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INVESTIGATION OF HEPATOCYTE SIGNALING PATHWAYS IN CHRONIC KIDNEY DISEASE. CLINICAL AND EXPERIMENTAL STUDIES.

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To my family

ABSTRACT

Chronic kidney disease (CKD) is defined as a loss of renal function from any cause and lasting for more than three months. The CKD phenotype is similar across multiple etiologies, suggesting that renal damage itself is a dominant factor. Thus, regardless of the cause even a moderate loss of renal function is associated with impaired hepatic metabolism of glucose, cholesterol, lipid particles, bile acids and plasma proteins. There are surprisingly few studies investigating a putative contribution of this impaired hepatic metabolism to the phenotype of CKD. The overall aim of the current thesis was thus to investigate a hypothetical impact on certain well-characterized hepatic signaling pathways in uremic patients and through both clinical and experimental studies.

In Paper I, we describe the release of FGF-19 to the blood, following a meal provocation rich in energy and fat given to CKD patients with severely impaired renal function as well as to age- and gender-matched healthy subjects. We report that the expected increase in postprandial FGF-19 appears to be blunted in CKD patients due to other reasons than delayed gastric emptying. Seven days of pre-treatment with either one of the natural anti-oxidants N-acetylcysteine or anthocyanins led to a partial normalization of postprandial FGF-19 release.

In Papers II and III, primary cultures of human hepatocytes were used to study intra-cellular metabolic signaling under uremic conditions *in vitro*. We designed and used a model entailing culture of these cells (isolated from resected livers) together with CKD patient or healthy sera. We found that hepatocytes exposed to uremic sera rapidly develop an unhealthy metabolism characterized by increased gluconeogenesis and lipogenesis accompanied by perturbations of several key cellular signaling networks. We found no effects of uremic sera on FGF-19 receptor signaling, bile acid synthesis or bile composition.

In Paper IV, we investigated the role of hepatic metabolism on systemic FGF-19 levels. Portal and systemic (peripheral arterial and central venous) blood concentrations of FGF-19 and bile acids were assessed in 75 non-CKD patients undergoing liver surgery. We found no differences of FGF-19 concentrations between portal and systemic blood. Furthermore, treatment of primary hepatocytes with FGF-19 *in vitro* inhibited CYP7A1 expression only at supra-physiological concentrations (2.3-fold decrease) while physiological concentrations of the bile acid CDCA elicited a 12-fold decrease. We conclude that systemic blood concentrations of FGF-19 can be considered to mirror portal concentrations at least in non-CKD populations.

LIST OF SCIENTIFIC PAPERS

I. **Li M,** Qureshi AR, Ellis E, Axelsson J.
Impaired postprandial fibroblast growth factor (FGF)-19 response in pa-tients with stage 5 chronic kidney diseases is ameliorated following antioxidative therapy.
Nephrol Dial Transplant. 2013 Nov;28 Suppl 4: iv212-9.

II. **Li M,** Ellis EC, Johansson H, Nowak G, Isaksson B, Gnocchi D, Parini P, Axelsson J.
Changes in gluconeogenesis and intracellular lipid accumulation characterize the uremic human hepatocyte ex vivo.
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III. **Meng Li,** Helene Johansson, Lisa-Mari Mörk, Helen Zemack, Bengt Isaksson, Ewa Ellis, Jonas Axelsson.
Bile acid metabolism in human primary hepatocytes under uremic conditions.
Manuscript.

IV. Helene Johansson, Lisa-Mari Mörk, **Meng Li**, Helen Zemack, Anita Lövgren Sandblom, Ingemar Björkhem, Jonas Höijer, Bo-Göran Ericzon, Carl Jorns, Stefan Gilg, Ernesto Sparrelid, Bengt Isaksson, Greg Nowak and Ewa Ellis.
Fibroblast growth factor 19 in portal and systemic blood.
Manuscript.

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LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ACACA	Acetyl-CoA carboxylase alpha
ADMA	Asymmetric dimethylarginine
AHR	Aryl hydrocarbon receptor
AKR1D1	Aldo-keto reductase family 1, member D1
Apo	Apolipoprotein
ASBT	Apical sodium-dependent bile transporter
ATF4	Activating transcription factor 4
AUC	Area under the curve
BSEP	Bile salt export pump
CA	Cholic acid
CAR	Constitutive androstane receptor
CDCA	Chenodeoxycholic acid
CHOP	C/EBP homologous protein
ChREBP	Carbohydrate-responsive element binding protein
CIDEC	Cell death-inducing DFFA-like effector C
CKD	Chronic kidney disease
CMPF	3-Carboxy-4-methyl-5-propyl-2-furanpropionate
CPT1A	Carnitine palmitoyltransferase 1A (Liver)
C/EBPs	CCAAT-enhancer-binding proteins

CVD	Cardiovascular disease
CYP450	Cytochrome P 450
CYP7A1	Cholesterol 7a-hydroxylase
CYP8B1	Hydroxylated by sterol 12α-hydroxylase
CYP27A1	Sterol-27 hydroxylase
DCA	Deoxycholic acid
DGAT	Diacylglycerol acyltransferase
eGFR	Estimated GFR
FABP	Fatty acid binding protein
FAS	Fatty acid synthase
FGF-19	Fibroblast growth factor 19
FGFR4	Fibroblast growth factor receptor 4
FOXO1	Forkhead box O1
FXR	Farnesoid X-receptor
G6pase/G6PC	Glucose 6-phosphatase
GFR	Glomerular filtration rate
GLUT2	Glucose transporter type 2
GRP78	Heat shock 70kDa protein 5
HD	Hemodialysis
HDL	High density lipoprotein
HMGCR	HMG-CoA reductase
HNF1A	HNF1 homeobox A

HNF4A	Hepatocyte nuclear factor 4, alpha
HSD11B/11βHSD	Hydroxysteroid (11-Beta) dehydrogenase
IR	Insulin resistance
IRS	Insulin receptor substrate 1
KLB/β-klotho	Klotho beta
LCA	Lithocholic acid
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low density lipoprotein
LIPC	Lipase, hepatic
LRP	Receptor-related protein
LXR	Liver X receptor
MDR1A	Multidrug resistance protein 1
Mrp	Multidrug resistance protein family member
MP865	Freeze-dried blueberries containing anthocyanidins
NAC	N-acetyl cysteine
Nrf2	Nuclear factor, erythroid 2-like 2
NTCP	Sodium (Na+)-taurocholate cotransporting polypeptide
OATP	Organic anion transporters
OST	Organic solute transporters
PAPSS2	3'-Phosphoadenosine 5'-phosphosulfate synthase 2
PB	Phenobarbital
PD	Peritoneal dialysis

PEPCK/PCK1	Phosphoenol pyruvate carboxykinase
PGC1α/PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PLIN2	Perilipin 2
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
PRKCE	Protein kinase c, epsilon
PXR	Pregnane X receptor
Rif	Rifampicin
SREBP-1c	Sterol regulatory element-binding protein-1c
SCARB1	Scavenger receptor B-1
SHP	Short heterodimeric partner
SULT2B1	Sulfotransferase family, cytosolic, 2B, member 1
TG	Triglyceride
UDCA	Ursodeoxycholic acid
UGT	UDP-glucuronosyltransferase
VDR	Vitamin D (1,25- dihydroxyvitamin D3) receptor
VLDL	Very low density lipoprotein
XBP1	X-box binding protein 1

1 INTRODUCTION

1.1 THE HUMAN KIDNEY

1.1.1 Renal anatomy

In humans the two kidneys are located outside the peritoneum, against the back of the abdominal cavity and on either side of the spine. The right kidney sits just below the liver and is therefore slightly smaller and lower than the left which sits approximately level with vertebras T12-Ll, below the diaphragm and behind the spleen. The kidneys are normally partially covered by the 11th and 12th ribs. Just above each kidney sits the adrenal gland, while the whole complex is surrounded by perirenal fat, pararenal fat and the renal fascias. In adult humans, an individual kidney is approximately 11–14 cm in length, 6 cm wide and 4 cm thick, weighing around 150 grams.

The kidneys are "bean-shaped" structures with a convex and a concave aspect. On the concave aspect sits the renal hilum, the recessed area housing the renal artery, the renal vein, lymphatic vessels and the ureter. The surface of each kidney consists of a fibrous capsule. Inside, the parenchyma of each kidney is divided in outer portion renal cortex and inner portion renal medulla, with 10-20 cone-shaped renal lobes, called a renal pyramid. The nephron forms the basic structural and functional unit of the kidney, which each contain approximately 1 million of these distributed across both the cortex and medulla, Below these two layers of nephrons lie first the minor and then the major calyces, leading the urine into the renal pelvis , whence it travels down the ureters and into the urinary bladder.

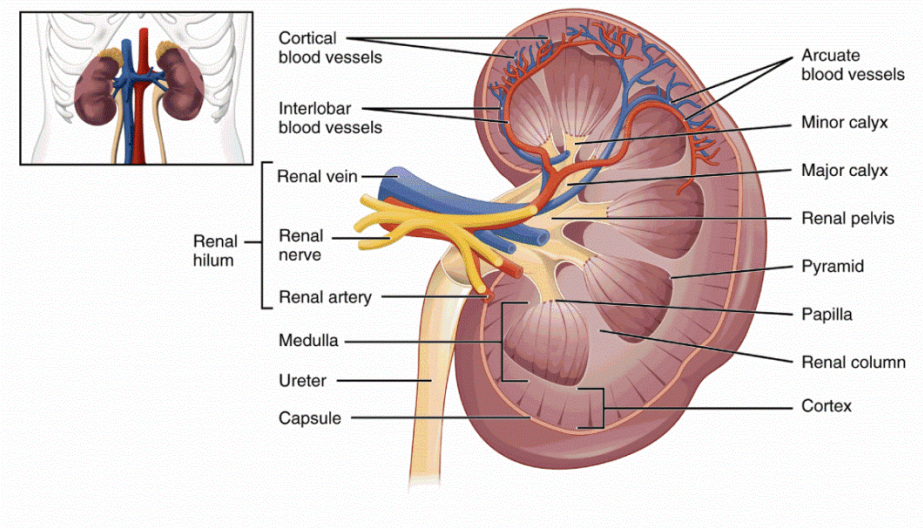

Figure 1. Cross-section through the kidney with key anatomical structures indicated. *Source: Anatomy & Physiology, Connexions Web site. http://cnx.org/content/col11496/1.6/ (Jun 19, 2013). Published under Creative Commons Attribution 3.0 license.*

The kidneys are supplied with oxygenated blood through the renal arteries, which in turn branch directly off the abdominal aorta. After entering the renal hilum, the blood flows into many segmental arteries and on to the interlobar arteries, arcuate arteries (which pass through the border of the renal cortex and medulla), interlobular arteries and finally into the afferent arterioles of each nephron. Following filtration in the glomerulus, efferent arterioles carry the unfiltered portion on into peritubular capillaries that supply the distal tubules and other structures of the renal cortex. The blood from these capillaries then drains into interlobular veins, which combine into larger venules, veins and finally back through the two renal veins into the inferior vena cava (**Figure 1** and **Figure 2**).

1.1.2 The nephron

The nephron is the functional filtration unit of the kidneys, each comprising a complex of interconnected capillary loops surrounding the double-walled glomerular (Bowman's) capsule and its' attached renal tubule – subdivided into the proximal tubule, the loop (of Henle) and the distal tubule that connects the tubule to the collecting duct that flows into the renal papilla.

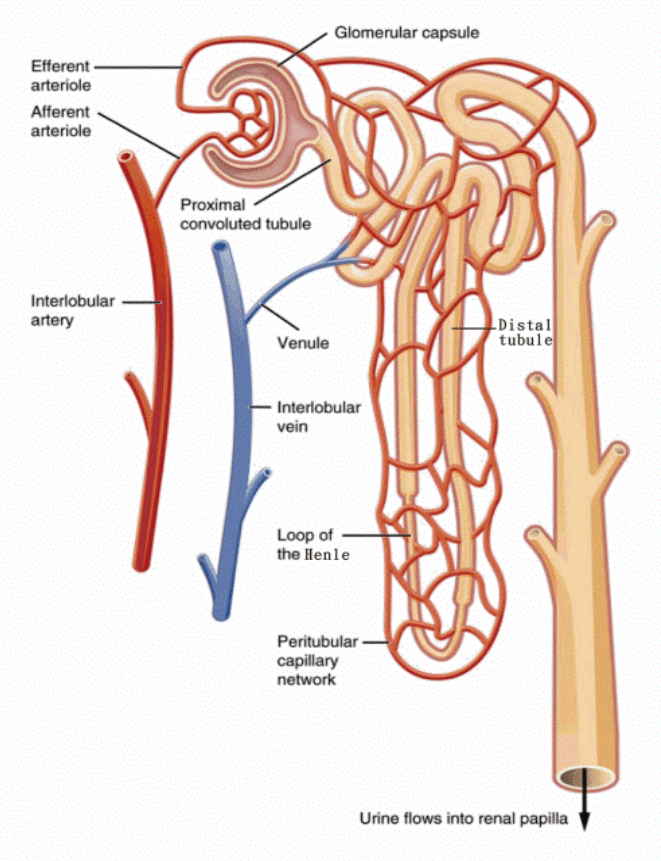

Figure 2. Nephron diagram. *Source: Modifed from Anatomy & Physiology, Connexions Web site. http://cnx.org/content/col11496/1.6/ (Jun 19, 2013). Published under Creative Commons Attribution 3.0 license.*

The glomerular capsule is a highly specialized structure containing the glomerular filter, whose main function is to filter blood plasma to produce glomerular filtrate. To do this, the capillaries passing into the glomerulus are lined by specialized endothelial cells, adjoining a special basement membrane with both covered by specialized podocytes whose appendages encircle the arteriole. All three structures contribute to the filtration barrier, and about 20% of the afferent blood plasma filters into the capsule and into the adjoining tubule. Membrane permeability differs for the various constituents

of plasma, and is likely determined by the ultra-structure of the layers, the transmembrane pressure and the size (and potentially charge) of each plasma molecule.

Thus, the composition of the filtered plasma ("primary urine") entering the tubules is very different from urine and these differences are explained by mechanisms such as passive and active tubular reabsorption and secretion, enabling a tight control of the final urine composition.

Glomerular filtration rate (GFR) describes the total volume of fluid filtered from the glomerular capillaries into the glomerular capsule per unit time and in all nephrons of an organism. GFR is usually assessed through a marker substance such as creatinine, but it should be kept in mind that results are then affected by the permeability of the membranes to the measured substance, as well as the net filtration pressure (stable over a wide range of blood pressures but not always), and any active reabsorption or secretion of the substance in the tubules. The normal range of human GFR (normalized to body surface area) is 90-130 ml/min/1.73m^2.

1.1.3 **Renal physiology**

The kidney participates in whole-body homeostasis, playing a central role for the maintenance of acid-base and fluid balances; regulation of sodium, potassium and other electrolytes; clearance of water-soluble waste products such as ammonia; the regulation of blood pressure; as well as recycling of glucose, amino acids and many other molecules.

Having a low molecular-weight and dissociated from plasma proteins, water and many salts are freely filtered into the primary urine, from whence more than 99% is usually reabsorbed during tubular passage. As an example, sodium re-absorption occurs at several stages of the tubule and is an active trans-cellular transport from the tubular lumen to the interstitial fluid mediated by Na^+/K^+-ATPase pumps. Water is reabsorbed both passively by osmosis (dependent upon sodium reabsorption) and actively through water channels (aquaporins). These are specialized proteins that allow water but few other compounds to cross the cell membrane, and are usually synthesized ahead of time and stored in specialised intracellular vesicles ready to be used when needed (eg. dehydration). Aquaporins thus play an important role in maintaining water balance in both the short and long term. Other solutes, such as glucose, amino acids and bicarbonate are likewise passively and actively reabsorbed or secreted, often as a consequence of the sodium gradient and through co-transport and counter-transport mechanisms.

The long-term maintenance of sodium homeostasis is of central importance to life, as sodium is the major osmolyte in extracellular water and thus a key regulator of cell shape, intravascular volume and blood pressure. A decrease in plasma volume or GFR triggers

renal juxtaglomerular cells to secrete renin, an enzyme that splits angiotensin I from angiotensinogen released by the liver. Angiotensin I is itself inactive, but is further cleaved into angiotensin II by angiotensin-converting enzyme (ACE). Angiotensin II in turn is a very potent regulator of blood pressure, acting both systemically on the vasculatures, locally in the renal arterioles and through stimulation of aldosterone release to promote the reabsorption of sodium and water in the kidney.

Besides maintaining a healthy balance of ingested water and ions, the kidneys clear a variety of waste products from the blood. These include waste from protein catabolism and nucleic acid metabolism (nitrogenous compounds). Urea is the main vehicle for nitrogen waste including ammonia. It is primarily synthesized in the liver whence it travels in the blood to the kidneys and enters the urine. Urea additionally plays an important role in tubular water and ion reabsorption as it participates in a number of counter-current transporters. Another commonly encountered waste product is creatinine, it is also synthesized primarily in the liver through methylation of glycocyamine formed by muscle metabolism. As it is easy to measure and relatively independent of diet it is commonly used as a surrogate marker for or a means to estimate GFR.

The kidneys also play an important role in maintaining the body's pool of amino acids. This is achieved through both active tubular secretion and re-uptake of amino acids, *de novo* amino acid synthesis and lysosomal of plasma proteins leaking into the primary urine and endocytised by the tubular epithelium [1]. Thus, the kidney is the major site for removal of circulating glutamine (30% of daily disposal), proline (60%), citrulline (100%), S-adenosylhomocysteine (100%) and cysteinyl-glycine (90%), as well as the central source of circulating serine (100%), cysteine (100%), arginine (50%), tyrosine (50%) and lysine (5-20%). Daily around 300 mmol of free amino acids cross the glomerular filter, with about 98% reabsorbed [1-4].

1.2 CHRONIC KIDNEY DISEASE/UREMIA

1.2.1 Definition and classification

Chronic kidney disease (CKD), also known as chronic renal disease, encompasses different pathophysiological processes associated with a progressive loss in kidney function over a period of months or years. To simplify communication and treatment studies, CKD has since about 15 years been defined as the persistent loss of at least one renal function for more than 3 months and divided into five stages based on eGFR and signs of structural damage to the renal filter (albuminuria). Stages 1-5 signify progressively worse renal health and declining estimated GFR (≥ 90 but eg. proteinuria;

60-89; 30-59; 15-29; <15 mL/min/1.73 m^2, respectively). These cut-offs have been chosen somewhat arbitrarily but based on a dramatic and gradual increase in the risk of mortality and progression to end-stage renal disease (ESRD) [5]. As a comparison, normal ageing is associated with an observed decline in GFR from a peak value of around 120 mL/min/1.73 m^2 in young adults to a mean of 70 mL/min/1.73 m^2 at age 70.

