)	1.8 (0.4-6.9)	0.44
CRP (mg/L)	14.3 (0.9-63.0)	4.4 (0.2-104.0)	<b>0.01</b>
NT-ProBNP (ng/L)	3442 (185-70000)	1811 (18-54028)	0.14
<b>Treatment</b>			
Anti-diabetic therapy (%)	16	27	0.22
Lipid-lowering therapy (%)	44	53	0.38
RAA %, (%)	32	46	0.18
Beta blocking agents, (%)	48	46	0.87
AAP (%)	64	52	0.27

**Abbreviations:** AVF, arteriovenous fistula; AVG, arteriovenous graft; BMI, body mass index; SBP, systolic blood pressure; PTH, parathyroid hormone; CRP, C-reactive protein; BMI, body mass index; PEW, protein-energy wasting; ADPKD, Autosomal dominant polycystic kidney disease; CV, cardiovascular; RAA, Renin-angiotensin-aldosterone system antagonists; WBC, white blood cell; nPCR, normalized protein catabolic rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AAP, Antiplatelet agents; NT-proBNP, N-terminal pro-brain natriuretic peptide, ZAG: Zinc-Alpha2-Glycoprotein, Ln-transformed: log-transformed.

Data are reported as mean  $\pm$  SD if the variable distribution is Gaussian or as median (minimum, maximum) otherwise. Differences between tertiles were tested using Kruskal–Wallis or chi-squared test as appropriate.

### Supplemental Table 3. Causes of deaths

Cause (n=49)	n (%)
Cardiovascular	17 (35)
Malignancy	6 (12)
Infectious	16 (33)
Other	10 (20)

### Supplemental Table 4. Cardiovascular events

Events (n= 62)	n (%)
<i>Non-fatal</i>	52 (84)
Cardiac	
Congestive cardiac failure	7 (13)
Angina pectoris/myocardial infarction	22 (42)
New-onset arrhythmia	2 (4)
Ischemic cerebrovascular accident	
Peripheral ischemia	16 (31)
<i>Fatal</i>	10 (16)

### Supplemental Table 5. Cause of death according to ZAG tertiles

Cause of death (n=49)	Tertile 1	Tertile 2	Tertile 3	P value
Cardiovascular	2	3	12	0.003
Malignancy	0	3	3	0.219
Infectious	7	4	5	0.617
Other	3	3	4	0.897

Differences between tertiles were tested using chi-squared test.

**Supplemental Table 6. AUC using ROC curve analysis to predict all-cause mortality and cardiovascular events by various parameters.**

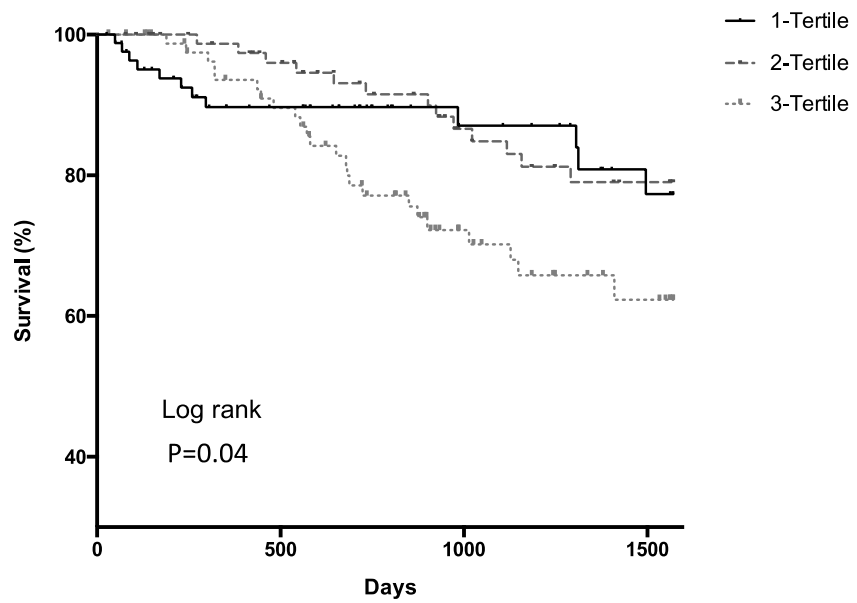
Variable	All cause of mortality				CV events					
	AUC	SE	95 %CI	p-value *	p-value \$	AUC	SE	95 %CI	p-value *	p-value \$
Ln-transformed ZAG	0.615	0.048	0.52 to 0.71	0.012		0.635	0.043	0.55 to 0.72	< 0.001	
Charlson co-morbidity	<b>0.789</b>	<b>0.030</b>	<b>0.73 to 0.85</b>	<b>&lt; 0.001</b>	<b>0.002</b>	<b>0.755</b>	<b>0.031</b>	<b>0.69 to 0.82</b>	<b>&lt; 0.001</b>	<b>0.024</b>
Charlson co-morbidity + ZAG	<b>0.798</b>	<b>0.029</b>	<b>0.74 to 0.85</b>	<b>&lt; 0.001</b>	<b>0.001</b>	<b>0.761</b>	<b>0.031</b>	<b>0.70 to 0.82</b>	<b>&lt; 0.001</b>	<b>0.017</b>
NT-proBNP	<b>0.693</b>	<b>0.044</b>	<b>0.61 to 0.78</b>	<b>&lt; 0.001</b>	<b>0.237</b>	<b>0.690</b>	<b>0.041</b>	<b>0.61 to 0.77</b>	<b>&lt; 0.001</b>	<b>0.349</b>
Serum albumin	0.583	0.046	0.50 to 0.67	0.074	0.630	0.557	0.042	0.47 to 0.64	0.182	0.199
CRP	<b>0.709</b>	<b>0.038</b>	<b>0.63 to 0.78</b>	<b>&lt; 0.001</b>	<b>0.132</b>	<b>0.635</b>	<b>0.040</b>	<b>0.56 to 0.71</b>	<b>0.002</b>	<b>0.997</b>
Vascular access	0.552	0.048	0.46 to 0.65	0.258	0.353	0.512	0.043	0.43 to 0.593	0.785	0.042
PEW	0.605	0.046	0.51 to 0.69	0.023	0.873	0.573	0.042	0.49 to 0.65	0.086	0.300
Hemoglobin	0.581	0.049	0.48 to 0.68	0.078	0.621	0.536	0.043	0.45 to 0.62	0.397	0.103
PTH	0.588	0.049	0.49 to 0.68	0.067	0.691	0.520	0.044	0.43 to 0.61	0.652	0.063
25-OH Vitamin D3	0.608	0.047	0.52 to 0.70	0.025	0.980	0.505	0.047	0.41 to 0.60	0.907	0.054
Age	<b>0.790</b>	<b>0.032</b>	<b>0.72 to 0.85</b>	<b>&lt; 0.001</b>	<b>0.003</b>	<b>0.710</b>	<b>0.035</b>	<b>0.64 to 0.78</b>	<b>&lt; 0.001</b>	<b>0.173</b>
Ferritin	0.558	0.049	0.46 to 0.65	0.228	0.402	0.517	0.044	0.43 to 0.60	0.712	0.054
nPCR	0.540	0.046	0.45 to 0.63	0.399	0.257	0.506	0.042	0.42 to 0.59	0.885	0.032
Dialysis doses (KTV)	0.531	0.047	0.44 to 0.62	0.505	0.212	0.511	0.040	0.43 to 0.59	0.799	0.035
Phosphorus	0.539	0.045	0.45 to 0.63	0.405	0.248	0.503	0.044	0.42 to 0.59	0.938	0.033
Dialyse vintage	0.575	0.044	0.49 to 0.66	0.102	0.541	0.528	0.044	0.44 to 0.61	0.509	0.083

**Abbreviations:** ROC: receiver operating characteristic curve; AUC: areas under the ROC curves; PTH, parathyroid hormone; CRP, C-reactive protein; PEW, protein-energy wasting; CV, cardiovascular; nPCR, normalized protein catabolic rate; NT-proBNP, N-terminal pro-brain natriuretic peptide, ZAG: Zinc-Alpha2-Glycoprotein, Ln-transformed: log-transformed

