Investigating the potential causes of Mesoamerican Nephropathy (MeN)

Evangelia-Theano Smpokou

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I, Evangelia-Theano Smpokou, confirm that the work presented in this thesis is my own. There have been collaborations with other institutions and students and these are stated in the text. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Evangelia-Theano Smpokou

Abstract

Mesoamerican Nephropathy (MeN) has emerged as a major public health concern in Central America. MeN is not associated with traditional chronic kidney disease (CKD) risk factors, and the cause remains unknown.

Our aim was to bring together evidence for and against potential causes of MeN. We used biosamples collected from 350 initially apparently healthy men and women aged 18-30 years from a population at risk of MeN to: (i) explore potential early biomarkers of kidney dysfunction, and (ii) quantify associations between possible nephrotoxins and change in renal function over a 2-year follow-up period. We quantified a range of serum and urinary markers of renal injury. Twelve metals and metalloids were analysed by inductively-coupled plasma-mass spectrometry. Twelve pesticides, their metabolites and two mycotoxins were analysed by liquid chromatography coupled to mass spectrometry. Differences in the creatinine-corrected urinary and serum concentrations of the measured exposures were examined between participants in different kidney function decline groups. Furthermore, the effect of heat and elevated fructose individually and in combination was assessed *in vitro*.

The levels of urinary RBP, NGAL and serum UA were significantly elevated in the established kidney dysfunction group but did not help identify those with a normal baseline but declining function. Elevated levels of aluminium and total arsenic were observed across the population but no differences were identified between the different groups. Finally, *in vitro* experiments showed that heat stress suppressed the mRNA expression of MCP-1 but caused an early increase in expression of fibrogenic genes. Elevated fructose led to early mRNA increase in MCP-1, KIM-1, TGF-β, COLA1 and KIM-1 secretion. However, a number of these molecules were suppressed after longer term exposure.

These findings provide evidence against the xenobiotic hypothesis investigated in this thesis as the primary cause(s) of MeN in Nicaragua. Future research priorities include examining alternative toxins not included in the

present study and identification of more accurate biomarkers for the early detection of MeN.

Impact Statement

Mesoamerican Nephropathy (MeN) mainly affects young agricultural workers in low income and developing countries. MeN is usually not diagnosed until the disease is advanced. Treatment options are limited and there is a high socioeconomic cost with high mortality. The aetiology still remains unknown although many hypotheses have been proposed.

Our study contributes to the general knowledge surrounding the still unexplained aetiology of MeN. To date most studies have focused on the acute effects of heat stress and dehydration pre- and post- sugarcane harvest. In our study serum and urine samples were collected from a longitudinal cohort study in Nicaragua in which 350 healthy men and women were followed for a period of 2 years and a series of analytes were measured and compared to renal function over time. The speed that the disease is developing in just 2 years in our study, highlights the importance of early accurate biomarkers for early detection and intervention to slow down its progression. Our results provide strong evidence against two of the main hypotheses (heavy metals and pesticides) as playing a primary role in the development of the MeN.

The work described in this PhD has an international impact across several fields: academia, clinical medicine and hopefully in the near future the law policing in workplaces. Additionally, the samples collected for our study, provide an important resource to test further hypotheses as to the cause of MeN (e.g., infectious diseases). Within academia the research methodology of collecting specimens across a time period can be used by other studies within CKDu as well as other large cohort studies. The main impact of the study will be to improve understanding of CKDu but also provide further information on the impact of various factors on kidney injury. This information will help to inform public health decisions as MeN has such a widespread effect on local populations. Our results in combination with other studies could lead to new laws and improvements in the quality of the environment and finally the quality of life of affected communities. Although this study is based on results from

Nicaragua it will have an impact on the other major countries where CKDu is prevalent: India, Sri Lanka, Guatemala, El Salvador and Costa Rica.

The outputs for these impacts will be through a mixture of journals and interaction with non-governmental and governmental groups. One journal article has already been published. Taken together, the overall body of work on the causes of CKDu should lead to legislation for better working practices as well as ensuring affected communities are provided with clean drinking water, sanitation and education.