Phenotypically, CKD stages 1 and 2 are usually not associated with overt symptoms of renal disease but carry an increased risk of mortality, hypertension and progressive kidney damage. At CKD stages 3 and 4 almost all organs are affected, with clinical complications of CKD including anemia, fatigability, hypertension, poor appetite, muscle catabolism, disorders in water and ion homeostasis, hyperlipidemia, acidosis, hypocalcemia, and osteoporosis. At stage 5 (ESRD), the accumulation of normally excreted toxins leads to marked disturbances of body function that usually necessitate renal replacement therapy for survival. Treatment options include hemodialysis (HD), peritoneal dialysis (PD), or kidney transplantation.

1.2.2 **Etiology and epidemiology**

Over the last 30 years CKD has become an important public health problem in most countries of the world [6, 7]. Partly as a result of increased obesity and diabetes, partly due to an increase in unhealthy living associated with increasing affluence, the incidence and prevalence of CKD is rising and some degree of the disease now affects more than 10% of the general population in many countries [8-13]. Population survey data from the U.S.A. indicate a prevalence of more than 6% of adults for stage 1 and 2 CKD, 4.5% for stage 3 and 4 and perhaps 2% for stage 5 [8-13]. Common causes of CKD there include diabetic nephropathy (damage to the kidneys caused by diabetes; 30-40% of ESRD in the U.S.A.) [14, 15], hypertension [16-18], glomerulonephritis (an inflammation of the glomeruli or small blood vessels in the kidneys), IgA nephropathy (characterized by idiopathic IgA deposition in the glomeruli)[19, 20], urinary tract obstruction, and congenital diseases such as adult polycystic kidney disease. Acute kidney injury (AKI), also an important cause of CKD, is defined as a sudden decrease in kidney function that develops over a few hours or days, often as a complication to trauma or surgical interventions, and associated with a high morbidity and mortality risk. Of patients with AKI, 30%-70% have been reported to go on to develop some degree of CKD [21, 22].

Almost uniquely among the diseases of major body organs, the advances in science and therapy over the past decades have not greatly benefited CKD patients, who continue to have a rate of morbidity and mortality far above the general population [23, 24]. Indeed, more than half of CKD patients are predicted to die before they reach stage 5, and the mortality risk of CKD is greatly increased at every stage. The rate of death in CKD in rich

nations divided by stage 3, 4, 5 has been estimated at 5, 10, and 15 times higher respectively than stage 1 and 2 CKD [23]. After starting dialysis, only about half of the patients will live beyond five years, mainly due to deaths from cardiovascular complications and infection [23, 24].

1.2.3 **Symptoms and metabolic alterations in uremia**

Fatigue is an often reported early symptom of declining GFR, as is the loss of appetite, pruritus and mental acuity. With the gradual loss of filtration capacity (shrinking GFR), patients develop problems excreting salts and water. Oedema causes the swelling of the limbs. At more advanced stages of CKD, patients are at risk from acidosis (leading to muscle catabolism and imbalances in salts that can cause arrhythmia, muscle cramps and nerve tingling) as well as hyperkalemia (can give rise to deadly cardiac arrhythmias). Protein-energy malnutrition is another complication common in CKD patients, especially at lower GFRs. In patients before dialysis protein intake is restricted to reduce symptoms and preserve renal function, while with maintenance dialysis even a normal dietary protein and energy intake is often inadequate to the need, which is elevated also due to losses in the dialysate [25].

In general and regardless of etiology, CKD is associated with disturbances in nutritional homeostasis. This includes altered circulating and muscle amino acids profile [26], hyperglycemia or normoglycemia with apparent hyperinsulinemia [27], and disturbances in blood lipids (elevated triglyceride) and cholesterol/lipoproteins (high ApoB and low ApoA-bearing particles, very high Lp(a)) [28]. Circulating levels of insulin are elevated already early in the course of CKD and apparent insulin resistance (IR) correlates with GFR [29]. Interestingly, after the initiation of renal replacement therapy, insulin levels drop somewhat but are not completely normalized [30] (**Table 1**).

Table 1. Brief summary of studies investigating metabolism in CKD patients.

Author	Model system	Findings in patients	Ref
Glucose and insulin			
Mulec H, *et al.* 1998	Insulin-dependent diabetic nephropathy patients	Hyperglycemia	[31]
Shinohara K, *et al.*2002	CKD stage 5	Insulin resistance (IR), a pathological situation in which cells fail to response to the normal actions of the hormone insulin	[32]
Chen J, *et al.*2003	CKD stage 3-4 without diabetes	Hyperinsulinemia	[27]
Becker B, *et al.*2005	CKD stage 1-5	IR	[33]
Miyamoto T, *et al.*2011	CKD stage 5	Hyperinsulinemia	[34]
Lipids			
Chan MK, *et al.* 1982	CKD stage 5	Hyperlipidemia, low HDL cholesterol	[35]
Cramp DG, *et al.* 1977	CKD patients without dialysis treatment	High plasma VLDL-TG concentration	[36]
Batista MC, *et al.* 2004	CKD stages 3-4	Low ApoA-I and high IDL and ApoB-100	[28]
Lee PH, *et al.* 2009	CKD stages 3-5	High serum TG level	[37]
Saland JM, *et al.* 2010	CKD stages 4-5 in children	High TG, low HDL–C	[38]
Attman PO, *et al.* 2011	CKD stages 1-4	High ApoB and ApoC-III	[39]
Wang X, *et al.* 2012	CKD stage 5	High plasma TG, VLDL-TG, and VLDL-apoB-100 concentrations	[40]
Amino acids			
Flügel-Link RM, *et al*. 1983	CKD stage 5	Low tyrosine, ratios of tyrosine to phenylalanine and valine to glycine	[41]
Bergström J, *et al*. 1990	CKD stage 5	Low threonine, serine and valine	[42]
Divino Filho JC, *et al*. 1997	CKD stage 5	Low leucine, valine, phenylalanine, tyrosine	[43]
Valli A, *et al*. 2008	CKD stage 5	High S-adenosylhomocysteine and S-adenosylhomocysteine	[44]
Fadel FI, *et al*. 2014	CKD stage 4-5	High plasma glutamine, homocysteine	[45]

Underscoring the links between declining renal function and dysmetabolism, a renal transplantation with a well-functioning graft is associated with a marked reduction in all symptoms, a near normalization of metabolism (except in patients who develop IR due to cortisone medication), and a dramatic drop in the risk of cardiovascular disease and mortality [46, 47]. However, it must be noted that new but less dramatic risks emerge with the need for lifelong immunosuppressive therapy – including corticosteroids, azathioprine, mycophenolate sodium, mycophenolate mofetil, cyclosporine, belatacept, tacrolimus, everolimus and rapamycin [48-50].

There is also a partial reversal of many of the symptoms listed above following the initiation of dialysis therapy, but the dramatically increased risk of morbidity and mortality remains unchanged or is even increased after initiation of dialysis [51]. Additionally, treatment with PD carries the risk of glucose overload due to absorption from the dialysate in the peritoneal cavity and studies have linked PD to the development of hyperglycemia and *de novo* diabetes [52, 53] in CKD patients. This treatment modality is also associated with higher circulating triglycerides (TG), low-density lipoprotein (LDL) cholesterol and Apo B levels as compared to those found in non-dialyzed patients with CKD stage 5 and HD patients [54]. Finally, it must be noted that the enumerated metabolic changes have not been linked to an increase in mortality risk in PD as compared to HD, which remains similarly high in both modalities.

1.2.4 **Uremic toxicity**

For unknown reasons, CKD is associated with systemic alterations in the functions of many organs that appear both quantitatively and qualitatively more linked to GFR decline than to the initial cause of renal damage. Due to the known purifying functions of the kidney and the fact that serum from renal patients can induce cellular dysfunction in a range of tissues [55], it has been hypothesized that the retention of progressively larger amounts of normally excreted toxic compounds is the explanation. These unknown compounds are called uremic toxins, and the state of CKD is thus often referred to as uremia [56-58]. Uremic toxins are likely to include both soluble and insoluble molecules of many classes, presenting a variety of properties which makes their accurate classification extremely difficult. The European Uremic Toxin Work Group (EUTox) has been created within the European Society for Artificial Organs (ESAO) to discuss and analyze matters related to the identification, characterization, analytic determination, and evaluation of biologic activity of uremic retention solutes. They recently enumerated 90 uremic toxins based on 857 publications between 1966 and 2002 [59], but also pointed out that newfound substances are added all the time [60-64].

The most common classification of uremic toxins is based on molecular weight. First, low-molecular-weight uremic toxins (<500 Da) that are soluble in water and thus easily removed by dialysis therapy; the EUTox listed 68 solutes in this group, including asymmetric dimethyl arginine (ADMA), creatinine, guanidine, oxalate, urea and uric acid [59]. Second, middle-molecular-weight molecules (> 500 Da) that are soluble in water; of which EUTox lists 22, including peptide hormones such as leptin, motilin and α1-acid glycoprotein. Third, protein-bound solutes, of which the EUTox lists 25; these have a high affinity for circulating transport proteins such as albumin, making them difficult to remove by dialysis. Their molecular weight is variable, but usually more than 500 Da, and the group includes advanced glycation end products (AGEs), indoxyl sulfate, phenolic compounds, and *p*-cresyl sulfate [59, 63-68]. Examples of uremic toxins according to this classification are given in **Table 2**, adapted from Vanholder *et al.*[59].

Urea and creatinine are classified as uremic toxins, but also function as biomarkers to estimate residual renal function. Importantly, studies have not linked either of these molecules to toxic effects, and it is likely that they are merely convenient markers of far more complex processes. Given the thousands of peptides found in healthy blood along with the hundreds of thousands of smaller metabolites, the EUTox classification is clearly outdated [69] and a new classification based on for example plasma proteomics or the impact of molecules on major cellular pathways is needed.

Table 2. Examples of types and sizes of different uremic toxic molecules.

Low molecular-weight water-soluble toxins		Middle molecules	Protein-bound
1-methyladenosine	N^2,N^2 dimethylguanosine	Adrenomedullin	2-methoxyresorcinol
1-methylguanosine	N^4-acetylcytidine	Atrial natriuretic peptide	3-deoxyglucosone
1-methylinosine	N^6-methyladenosine	β_2-microglobulin	CMPF
ADMA	N^6threonylcarbamoyladenosine	β-endorphin	Fructoselysine
α-keto-δ-guanidinovaleric acid	Orotic acid	Cholecystokinin	Glyoxal
α-N-acetylarginine	Orotidine	Clara cell protein (CC16)	Hippuric acid
Arab(in)itol	Oxalate	Complement factor D	Homocysteine
Argininic acid	Phenylacetylglutamine	Cystatin C	Hydroquinone
Benzylalcohol	Pseudouridine	Degranulation inhibiting protein I^c	Indole-3-acetic acid
β-guanidinopropionic acid	SDMA	Delta-sleep inducing peptide	Indoxyl sulfate
β-lipotropin	Sorbitol	Endothelin	Kinurenine
Creatine	Taurocyamine	Hyaluronic acid	Kynurenic acid
Creatinine	Threitol	Interleukin-1β	Leptin
Cytidine	Thymine	Interleukin-6	Melatonin
Dimethylglycine	Uracil	κ-Ig light chain	Methylglyoxal
Erythritol	Urea	λ-Ig light chain	N^{ε}–(carboxymethyl)lysine
γ-guanidinobutyric acid	Uric acid	Leptin	*p*-cresol
Guanidine	Uridine	Methionine-enkephalin	Pentosidine
Guanidinoacetic acid	Xanthine	Neuropeptide	Phenol
Guanidonosuccinic acid	Xanthosine	Parathyroid hormone	P-OHhippuric axid
Hypoxanthine		Retinol-binding protein	Putrescine
Malondialdehyde		Tumor necrosis factor-α	Quinolinic acid
Mannitol			Retinol-binding protein
Methylguanidine			Spermidine
Myoinositol			Spermine

1.3 THE HUMAN LIVER

1.3.1 Liver anatomy

The liver is the largest organ in the healthy human, normally weighting between 1.4–1.7 kg. It is located in the upper right quadrant of the abdominal cavity, below the diaphragm and above the bowel. The liver receives an ample blood supply from two blood vessels, the hepatic artery that carries oxygen-rich blood from the aorta, and the portal vein which carries nutrients and other ingested substances from the intestines. The basic and functional units of the liver are the lobules, each of which is made up of millions of hepatocytes organized along with other cell types around a liver vein and a bile duct. Classically, the liver is grossly divided into six uneven lobes; a large right and a small left lobe at the anterior surface (diaphragmatic) and two additional lobes located between the right and left lobes shown from the inferior surface (visceral surface), the caudate and quadrate lobes. However, beside this classic anatomical division into lobes, a surgically relevant classification which divides the liver into eight segments based on blood supply as proposed by the French surgeon Claude Couinaud is often used (**Figure 3**).

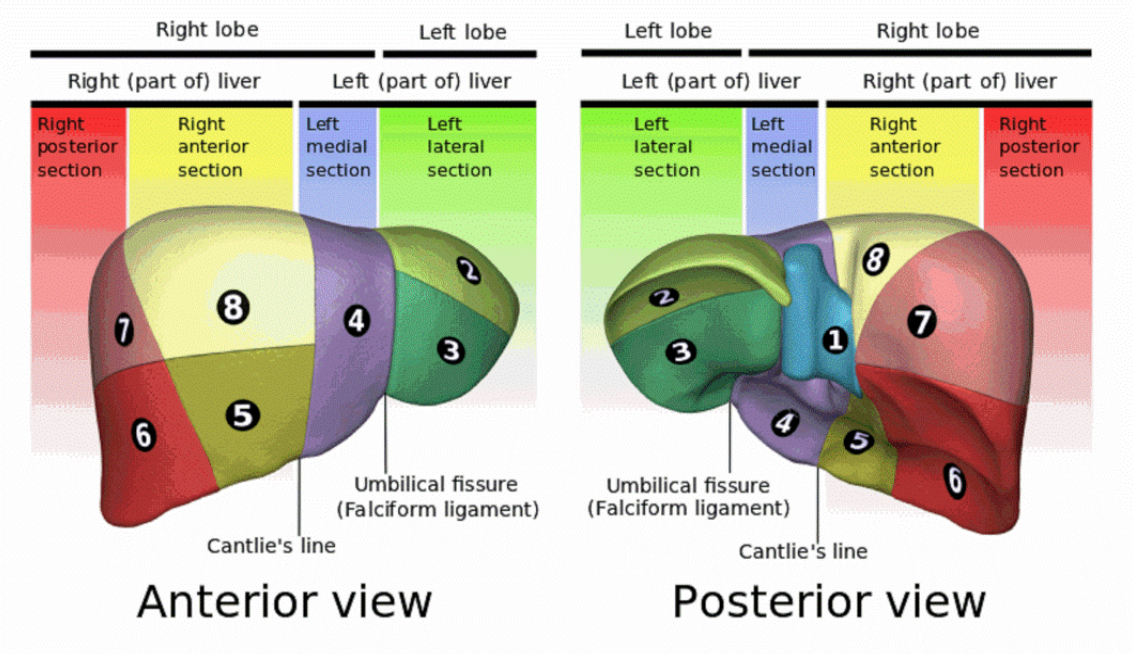

Figure 3. Couinaud classification system. *Source: Polygon data is generated by Database Center for Life Science (DBCLS). Published under Creative Commons Attribution-Share Alike 2.1 Japan license.*

1.3.2 **Liver physiology**

With the intestinal efferent blood draining mainly through the portal vein, the liver plays a central role in metabolism. As the first organ to encounter the full range of absorbed molecules, the liver is a central site of detoxification, metabolism and storage. It plays vital roles in the homeostasis of carbohydrates, lipids, cholesterol, lipoproteins, proteins and amino acids, along with many vitamins and minerals.

1.3.2.1 Glucose metabolism

The liver maintains plasma glucose in a stable and narrow range through several complimentary mechanisms: after a meal, endogenous glucose release (from liver production and kidney reabsorption) decreases to a very low level due to pancreatic release of insulin, causing the liver to take up approximately 25-35% of the absorbed glucose and convert it to glycogen (glycogenesis) or fat (lipogenesis). During fasting this stored glycogen can easily be re-converted to glucose (glycogenolysis) and released, but the liver can also employ secondary sources such as lactate, pyruvate, amino acids and glycerol to manufacture new glucose (gluconeogenesis) for the same purpose.

Gluconeogenesis is regulated mainly by transcriptional activation of the key enzymes phosphoenol pyruvate carboxykinase (PEPCK), fructose 1, 6-bisphosphatase and glucose 6-phosphatase (G6Pase). Of these, the PEPCK promoter is well-known to be induced by circulating signals acting through second-messengers (glucagon, glucocorticoids, thyroid hormone) [70] as well as by transcription factors such as FoxO1 and hepatocyte nuclear factor (HNF)-4α. Peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α/PPARGC1A) acts as a master regulator and directly binds to HNF-4α or FoxO1 to regulated their interaction with target genes [71] (**Figure 4**). Normally, insulin suppresses PEPCK and G6Pase gene expression via the second-messenger phosphatidylinositol 3-kinase (PI 3-kinase) pathway [72]. Of note, interleukin (IL)-6, a major activator of signal transducer and activator of transcription (STAT)-3, has been reported to markedly reduce PEPCK and G6Pase gene expression [73, 74].

1.3.2.2 Lipid homeostasis

The liver plays key roles in several pathways involving lipid metabolism. It is a key determinant of metabolism of fatty acids, lipoprotein turnover and release, as well as cholesterol synthesis and degradation. In the exogenous pathway, dietary fats are emulsified and hydrolyzed by bile acids in the intestinal lumen, from whence they are then absorbed by enterocytes and packed into nascent chylomicrons (NCs) that enter the enterohepatic circulation via the lymphatic system [75].

At first bearing ApoA-I and ApoA-IV, the NC matures through interaction with HDL and thus aquires ApoC-II and ApoE as well as the ability to activate lipoprotein lipase (LPL), which catalyzes the hydrolysis of chylomicron TG into free fatty acids and glycerol [76]. Most of fatty acids are normally transported in this manner to either myocytes where they are used for energy production or else into adipocytes for energy storage. The chylomicron remnant then continues to the liver whence it is cleared through interaction with the LDL receptor, LDL receptor-related protein (LRP) and scavenger receptor B-1 (SCARB1).

The liver can also synthesize TG *de novo* from free fatty acids and glycerol. In the endogenous pathway, these are combined with cholesteryl esters and ApoB-100 to form very low density lipoprotein (VLDL), which is able to transport its' cargo to the periphery as described above. Excess cholesterol is transported from the periphery in HDL to the liver.