\* vs. line of equality (AUC = 0.5) and \$ vs. ZAG AUC

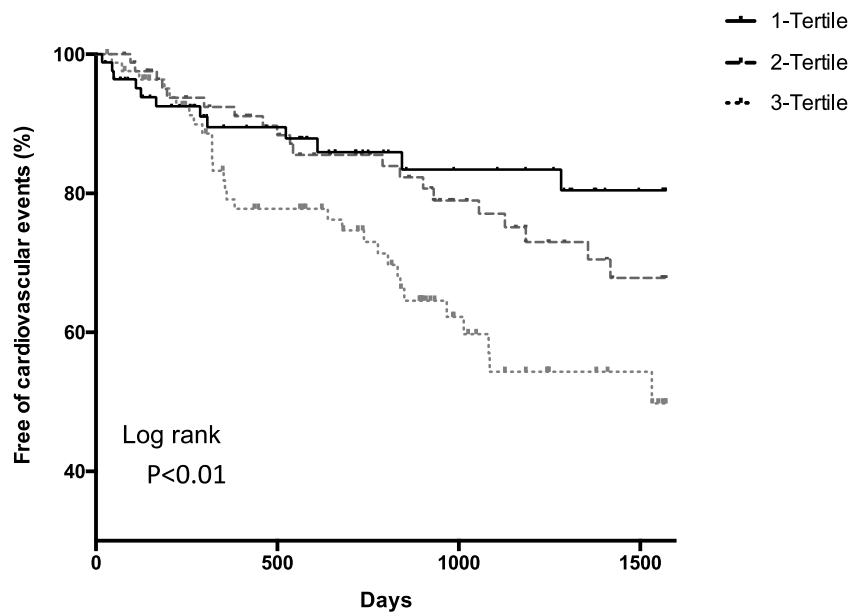


Figure 1



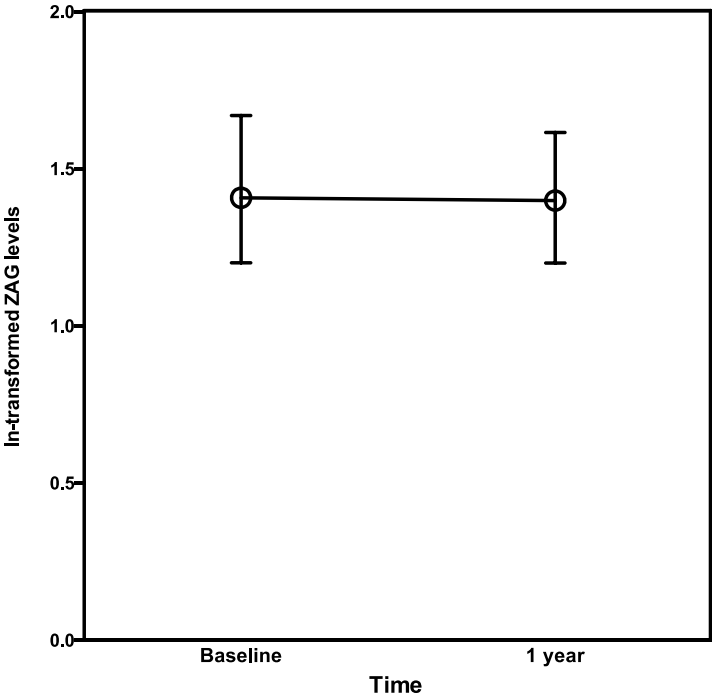
	Number of patients at risk			
	0 day	500 days	1000 days	1500 days
Tertile 1	84	60	33	23
Tertile 2	84	70	50	35
Tertile 3	84	68	36	18

Figure 2

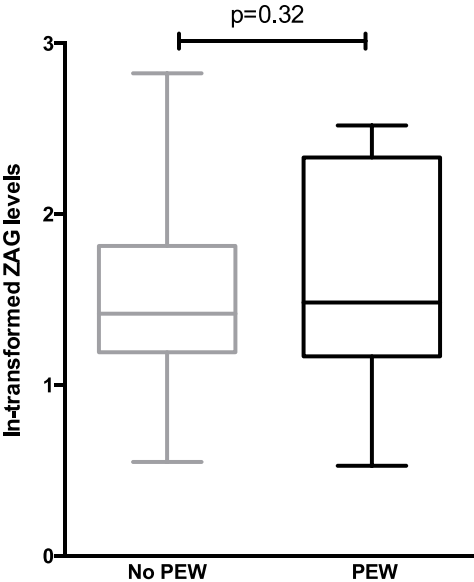


	Number of patients at risk			
	0 day	500 days	1000 days	1500 days
Tertile 1	84	56	32	21
Tertile 2	84	66	43	26
Tertile 3	84	56	26	13