Acknowledgements

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List of Abbreviations

AA	Aristolochic Acid
ACR	Albumin to Creatinine Ratio
ADP	Adenosine diphosphate
AKI	Acute kidney Injury
Al	Aluminium
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
As	Arsenic
AST	Aspartate Aminotransferase
ATP	Adenosine triphosphate
Au	Gold
B	Boron
B-2M	Beta-2-Microglobulin
BA	Boric Acid
BCG	Bromocresol Green
BEN	Balkan Endemic Nephropathy
BIL	Bilirubin
BLO	Blood
bp	Base pair
Cd	Cadmium
CFCA	Chloro-3,3,3-trifluoro-1-propen-1-yl]-2,2-dimethylcyclopropanecarboxylic acid
CFCA	Chloro-3,3,3-trifluoro-1-propen-1-yl]-2,2-dimethylcyclopropanecarboxylic acid
CINAC	Chronic interstitial nephritis in agricultural communities
CIT	Citrinin
CKD	Chronic Kidney Disease
CKDnt	Chronic Kidney Disease of non-traditional cause
CKDu	Chronic Kidney Disease of unknown origin
Co	Cobalt
Cr	Chromium
Cu	Copper
Cys-C	Cystatin-C
2,4 D	2,4-dichlorophenoxyacetic acid
DCCA	3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle medium
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme-linked immunosorbent assay
ESRD	End-Stage Renal Disease
ETU	Ethylenethiourea
FBS	Fetal Basal Serum
4F-3PBA	4-fluoro-3-phenoxybenzoic acid

Fe	Iron
Ge	Germanium
GFR	Glomerular Filtration Rate
GLU	Glucose
Govt.	Government
GWAS	Genome wide Association study
γGT	Gamma-glutamyl transferase
Hg	Mercury
HK-2	Human Kidney-2 cell line
HKC-8	Human kidney proximal tubular
In	Indium
ICD10	International Classification of Diseases (Version 2010)
ICP-MS	Inductively Coupled plasma Mass spectrometry
IFCC	International Federation of Clinical Chemistry
IL-1β	Interleukin 1-β
IMP	Inosine monophosphate
KDIGO	Kidney Disease: Improving Global Outcomes
KET	Ketones
KIM-1	Kidney Injury Molecule-1
LEU	Leucocytes
LC-MS	Liquid Chromatography-Mass spectrometry
LDH	Lactate dehydrogenase
LIN	La Isla Network
LOD	Limit of detection
LOQ	Limit of quantification
LSHTM	London School of Hygiene and Tropical Medicine
MCP-1	Monocyte Chemoattractant Protein-1
MCPA	4-chloro-2-methylphenoxy acetic acid
MDH	malate dehydrogenase
MeN	Mesoamerican Nephropathy
Mn	Manganese
NAG	N-acetyl-β-D-glycosaminidase
N180	ICD10 code for End stage renal disease
NAD^+	Nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydride
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NHANES	NHANES
NHS	National Health System
NIT	Nitrates
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OD	Optical Density
5-OH-TBZ	5-hydroxy-tiabendazole
OH-PYR	3-hydroxy-pyrimetanil
OSHA	Occupational Safety and Health Administration
OTA	Ochratoxin A
3-PBA	3-phenoxybenzoic acid
PAHO	Pan American Health Organization
Pb	Lead
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain reaction

Pt	Platinum
QC	Quality Control
RBP	Retinol-binding protein
Rh	Rhodium
RRT	Renal Replacement Therapy
sCr	Serum creatinine
sCys-C	Serum cystatin-C
SDHA	Dehydrogenase
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Se	Selenium
SG	Specific Gravity
Si	Silicon
SNP	Single Nucleotide Polymorphism
Sr	Strontium
TGF-β	Transforming Growth Factor-β
TCP	3,5,6-Trichloro-2-pyridinol, related with metabolism of insecticide chlorpyrifos
TEB-OH	hydroxy-tebuconazole
TEMED	Tetramethylethylenediamine
TQD	Tandem Quadrupole detection
UA	Uric acid
UHPLC	Ultimate High-Performance Liquid Chromatography
UNAN	National Autonomous University of Nicaragua
URO	Urobilinogen
WBGT	Wet Bulb Globe Temperature
Y	Yttrium

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Conferences and publications

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at risk of Mesoamerican Nephropathy (MeN). 11th International Symposium on Biological Monitoring in Occupational and Environmental Health (ISBM-11), Leuven, Belgium, 28th -30th August 2019.