Thus, fatty acids in the liver derive from either endogenous lipogenesis, direct uptake in the gut or lipolysis and transport from storage tissues such as adipocytes. Free fatty acids in the blood, for example bound to albumin, may also be taken up by hepatocytes through transport proteins such as fatty acid binding protein (FABP). Regardless of the substrate source, hepatic fatty acids synthesis is catalyzed by two key enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), both of which are regulated by the transcription factors sterol regulatory element-binding protein(SREBP)-1c and carbohydrate-responsive element binding protein (ChREBP) [77] along with the nuclear receptors PPARα and liver X receptor (LXR)-α [78-80]. Likewise, TG is generated by the enzyme diacylglycerol acyltransferase (DGAT) under the control of C/EBPα or PPARγ [81, 82].

Finally, oxidation of fatty acids in the liver occurs in mitochondria, peroxisomes and microsomes depending on the chain length. Carnitine palmitoyltransferase (CPT)-1 is the rate-limiting step of mitochondrial fatty acid β-oxidation, through its' control of long-chain acyl-CoA transport across the mitochondrial membrane [83] (**Figure 4**).

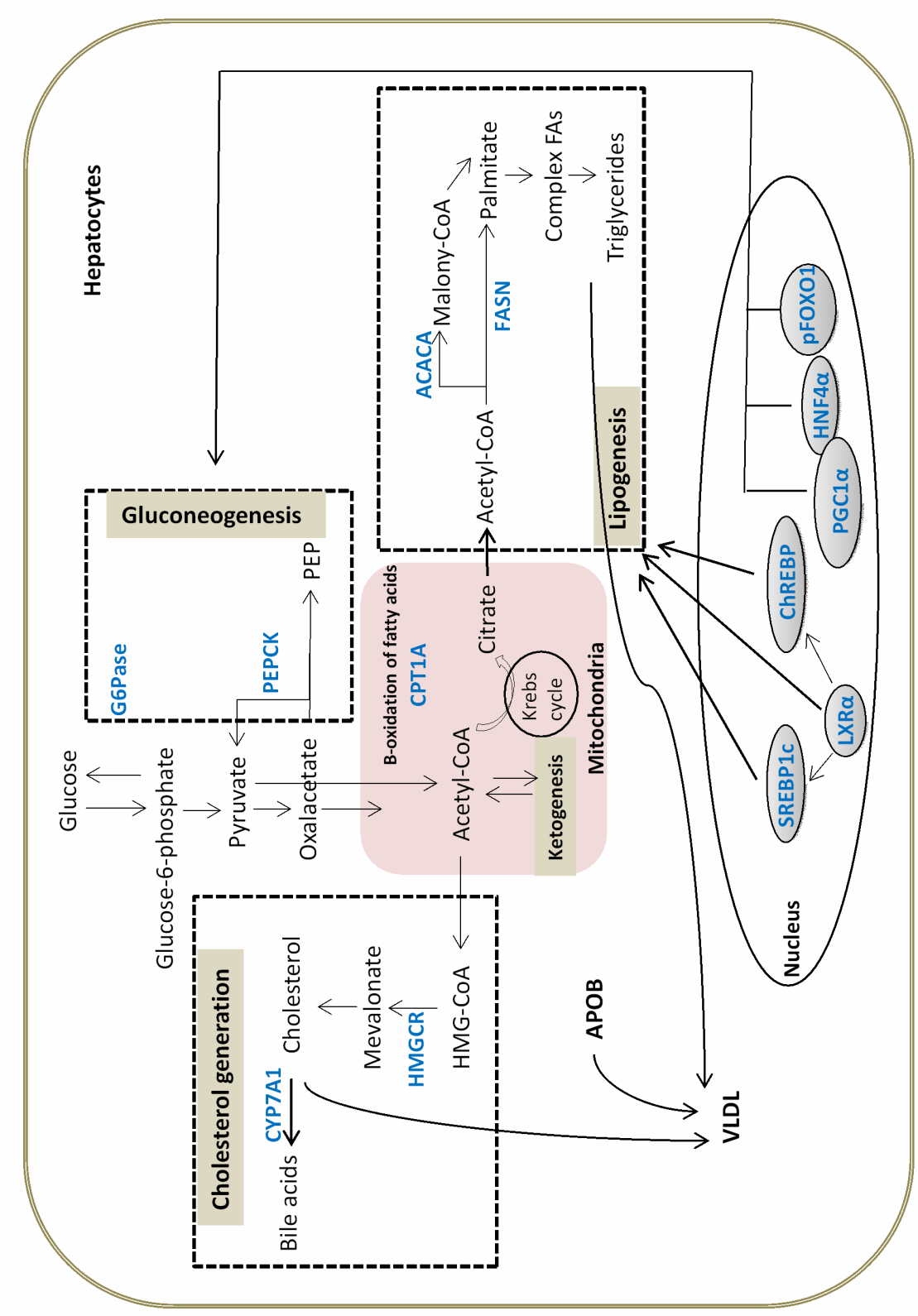

Figure 4. Glucose and lipid metabolism in hepatocytes.

1.3.2.3 Cholesterol and bile acids homeostasis

Cholesterol is a critical component of the cell membrane, steroid hormones and vitamin D. It also functions as a signaling molecule that interacts with several nuclear hormone receptors. About 20–25% of the total daily cholesterol production occurs in the liver. This synthesis begins when two-carbon acetate groups from acetyl-CoA condense to form acetoacetyl-CoA, which is then converted into 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). Next, HMG-CoA is irreversibly converted into mevalonate by HMG-CoA reductase (HMGCR), the rate-limiting enzyme of this pathway. Cholesterol is also a precursor for bile acids. Primary bile acids are exclusively synthesized in the liver, from where they are mainly released to the bile but also to the bloodstream. As many bile acid metabolites are cytotoxic synthesis, storage and release are tightly controlled, mainly through transcription of the rate-limiting enzyme in bile acid synthesis, cholesterol 7a-hydroxylase (CYP7A1) [84] .

1.3.2.4 Amino acids and proteins

Most of the plasma proteins found abundantly in healthy plasma are synthesized exclusively in the liver. These include as albumin, α-1-microglobulin and α-1-antitrypsin, as well as most coagulation factors and the inhibitor of calcium complex formation, AHSG. The hepatocytes are also the primary site of production for substrates of protein metabolism including non-essential amino acids, deamination and transamination reactions, the removal of excess ammonia via the urea cycle, and the production of ketone bodies during prolonged starvation.

Ammonia is a waste product of oxidative de-amination reactions. Due to its' toxicity at even low concentrations, it is rapidly and effectively removed from the body in the urea cycle (also named ornithine cycle). This sequence of reactions mediates the conversion of ammonia into non-toxic urea through a series of biochemical reactions that all occur in the liver (**Figure 5**). Urea is subsequently released into the bloodstream to be passively and actively secreted by the kidneys.

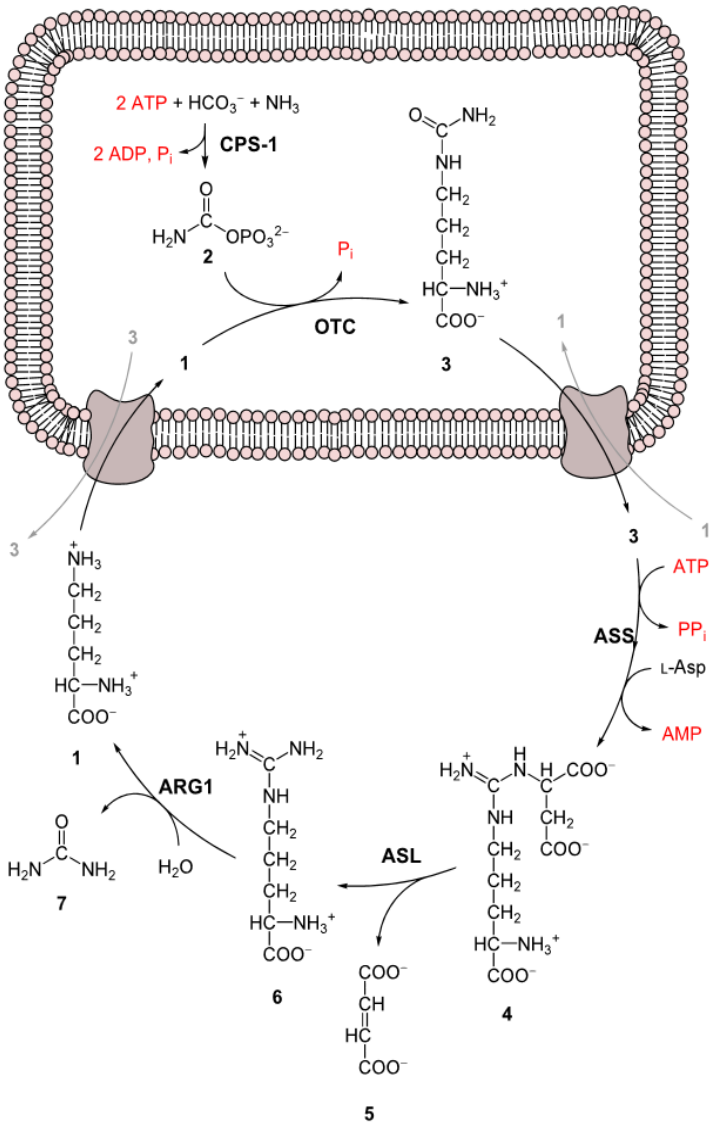

Figure 5. The reactions of the urea cycle.

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As shown in **Figure 5**, urea synthesis entails NH_3^+, HCO_3^- and ATP combining to form carbamyl-phosphate, which in turn reacts with ornithine to form citrulline. The last reaction occurs in the mitochondria, were it is catalyzed by ornithine transcarbamylase (OTC). Citrulline is then released back into the cytosol to react with aspartate to form argininosuccinate, a reaction catalysed by argininosuccinate synthetase (AS). Next, argininosuccinate is cleaved to form fumarate and arginine by argininosuccinate lyase (AL). Fumarate is oxidized in the tricarboxylic acid (TCA) cycle, while arginine is cleaved to form ornithine and urea. Urea is then dissolved into the blood stream and transported to the kidneys. There, it is passively filtered through the glomerular filter as well as actively excreted into the tubular epithelium. Interestingly, these same renal cells are also involved in arginine synthesis and reabsorption and both AS and AL are highly expressed in the kidneys [85].

1.3.2.5 Xenobiotics

Finally, the liver metabolizes many xenobiotic compounds. Often this involves biotransformation of chemical structures to remove biological activity and increase water solubility, while final clearance is often through the kidneys. For simplicity, the reactions involved are often divided into three phases: Phase I (modification), Phase II (conjugation), and Phase III (excretion). Phase I reactions occur mainly in the liver but

also in other tissue, including the kidneys, lungs, gastrointestinal epithelium and the skin. A variety of enzymes catalyze Phase I reactions, including cytochrome P (CYP) 450 oxidases CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. The purpose of these reactions is to convert xenobiotics to more water soluble metabolites by unmasking or inserting a polar group, such as -OH, -SH, or -NH2. In Phase II, the modified compounds are conjugated with charged molecules such as glutathione or sulfate to form yet more polar groups, for example carboxyl (-COOH), hydroxyl (-OH), amino (NH2) and sulfhydryl (-SH) groups. A large number of enzymes may be involved but the most well studied is glutathione S-transferase [86]. These reactions increase water solubility. Finally, in Phase III the conjugated xenobiotics are further deactivated through the addition of acetylated conjugates. This renders the molecule accessible for members of the multidrug resistance protein (Mrp) family, a member of the ATP-binding cassette transporters superfamily that aids the removal of the molecule to the circulation [87-89].

1.3.3 Liver resection

Liver resection, also referred to as hepatectomy, is the surgical removal of a portion or all of the liver, often to remove a benign (eg. hepatocellular adenoma, hepatic hemangioma and focal nodular hyperplasia) or malignant (hepatocellular cancer) growth. Liver resection may also be performed in cases of metastasis from non-hepatic cancers to the liver (eg. colorectal cancer). It is reported that around 10-25% of colorectal cancer patients have metastatic liver disease [90-92]. Finally, in orthotopic liver transplantation surgery the recipient's liver is removed and replaced by a donor liver from a deceased donor, or parts of the liver from a living donor. The ex-planted liver tissue may be used immediately or stored for a short time in so-called University of Wisconsin preservation solution. Indeed, human hepatocytes have been isolated from liver tissue after storage for up to 48 h at 4°C at a similar yield, viability and plating efficiency as those isolated from fresh tissue [93].

1.4 HEPATOCYTES

1.4.1 Hepatocyte structure and function

Hepatocytes are the main functional cells of the liver, and are involved in all of the various metabolic, endocrine and secretory processes that take place there. They are also the site of synthesis of serum proteins such as albumin, most blood clotting factors, lipoproteins, bile salts and phospholipids. Hepatocytes, also called hepatic parenchymal cells, account for approximately 80% of the total liver volume and 60-65% of the total cell count. Functionally, the hepatocytes are arranged in hexagonal plates that anastomose

with one another, each intersected at right angles with a vein. Hepatocytes *in situ* are polygonal in shape, while their surfaces border either the sinusoids (sinusoidal face, location of the blood-flow to the central vein) or neighboring hepatocytes (lateral faces). Through the blood-filled sinusoids a variety of substances are transported. Additionally, the lateral faces of several hepatocytes, connected by tight junctions, form a bile canaliculi that collects newly released bile and delivers it into the bile ducts and ultimately to the gallbladder. Microvilli are abundantly present on the sinusoidal faces and greatly increase the cell surface area there, while they are only sparsely seen in the bile canaliculi.

The remaining cells in the liver, called non-parenchymal cells (NPCs), include Kupffer cells, sinusoidal endothelial cells, stellate cells, cholangiocytes (epithelial cells) and intrahepatic lymphocytes. NPCs are thought to exert both positive and negative influence on hepatocyte proliferation and function [94], in addition to forming a physical barrier between hepatocytes and the blood circulating within the sinusoids [95] (**Figure 6**).

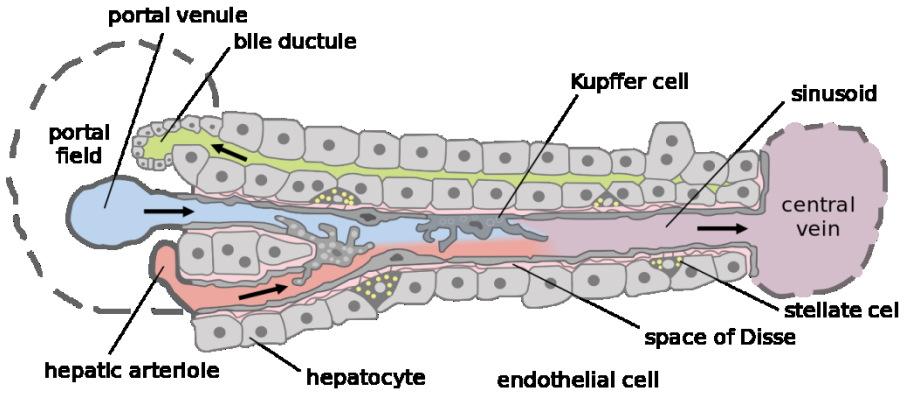

Figure 6. Microscopic anatomy in liver. *Source: WikiMedia. Published under Creative Commons Generic 2.5 license.*

1.4.2 *In vitro* systems for hepatocyte studies

Human primary hepatocytes in culture have been reported to be the most relevant cell culture system to study liver-specific molecular mechanisms of glucose, lipid and bile acids metabolism [96]. However, due to the scarcity of fresh human liver samples, complicated isolation procedures, short survival time and high costs involved in working with primary cultures, the human hepatoma cell lines HepG2 and HuH7 are frequently used instead. However, these hepatoma cell lines are derived from single donors and have spent a long time as immortalized cells in culture [96]. These phenotypic changes may lead to erroneous conclusions. For example, both HepG2 and HuH7 have been shown to lack expression and activity of certain CYP450 and Phase II detoxification enzymes

present in human hepatocytes [97-99]. HepG2 cells secrete bile acid precursors and unconjugated bile acids that are not found in adult humans, and are thus unsuited to the study of bile acids conjugation reactions and transport [100-103].

Primary mouse or rat hepatocytes are also an alternative for *in vitro* liver research. They have been used in the few previous studies of uremic liver, but suffer from important limitations due to interspecies differences. Thus, many aspects of cholesterol and TG synthesis [104], CYP450 detoxification [105], gluconeogenesis [106-108], and apolipoprotein expression [109] are known to differ between man and rodent. However, it was rat livers that in 1969 were used by Berry and Friend [110] to develop the collagenase perfusion method still used to isolate viable hepatocytes. This discovery was the basis for later improvements and adaptions to isolate primary human hepatocytes [111-113]. Today, the two-step perfusion procedure used in our studies is the most common and widely accepted [114].

1.4.3 Human primary hepatocytes culture

As described hepatocytes have an apical and a basal aspect, and attachment to an extracellular matrix (ECM) is necessary to maintain these *ex vivo*. Failure to attach or the lack of the right ECM components causes decreased cellular polarity accompanied by the loss of transcription of many liver-specific genes [115, 116]. Current protocols therefore attempt to monitor and maintain the polygonal shape and specific functions in cultures of isolated primary hepatocytes, most often through the coating of culture plates with rat tail collagen, sometimes with the addition of Matrigel (a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells) [117-119].

Human hepatocytes cultured in this way exhibit a continuous monolayered growth with both constitutive and drug-induced microsomal CYP3A4 expression, the formation of bile canaliculi, active DNA synthesis and high expression levels of cytoskeletal mRNAs and proteins (actin, tubulin, cytokeratins, vinculin, alpha-actinin, and desmoplakin) [118, 120, 121]. The effect of Matrigel on human primary hepatocytes in culture is disputed. Although human primary hepatocytes grown on a collagen and Matrigel substrate exhibit a clearer morphology of tight junctions, gap junction and bile canaliculi than do cells grown on collagen alone [118-120], no differences in the expression of P450 enzymes have been reported at high cell densities, while low density cells grown on collagen and Matrigel exhibited lower basal CYP3A4 expression than cells on collagen alone [118, 122].

Using the above techniques, *in vitro* cultures of primary hepatocytes with stable phenotype can be reproducibly maintained for 10-14 days. Attempts have been made to extend culture times to several weeks [123-126], including through the addition of serum

or hepatocyte growth factor (HGF) and epidermal growth factor (EGF), adult human hepatocytes could survive at least 35 days with retain their basic phenotypical characteristics, such as liver specific proteins secretion (albumin, ApoA1 and ApoB100) and CYP1-4 family proteins expression [124], or by using collagen gel sandwich systems to keep cells up to 78 days, the polygonal morphology and high levels of albumin secretion were maintained throughout the culture period [123].