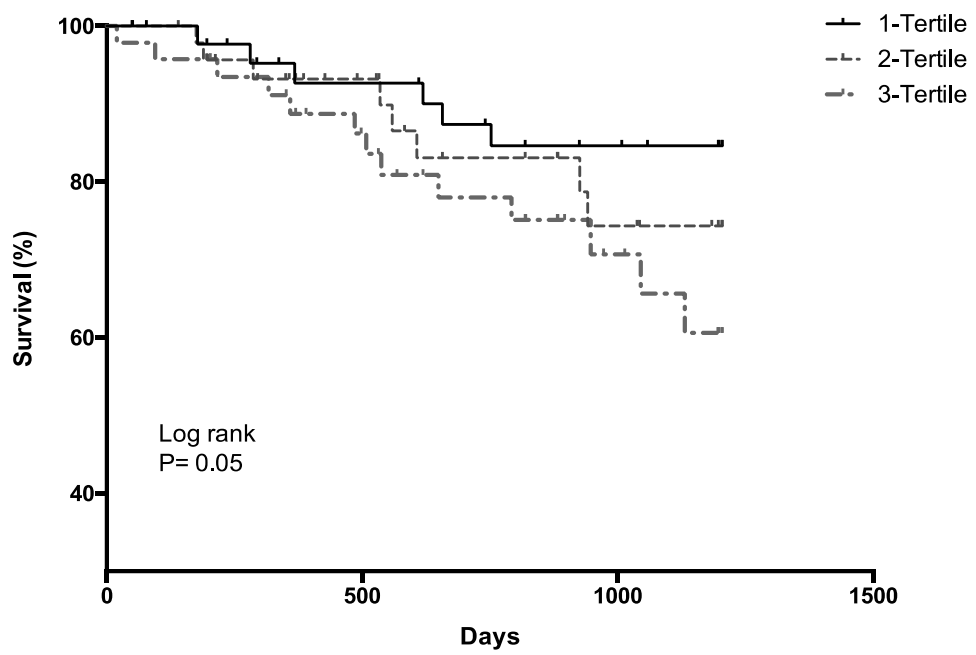
Supplementary Figure 1

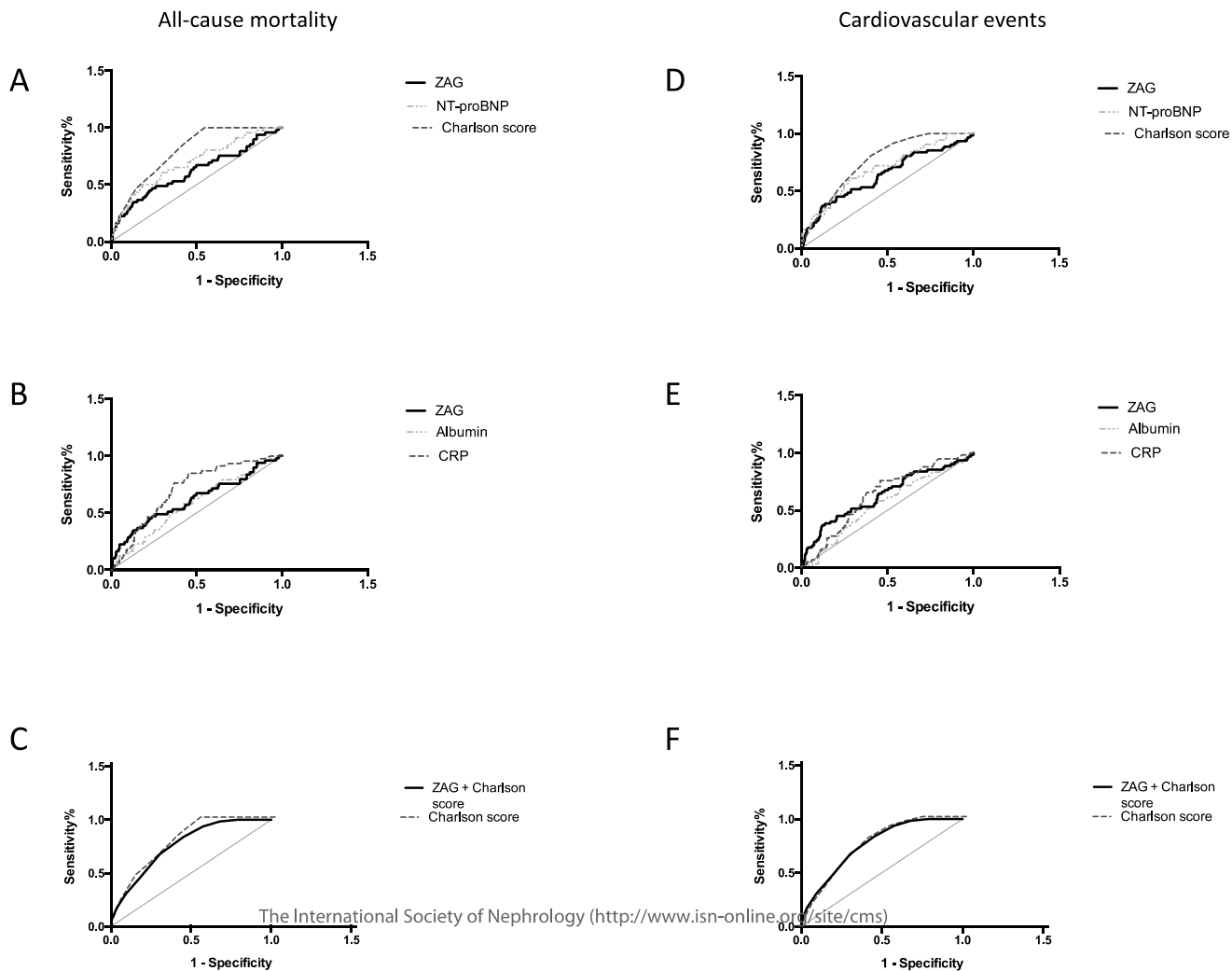


Supplementary Figure 2



Supplementary Figure 3





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## 5 Insulin resistance

*Moderate uraemia does not always result in insulin resistance in mice*

*Line S. Bisgaard, **Dan Yi** et al, submitted to *Clinical and Experimental Nephrology* in April 2018.*

Chronic kidney disease (CKD) caused accumulation of waste products in plasma, which leads to uremia. CKD markedly increases the risk of death. 5-year survival rate in patients with dialysis approaches to half at most. The mortality of these patients is primarily caused by cardiovascular disease (CVD). CKD, especially end stage renal disease (ESRD) is also a risk factor for cardiovascular (CV) diseases. Insulin resistance (IR) is known to be an independent risk factor for cardiovascular morbidity and mortality in CKD patients, and the rate of death among hemodialysis patients increases with increased IR. In return, CKD is also associated with development of insulin resistance.

Animal model of CKD is an effective and mainstay way to simulate CKD in human beings. The most frequently used animal models to study CKD are surgically nephrectomized rodents and chemically adenine diet models. Some animal studies have showed that the induction of moderate uraemia could lead to IR in normocholesterolemic rodents. In our research on CKD, we also performed many CKD animal models. However, we found that uremia is not always associated with the development of IR in some animal models that we have used during the study of CKD.

This article summarized the CKD models we used with glucose and insulin tolerance tests and discussed all the possible reasons that IR was not observed in any of the different uremic models investigated, including the underestimated factors in the process when we performed these CKD animal models. This study was substantiated to include investigation of IR in normocholesterolemic surgically as well as chemically induced mouse models of CKD. Our data clearly showed that insulin resistance does not always accompany moderate uraemia in mice. This is meaningful and helpful for the future researchers who will be focus on IR with CKD to choose a better way of CKD animal models and promote their in vivo study. This article is valuable for them to refer to.

## **Moderate uremia does not always result in insulin resistance in mice**

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**Short title:** Insulin resistance is not induced in uremic mice.

**Word count:** 2891. This paper contains 2 Figures and 2 Tables.

**Number of references:** 18

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## **Abstract**

**Background:** Chronic kidney disease leads to accumulation of waste products in plasma, i.e. uremia. In human patients, uremia is associated with increased risk of early cardiovascular death, increased atherosclerosis, and development of insulin resistance. Good reproducible animal models encompassing all of these traits are required to enable analyses of drug efficacy as well as disease mechanisms accelerating insulin resistance and atherosclerosis in CKD. It has previously been reported that induction of moderate uremia leads to development of insulin resistance in wildtype mice, which do not develop atherosclerosis. The aim of the present study was to develop a mouse model to analyse effects of uremia on both insulin resistance and atherosclerosis in one setting. **Methods:** Moderate uremia was induced surgically (by 5/6 nephrectomy) in hyperlipidemic atherosclerosis-prone mice and surgically or chemically (using adenine-enriched diet) in normolipidemic wildtype mice. Insulin resistance was determined by glucose and/or insulin tolerance tests in uremic mice and corresponding controls. **Results:** Much to our surprise, uremic mice did not develop insulin resistance in neither hyper- nor normolipidemic settings, and irrespective of whether uremia was induced surgically or chemically. **Conclusion:** Moderate uremia does not always result in insulin resistance in mice.

**Keywords:** CKD, uremia, insulin resistance.

## **Introduction**

Chronic kidney disease (CKD) leads to accumulation of waste products in



plasma (uremia). CKD markedly increases the risk of death; the 5-year survival rate in patients on dialysis is only ~50 %, primarily due to cardiovascular disease (CVD) [1]. An important contributor to this increased risk is accelerated atherosclerosis. To enable sufficient treatment of CKD patients, it is essential to understand why/how CKD accelerates atherogenesis. Already at early, subclinical, stages of human disease progression, CKD is associated with development of insulin resistance (IR). Also, in normocholesterolemic rodents, induction of moderate uremia has been shown to result in IR [2-5]. IR is known to be an independent risk factor for cardiovascular morbidity and mortality in CKD patients and the rate of death among haemodialysis patients increases with increased IR [6]. We hypothesised that insulin resistance might contribute to accelerated atherosclerosis in CKD. Thus, we initially aimed at establishing a mouse model combining moderate uremia (5/6-nephrectomy) and atherosclerosis (in LDLr<sup>-/-</sup> mice) to investigate whether uremia increased IR in settings of hypercholesterolemia. Since uremia did not lead to insulin in LDLr<sup>-/-</sup> mice, the study was substantiated to include investigation of IR in normocholesterolemic surgically as well as chemically-induced mouse models of CKD.

## **Methods**

### *Ethical statement*

5/6 nephrectomy experiments were performed in Denmark (Univ. of Copenhagen) according to the principles stated in the Danish law on animal experiments and were approved by the Animal Experiment's Inspectorate, Ministry of

Justice, Denmark (authorization n°2013-15-2934-00843). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the European Parliament (EU directive 2010/63/EU).

Chemical nephrectomy experiments were performed in France (Univ. of Lyon) under the authorization n°69-266-0501 and were in agreement with the guidelines laid down by the French Ministry of Agriculture (n° 2013-118) and the European Union Council Directive for the protection of animals used for scientific purposes of September 22nd, 2010 (EU directive 2010/63/EU).

#### *5/6 subtotal nephrectomy*

Female LDLr<sup>-/-</sup> mice (B6.129S7-Ldlrtm1Her/J, The Jackson Laboratory, Bar Harbor, USA) or C57Bl6/J mice (C57BL/6Ntac, Taconic, Ejby, Denmark) were housed under a 12 hour light/dark cycle with water and food ad libitum. Moderate uremia was induced by 5/6 nephrectomy in LDLr<sup>-/-</sup> mice in a two-step operation as previously described [7]. Mice were anesthetized with Zoletil (Tiletamin 1,63 mg/mL, Zolazepam 1,63 mg/mL, Xylazin, 2,61 mg/mL, Butorphanoltartrat 0,065 mg/mL) at a dose of 0.01 mL/g mouse, and analgesia was administered by subcutaneous injection of buprenorphine (0.05 mg/kg mouse). In wt C57BL/6 mice, 5/6 nephrectomy was performed in one step. For both LDLr<sup>-/-</sup> and wt studies, control mice were subjected to sham-operations. Atherosclerosis and kidney inflammation in the LDLr<sup>-/-</sup> mice have been accessed and published elsewhere [8].

In LDLr<sup>-/-</sup> mice, fasting plasma was collected two weeks after the 2<sup>nd</sup> operation,

while intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT) were performed three and four weeks, respectively, after the 2<sup>nd</sup> operation. Seven weeks after the 2<sup>nd</sup> operation, LDLr<sup>-/-</sup> mice were put on a cholate-free Western type diet with 0.3 % cholesterol and 4.25 % fat (D01061402, Brogaarden). A 2<sup>nd</sup> IPGTT was performed after eight weeks on diet and blood samples were collected after 9½ weeks. In wt C57BL6 mice, fasting plasma was collected four weeks after induction of uremia and IPGTT or IPITT was performed six or eight weeks later, respectively. Blood was collected in either EDTA or heparinized microtubes and centrifuged at 4000 rpm for ten minutes at 4°C.

*Chemical nephrectomy: adenine induced uremia*

C57BL/6JRj mice were purchased from Janvier SA (Le Genest-Saint-Isle, France) and housed in an air-conditioned room with a controlled environment of 21 ± 0.5°C and 60-70% humidity, under a 12-h light/dark cycle with free access to food (2016C, 12.6 kJ/g, Harlan, Gannat, France) and water.

CKD was induced in mice using an adenine rich-diet. Twenty mice were randomized to either a CKD or a control group. The animals assigned to the CKD group were fed chow-containing 0.25% (w/w) adenine on A04 basis (SAFE, Augy, France) for four weeks and followed for an additional three weeks on a regular diet (A04, 13.4 kJ/g, SAFE). The control animals were fed regular chow (A04) throughout the observation period. After three weeks of uremia, IPGTT and IPITT were performed. After four weeks of uremia, mice were deeply anesthetized with sodium

pentobarbital (70 mg/kg intraperitoneally). Blood (750 $\mu$ L) was collected through cardiac puncture in heparinized tubes, centrifuged for two minutes at 3500g to separate plasma, snap-frozen in liquid nitrogen and stored at -80°C until analysis.

#### *Insulin and glucose tolerance tests*

Mice were fasted for six hours prior to IPGTT/IPITT. LDLr<sup>-/-</sup> mice were injected with either 1 g/kg mouse of glucose (IPGTT) or 0.75 U insulin/kg mouse (IPITT), while wt or adenine mice were injected with 1 g glucose/kg mouse, or 0.5 U insulin/kg mouse for IPITT. Blood samples (5  $\mu$ L) were collected from the tip of the tail at baseline and 15, 30, 60, and 120 min after glucose or insulin injection. The glucose concentration was subsequently measured on a Biosen, autoanalyser (EKF Diagnostics GmbH, Magdeburg, Germany) according to the manufacturer's instructions. For adenine mice experiment, plasma glucose was measured using a glucometer (Accu-chek performa, Roche, Meylan, France).

#### *Plasma measurements*

For 5/6 nephrectomy experiments, plasma urea, creatinine, cholesterol, total calcium, and phosphate were measured with a Modular P Hitachi automatic analyzer (Roche A/S) and reagents from Roche A/S or a Cobas® 8000 modular analyzer series (Roche A/S). Insulin was measured by the assay department at Novo Nordisk by a sandwich assay using the LOCI principle[9]. For adenine experiments, the plasma concentration of urea, total cholesterol, and triacylglycerols were determined using

commercial enzymatic assays (bioMérieux, Marcy-L'Etoile, France), while plasma insulin was measured using an immunoassay (Mouse insulin, Bertin Pharma, Artigue, France).

### *Statistical analysis*

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego). Comparisons were done with Student's *t* test. For IPITT and IPGTT, the area under the curve was determined for each animal followed by Student's *t* test.  $p < 0.05$  was considered significant.

## **Results**

### *Surgically induced moderate uremia does not induce insulin resistance in mice.*

To assess whether uremic LDLr <sup>-/-</sup> mice develop insulin resistance, uremia was induced by 5/6 nephrectomy (NX) in a two-step operation. As expected, this led to moderate uremia as determined by a more than two-fold increase in urea and a ~four-fold increase in creatinine in NX compared to SHAM mice measured 2 weeks after the second operation (Table 1A). Also, a small, but significant, increase in plasma cholesterol was detected in NX mice (Table 1A). To assess insulin and glucose tolerance, we measured insulin levels and performed IPGTT and IPITT after six hours of fasting. Despite the moderate uremia, there were no differences in plasma insulin levels between SHAM and NX mice (Table 1A). In line with this, both IPGTT and IPITT showed similar results in SHAM and NX mice (Fig. 1A and B).

The mice were subsequently put on a high cholesterol diet to induce severe hypercholesterolemia and thereby accelerate atherogenesis. Plasma measurements after eight weeks on the atherogenic diet confirmed that NX mice remained moderately uremic (urea ~2.5 fold and creatinine ~1.8 fold higher in NX mice), while the difference in cholesterol levels originally observed on chow diet was abolished on the high cholesterol diet (Table 1B). To investigate whether the diet could influence the insulin sensitivity, IPGTT was performed after eight weeks on the high cholesterol diet. Again, we did not detect any differences between SHAM and NX mice (Fig. 1E).

Next, IPGTT and IPITT analyses were performed in surgically induced uremic, normocholesterolemic C57BL/6 wt mice. These analyses unequivocally confirmed similar IPGTT and IPITT in NX and SHAM wt mice (Fig. 1C and D). Furthermore, fasting insulin levels were identical in SHAM and NX wt mice (Table 1C). Overall, the results show that, at least in our hands, neither LDLr<sup>-/-</sup> mice nor C57BL6 wt mice develop insulin resistance as a consequence of moderate uremia induced by 5/6 NX.

*Chemically induced severe uremia does not induce insulin resistance.*

To further explore the potential relationship between uremia and insulin resistance, we implemented a non-surgical model of CKD using an adenine-rich diet. Biometric and biochemical data for control and adenine-fed mice data are summarized in Table 2. Body weight gain was significantly reduced in adenine-fed mice and most organ weights were decreased. Especially, Adenine-fed mice exhibited a striking decrease in white adipose tissue accretion (-50%, P<0.001). As expected,

the plasma urea concentration was elevated (four-fold,  $P < 0.001$ ) indicating severe uremia in mice receiving the adenine-rich diet. Furthermore, plasma triacylglycerol concentrations were within the range of control mice while plasma cholesterol levels were increased (+ 21%,  $P < 0.05$ ) in uremic compared to control mice (Table 2). There were no differences in either plasma glucose or fasting insulin levels in adenine-fed mice compared to control mice, indicating normal glycaemic control. This was confirmed by IPGTT and IPITT analysis. Thus, no differences were observed between the adenine-fed mice and control mice during IPGTT and IPITT (Fig. 2), unambiguously excluding the development of insulin resistance.

## **Discussion**

Development of atherosclerosis is accelerated in patients with CKD, but the mechanisms leading to the acceleration are still not clear. Insulin resistance has been shown to be an independent risk factor for cardiovascular mortality [10, 11], and insulin resistance has been detected in CKD patients already at early stages [12]. Thus, our aim was to establish whether insulin resistance could explain (some of) the accelerated atherosclerosis seen in CKD. To do this, we set out to establish a model that both developed atherosclerosis and insulin resistance. We did, however, not detect insulin resistance in LDLr<sup>-/-</sup> mice neither on normal or high cholesterol diet. The lack of insulin resistance was confirmed in wt mice. Finally, we tested whether adenine-induced CKD could be a potential model for uremia induced insulin resistance, but again we found a normal glycaemic control in the uremic mice.

These results are rather surprising, since both Koppe *et al* and D'Apolito *et al* have reported that uremic C57BL6 mice are insulin resistant [4, 5]. D'Apolito *et al* argue that elevated urea levels alone can induce insulin resistance [5]. However, since the fold change in urea levels is comparable between their study and our study, increased urea may not be the sole factor mediating IR in settings of uremia in mice. Several factors such as the strain, the gender, or the procedure used to induce CKD could account for such discrepancies between animal studies. The genetic background, i.e. the strain of the mice, deserves some comments. Indeed, uremic C57BL/6JRj (through 5/6 NX) were found to be insulin resistant [4] and hyper-insulinemic, while, in contrast, uremic C57BL/6N mice (also through 5/6 NX) were found to be hypo-insulinemic [13], pinpointing the pivotal role of the genetic background. In good agreement, different C57BL/6 substrains proved to respond differentially to diet induced obesity (DIO) [14]. These observations strongly support the importance of the genetic background in metabolic research.