1.5 BILE ACIDS

In addition to their role in solubilizing lipids in the intestine, regulating cholesterol excretion and participating in the enterohepatic circulation, recent findings suggest that bile acids also act both locally and systemically to modulate multiple signaling pathways involved in the sensing and metabolism of lipids, glucose and energy [127, 128]. Impaired regulation and expression of bile acid synthesis and transporters may play a role in a wide range of human diseases, including fatty liver disease, diabetes, obesity and irritable bowel syndrome [129-131].

1.5.1 Chemical composition

Bile acids are water-soluble, amphipathic molecules synthesized from cholesterol in the liver in order to facilitate the uptake of lipophilic nutrients and regulate cholesterol homeostasis [127, 132]. Structurally, bile acids are composed of four rings of steroid structure, terminating in a five- or eight-carbon side-chain of a carboxylic acid [133]. The four rings are termed A, B, C, and D from left to right, with the D-ring always having one carbon less than the other three (**Figure 7**). The hydroxyl groups can be located up (termed β) or down (termed α). All bile acids have a 3-hydroxyl group which is derived from cholesterol [133].

1.5.2 Bile acid synthesis

The human bile acid pool consists of the primary bile acids chenodeoxycholic acid (CDCA) and cholic acid (CA) synthesized from cholesterol in the liver, along with the secondary bile acids - deoxycholic acid (DCA, from CA), lithocholic acid (LCA), isolithocholic acid (ILCA) [134] and allolithocholic acid (ALCA) [135] (all from CDCA) - created in the intestinal lumen through the actions of bacterial enzymes. Other vertebrate species have bile acids, but these have evolved differently along with the gut microbiota. Mainly, the differences are in the configuration of steroid nucleus and side-chains, including hyocholic acid in pigs [136], α- and β-muricholic acid (MCA) in rodents, and ursodeoxycholic acid (UDCA) in bears [137].

Also, even before the primary bile acids are secreted from the hepatocyte into the lumen of the bile canaliculi, more than 98% are conjugated via an amide bond at the terminal (C24) carboxyl group to one of the amino acids glycine or taurine. The resulting molecule is termed conjugated bile acid and the conjugation makes them more readily excretable into the bile and renders them less cytotoxic [138].

The synthesis of bile acids involves a total of 17 individual enzymes that catalyze a complex array of reactions in the endoplasmic reticulum, mitochondria, cytosol and peroxisomes [137]. Synthesis takes place through one of two pathways, the classic and the alternative pathway. In the classic pathway of bile acid synthesis, which accounts for approximately 90% of total bile acid production, hydroxylation of cholesterol at the 7α position is the first step. This reaction is rate-limiting and catalyzed by CYP7A1 [139]. 7α-hydroxycholesterol is then converted into 7α-hydroxy-4-cholesten-3-one (C4) by 3b-hydroxy-D5-C27-steroid dehydroxylase, which in turn can be hydroxylated by sterol 12α-hydroxylase (CYP8B1) to form CA or directly used to generate CDCA, and the CA to CDCA ratio is thus a marker of CYP8B1 function. In the alternative pathway, which accounts for less than 10% of the total bile acids produced under normal conditions, the C27 of cholesterol is oxidized in a reaction that is catalyzed by the mitochondrial enzyme sterol-27 hydroxylase (CYP27A1), followed by hydroxylation at the C-7 position by oxysterol 7α-hydroxylase (CYP7B1) to form oxidation products that are then converted to CDCA [140-143]. The major bile acid synthesis and conjugation are shown in **Figure 7**.

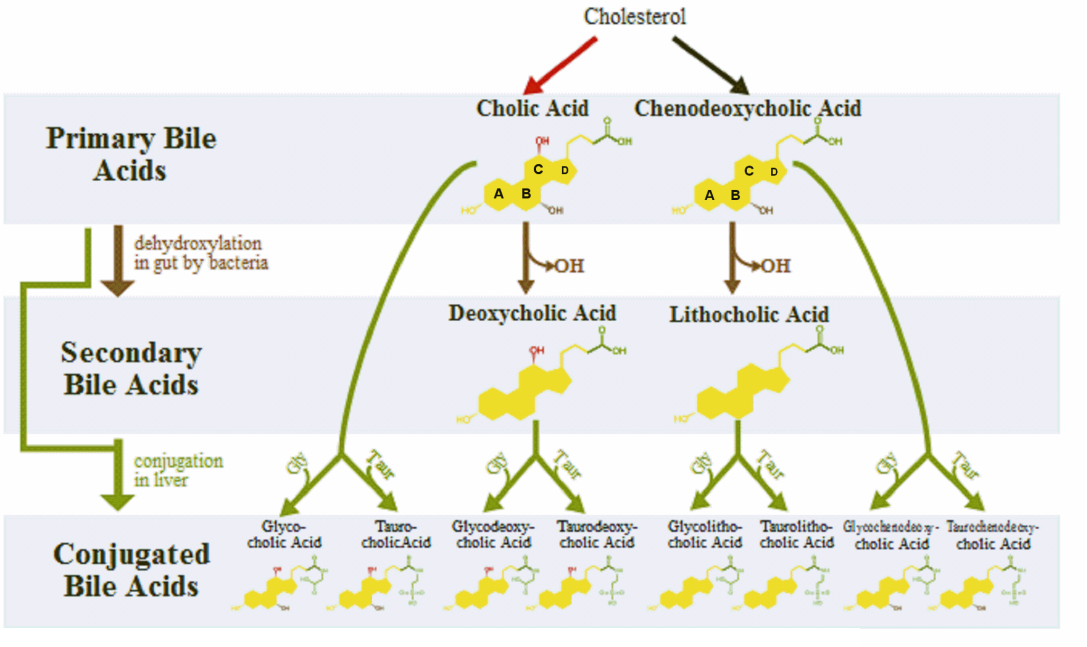

Figure 7. Major primary and secondary bile acid synthesis and conjugation.
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1.5.3 **Bile acid transport**

Following conjugation, bile salts synthesized in the hepatocyte are secreted into the bile canaliculi whence they flow into the larger bile ducts and finally enter the gallbladder. At the canaliculi membrane of hepatocytes, the ATP-binding cassette (ABC) transporter family member bile salt export pump (BSEP) is the major transport protein mediating efflux of bile salts, working against a concentration gradient with 100-1000 fold higher concentrations in the bile than in hepatocytes [144, 145]. In addition, Mrp2 and MDR1A also export bile acids into the bile, along with a range of organic anions such as bilirubin glucuronides, glutathione-S-conjugates and drugs [88, 146]. Hepatocytes also export bile acids into the systemic circulation through both OST-α and OST-β as well as Mrp3 and Mrp4 [147-150].

Bile salts are stored in the gall bladder, and food intake stimulates gallbladder contraction to release bile into the duodenum. In the gut, bacteria modify the majority of bile salts to generate secondary bile acids. Around 95% of the resulting bile acids (modified and unmodified) are then reabsorbed in the ileum through the enterocyte apical sodium-dependent bile transporter (ASBT) which is highly expressed in the brush border membrane. On the basolateral cell membrane of the same cells bile acids are re-exported into the blood stream (transcytosis) by organic solute transporters (OST)-α and -β, allowing their return to the liver via the portal circulation [148, 151]. There the recycled bile acids are taken up by hepatocytes using sodium (Na+)-taurocholate cotransporting polypeptide (NTCP) [152] and organic anion transporters (OATPs) such as OAT1B1 and OAT1B3 [153]. While NTCP is the primary conduit for conjugated bile acids, OATPs carry conjugated and unconjugated bile acids as well as cardiac glycosides, steroids, peptides *etc* [154-157].

The entire process is referred to as the enterohepatic circulation of bile acids, and results in a loss of only about 5% of secreted bile acids into the feces. This fecal loss is compensated by *de novo* synthesis in the liver as described above. Of the reabsorbed bile acids, 90% are taken up by hepatocytes already on their first passage through the portal vein. Those that escape the liver and enter the systemic circulation are to some degree cleared by the kidney, as are bile acids exported by the hepatocytes directly into the bloodstream. However, healthy human urine contains only minute amounts of bile acids [158], as most bile acids filtered are reabsorbed in the renal proximal tubular epithelium, mainly through its' expression of ASBT. In addition OST-α, -β and Mrp3 are expressed basolaterally in these cells, and are thought to mediate the re-export of bile acids back into the systemic circulation [159]. An overview of the circulation of bile acids is shown in **Figure 8**.

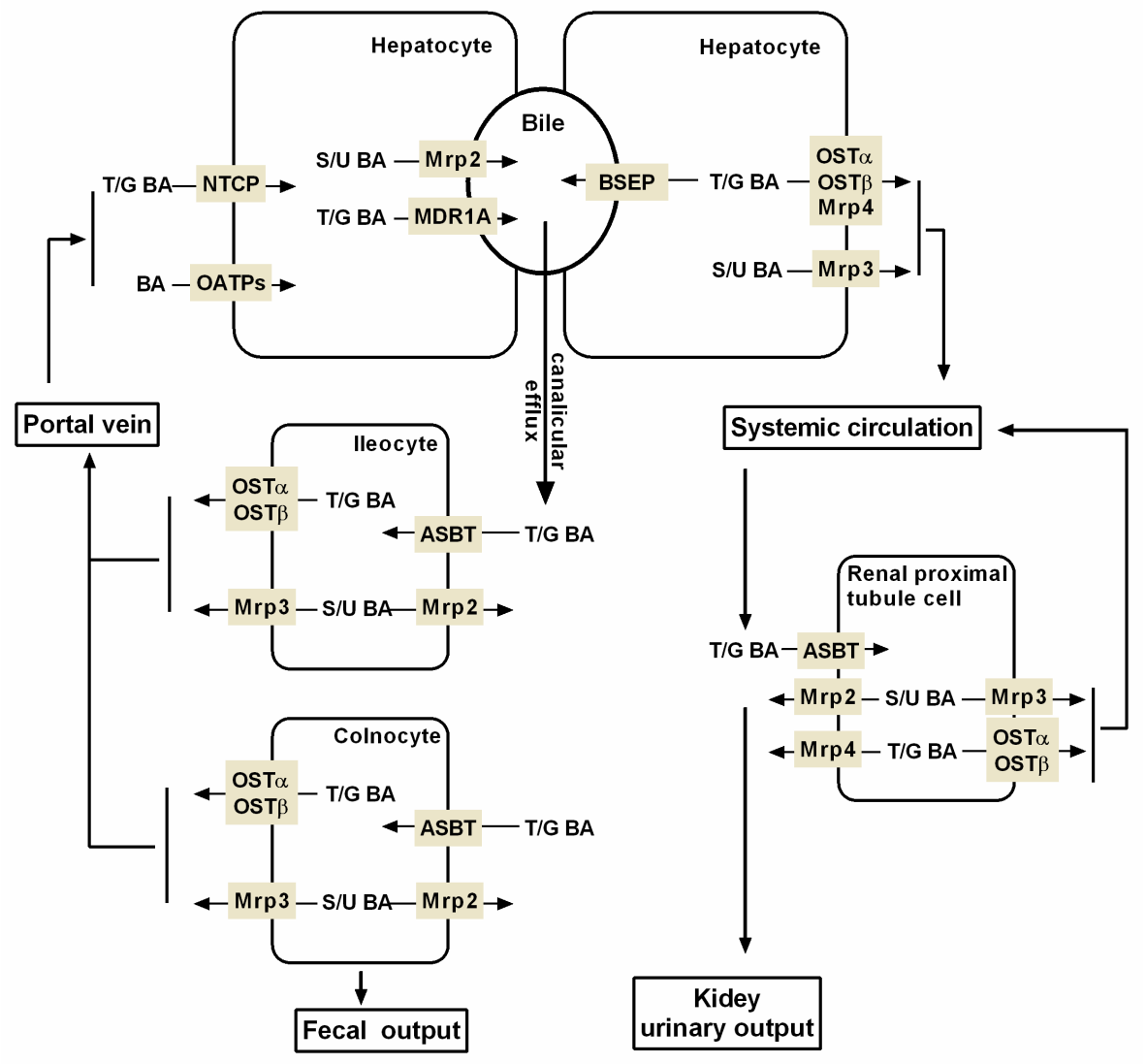

Figure 8. Circulation of bile acid. *T/G BA: tauro- or glycol-conjugated bile acids; S/U BA: sulphated or glucuronidated bile acids.*

1.5.4 **Bile acid regulation**

The amphipathic properties of bile acids make them able to integrate into cell membranes and lipid particles, a property that renders them cytotoxic at higher concentrations either intracellularly or extracellularly [160-162]. Thus the transcription of enzymes involved in synthesizing bile acids is tightly regulated, mainly by nuclear hormone receptors and other transcription factors that work together to maintain bile acid homeostasis [137]. In the hepatocytes, bile acids are natural ligands of the farnesoid X-receptor (FXR), a key nuclear receptor that activates multiple pathways of bile acid, glucose and lipid homeostasis as well as regulating certain inflammatory responses [127, 163-165]. Upon binding to bile acids, FXR induces the transcription of the short heterodimeric partner (SHP) gene, which in turn acts to suppress CYP7A1 [166, 167]. Likewise, NTCP has been reported to be regulated by bile acid-activated FXR via induction of SHP in primary rat hepatocytes, HepG2 and Cos cell lines [168]. Also, FXR has been shown to directly regulate OATP1B1 and OATP1B3 expression in Huh-7 or HepG2 cell line [169]. Hepatocytes nuclear factor-1α (HNF1α) has been reported to repress CYP27 transcriptional activity by binding to its promoter [170].

Many of the enzymes and transporter proteins that metabolize and transport bile acids are intimately involved in the processing of xenobiotics [171]. Perhaps for this reason, many of these proteins are regulated by three well-studied ligand-activated nuclear receptor transcription factors: pregnane X receptor (PXR), constitutive androstane receptor (CAR) and vitamin D (1, 25- dihydroxyvitamin D3) receptor (VDR). Thus treatment of human hepatocytes with a PXR ligand (rifampicin) has been shown to increase OATP1B1 and decreased OATP1B3 and BSEP mRNAs [172]. However, similar results were not seen for Mrp2 and 3, and later studies have indicated that their regulation is complex and may involve one or more of PXR, CAR, FXR and VDR [173-176]. Mrp4 expression increases after activation of CAR in both primary human hepatocytes and HepG2 cells [177].

Data also support roles for PXR, CAR and VDR in the regulation of multiple genes involved in both Phase I and II reactions of drug metabolism, including CYP1A, CYP2B, CYP3A, sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs) [178]. Finally, OST-α and -β are both up-regulated by FXR in Huh7 and HepG2 cell lines, and this increase could be blocked through pre-treatment with FXR siRNA [177]. In summary, a role for NR in the regulation of bile acid synthesis and transports is evident, but the exact mechanisms and links to specific pathological conditions still need to be further elucidated [171].

1.6 HEPATIC METABOLISM IN UREMIA

1.6.1 Glucose

The liver plays a unique role in carbohydrate metabolism through its ability to take up and store glucose as glycogen, or to make new glucose through gluconeogenesis as required. In the postprandial state, blood glucose is taken up via the glucose transporter type 2 (GLUT2) on hepatocytes, following immediately by either oxidization for the production of energy (ATP), glycolysis catalyzed by glucokinase or utilization for glycogen synthesis by glycogen synthase. Conversely, in the fasting state the liver generates glucose both through the breakdown of stored glycogen and *de novo* synthesis (gluconeogenesis).

In 1910, Newbauter *et al.* [179] first described hyperglycemia in the setting of uremia. Since then abnormalities in carbohydrate metabolism in CKD patients have been reported by many investigators [180-182]. Abnormal results on an oral glucose tolerance test (OGTT) may occur in over 50% of individuals with CKD stages 3-5 [180, 183]. Despite this the vast majority of non-diabetic patients with uremia are euglycemic when sampled fasting, but many exhibit increased circulating insulin levels and an impaired glucose disposal rate on intake [29, 34, 184].The mechanisms underlying this impaired glucose metabolism have been studied by a few researchers, who have proposed causative factors including metabolic acidosis, toxic substance accumulation (eg. free fatty acids, antagonistic insulin hormones, pseudouridine, *etc*) or an increase in gluconeogenesis [184-186].

Insulin is an anabolic hormone produced by β-cells in the pancreas. It is the qualitatively most important regulator of carbohydrate disposal in the postprandial state; it is also involved in lipid metabolism. Key effects of insulin include an increase of glucose storage as glycogen in muscles and in the liver, inhibition of glucose synthesis by the liver, and promotion of lipid storage as TG in adipose tissue. As noted, insulin levels are often elevated in the setting of uremia. The reasons are unknown, but may include a reduced clearance in the proximal tubules and increased sympathetic nerve activity [187, 188].

In the 1980s, DeFronzo *et al*. described IR (separate from hyperglycemia) in CKD patients [29]. Following this report, several studies have used cellular and animal models to increase our understanding of the mechanisms but key facts are still missing. Friedman *et al.* [189] studied CKD patients using hyperinsulinemic euglycemic clamps and reported that the increase in insulin-stimulated glucose transport is significantly reduced (by 50%) in muscles of these patients as compared to controls. Subsequent studies have shown decreased tyrosine phosphorylation of IRS-1, abnormalities of PI3K signaling, as well as a decreased serine phosphorylation of downstream PKB/Akt in rodent CKD models [190, 191].

Regarding hepatic glucose production in uremic patients, there is no consensus. Friedman *et al*. [189] and DeFronzo, RA [192] both found no effect of uremia on hepatic glucose production using hyperinsulinemic euglycemic clamps, and studies of liver slices from uremic rats likewise uncovered no defects in hepatic glycogen metabolism [193]. However, Schmitz *et al.* [194] found that hepatic glucose production was reduced by hyperglycemia in controls but not in uremic patients, indicated an impaired suppression in these patients. Likewise, cultured primary rat hepatocytes have been reported to exhibit a blunted response in glucogenesis upon insulin stimulation [195]. More recently, 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1), a determinant of intracellular cortisone signaling, was found to promote IR in the livers of uremic in mice, leading to increased hepatic gluconeogenesis and lipogenesis [196].

There are also data that link the accumulation of specific uremic toxins to dysmetabolism of glucose and lipids. Urea (putatively by inducing oxidative stress) [197], ADMA [198] and uric acid [199] have all been reported to associate with IR in CKD patients. Of special interest, P-cresyl sulfate (a protein-bound uremic toxin) was recently shown to directly alter insulin signaling through activation of the extracellular signal-regulated kinases (ERK1/2) [200]. Clearly, future studies are needed to better characterize the nature, consequences and causes of IR in CKD.