Female mice are generally thought to be more insulin resistant than male mice. Unfortunately, mouse gender is not clearly specified in either of the previously published studies. Thus, we cannot deduce whether the use of female mice in our study could explain the lack of NX effect on IR. Finally, it could be speculated that the manner in which moderate uremia is induced may play a role. In our study, the LDLr<sup>-/-</sup> mice were operated in a two-step procedure, with two weeks separation; while there is only one week between the two operations in D'Apolitos study [5]. The sequence of the two stages of the 5/6 NX process might also contribute to these



differences. Indeed, if subtotal nephrectomy (i.e. removal of 2/3 of one kidney) precedes the unilateral nephrectomy, it allows the kidney remnant to heal, while the animals exhibit only a limited decrease of glomerular filtration rate. In contrast, if unilateral nephrectomy precedes the subtotal nephrectomy, mice become immediately uremic post-surgically. As a consequence, the healing of the kidney remnant following the removal of 2/3 of the remaining kidney happens in a uremic environment. Generally speaking, the mortality rate of 5/6 NX is higher if unilateral nephrectomy precedes the subtotal nephrectomy. In Koppe *et al*, subtotal nephrectomy was performed as the first stage, and then one week later, unilateral nephrectomy was performed [4].

Chemical nephrectomy using an adenine-enriched diet has been shown to be a robust model of CKD in mice and rats. Adenine diet induces a substantive kidney failure [15, 16], depending on the duration and the concentration of adenine in the diet [17]. Adenine induced renal failure trigger a major weight loss associated to a decrease in fat accretion. Indeed, adenine strongly alters the palatability of the food, thus leading to a very significant decrease in food intake. This strong decrease in fat accretion could prevent the development of IR, making adenine diet an inappropriate model to study metabolic disturbances associated with CKD.

Our results clearly show that insulin resistance does not always accompany moderate uremia in mice. Several important, but often underestimated, factors such as the strain, the gender, or the exact procedure used to induce CKD could account for the discrepancies between animal studies. The limited or insufficient description of

the procedures and of the biological characteristics of the animals (e.g. strain, gender, age etc) makes it difficult to understand the discrepancies between the different studies. The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines, proposed as a tool to improve the reporting of research using animals [18], could help researchers to more accurately document their research and promote more reproducible research.

### **Compliance with Ethical Standards**

Conflict of interest: LSB, SER, CM, AR, and LFIN have been or are employed at Novo Nordisk A/S. TXP and LFIN are currently employed by Gubra. The other authors have declared that no conflict of interest exists.

Ethical approval: All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (IRB approval number n°2013-15-2934-00843 and n° 2013-118). See methods section for more information.

### **Acknowledgements**

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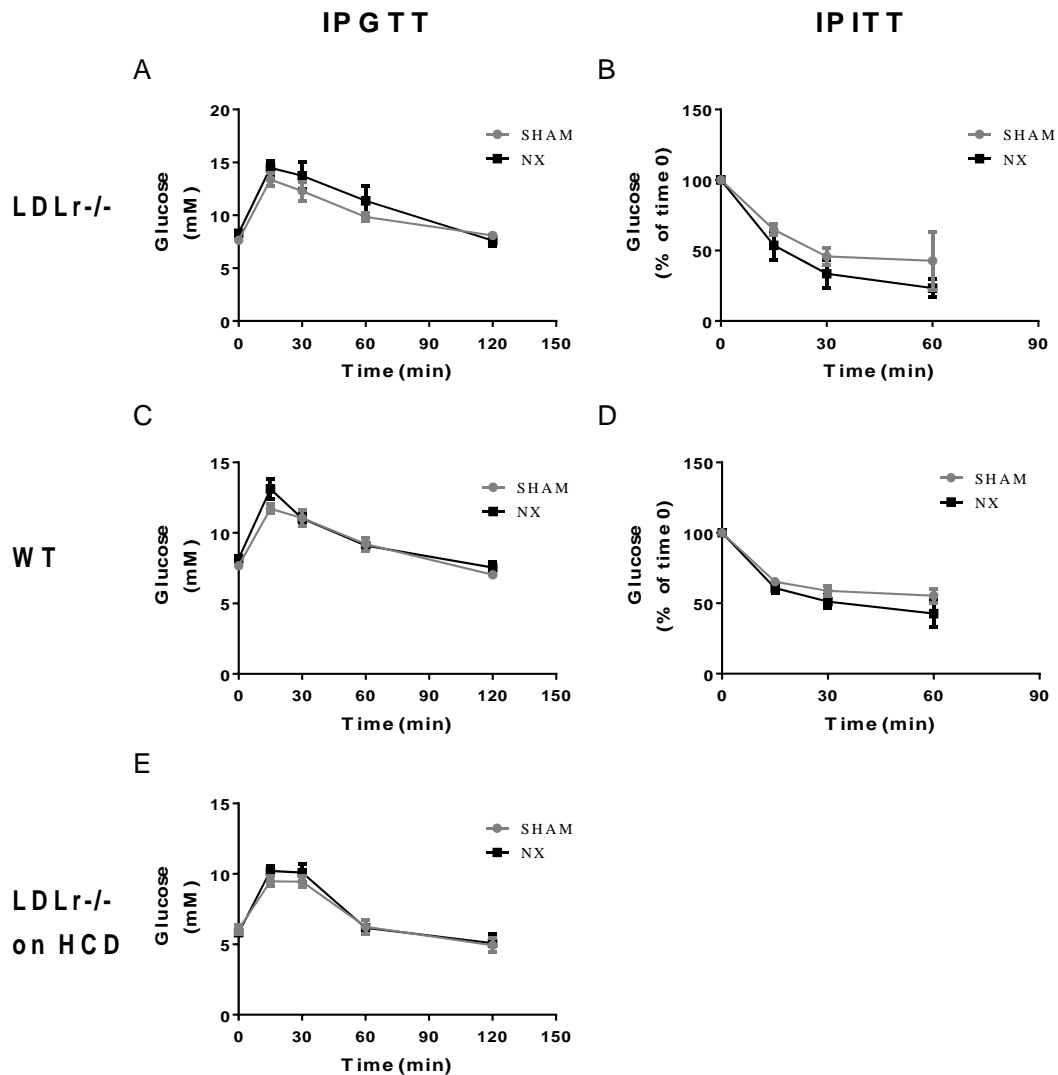
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## Figure legends

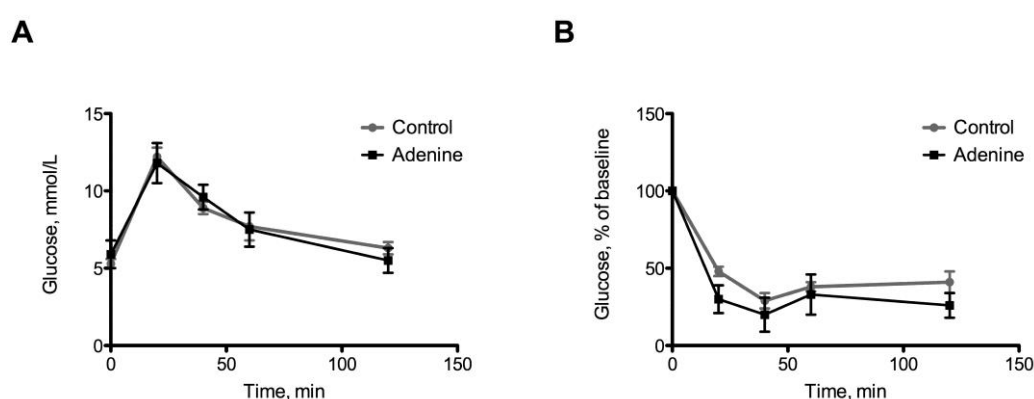


**Fig. 1 Glucose and insulin tolerance tests in NX and SHAM mice**

Intraperitoneal glucose tolerance test (IPGTT) (a, b and e). Plasma glucose was measured in blood samples at baseline and 15, 30, 60, and 120 min after i.p. injection with glucose (1 g/kg) in (a) LDLr-/- (N = 10 SHAM, 10 NX), (c) wt (N = 10 SHAM, 5 NX), and (e) LDLr-/- mice on a high cholesterol diet (N = 9 SHAM, 10 NX).

Insulin tolerance test (b and d). Plasma glucose was measured in blood samples at baseline, 15, 30, and 60 min after i.p. injection with insulin (0.75 U/kg (b) and 0.5 U/kg (d)) in (b) LDLr-/- (N = 4

SHAM, 3 NX) and (d) wt mice (N = 7 SHAM, 4 NX). A number of mice went into hypoglycaemia during the ITT and had to be given a glucose bolus. These mice were excluded from the analyses. Data are depicted as mean $\pm$ SEM. The area under the curve (AUC) was determined for each animal individually followed by Student's *t* test.



**Fig. 2 Glucose and insulin tolerance tests in adenine and control mice**

Intraperitoneal glucose tolerance test (IPGTT) (a). Plasma glucose was measured in blood samples at baseline and 20, 40, 60, and 120 min after i.p. injection with glucose (1 g/kg) in control (n = 10) or adenine mice (n=9). No difference between control and CKD mice was found to be significant at the  $P < 0.05$  level (2-way ANOVA).

Intraperitoneal insulin tolerance test (IPITT) (b). Plasma glucose was measured in blood samples at baseline 20, 40, 60, and 120 min after i.p. injection of insulin (0.5 UI/kg) in control (n = 10) or adenine mice (n=9). Data are depicted as mean  $\pm$  SEM. The area under the curve (AUC) was determined for each animal individually and compared using Student's *t* test. No difference between control and adenine mice was

found to be significant at the  $P < 0.05$  level.

**Table 1 Plasma biochemistry and insulin levels in LDLr<sup>-/-</sup> (A), LDLr<sup>-/-</sup> mice on high cholesterol diet (B) and wt mice (C)**

	A		B		C	
	LDLr <sup>-/-</sup>		LDLr <sup>-/-</sup>		WT	
	SHAM	NX	SHAM	NX	SHAM	NX
N	10	10	9	10	10	5
Weeks of uremia	2	2	9	9	4	4
diet	chow	chow	HCD	HCD	chow	chow
Urea (mmol/L)	8.9 ± 0.4	26.1 ± 2.3 <sup>****</sup>	7.8 ± 0.5	19.7 ± 1.5 <sup>****</sup>	6.8 ± 0.4	14.8 ± 0.5 <sup>****</sup>
Creatinine (μmol/L)	8.0 ± 0.7	35.4 ± 4.2 <sup>**</sup>	12.9 ± 0.9	23.2 ± 0.8 <sup>****</sup>	12.3 ± 2.3	30.3 ± 5.5 <sup>**</sup>
Cholesterol (mmol/L)	8.0 ± 0.7	9.6 ± 0.5 <sup>*</sup>	30.8 ± 2.3	32.3 ± 2.5	3.3 ± 0.1	3.5 ± 0.2
Insulin (pmol/L)	33.0 ± 5.5	32.0 ± 3.0	n.d	n.d	26.0 ± 2.8	23.9 ± 1.5

Values represent mean ± SEM, Student's *t* test, <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$ , <sup>\*\*\*\*</sup> $P < 0.0001$

SHAM: sham operated ctrl mice, NX: 5/6-nephrectomized mice, HCD: high cholesterol diet

**Table 2 Biometry and plasma biochemistry in mice fed a control or an adenine diet 0.25% (w/w) to induce CKD**

	Control	CKD	Change
N	10	10	
Weeks of uremia	3	3	
Diet	Standard	Adenine	
<b>Biometry</b>			
Body weight, g	25 ± 1	15 ± 1	-40% <sup>***</sup>
Liver, mg	1361 ± 67	771 ± 32	-43% <sup>***</sup>
Kidney, mg	281 ± 10	119 ± 4	-58% <sup>***</sup>
Heart, mg/10g	125 ± 5	45 ± 1	-25% <sup>***</sup>
Gastrocnemius, mg	126 ± 4	77 ± 3	-39% <sup>***</sup>
WAT, mg	530 ± 48	272 ± 33	-49% <sup>***</sup>
<b>Biochemistry</b>			
Urea, mmol/L	5.1 ± 0.3	25.7 ± 1.1	+403% <sup>***</sup>
Glucose, mmol/L	5.2 ± 0.3	5.8 ± 0.4	+11%
Insulin, pmol/L	33.2 ± 4.6	37.1 ± 6.4	+12%
Cholesterol, mmol/L	3.26 ± 0.29	4.16 ± 0.26	+27% <sup>*</sup>
Triacylglycerols, mmol/L	1.14 ± 0.10	1.35 ± 0.16	+18%

Data are mean ± SEM and variation compared to control group. <sup>\*</sup> $P < 0.05$ , <sup>\*\*\*</sup> $P < 0.005$ .

Significant difference between control and adenine mice. Abbreviation: WAT, white adipose tissue.



## General discussion

Chronic kidney disease (CKD) is associated with the accumulation of a large variety of uremic toxins. In renal diseases, many compounds that are not efficiently removed by the kidneys can be associated with negative outcomes in end stage renal disease (ESRD) patients. Among these uremic compounds, some of them belong to the group of protein bound uremic toxins (PBUTs) due to their tight interactions with plasma proteins especially serum albumin. Protein bound uremic toxins have received extensive attention, mainly because of their high toxicity and poor removal by conventional dialysis. Protein bound uremic toxins, such as in indoxyl sulfate (IS), phenylacetic acid (PAA), p-cresyl glucuronide (p-CG), may contribute significantly to the progression of chronic kidney disease (CKD) and to the increased risk of cardiovascular disease (CVD) observed in CKD patients. Due to their tight binding to plasma proteins, these hydrophobic uremic toxins are poorly cleared using conventional dialysis and thus strongly accumulate in end stage renal disease patients.

Protein bound uremic toxins are retained in uremia and may promote deleterious effects in biological systems. As they accumulate in plasma, they may affect drug binding to plasma proteins and result in altered pharmacokinetics and pharmacodynamics of the drug. Human serum albumin (HSA) is the most abundant protein in human plasma (i.e. 60% of all plasma proteins). With an extraordinary ligand binding capacity, HSA binds a wide variety of drugs and carries many endogenous and exogenous compounds. Different classes of ligands bind to HSA at multiple sites. The pioneering work of Sudlow et al. showed that most drugs bind

with high affinity to one or two sites on human serum albumin, which are called Sudlow's sites I and II. Most drugs and uremic toxins bind to one or two of these two binding sites. For example, Indoxyl sulfate (IS) and p-cresyl sulfate (p-CS) bind to Sudlow's binding site II while 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF) bind to site I. For this reason, investigation of the binding site, the binding protein and the binding mode of uremic toxins is very useful to help decipher pharmacokinetics of protein bound drugs, improve the therapy of hemodialysis (HD), and optimize the treatment of renal patients. Total plasma level of PBUTs consist of a protein bound fraction and an unbound biologically active fraction, the latter being the only one to be removed using dialysis. Protein bound uremic toxins are therefore recognized as important targets of therapeutic removal.

However, the binding properties of PAA and p-CG have received little attention in the scientific literature. Our study first evaluated the binding parameters and provides important quantitative data on the binding affinity of PAA<sup>130</sup> and p-CG to HSA (**Table 16**).

	p-CS <sup>103</sup>	IS <sup>103</sup>	PAA <sup>130</sup>	p-CG <sup>131</sup>
K <sub>I</sub> , M <sup>-1</sup>	1×10 <sup>5</sup>	0.98×10 <sup>5</sup>	3.38×10 <sup>4</sup>	2.77×10 <sup>3</sup>
HSA bound	80%	78%	26%	23%

**Table 16.** Binding parameters of p-CS, IS, PAA and p-CG to HSA.

Compared with p-CS and IS, the binding constants of PAA and p-CG are much lower. PAA and p-CG are therefore only weakly bound to proteins. Indeed, 75% of these toxins circulate in human plasma in their free form, which can be removed by hemodialysis. This observation could challenge the classification of PAA and p-CG

by EUTox as protein bound uremic toxins. The values are lower than several measurement performed *in vivo*. On the one hand, Itoh et al reported the protein binding of PAA to be 60%<sup>132</sup> while Boehringer et al reported the protein bound fraction of PAA to be 59%<sup>133</sup>. On the other hand, Meert et al. determined the protein binding percentage of p-CG in plasma obtained from HD patients to be 8.3%<sup>134</sup> while Liabeuf et al. found it to be 9.3%<sup>135</sup>. It is worth noting that our binding experiments were only performed with purified serumalbumin solution and that we cannot exclude that PAA or p-CG could bind to other plasma proteins (i.e. other than albumin). A second point is that serumalbumin could undergo several post translationnal modification in uremia (e.g. guanidinylation, carbamylation, carbonylation,...) that could alter its affinity for uremic toxins. Meijers et al. pinpointed that CKD patients commonly exhibited a “dysfunctional” albumin regarding the binding of uremic toxins.<sup>136</sup> Serumalbumin isolated from plasma CKD patients is post-translationally modified by guanidinylation of some lysine residues. It was demonstrated as a proof of concept, that *in vitro* guanidinylation of native serum albumin results in a blunted binding capacity for indoxyl-sulfate<sup>137</sup>. These topics however deserve further studies using plasma from healthy or CKD patients spiked with these toxins.