1.6.2 Lipids and cholesterol

Dyslipidemia – especially an elevated TG, a reduced HDL and an increased VLDL concentration [34, 201] - is common in later-stage CKD, and not only together with proteinuria. Elevated blood cholesterol and TG have also been linked to an increased risk of CVD in CKD patients at both the pre-dialytic and dialytic stage [202]. Despite this association and the relationship between GFR and dyslipidemia [201], relative few studies have examined the role of hepatic lipid metabolism in the setting of uremia.

In 1999, Vaziri ND *et al.* [203] found down-regulation of hepatic Apo A-I gene expression in 5/6 nephrectomized rats, a finding consistent with the known decrease in plasma Apo A-I concentration in CKD patients. Han *et al*. [204] used a rat model of the nephrotic syndrome (characterized by proteinuria without reduced GFR, hypercholesterolemia and hypertriglyceridemia) to report increased hepatic SREBP-1, LXR α/β, FAS, ACC and HMG-CoA-reductase, as well as reduced CPT and L-FABP, on protein level. They suggested that increased hepatic fatty acid production and reduced fatty acid uptake by the liver may contribute to hypertriglyceridemia in the nephrotic syndrome. Kyubok *et al.* [205] reported a similar result in 5/6 nephrectomized rats, and suggested a ChREBP-mediated up-regulation of lipogenic enzymes (FAS, ACC) and downregulation of PPARα-regulated fatty acid oxidation (CPT-1A) and DGAT. Wang X

et al. [40] found the secretion rates of VLDL-TG and VLDL-apoB-100 were not changed between CKD patients and healthy controls, the hypertriglyceridemia in patients was associated with the impaired plasma clearance. Finally, recently Stegmayr *et al.* [206] found that the administration of heparin anticoagulation to HD patients promptly resulted in a release of both lipoprotein and hepatic lipase to the blood. They hypothesized that liver loss of these enzymes may result in a transient uptake deficiency engendering hypertriglyceridemia.

In another recent study, Ananda *et al.* [196] reported elevations in TG, total cholesterol and non-esterified fatty acids in uremic rats which also had high hepatic expression of ACC, FAS, SREBP1c, HMGCR and 11βHSD1. They suggested an increase in *de novo* lipogenesis and cholesterol synthesis in the liver and showed that inhibition of 11βHSD1 resulted in an improvement of dyslipidemia, suppressed hepatic gluconeogenesis and improved systemic insulin sensitivity.

1.6.3 **Alterations in xenobiotic metabolism**

CKD patients are often heavily medicated, while reduced renal function is well-known as a risk factor for adverse drug reactions [207-209]. Over 90% of common drugs are metabolized by CYP enzymes in the liver, whence they exit to be transported as inactive, water soluble compounds and excreted in the urine. In CKD, even without liver disease, drug clearance is impaired in proportion to the reduction in GFR [210]. This appears to primarily be the result of impaired hepatic CYP450 reactions [211], but is likely also affected by factors such as the decrease in plasma protein to bind drugs, reduced hepatic conjugation reactions (primarily glucuronidation and acetylation [212-215]) and the accumulation of bioactive metabolites destined for renal clearance that may exhort feed-back inhibition.

In animal models of uremia, protein levels of several P450 members including CYP2C6, CYP2C11, and CYP3A2 are all reduced along with their activities. Levels of hepatic CYP3A are also low [216, 217]. In addition, CKD has been associated with reduced hepatic expression of OATP transporters involved in drug absorption [218]. For example, a decrease in OATP2 expression was induced in primary rat hepatocytes after treatment with sera from 5/6 nephrectomized rats [218]. Likewise, sera from ESRD patients reduced mRNA levels of OATP1B1 and 2B1 in Hep3B cells as compared with healthy serum [219]. Moreover an *in vivo* study found an impaired rate of hepatic xenobiotic uptake (approximately 20% lower) in 5/6th nephrectomized as compared to control rats, consistent with the previously reported finds of OATP downregulation [220]. In another study, primary rat hepatocytes exposed to uremic serum decreased CYP2C and 3A on both mRNA and protein levels as compared to those exposed to healthy serum [217]. The

fractionation of these sera revealed that the greatest decrease occurred with cell exposure to the 10-15 kDa fraction [221] .

Specific uremic toxins have also been reported to directly inhibit CYP metabolism *in vitro*. A combination of the four uremic toxins benzyl alcohol, p-cresol, indoxyl sulfate and hippuric acid co-incubated with human liver microsomes resulted in a decrease of more than 50% in the activities of CYP1A2, CYP2C9, CYP2E1, CYP3A4, UGT1A1, UGT1A9 and UGT2B7. P-cresol was found to be the most potent individual inhibitor of the four [222]. Volpe *et al.* In a similar study showed that clinically relevant concentrations of CMPF, hippuric acid and p-cresol inhibit CYP3A4 metabolism in human liver microsomes [223]. Another study combined CMPF, 3-indoxyl sulfate, indole-3-acetic acid and hippuric acid and also reported a decrease in CYP3A4 mRNA for the four together but not when tested one by one. The authors speculate that this may indicate a cooperative mechanism of action for these uremic toxins [224].

1.7 FIBROBLAST GROWTH FACTOR (FGF) 19

1.7.1 The FGF family

The family of FGF is well known as regulators of developmental processes including differentiation, embryonic development, and angiogenesis [225]. Twenty-two human FGF family members are currently known. Of these, only FGF-19, 21 and 23 lack a heparin-binding domain and instead utilize a co-receptor from the Klotho gene family to position the ligand in relation to its' cognate FGF receptor [226]. This reduced affinity for heparin is postulated to prevent these three FGFs from adhering to extracellular membranes, thus allowing them to circulate and reach distant sites of action in an endocrine manner.

The study of FGF-19 in man has been slow to take off. Most of what we believe to be true for FGF-19 comes from studies of the mouse orthologue, Fgf-15. However, it should be noted that the homology between mouse Fgf-15 and human FGF-19 is only 40%, while most other FGF orthologues share more than 90% amino acid identity [227, 228]. Furthermore, Fgf-15 mRNA is mainly expressed in the ileum of adult mice while human FGF-19 mRNA has been reported to be expressed in significant quantities in fetal brain, skin, and retina, as well as in adult gallbladder, liver, kidney, spleen, heart, and leukocytes [229, 230]. Of the other two endocrine FGFs in humans, FGF21 is mainly expressed in pancreas and testis where it is regulated by PPARα, but it may also be found in the liver after fasting or under a ketogenic diet. Human FGF23 is mainly produced by osteocytes and osteoblasts and is regulated by VDR [231]. All three of these FGFs also exhibit large

individual variations, with median fasting levels (pg/mL) of 115 (47.4-349; FGF-19), 156 (28.8-1844; FGF21), and 42.5 (21.2-87.0; FGF23) respectively [227, 232-234].

1.7.2 FGF-19 signaling and metabolism

In humans, FGF-19 has been suggested to function as an enterohepatic signal exerting negative feed-back on the hepatic synthesis of bile acids. After a meal, bile acids released into the intestines stimulate the expression and/or release of FGF-19 by enterocytes, resulting in increased circulating levels. Holt *et al.* in 2003 treated human primary hepatocytes with the FXR agonists GW4064 and CDCA and demonstrated a large increase in FGF-19 mRNA expression, which were undetectable at baseline. The same group also uncovered a functional FXR responsive element in the FGF-19 promoter [235, 236].

For signal transduction FGF-19 probably requires the presence of both its receptor FGFR4 and the transmembrane protein β-Klotho, abundantly expressed in the liver. The Fgf-15-FGFR4-β-Klotho complex has been reported to be a strong suppressor of CYP7A1 gene expression, and consequently of bile acid synthesis in the liver [236, 237]. Study also shown that the FGF-19-FGFR4-β-Klotho complex activates the MAPK/ERK1/2 pathway in human primary hepatocytes, but the downstream mechanism that results in CYP7A1 inhibition is still unclear [238] while the levels of FGF-19 needed to suppress were approximately 10-fold higher than those reported to occur in man [235, 239]. However, clinical studies have confirmed a role for FGF-19 in regulation of bile acid synthesis *in vivo*. Lundasen *et al.* [240] found that humans exhibit a diurnal rhythm of FGF-19 secretion, with peaks appearing 90-120 min after a postprandial rise in serum bile acid levels and always followed by a decline in bile acid synthesis.

In addition to the regulation of bile acid synthesis, researchers found that FGF-19 also plays a role in gallbladder filling [241] as well as putatively in glucose and lipid metabolism. Two studies have reported an increased metabolic rate following FGF-19 administration to an obese mouse model, a change accompanied by decreased liver TG content and a lower circulating cholesterol level [242, 243]. Recent data also implicate mouse Fgf-15 signaling in regulation of a physiologically important, insulin-independent endocrine pathway that stimulates hepatic glycogen production and protein synthesis, but not lipogenesis [244]. However, in humans serum FGF-19 levels have been reported to vary independently of lipids [234]. Finally, in mice Fgf-15 can also reduce the phosphorylation of CREB, thereby inhibiting PGC-1α binding to G6pase and PEPCK, repressing gluconeogenesis [245].

1.7.3 **FGF-19 in uremia**

Most cytokines and peptide hormones are elevated in CKD [246]. The molecular weight of FGF-19 is around 20 kDa, and hence circulating FGF-19 should have a relatively low sieving coefficient at the glomerular filter and a large proportion enters the renal tubules for endocytotic clearance. Indeed, few studies have reported FGF-19 in fasting humans (see above), while the presence of Fgf-15 in rat blood plasma remains unconfirmed [247]. In CKD only one previous report examining FGF-19 in the circulation exists. In a cross-sectional study of 60 fasting chronic hemodialysis patients and 60 controls, Reiche *et al.* found approximately 1.5 times higher concentrations in patients, where FGF-19 levels correlated negatively with biomarkers of inflammation [248].

2 AIMS

The overall aim of this thesis was to investigate putative hepatic signals and processes that may contribute to the described metabolic complications of chronic renal disease.

The specific aims of the included papers were:

- To investigate postprandial FGF-19 response in CKD patients in relation to insulin, glucose and TG levels (Paper I).

- To characterize the key pathways known or suspected to mediate and regulate the metabolism of glucose and lipids in human hepatocytes under uremic and healthy conditions (Paper II).

- To explore changes to bile acid metabolism pathways in human hepatocytes under uremic and healthy conditions and relate them to nuclear receptor and FGF-19 (Paper III).

- To assess physiological levels of FGF-19 and bile acids in the portal and systemic circulation (Paper IV).

3 METHODOLOGY

3.1 ETHICS

All studies were approved by the original ethics committee and informed consent was obtained from all patients. The thesis in its entirety conforms to the declaration of Helsinki as amended.

3.2 TISSUE DONORS

3.2.1 Patients

In **Paper I**, we enrolled ten non-diabetic CKD patients receiving maintenance hemodialysis (HD) since at least 3 months, thrice weekly and for at least 3.5 h per treatment. During the 8 weeks trial, 4 subjects dropped out (3 due to unrelated hospitalizations and one due to a failure to tolerate the test meal). Ten unrelated non-diabetic healthy subject (HS) were recruited by advertisement to serve as the control group. One control dropped-out by choice. Exclusion criteria were age under 20 or over 70 years, ongoing treatment for infection or inflammatory disease, mental illness, chronic bowel disease, a history of cholecystectomy and/or malabsorption and known liver disease. Following baseline assessment, each patient was treated with each one of N-acetyl cysteine (NAC) (dosage 600 mg per os b.i.d. = 1200 mg daily), freeze-dried blueberries (MP865; dosage 240 mg per os b.i.d. = 480 mg daily) or lactose placebo (dosage 200 mg per os b.i.d. = 400 mg. daily) in a double-blinded manner and random order with a 7 days treatment followed by a 7 days washout repeated three times.

At the end of each treatment period (day 7) and following an overnight fast of at least 8 hours, each patient was given a standardized high fat (from cream), carbohydrate-rich (sucrose) meal (700 kcal/m^2 of body surface area) followed by blood sampling at 6 time points over 4 h (see method below). The study design is illustrated in **Figure 9**.

In **Paper IV**, 75 patients were enrolled from those undergoing hepatic surgery at the Transplantation Unit of the Karolinska University Hospital in Huddinge. These surgeries constituted liver transplantation, living donation or liver resection. Patients with hepatitis B or C were excluded.

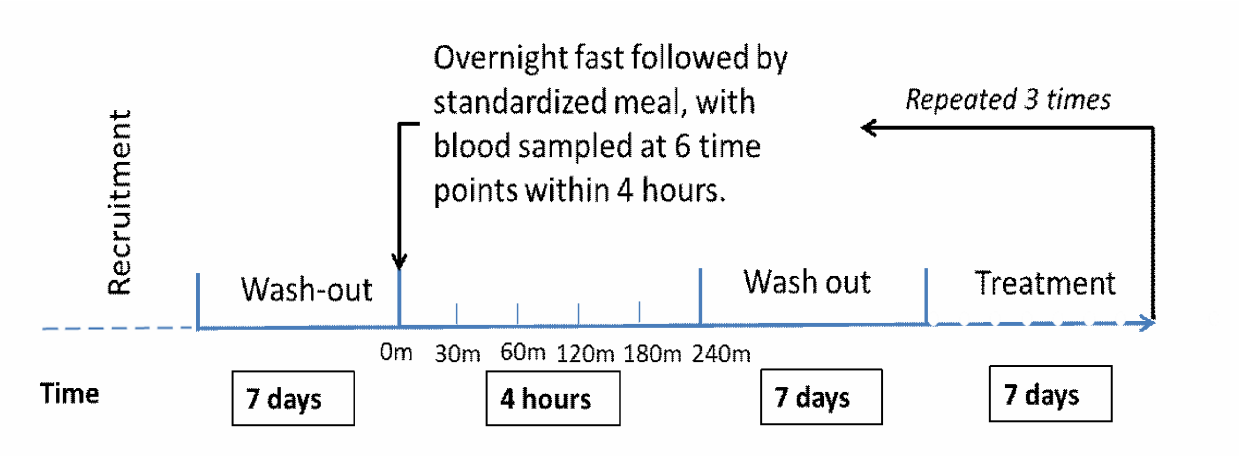

Figure 9. Illustration of study design used in Paper I.

During surgery, liver and gallbladder knife biopsies as well as bile from the gallbladder were collected. Portal and systemic (central venous and peripheral arterial) blood samples were taken as seen in **Figure 10**.

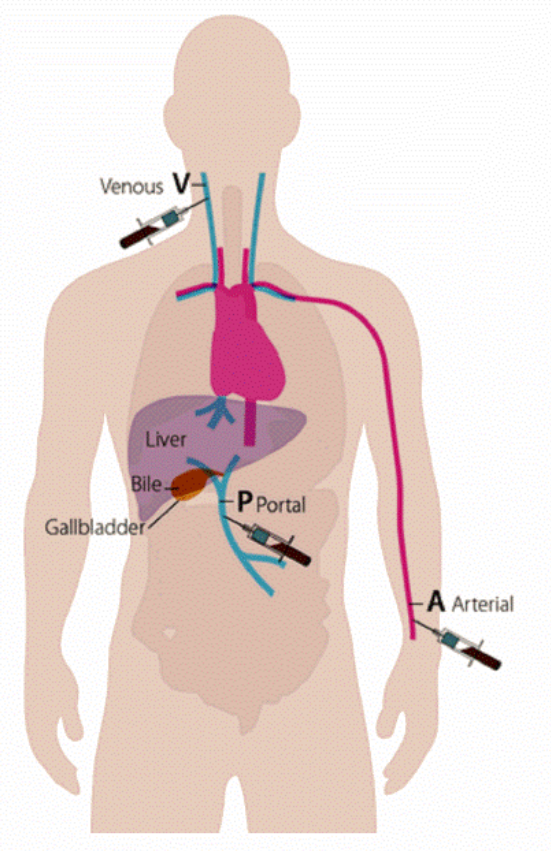

Figure 10. Schematic illustration of where blood samples were taken during liver surgery.

3.2.2 Uremic sera

For **Paper II** and **Paper III**, serum samples were collected from eight patients with severe CKD (GFR 9.4±1.6 mL/min/1.73 m^2) and treated with maintenance HD since at least 3 months, thrice weekly and for at least 3.5 h per treatment. Also from eight age- and sex-matched controls (GFR >80 mL/min/1.73 m^2) recruited by advertisement. Exclusion criteria were: 1) age under 40 or over 75 years; 2) diagnosed chronic disease in any organ other than the kidney; 3) ongoing immune-modulatory or other unconventional pharmacological therapy; and 4) an inability to give informed consent. Pre-made standard vacuum tubes (Vacutainer®, Becton Dickinson) were used to draw venous blood from the arm. The blood was sedimented at room temperature for 20 min, then centrifuged at 2,000 X g for 10 min at 5° C. The supernatant was immediately pipetted into clean polypropylene tubes in 0.5 mL aliquots and frozen at –70 °C. Pooled sera from 8 healthy and 8 patients were prepared separately.

Part of the sera was next dialyzed against DMEM over a 2 kDa molecular weight cut-off filter using Slide-A-Lyzer G2 (#7721, Thermo Fisher Scientific) run at +4° C for 24 h (1:100 v/v). The resulting filtrate was then frozen at -70° C for later use.

3.2.3 Liver donors

Freshly resected liver tissues were obtained between years 2012-2015 from patients undergoing surgery for metastatic cancer or from donor livers that could not be used for transplantation. Liver tissue was transported to the laboratory from the operation rooms in cold Eagle's Minimum Essential Medium (EMEM) within a short time from devascularization. In some cases the tissue was kept in University of Wisconsin preservation solution at +4° C overnight. Donor information regarding age, sex and the reason for surgery is summarized in **Table 3** below**.**

Table 3. Human liver donor demographics.