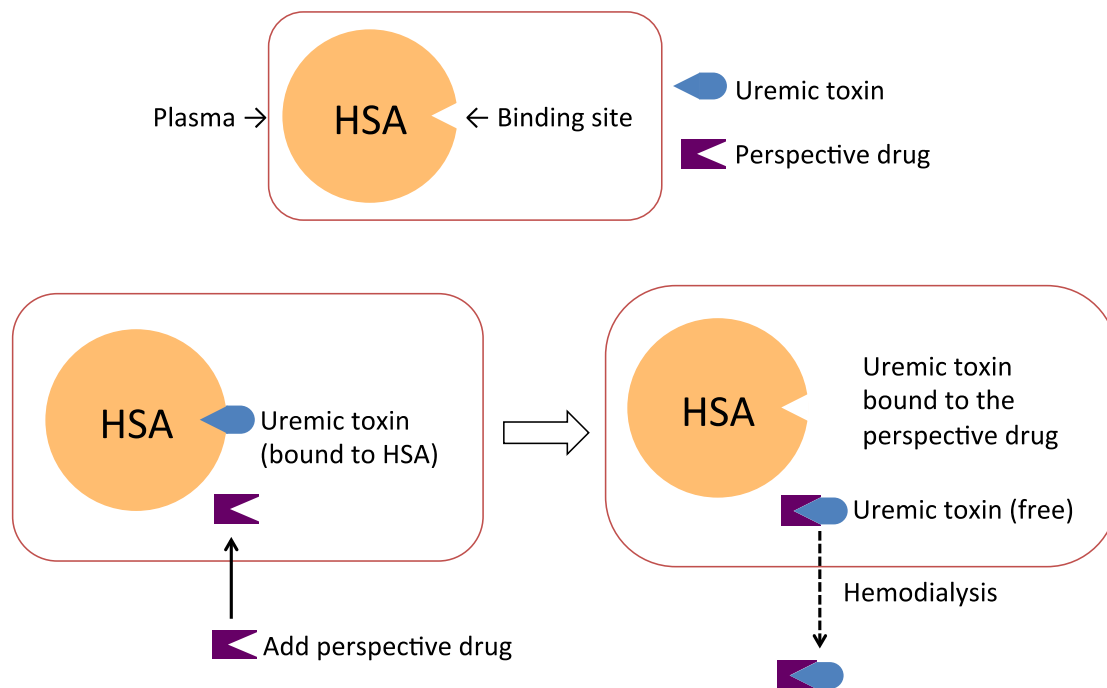
Current HD technics do not efficiently remove protein bound uremic toxins because of their strong interaction with plasma proteins. A better understanding of the interactions of PBUTs with HSA and plasma proteins is needed to develop new strategies of dialysis aimed at removing these uremic compounds. Thus, novel dialysis methods should be developed to remove these protein bound uremic toxins

more efficiently in order to prevent uremic complications. Therefore, we summarized and discussed in a review the interest of chemicals displacers as a valuable option to enhance PBUTs removal in CKD patients.<sup>78</sup> In most cases, the binding of uremic toxins to plasma proteins is reversible obeying the “law of mass action”, although the binding affinity of some uremic toxins, like IS and p-CS, is quite strong. Because the type of binding between them involve non-covalent binding such as for instance electrostatic or hydrophobic interactions, Van de Waals forces or hydrogen bonds. These properties lead us focusing on albumin binding sites and the strategies of displacement of those toxins. Depending on the affinity of the uremic toxin for plasma proteins, a variable proportion of the toxin may bind to plasma proteins, with the remaining fraction being free and thus diffusible. The binding affinity on albumin is also variable. IS and p-CS exhibit tight binding properties requiring high affinity and concentrated displacers. In contrast, hippuric acid, CMPF and PAA are only weakly bound on albumin respectively on Sudlow site I for the two firsts and site II for the latter allowing an easier displacement.

Hence, we designed and synthesized some compounds that were expected to behave as displacers and displace PBUTs. However, the binding affinity of most PBUTs to HSA is too high to be easily displaced. None of these candidates we selected could bind to HSA with a higher affinity than p-CS, although N-phenyl glycine could approach to a very high binding proportion to site II. Even if high concentrations of displacers during hemodialysis may increase the binding affinity of these candidates, in vitro competition experiment is needed to unambiguously

determine the binding characteristics of these selected candidates in presence of uremic toxins. Furthermore, the toxicity of the displacers needs to be entirely and clearly tested before the implementation of in vivo experiment or clinical studies. Otherwise, we will use artificial toxic compounds to displace the “natural” PBUTs, and with a tighter binding affinity than PBUTs, these compounds must cause terrible problems and hardly to remove.

Because it is still not easy to remove PBUTs by the displacement method due to their tight binding to HSA, we conceived a perspective method with a brand new strategy of hemodialysis during the study of the structure of HSA. The strong binding affinity between PBUTs and HSA is the crucial reason for their poor removal and makes the problem to be trickier. But this high binding affinity could also be utilized to conceive a new strategy of “displacement”. The displacer is designed to displace HSA, rather than PBUTs. In this way, the displacer will bind with PBUTs and then the displacer-toxin complex can be removed together by hemodialysis. The structure of HSA, especially the binding sites on HSA, provides us a perfect and ready-made model of the potential candidate. This kind of compounds could be designed with a similar structure of the binding site on HSA. The probable process is shown in **Figure 46**.



**Figure 46.** The probable process of the prespective drug removing PBUTs.

However, there is still long way to implement strategy. The structure of HSA is complex and there are abundant binding sites on HSA. A better understanding of the structure, binding sites and binding properties of HSA is necessary and useful for CKD drug exploration. The development of biophysical technologies, especially the improvement of structural biology, will contribute to the exploration of structure and binding sites of HSA. Then, the drug with similar binding sites of HSA or with a higher affinity bound to PBUTs than HSA bound to PBUTs could be designed. This will finally contribute to the improvement of hemodialysis technics.

In hemodialysis patients, one middle-molecule uremic toxin, zinc-alpha2-glycoprotein (ZAG), a potent cachectic factor and lipid mobilizing factor, caught our attention because we found it increased during CKD. We demonstrated

that plasma ZAG concentration predicted clinical outcomes in hemodialysis patients. We used a cohort of 252 patients on HD for at least 3 months, without progressive cancer, followed up for more than 3 years. Mortality and cardiovascular events have been registered during the mean study period of 954 days. Our study first demonstrated that high serum ZAG concentration is a predictor of all and CV caused mortality; Serum ZAG concentrations were directly associated with overall mortality and CV events; ZAG has been detected in several malignancies, such as cancer. Thus, the observed relationship between ZAG and mortality may however reflect the presence of cancer not detected at the time of inclusion. The strengths of this prospective study include the large number of patients and the long-term follow-up. However, this study also has several limits. The main limitations are the lack of ZAG measurements at follow-up and the use of a single baseline measurement to predict events several years ahead. Indeed, there is no data about the evolution of ZAG concentration during HD. We did a follow-up survey and found ZAG concentration did not change in these patients after one year.

Another concern is that the potential misclassification of causes of death or CV events is possible, but this did not influence the relationship between ZAG and all-cause mortality. In addition, the patients that we studied in this cohort are from the same hemodialysis center from France, and the applicability of the study findings across nationalities remains unclear. Our present study first demonstrated that the potential clinical utility of ZAG levels for risk-stratifying patients with ESRD, with patients with highest levels being at the highest risk. Notably, these associations are

independent of other factors, including nutrition, CV risk factors, and inflammation markers. Further studies are needed to better elucidate the role of this adipokine in human diseases.