Donor ID	Age (yrs)	Gender	Diagnosis	Viability
HF171	68	Female	Metastasis (small bowel cancer)	81%
HF174	86	Male	Metastasis (colorectal cancer)	70%
HF175	78	Male	Hepatocellular carcinoma	50%
HF179	69	Female	Metastasis (colorectal cancer)	65%
HF180	16	Male	Healthy donor	77%
HF186	57	Male	Metastasis (colorectal cancer)	77%
HF187	42	Female	Leiomyosarcoma	78%
VF13	47	Female	Cardiac arrest	75%
HF195	45	Female	Metastasis (colorectal cancer)	67%
HF196	72	Male	Metastasis (colorectal cancer)	79%
HF202	69	Female	Metastasis (colorectal cancer)	69%
HF208	32	Female	Cholangiocellular cancer	74%
HF211	79	Male	Metastasis (colorectal cancer)	75%
HF214	70	Female	Cholangiocellular cancer	74%
HF217	73	Male	Metastasis (colorectal cancer)	70%
HF218	57	Female	Metastasis (colorectal cancer)	84%
HF244	48	Female	Metastasis (colorectal cancer)	90%
HF319	64	Female	Alcoholic chirrosis, EX-plant	73%
HF320	68	Male	Metastasis (colorectal cancer)	69%
HF321	53	Female	Metastasis (colorectal cancer)	67%
HF322	25	Female	Metastasis (colorectal cancer)	85%
HF356	74	Female	Metastasis (colorectal cancer)	74%
HF360	74	Male	Metastasis (colorectal cancer)	74%
HF361	68	Female	Donor (cerebellar hemorrhage)	73%
HF362	26	Female	Progressive Familial Intrahepatic Cholestasis	71%

3.3 ISOLATION OF PRIMARY HUMAN HEPATOCYTES

Hepatocyte isolation was performed in a two-step perfusion procedure as previously described by Strom *et al* [239]. Briefly, the liver tissue was first perfused using catheters sutured into the main hepatic vessels and connected to a peristaltic pump. Hank's balanced salt solution (HBSS) with 0.5 mM EGTA was used to remove calcium and irreversibly disrupt the desmosomal connections between cells, followed by a washing step with only HBSS. The liver tissue was then was perfused with 250 mg/L collagenase (Collagenase XI, Sigma) dissolved in EMEM until the tissue was completely digested (approximately 20 min). All perfusion solutions were preheated and maintained at 37^{o}C to preserve enzyme activity. Next, the liver capsule was cut using scissors and the parenchymal tissue chopped in ice-cold medium to release the hepatocytes. These were isolated using filtration and washed three times by centrifugation at 50 g for 5 min to remove any remaining collagenase and non-parenchymal cells. The trypan blue test was used to calculate cell viability and concentrations before the cells were plated.

3.4 CULTURE OF HUMAN PRIMARY HEPATOCYTES

Viable cells were seeded onto collagen-coated (collagen made in-house from rat tail [249]) 6-well plates at a density of 1.5 million cells/ well and cultured under standard conditions in William's E medium supplemented with HEPES (25 mM), glutamine (2 mM), insulin (1.2 nM), dexamethasone (100 nM), amphotericin B (0.05 μg/mL) and gentamicin (0.05 mg/mL). Five percent FBS was added for the first 2 hours after seeding, thereafter serum-free medium was used until the experiments.

Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2:95% air and medium changed daily during the experiment (total time 5 days). Based on a dose- and time-response analysis (**Paper II & III**), thus 5% sera and 24h incubation time was selected. Briefly, in this analysis primary human hepatocytes were treated with 0%, 5%, 10% or 20% sera for 1h, 6h and 24h, followed by assessment of mRNA levels of G6PC, PCK1, CYP1A1, FGF-19 and CYP7A1. The lowest serum concentration giving the strongest response was chosen along with the culture time that yielded the greatest changes. For this and all subsequent experiments we added the test sera to the culture medium on day 4. Cells were harvested on day 5 for isolation of RNA and protein and medium was frozen for later analysis. In **Paper II,** insulin treatment was performed during 6 h before harvesting RNA and for 15 min before harvesting protein (**Figure 11)**. Also in **Paper III**, some experiments entailed culturing cells with either CDCA or GW4064 for 24h before harvesting RNA and protein. Thus, sera treatment and FXR-agonist were added simultaneously.

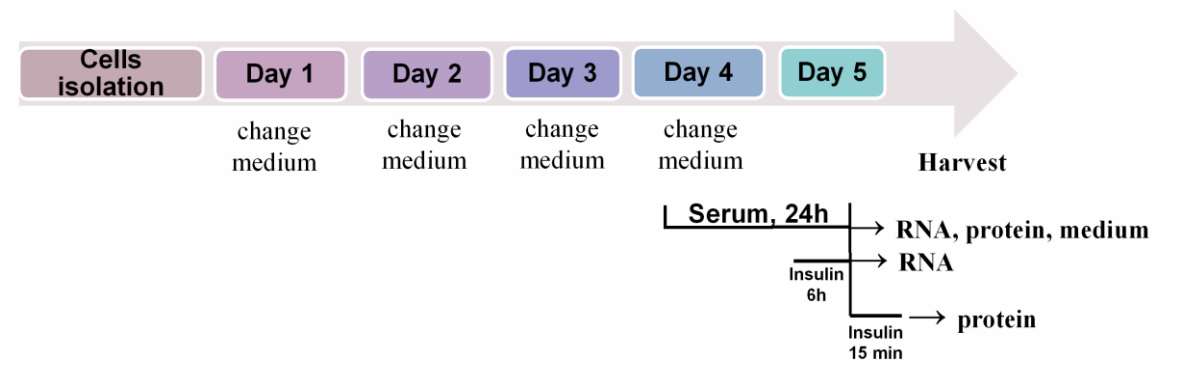

Figure 11. Schematic diagram of the cell experiments.

3.5 RNA ISOLATION AND REAL TIME PCR ANALYSIS

Total RNA was isolated from harvested hepatocytes using TRIzol Reagent (#15596-018; Invitrogen) and according to the manufacturer's instructions, and then stored at -70 ^{o}C. The RNA was quantified and quality assessed with a NanoDrop ND-100 spectrophotometer. Concentration was determined at 260 nm and purity by 260/280 nm ratio. Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitors (#4374966, Invitrogen). Real-time PCR assays of relative mRNA expression were performed using TaqMan Real Time PCR (StepOnePlus, Applied Biosystems). TaqMan PCR probes were bought from TaqMan Gene Expression Assays (Applied Biosystems) as described in **Paper II, III and IV**. Data were calculated by linearization of measured Ct values corrected by those of the housekeeping gene Cyclophillin A in the same cDNA preparation.

3.6 GLUCOSE PRODUCTION MEASUREMENT

Glucose production was measured as previously described [250, 251]. Briefly, primary human hepatocytes were washed with warm PBS to remove glucose. Cells were then pre-treated with 100 nM in serum-free Dulbecco's modified Eagles' medium (DMEM) for 15 min, followed by stimulation with cAMP (100 μM) and dexamethasone (50 nM) for 3 h in serum-free DMEM with or without gluconeogenic substrate (2 mM sodium pyruvate). Cell culture media was harvested and glucose concentration assessed by Konelab 20XT centrifugal analyser (Thermo Electron Corp.). The glucose concentration in each case was normalized to cellular protein concentrations. The total glucose production from both glycogenolysis and gluconeogenesis was measured in the presence of gluconeogenic substrate (sodium pyruvate, # 11360-039, Thermo Fisher Scientific), while glycogenolysis was measured in the absence of pyruvate. Glucose production via

gluconeogenesis was calculated as the difference between total glucose production and glycogenolysis.

3.7 WESTERN BLOT ANALYSIS

Primary human hepatocytes were washed twice in ice-cold PBS, then lysed and stored at -70° C. A Bio Rad protein assay kit (#500-0006, Bio Rad) was used to determine protein concentrations. After SDS-PAGE, proteins were transferred onto the nitrocellulose membranes using the iBlot dry blotting system (#IB23001, Thermo-Fisher Scientific) or using a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with the StartingBlock blocking buffer (##37543, Thermo-Fisher Scientific) for 30 min at room temperature. The membranes were then incubated with the primary antibody overnight at 4° C, and with the secondary antibody for 2 h at room temperature. The readout was detected using SuperSignal West Dura Extended Duration Substrate (#34075, Thermo-Fisher Scientific) according to the manufacturer's instructions, with the chemiluminescent signals captured by a Vilber Lourmat UV-instrument. Image J software (U.S. National Institutes of Health) was then used to quantify band intensity.

Antibodies for blotting were purchased from: Akt2 Rabbit mAb (#3063, Cell Signaling Technology(CST)); pAkt (Ser473) rabbit mAb (#4060, CST); pAkt (Thr450) Rabbit mAb (#12178, CST); FoxO1Rabbit mAb (#2880, CST); pFoxO1 (Ser256) antibody (#9461, CST); FoxO3a Rabbit mAb (#12829, CST); pFoxO1 (Thr24)/FoxO3a(Thr32) antibody (#9464,CST); Pan-Actin Rabbit mAb (#8456, CST); anti-rabbit IgG HRP-linked secondary antibody (#7074, CST); anti-AKR1D1 (#ab113503; Abcam), anti-OSTA (#ab103442; Abcam), anti-OST-beta (#ab121285; Abcam), anti-CYP8B1 (# ab129845; Abcam), anti-beta Tubulin (# ab179513; Abcam), anti-NTCP (#ab133670; Abcam), and anti-BSEP (#GTX102608; GeneTex).

3.8 PHOSPHORYLATION PROFILING ASSAY

In a subset of samples (**Paper II**), the Proteome Profiler Human Phosphokinase array (#ARY003, R&D Systems) was used to assess kinase phosphorylation at 43 well-characterized sites linked to intracellular signaling and according to the manufacturers' instructions. Briefly, proteins were lysed using the kit lysis buffer, and then incubated with the antibody-spotted membranes overnight at 4°C. Following repeated washing, the detection antibody cocktails were incubated with the membranes for 2 h at room temperature. Phosphorylated kinases that bound to the membranes were visualized by streptavidin–HRP chemiluminescence using the Vilber Lourmat UV-instrument.

3.9 ENZYME-LINKED IMMUNOASSAYS (ELISA)

The concentration of ApoB in the cell medium was measured using the Human ApoB ELISA kit (#3715-1HP-2; Mabtech), whereas total-, phospho (Ser612) - and phospho (panTyr)-IRS-1 were analyzed with PathScan IRS-1 sandwich ELISA kits (#7328, #7332C and #7133C; CST) according to the manufacturer's instructions.

3.10 LIPID STAINING

Intracellular lipid accumulation was assessed using the LipidTOX Green neutral lipid stain (#H34475, Thermo-Fisher Scientific) according to the manufacturer's instructions. Cells were fixed with 4% formaldehyde for 20 min at room temperature, washed three times with PBS, and then incubated with the dye for 40 min at room temperature. Nuclei were stained using NucBlue Live ReadyProbes reagent (# R37605, Thermo-Fisher Scientific). Stained tissues were visualized on an Olympus IX 71 inverted epi-fluorescence microscope.

3.11 LIPID EXTRACTION AND QUANTIFICATION

Cells were washed twice with PBS then incubated with hexan: isopropanol (3:2) for 1 h at room temperature on an orbital shaker. After transfer into glass tubes, drying was performed under N_2 at 40°C. The dissolved dried lipids were extracted in $CHCl_3$ and dried under N_2 at 40°C. A mixture of $CHCl_3$ and 1% Tritox-X-100 was then added and the samples were evaporated under N_2 at 40°C. The dried lipid extracts were dissolved in water and clarified by heating at 60°C. TG concentration was then assessed by a commercial colorimetric kit (#11730711216, Roche Diagnostics GmbH), with absorbance read at 492nm using a Tecan plate reader. The reading was normalized to total cell protein concentration.

3.12 BILE ACIDS EXTRACTION AND ANALYSIS BY GC-MS

Total bile acids (LCA, DCA, CDCA, CA and UDCA) were extracted from serum and bile as previously described [252]. Briefly, serum was mixed with deuterium-labelled internal standard (D_4-CA, D_2-CDCA, D_4-DCA and D_4-LCA) and hydrolyzed with 1M potassium chloride overnight at 120°C. Bile acids were extracted by basic ether extraction followed by acidic ether extraction, then washed until neutral with water. The extracted bile acids were methylated with trimethylsilyl diazomethane then derivatized using hexamethyldisilazane and trimethylchlorosilane in pyridine. Samples were quantified with GC-MS (6890 Network GC system/5973 Network mass selective detector, Agilent Technologies) and analyzed using the MassHunter Workstation software (Agilent Technologies).

3.13 BILE ACID EXTRACTION AND ANALYSIS BY HPLC-MS/MS

To measure conjugated and free bile acids, serum and cell culture medium were processed according to a modified protocol initially described by Tagliacozzi et al [253]. Samples were mixed with internal standards consisting of D_5-CA, D_4- UDCA, D_4-LCA, D_4- TCA, D_4- GCA D_4-GUDCA, D_4-GCDCA, D_4-DCA and D_4-GLCA. The mixture was centrifuged at 13 000 x g for 15 min and the upper phase was transferred to a glass centrifuge tube and evaporated under N_2. The residue was dissolved in MeOH and the tubes rinsed with a solution containing 40% MeOH, 0.02% formic acid and 10 mM ammonium acetate. The sample solution was transferred to Water's vials containing 3 parts 100% MeOH and 1 part 40% MeOH, 0.02% formic acid and 10 mM ammonium acetate. A Waters LC-MS/MS Micromass Quattro Micro 2695, equipped with a C18 reverse- phase column and ESI in negative mode was used for analysis. The result was evaluated with MassLynx software (Waters).

3.14 RNA SCOPE

RNAScope 2.0 kit and RNA probe (Hs-FGF-19, cat# 553981, Advanced Cell Diagnostics) were used to detect RNA expression in tissue sections by *in situ* hybridization. Briefly, liver and gallbladder tissues were fixed in formalin, dehydrated, paraffin embedded and sectioned onto glass slides. The sections were then baked at 60^{o}C and deparaffinized before pretreatment, hybridization and amplification according to the manufacturer's protocol. Next, signal was detected with 3, 3'-diaminobenzidine (DAB) and slides were counterstained with Gill's Hematoxylin I (Sigma-Aldrich). Finally samples were dehydrated and mounted with Mountex (Histolab) before detection of FGF-19 using an Olympus 1X71 microscope. The limit for background staining was determined as one dot per every ten cells according to the manufacturer's guidelines.

3.15 OTHER LABORATORY ANALYSIS

Serum glucose, insulin, urea and total protein were evaluated using a Konelab 20XT centrifugal analyser (Thermo Electron Corp.). VLDL-TG and VLDL-total cholesterol in cell culture medium were measured by fast protein liquid chromatography (FPLC) as previously described [254].

3.16 STATISTICAL ANALYSES

Data are presented as mean ± SEM. The significance of differences between groups was tested using paired Student's t-tests after log transformation, or one-way ANOVA as appropriate. Statistical significance was set at $p<0.05$. All statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software Inc).

4 RESULT AND DISCUSSION

4.1 ALTERED POSTPRANDIAL FGF-19 RESPONSE IN CKD

In **Paper I**, we investigated the links between time-dependent stimulated FGF-19 release and postprandial glucose, lipids, oxidative stress and inflammation in six CKD stage 5 patients on maintenance HD. Following an overnight fast, HD patients and HS received a standardized fat- and carbohydrate-rich meal, followed by 7 days treatment with an antioxidant compound: NAC or MP865 (freeze-dried blueberries) as well as placebo (lactose) and the meal procedure was repeated. A randomized (3 treatments but random order) and double-blind design was used (Eudra Clinical Trial Number 2005-004403-12).

The main finding of this paper was a delayed or blunted plasma FGF-19 response in CKD patients followed for 4 hours postprandially, with a significant improvement (but not normalization) seen following antioxidants therapy (NAC or MP865). This is an interesting observation that requires further investigation.

N-acetyl cysteine is a derivative of the amino acid L-cysteine, and has previously been reported to attenuate indoxyl sulfate-induced cell proliferation and tissue factor expression of vascular smooth muscle cells *in vitro* [255], reduce endoplasmic reticulum stress in macrophages exposed to uremic albumin [256], and to retard the decline of renal function in CKD patients over shorter time periods [257].

Meanwhile, MP865 (freeze-dried blueberries) contain at least the active ingredient anthocyanins. These polyphenols are found in a range of colored berries and are known to function *in vitro* as potent scavenger of oxygen radicals [258]. However, the exact role of this capacity *in vivo* remains unproven, and recent data also link anthocyanin to effects on mitochondria leading to a reduction in cytosolic cytochrome c and improved oxidative phosphorylation in ischemia-damaged mitochondria [259].

As either of the two compounds positively affected patient FGF-19 response to the same degree (basal versus NAC: −0.28, SE = 0.11, P = 0.01; basal versus MP865: −0.23, SE = 0.1, P = 0.03), one may speculate that their anti-oxidative properties may be involved. Indeed, CKD is well-known as a disease with a high burden of oxidative stress and in the study cohort we measured a high level of advanced oxidation protein products (AOPP) in the patients (although this value was unaffected by therapy and did not increase following the meal provocation).

In **Paper I** we also found the area under the curve (AUC) of FGF-19 to correlate with the AUCs for C-peptide and insulin over the same time, but not those of TG or glucose (**Figure 12**). These data do not fit with the few previous reports suggesting a role for

FGF-15/19 as a postprandial regulator of hepatic lipid and glucose homeostasis. For example, knock-in of FGF-19 in mice led to increased basal metabolic rate and lower TG levels in the liver [242], with a similar result has also observed in mice treated with FGF-19 [243]. When considering our data in relation to the earlier reports it must be kept in mind that our data were in humans.

Another potential confounder when interpreting our data on FGF-19 in the post-prandial state of CKD patients (**Paper I**) is the possible delay in gastric emptying known to occur in this group [260]. However, our data does not support such a delay in emptying. As shown in **Figure 12,** blood glucose and insulin rose rapidly in both groups, suggesting uptake from the meal. Furthermore, in both groups TG rose within 120 min of the meal and continued to rise for the duration of the follow-up (LDL did not change significantly during the study [34]). Thus, our data suggests both a timely and normal absorption of glucose (which normally starts in the mouth but takes place mainly in the intestinal epithelium and during the first 30 minute postprandially [261]) and fat (which is dependent on bile acids in the small intestine and on intestinal epithelium transcytosis). Regarding insulin, the healthy subjects exhibited a transient and rapid peak that left concentrations back at baseline levels at 240 min. Meanwhile the patients exhibited a higher rise and longer duration in insulin, but were still able to maintain blood glucose levels that were approximately the same as those in the controls or slightly higher.

Finally, regarding FGF-19, published studies of healthy subjects usually report a postprandial peak in serum levels 90-120 min after a postprandial rise in serum bile acids [240]. In **Paper IV**, bile acid and FGF-19 correlated strongly in both the portal vein and in the systemic circulation, but were not followed longitudinally. However, these patients were fasting and undergoing surgery so it is unclear if such longitudinal data would be comparable to these previous studies. In data not included in **Paper I**, we assessed plasma bile acids in all patients and controls only after the MP865 treatment period. As shown in **Figure 13,** in this analysis our control subjects had the expected response with a peak in plasma bile acid at 120 min and a subsequent rise in FGF-19. Patients meanwhile also exhibited a rise in bile acid approximately simultaneously, but their FGF-19 levels never rose convincingly at all. Taken together, our data suggests that reduced FGF-19 release by synthesizing cells is the most likely explanation for the observed reduction in circulating levels in CKD.

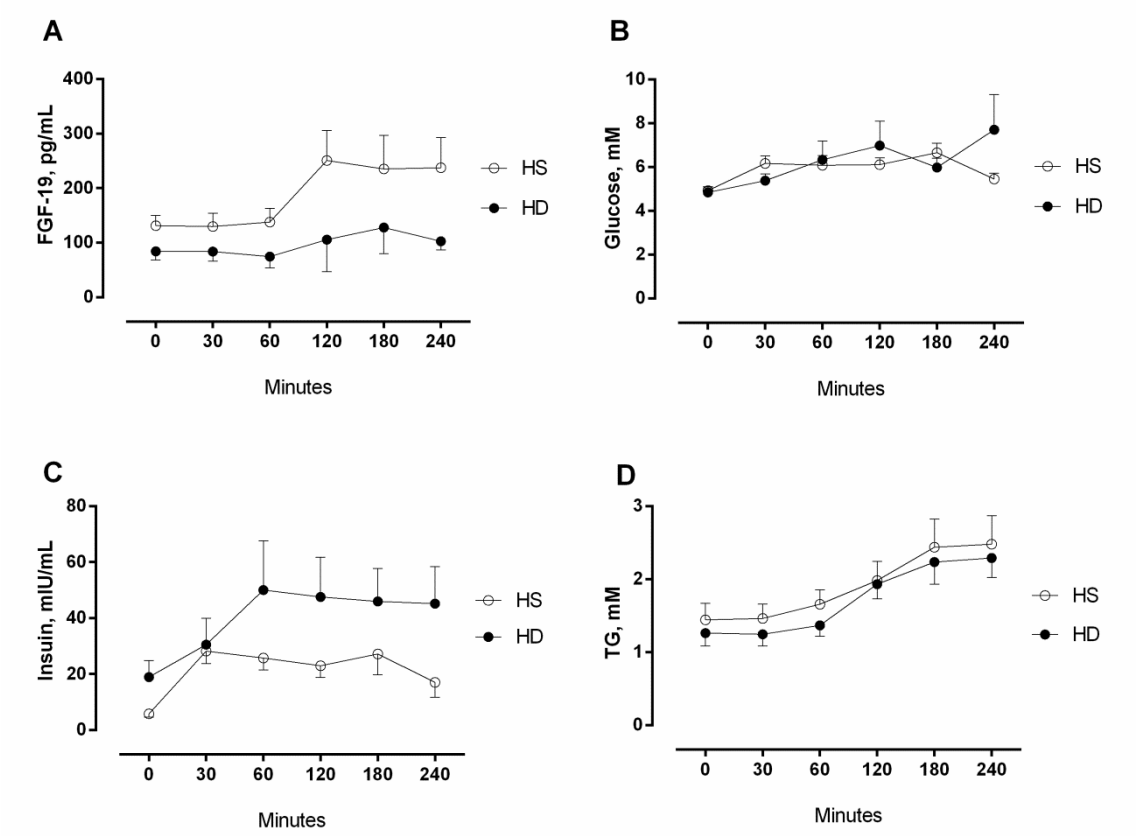

Figure 12. Postprandial level of (A) FGF-19, (B) glucose, (C) insulin, (D) TG. Grouped by healthy subjects (HS) or CKD patients on hemodialysis (HD).

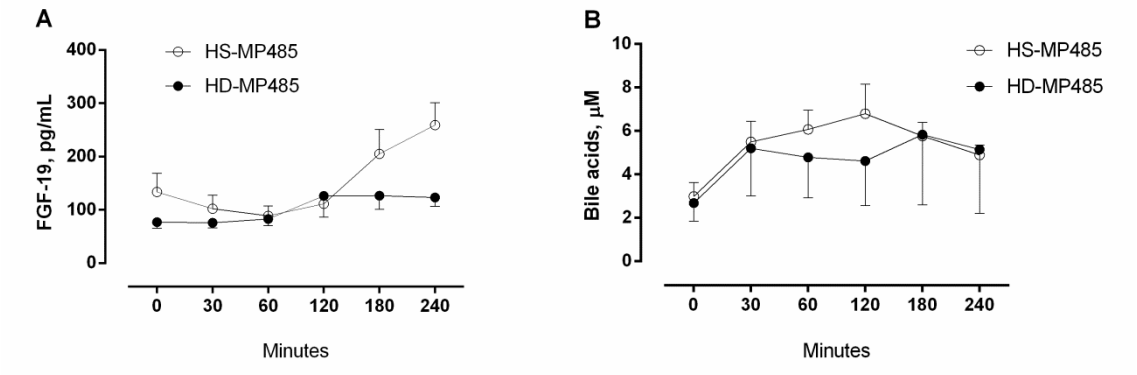

Figure 13. Postprandial FGF-19 and bile acids following 7 days of MP865 therapy. (A) FGF-19; (B) Total bile acids. Grouped by healthy subjects (HS) or CKD patients on hemodialysis (HD).

4.2 HEPATIC FGF-19 SIGNALING IS UNALTERED IN CKD

Bile acids are known to induce enterocytes to produce and release FGF-19 into the portal circulation, whence it reaches the liver. There it likely binds to its' receptor FGFR4 through a stabilizing co-factor, β-klotho, as reviewed above. This FGF-19-FGFR4-β-Klotho complex is believed to initiate a strong suppression of CYP7A1 through c-Jun N-terminal kinase (JNK)-signaling [235-237]. Recently, FGF-19 was also described as a direct target of nuclear FXR, while a functional FXR responsive element has been described in FGF-19 promoter [235, 236].

In **Paper III** we were unable to observe any differences in mRNA levels of the FGF-19, receptors FGFR4 and β-Klotho or of CYP7A1 between human primary hepatocytes treated with uremic or healthy sera (**Figure 14**). And there was also no difference in phosphorylation of the intracellular signaling molecules JNK (neither at amino acids T183/Y185 nor at T221/Y223) and c-Jun (at S63) (**Paper II**). This suggests that the altered FGF-19 signaling is not of hepatic origin in uremia, at least *in vitro*.

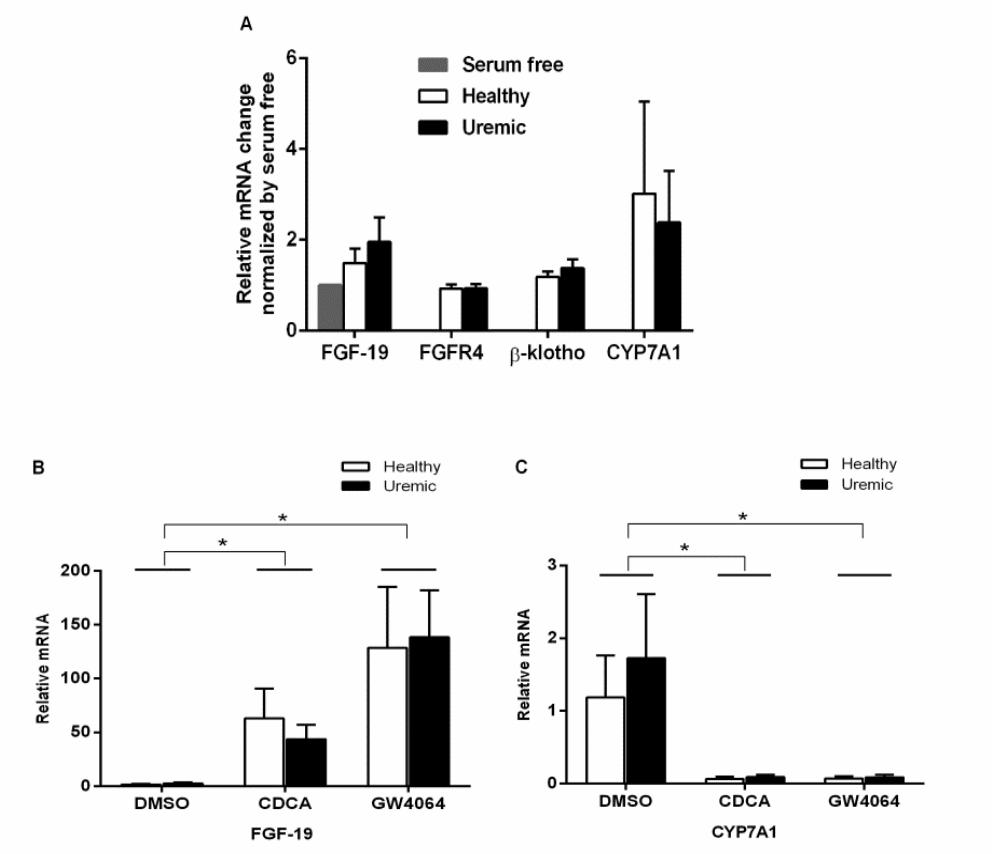

Figure 14. The *in vitro* effects of healthy uremic sera on mRNA of key components of the FGF-19 pathway with our without FXR agonism. (A FGF-19 signaling pathway; (B) FGF-19 mRNA expression after FXR agonists treatment; (C) CYP7A1 mRNA expression after FXR agonists treatment. Data are presented as mean ± SEM. * $p < 0.05$

Hypothesizing that FXR-signaling may be required for a robust FGF-19 response *in vivo*, we also investigated this pathway after pre-treatment with either the natural FXR-ligand CDCA or the synthetic agonist GW4064. Treatment with either agonist induced a rise in FGF-19 mRNA and a decrease in CYP7A1 mRNA as previously reported [235], but there were no differences between the sera treatment. We conclude that hepatic FGF-19 production is neither increased nor inhibited by uremic sera *in vitro*, nor is FGFR4/β-Klotho signaling disrupted. As FGF-19 *in vivo* is highly expressed in gall bladder and circulating FGF-19 may originate from gall bladder epithelium, further studies of uremic bile are needed.

4.3 CIRCULATING FGF-19 REFLECTS PORTAL CONCENTRATIONS

To better understand the physiological role of FGF-19 in humans, we next assessed FGF-19 and bile acid concentration in portal and systemic (peripheral arterial and central venous) blood and bile from 75 non-CKD patients undergoing liver surgery (**Paper IV**). As expected, both bile acid and insulin exhibited an obvious concentration gradient across the liver (portal vein compare to either arterial or peripheral venous blood). We found no such differences in FGF-19 concentrations (**Figure 15A**). Fasting before surgery may be a confounder as both FGF-19 and bile acid concentrations in blood have been reported to decrease during fasting [240], but as all subjects had measureable levels of bile acids and insulin we do not believe this to be an important explanation. We conclude that peripheral measurements of FGF-19 concentrations likely reflect also portal FGF-19 levels.

In **Paper IV** FGF-19 mRNA was very low or undetectable in liver biopsies, but high in gallbladder samples. Conversely, mRNA of CYP7A1, FXR, FGFR4, KLB and SHP were all significantly more abundant in the liver than in the gallbladder. Using RNA scope *in situ* hybridization staining on these tissues, we found FGF-19 mRNA only in the epithelial cells of the gallbladder (**Figure 16**). While Fgf-15 is predominantly expressed in the ileum of mice [236], FGF-19 expression in humans has primarily been found in the gallbladder, with only low levels in the ileum [262]. Our data offers confirmation of the previous human study [262] and reporting 60-fold higher concentrations of FGF-19 in bile than in serum (**Figure 15B**). The origins of circulating FGF-19 and its physiological role remain unclear. We conclude that hepatic FGF-19 release is not a major contributor to circulating levels in non-CKD individuals (and likely not in CKD-patients either).

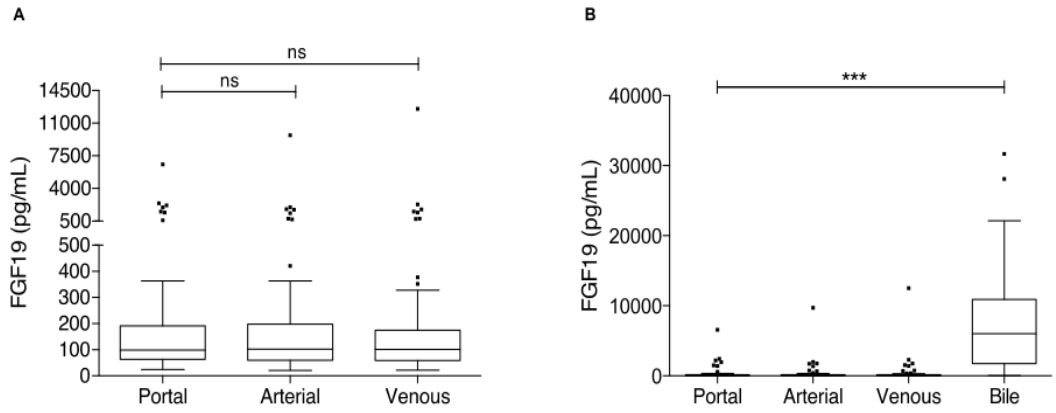

Figure 15. FGF-19 concentration in portal, systemic blood and bile. (**A**) FGF-19 concentration in blood (n=75) and (**B**) bile (n=54). Data are presented as median with IQR, with statistical comparisons performed by median regression with cluster robust standard errors. *** P<0.001, ns – not significant.

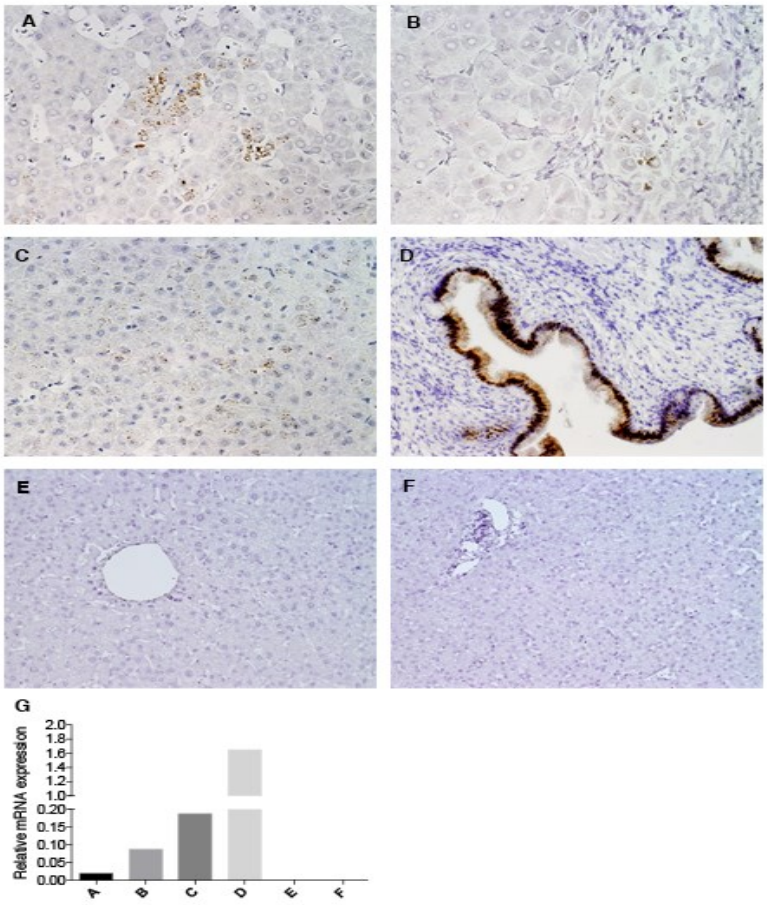

Figure 16. FGF-19 mRNA expression in liver and gallbladder. Representative *in situ* hybridization staining of sections from **(A-C)** liver tissue positive for FGF-19, original magnification 400x, **(D)** Gallbladder, original magnification 200x, and **(E-F)** liver tissue negative for FGF-19, original magnification 200x. **(G)** FGF-19 expression from each specimen measured by qPCR.

4.4 HEPATIC INSULIN RESISTANCE OCCURS IN CKD

To explore insulin-mediated glucose metabolism in our *in vitro* uremic hepatocyte model, cells were isolated from liver tissue and then treated with 5% pooled sera from patients (uremic group) and healthy (group) subjects **(Paper II)**.

We found a marked increase in glucose production through gluconeogenesis in uremic cells as compared to healthy. In healthy cells, addition of supra-physiological concentrations of insulin returned gluconeogenesis to baseline levels but there was virtually no change in cells treated with uremic sera (**Figure 17**).

At the same time, we observed an increase in mRNA levels of gluconeogenic genes PEPCK (PCK1), G6Pase (G6PC) and their upstream regulators PGC1α (PPARGC1A) and HNF4A. Moreover, Akt phosphorylation at S473 decreased following uremic sera treatment, whereas no differences were observed in pAkt T450, pFOXO1 S256, pFOXO3a T32, pIRS-1 pan-Tyr or pIRS-1 S612 (**Figure 18**). Taken together, we believe our data suggest that insulin resistance develops in hepatocytes following exposure to uremic sera.

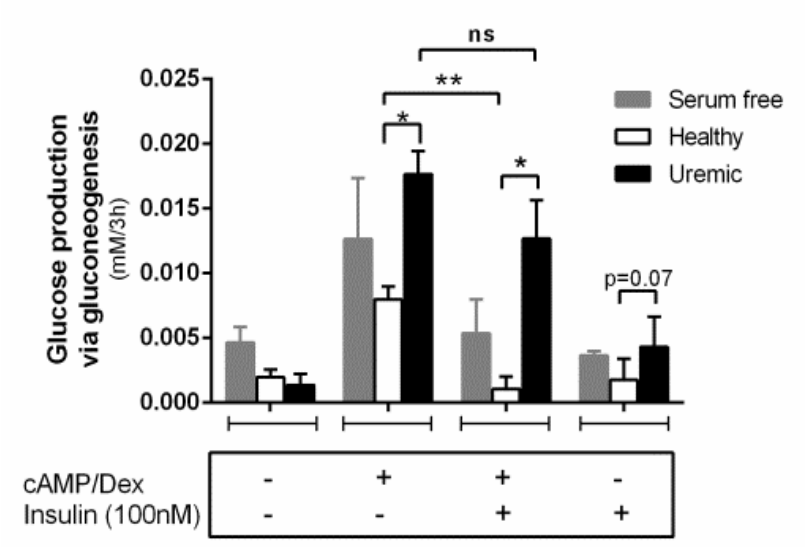

Figure 17. Increased glucose production via gluconeogenesis under uremic conditions. Data are presented as mean ± SEM. * $p<0.05$, ** $p < 0.01$.