In addition, we studied another protein bound uremic toxin, indoxyl sulfate, which is one of the more toxic uremic toxins in CKD patients. In vitro experiment, we found that IS could disrupt insulin signaling pathways and develop insulin resistance associated with CKD through activation of AhR. However, no major difference in fasting glucose or in insulin tolerance test were found with administration by IS in vivo study. IS shows a very different characteristics from p-CS that contributes to insulin resistance<sup>81</sup>. This indicates that the effect and target of IS may be different from p-CS. In vivo experiment is more comprehensive than in vitro experiment. In vitro method could be used to study the insulin signaling pathway in cells, but the condition of in vivo experiment is more complex and the whole health system should be taken into account. The probable reason might be that we used the adenine diet CKD model, which lead to the decrease in body weight especially the lost in white adipose tissue (WAT). Insulin resistance (IR) is closely related to obesity and white adipose tissue.<sup>138, 139</sup> We have developed and validated the adenine diet model, but it seems not a good model to study insulin resistance associated with CKD. For the chronic administration of IS, the healthy kidney could remove and excrete IS in urine to avoid IR. This is different from the administration of the injection with p-CS, which could lead to IR<sup>81</sup>. Thus, IS and p-CS have a very different metabolism pathways. Maybe the effect of IS is more complex and it may trigger the mechanism of



compensation to avoid IR. In conclusion, the role of indoxyl sulfate in IR and CKD remain to elucidate in the future.



## Conclusion and perspective

Our work were focused on uremic toxins and chronic kidney disease (CKD). Indoxyl sulfate (IS), a major protein bound uremic toxin (PBUT) in CKD patients, appears to be a very important uremic retention solute that could contribute to insulin resistance (IR) and cardiovascular risk in patients with CKD. In vitro study showed that IS, through activation of AhR, disrupted insulin signaling pathways and could contribute to insulin resistance associated with CKD. However, IS showed a very different metabolism from p-CS. We did not find IR in fasting glucose or in insulin tolerance test in vivo experiment. During the study of IS, we developed and validated a non-surgical animal model of CKD, which is adenine rich diet model. Adenine induced proteinuria and diuresis in mice, but did not result in the development of insulin resistance in mice.

We determined the binding properties of two other protein bound uremic toxins to human serum albumin (HSA), a major drug carrier protein in plasma. Our study provided important quantitative data on the binding affinity of phenylacetic acid (PAA) and p-cresyl glucuronide (p-CG) for HSA. Clinically, we focused on zinc-alpha2-glycoprotein (ZAG), a middle molecule uremic toxin that accumulated in CKD patients under hemodialysis (HD). We demonstrated for the first time the potential clinical utility of ZAG levels to be an independent factor associated with an excess overall mortality and cardiovascular mortality risk in HD patients.

Current HD methods could remove small water soluble compounds and middle molecules, but do not efficiently remove PBUTs because of their strong interaction

with plasma proteins. We developed a new strategy to improve PBUTs clearance in HD with chemical displacers. Some designed compounds were synthesized, and some of them showed the potential to be displacers with high affinity binding to HSA. A better understanding of the PBUTs and the structure and binding sites of HSA is mandatory to develop new dialysis strategies in order to remove this kind of compounds.

Our findings contribute to the basic research of CKD and uremic toxins; provide new idea and method for the clearance of PBUTs. For the future study, the effect of IS on common CKD animal model with 5/6 nephrectomy should be tested in order to investigate if IS could induce IR in the most common used CKD models. We reported that (in contrast to p-CS) IS did not contribute to IR in mice with adenine induced kidney failure. As previously discussed, adenine diet may not be the best model to study dysmetabolism associated with CKD. These experiments could therefore be reproduced in the canonical model of 5/6 nephrectomy. It would indeed be interesting to study the difference between these two models of CKD. The specific advantages and disadvantages of the two methods will provide valuable information for the potential researchers focused on CKD to make the best choice. If so, aryl hydrocarbon receptor (AhR) mutant animal could be used to determine if IS could induce IR through activation of AhR in animal. The relationship between CKD and cardiovascular disease is not simply equal to the relationship between uremic toxins and insulin resistance. Abundant work is still in ahead to discover it. With the result of clinical study of ZAG, future experimental studies could be performed to better

elucidate the role of ZAG and the pathophysiological mechanisms in cardiovascular events in patients with CKD and some other human diseases.

The concept of uremic toxins and the new discoveries of PBUTs are evolving constantly. The determination of the properties of these uremic solutes is necessary and important to understand their clearance and for CKD drug discovery. More displacers should be designed and tested for the displacement capacity of binding site II on HSA. Computer-aided drug design (CADD) and docking software are useful and efficient tools to help us know the interaction between our candidates and HSA in theory, and select the optimal structures or derivatives.

As protein bound uremic toxins have a strong affinity for binding to HSA, a new strategy is just to utilize their binding properties. A future perspective of removal of uremic toxins can be a displacement of albumin in the protein-toxin complex, rather than uremic toxins. As introduced in the preceding sections, small water-soluble compounds (<500 Da) can be easily removed by dialysis. Most protein bound uremic toxins are smaller than 500 Da. Thus, a drug as a small “displacer” could be designed to “displace” albumin and bind to the toxins. Then, the displacer-toxin complex could be removed by hemodialysis. This method needs a better understanding of the structure, binding sites and binding properties of HSA. In this perspective method, the so-called “displacer” plays a role as a kind of “soluble receptors” of the uremic toxins. So the displacer could be designed by the structure of binding sites on HSA. With these similar “artificial binding sites”, different PBUTs, which bind to different sites on HSA in a dynamic equilibrium, can leave HSA and bind to the small “receptor”

and become “free”. This drug could act like a magnet to attract protein-bound uremic toxins binding on it and finally be removed by hemodialysis together as a drug-toxin complex.

With the development of biochemistry, molecular biology, biophysics and physical technologies, a better understanding of the structure, binding sites and binding properties of HSA is feasible; the breakthrough of high-affinity displacers is possible; new and creative strategies of HD technics will be progressed to expand a brand new therapy of CKD.

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## FOLIO ADMINISTRATIF

### THESE DE L'UNIVERSITE DE LYON OPEREE AU SEIN DE L'INSA LYON

NOM : YI  
(avec précision du nom de jeune fille, le cas échéant)

DATE de SOUTENANCE : 28/06/2018

Prénoms : Dan

TITRE : Contribution to the Study of Uremic Toxins in the Context of Chronic Kidney Disease

NATURE : Doctorat

Numéro d'ordre : 2018LYSEI054

Ecole doctorale : Ecole doctorale interdisciplinaire sciences-santé, ED 205

Spécialité : Physiologie

#### RESUME :

L'insuffisance rénale chronique (IRC) est une affection caractérisée par une perte progressive de la fonction rénale. L'IRC est associée à l'accumulation de diverses toxines urémiques. Les toxines urémiques ou solutés de rétention de l'urémie sont des composés qui s'accumulent chez les patients atteints d'IRC en raison d'un défaut de clairance rénale et qui exercent des effets biologiques délétères. Les hémodialyses éliminent mal les toxines urémiques liées aux protéines (PBUT), en raison de leur liaison aux protéines plasmatiques, en particulier la sérumalbumine humaine. En conséquence, les toxines urémiques liées aux protéines s'accumulent chez les patients atteints d'IRC et leur concentration ne peut que difficilement être diminuée chez les patients atteints d'insuffisance rénale terminale (IRT). Mes travaux sont principalement centrés sur les toxines urémiques, en particulier les toxines urémiques liées aux protéines, comme l'indoxyl-sulfate (IS), l'acide phénylacétique (PAA) et le p-crésyl-glucuronide (p-CG); et la zinc-alpha2-glycoprotéine (ZAG) qui est une « middle molécule ». Nous avons étudié le rôle de l'IS dans le développement de la résistance à l'insuline et d'autres troubles métaboliques associés à l'IRC, ainsi que ses effets sur l'inflammation et le stress oxydant. Nous avons exploré les propriétés de liaison du PAA et du p-CG à la sérumalbumine, qui est la plus abondante protéine dans le plasma humain. Enfin, nous avons essayé de développer une nouvelle stratégie d'élimination des PBUT, à l'aide de déplaceurs/compétiteurs chimique.

MOTS-CLÉS : Chronic kidney disease, Uremic toxins, Human serum albumin, Hemodialysis

Laboratoire (s) de recherche : Laboratoire de Cardiovasculaire, Métabolisme, Diabétologie et Nutrition (CarMeN)

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