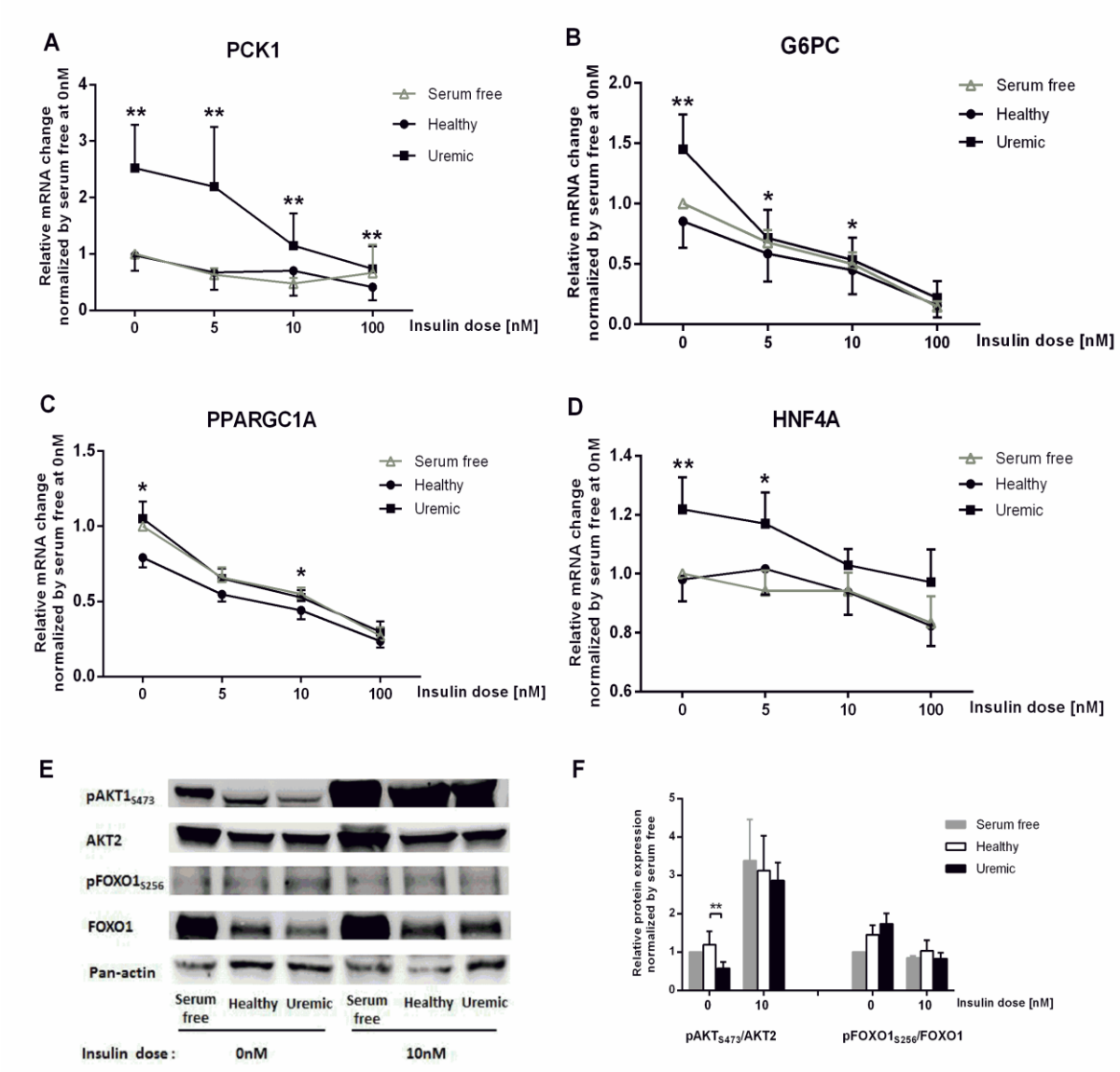

Figure 18. Effect of uremic sera on hepatic glucose metabolism genes. mRNA expression of gluconeogenetic genes (A) PCK1; (B) G6PC; (C) PPARGC1A; (D) HNF4A. (E) western blot; (F) quantification of blot densities. Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

Insulin resistance has recently been reported to occur in cultured hepatocytes from uremic rats (5/6th subtotal nephrectomy) [195] while an earlier study described a 25% to 45% decrease in gluconeogenesis in the livers of rats with renal failure [263]. However, another study of a similar rat model found gluconeogenesis to be increased and speculated that this was an adaptive response to glycogen depletion [263]. In patients, glucose uptake has been reported normal in the liver but reduced by 60% in the leg [263].

Our data also addresses the mechanism(s) behind the observed insulin resistance, but offers no clear answer. Hepatocyte mRNA of HSD11B1 (11βHSD1), but not of HSD11B2 (11βHSD2), increased under uremic conditions. The HSDs are catalytic enzymes that regulate intra-cellular glucocorticoid production by converting cortisone into cortisol (HSD11B1) or back (HSD11B2) [196]. Our data on HSD11B1 are of interest partly due to the large observed differences in hepatocyte responses to cAMP and dexamethasone depending on whether they had been cultured with uremic sera or not (**Figure 17**). Dexamethasone is a synthetic glucocorticoid estimated to be 50 times more potent than cortisol. They are also of interest since a recent study [196] in a rat model of CKD reported an increase in HSD11B1 at both the mRNA, protein and activity levels which also correlated with increases in hepatic gluconeogenesis and lipogenesis. While glucocorticoids are well-known to induce gluconeogenic genes (including PEPCK and G6pase) at the transcriptional level, the exact mechanisms leading to elevated HSD11B1 under uremic conditions remains to be determined. Also remaining to be performed are interventional experiments deigned to assess if the elevation in HSD11B1 is a causal factor or merely a reflection of such a factor. Our data offers some support for a direct mediating role by showing that uremic sera can increase phosphorylation of p53 at the p38-specific S15 and S46 sites in human hepatocytes. In HepG2 cells pro-inflammatory factors including TNFα can induce transcription of HSD11B1 via the p38 MAPK-C/EBPβ signaling pathway [264].

4.5 UREMIC HEPATOCYTES ACCUMULATE LIPIDS

Following exposure over 24 hours to 5% uremic sera, we found that our primary human hepatocytes accumulated both intracellular and extracellular lipids. Concentrations of VLDL-TG in the media rose (**Paper II**), while fluorescent staining revealed an intracellular lipid content that was 56% higher in cells treated with uremic sera. Furthermore, when intracellular lipids were extracted, we found these to be 49% higher in uremic cells (**Figure 19**). Clinically, VLDL is frequently elevated in the circulation of CKD patients, who also exhibit an altered plasma composition of lipoproteins comprising low HDL, low ApoA and high ApoB [28, 35, 39]. In our model ApoB mRNA increased in uremic sera-treated cells, but ApoB protein levels in the media did not. Apart from a lack of one or more substrates (including cholesterol), these results may also reflect a possible role of post-translational regulation of ApoB [265].

Compared to cells cultured with healthy sera, uremic cells also exhibited higher mRNA levels of lipogenic genes such as SREBF1, FASN, ChREBP, DGAT1, and CIDEC. Unexpectedly the same was found for several genes involved in lipolysis (LIPC, PPARα, CPT1A, FABP1) and lipoprotein uptake (LDLR, LRP1). These results are different from a recent report of uremic rats [205], where CPT1A and DGAT were reported to be decreased.

Patient data on TG production in CKD suggest high levels. For example, Cramp DG *et al.* [36] studied 13 CKD patients not yet on dialysis and found increased hepatic production of TG. Also, others [35, 266] have found a high prevalence of hypertriglyceridemia. However, in **Paper I**, we found no difference in baseline TG between patients and controls. Thus, further studies are needed before we can complete know the mechanism of dyslipidemia in CKD.

Ideally, lipid metabolism could be assessed by studying fresh biopsies and primary hepatocytes obtained from CKD patients. However, multiple reasons make such a sampling difficult. Thus, we are pleased that several aspects of lipid metabolism known to be altered in CKD patients were also disturbed in our model. These disturbances were seen already at a 1:20 dilution of uremic sera, and were not changed by prior dialysis of these. We hope that our model can be used to further elucidate pathways perturbed by CKD.

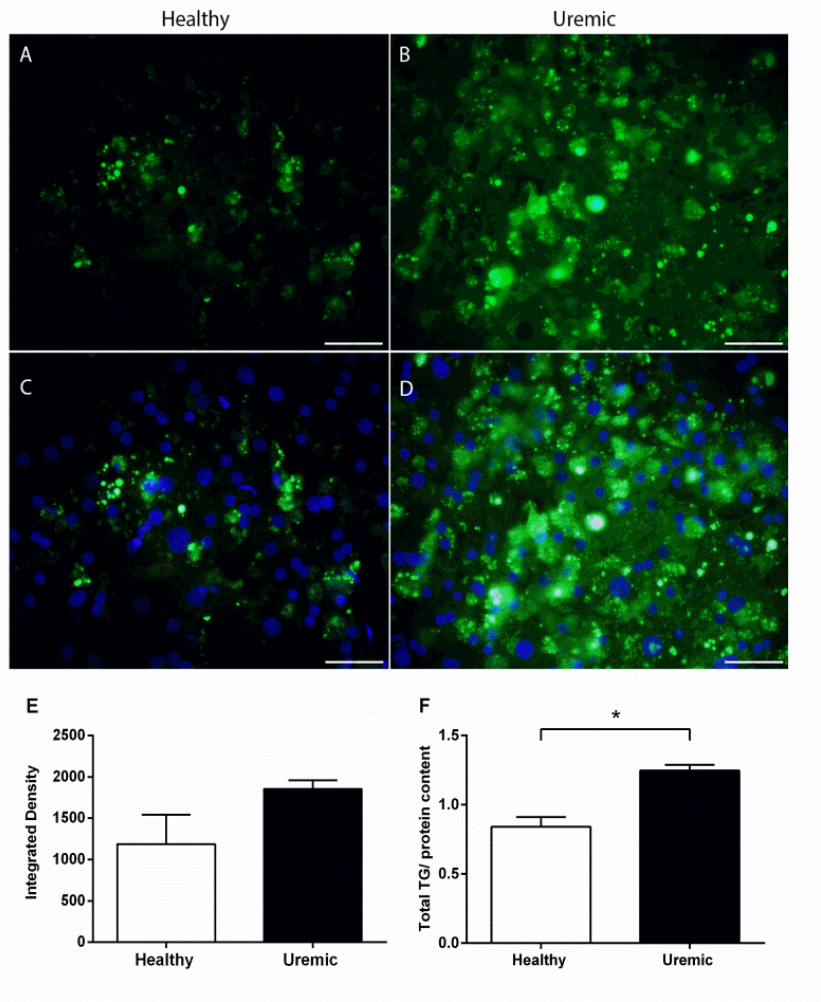

Figure 19. Intracellular lipid accumulation in uremic hepatocytes. Cells were treated with healthy (**4A, 4B**) or uremic sera (**4C, 4D**), following fluorescence staining for neutral lipid and nucleus. Green fluorescence reveals neutral lipid droplets, whereas the blue are nuclei. Integrated densities for each treatment group are shown in (**4E**). Direct cell lipid extraction and quantification was also performed and the results are given in **4F**. Scale bar is 50µm in a–d; inserts illustrate original magnification × 40.

4.6 FEW CHANGES IN BILE ACID METABOLISM IN CKD

To evaluate circulating bile acids in CKD patients and to link these findings to bile acid metabolism, we preformed analyses of the primary human hepatocyte model using *in vitro* exposure to uremic sera **(Paper III)**. Using GC-MS/MS, we were unable to see any differences between the bile acid content of the serum. However, using a highly sensitive method based on LC-MS/MS, we found that patient sera on average contain higher levels of tauro-conjugated BA and higher levels of sulphated-LCA, as well as a higher proportion of easily soluble trihydroxy- bile acid forms. Analyzing cell culture media before and after experiments, we found no differences in primary bile acid content although concentrations were in general very low.

At mRNA and protein level, we were also unable to detect any differences in key bile acid synthesizing enzymes, including CYP7A1, CYPB81, CYP27A1 and AKR1D1. However, several transporters implicated in bile acid shunting were higher in cells treated with uremic sera. These included NTCP, OST-α, and OST-β while OATP1B3 levels decreased. Our results thus confirm a recent report of similar findings in uremic rats [267]. To elucidate if the changes in bile acid transport genes could be physiological, we treated cells with two FXR-agonists, CDCA and GW4064. As expected, FXR-controlled genes were markedly induced in both groups, including SHP, OST- α, and OST-β while CYP7A1 decreased. Comparing the two sera groups, uremic sera-treated cells had a significantly higher increase in SHP and OST- α after both CDCA and GW4064 treatment, as well as in OST-β under CDCA treatment. We conclude that central elements regulating the synthesis and release of bile acids appear to be unchanged in cells exposed to uremic sera. The observed increase in organic solute transporters' mRNA before and after FXR agonist may or may not be of relevance. We speculate that it may reflect a general increase in cellular stress in the uremic group, as a recent study [268] found that the nuclear receptors FXR and HIF-1α bind in close proximity to the OST-α gene promoter and produced synergistic effects on OST-α expression. Also, ammonia was recently shown to induce a normoxic accumulation of HIF1α *in vitro*[269], which may be one factor linking uremic toxicity to HIF-priming of target genes and a later accentuated response to FXR agonist.

Finally, we investigated mRNAs of hepatic nuclear receptors after sera treatments. Our results showed significantly changes in uremic treated cells, but not in healthy cells. For example, mRNA of FXR, SHP and CAR increased while VDR mRNA decreased. No differences at the mRNA level were found for PXR, HNF-1α or AHR. Besides demonstrating the general nature of the metabolic disturbances induced by uremic sera, the above data are also of interest when designing future studies to elucidate the potential causes of dysmetabolism in uremic hepatocytes. Several nuclear receptors are thought to

interact directly with bile acids to modulate hepatocyte metabolism, including FXR, PXR, CAR and VDR [270-272]. Indeed, as reviewed above FXR is thought to be a key regulator of bile acid synthesis, CAR and PXR were reported to function as the xenobiotic-sensing receptors [273]. Recently, CAR was also reported to directly or indirectly regulate the expression of 11HSDB1 (which is also induced by 7-oxo-LCA) [274]. As we found elevated 11HSDB1 mRNA in our uremic hepatocytes along with enhanced glucocorticoid responses, IR (putatively induced by increased glucocorticoid signaling) and higher levels of hydroxylated bile acid (another putative target of 11HSDB1 [275]) it seems natural to design experiments to look at CAR-mediated 11β -HSD1 signaling , downstream targets and a hypothetical interaction of CAR with one or more uremic toxins.

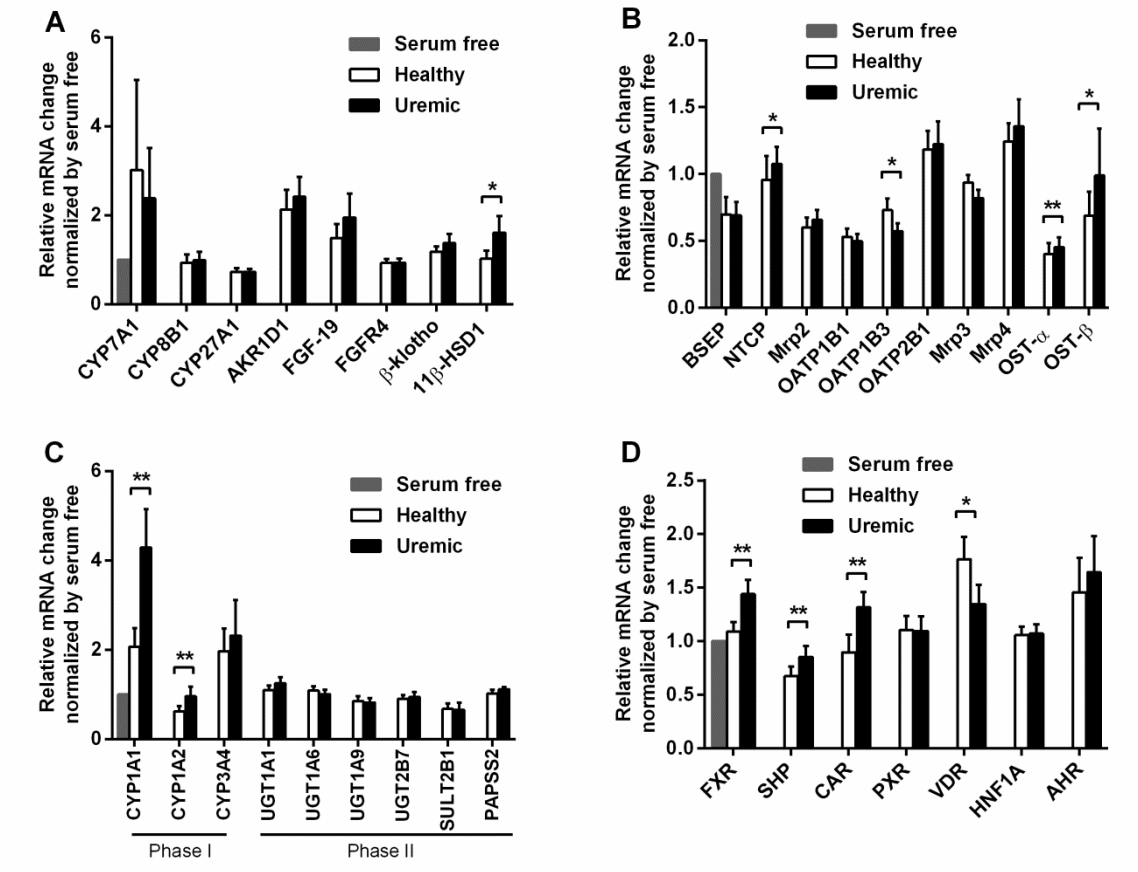

Figure 20. mRNA expression in hepatocytes exposed to healthy and uremic sera. **(A)** Genes involved in bile acid synthesis; **(B)** Genes involved in bile acid transporter; **(C)** Genes of xenobiotic-metabolizing enzymes; **(D)** Nuclear receptors genes. Data are presented as means ± SEM; * $p < 0.05$; ** $p < 0.01$.

5 GENERAL CONCLUSIONS

- Peripheral blood level of FGF-19 likely reflect portal FGF-19 concentration in the portal vein.

- Advanced CKD may be associated with a blunted postprandial FGF-19 response that is partially normalised following 7 days of either one of the anti-oxidative treatments N-acetyl cysteine or freeze-dried blueberries.

- In the liver, FGF-19 production appears to be neither increased nor inhibited by uremic sera *in vitro*, nor is FGFR4/β-Klotho signaling disrupted.

- Human primary hepatocytes treated with uremic sera develop insulin resistance leading to increased gluconeogenesis.

- *In vitro* culture of human primary hepatocytes with uremic sera leads to increased lipogenesis and the accumulation of significantly more intra- and extracellular lipids as compared to culturing the same cells with healthy sera.

- The central elements regulating the synthesis and release of bile acids appear to be unchanged in cells exposed to uremic sera.

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