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Chapter 1: Introduction

1.1 Background

1.1.1 Normal function of the kidneys

The kidneys are two bean-shaped organs, each about the size of an adult fist, found on the left and right sides of the spine at the lowest level of the rib cage (Figure 1.1) and they process 1.25 L/min or 25% of the resting cardiac output via the arterial blood supply (1).

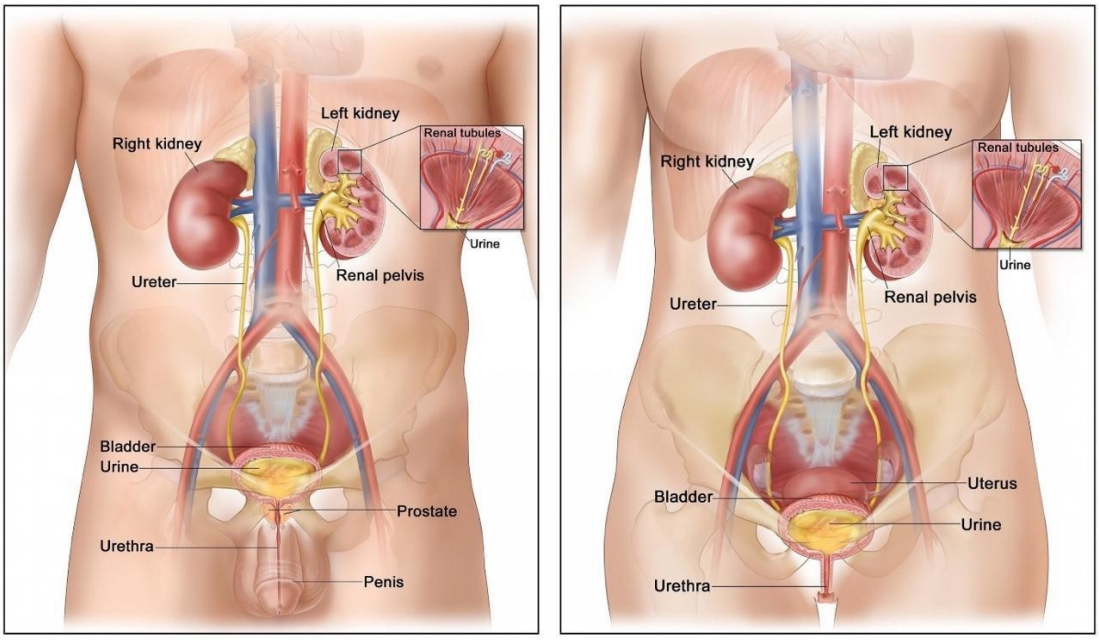

Figure 1.1: Anatomy of the male and female urinary system. National Cancer Institute © 2010 Terese Winslow LLC, U.S. Govt. has certain rights.

The main functions of the kidneys are to regulate the composition of the extracellular fluid, filter the blood to produce urine for the excretion of toxic products of metabolic processes, but also to maintain homeostasis of electrolytes, blood pressure and the acid-base balance. Moreover, kidneys activate hormones such as calcitriol, and produce erythropoietin and renin that are vital for sodium regulation through the secretion of aldosterone, the production of red blood cells and regulation of blood pressure, respectively. Finally, the synthesis of 1,25-dihydroxycholecalciferol (calcitriol) from vitamin D leads to the absorption of calcium from the gut and calcification of the bones (2,3). Urine also contains solutes, the major ones being urea, sodium,

phosphate and chloride. The volume and osmolality of the urine varies from day to day and depends on fluid intake, diet and loss through sweat and faeces.

The kidneys are made up of ~1.25 million nephrons each. A nephron is the functional unit of the kidney and consist of a glomerulus where blood is filtered and a tubule, responsible for the reabsorption of essential elements and the secretion of urea, creatinine, H^+, K^+, NH_3, and a wide range of endogenous (and exogenous) low-molecular weight molecules (4). The main functional unit of the nephron is the glomerulus and the tubules. A healthy glomerulus is shown in Figure 1.2.

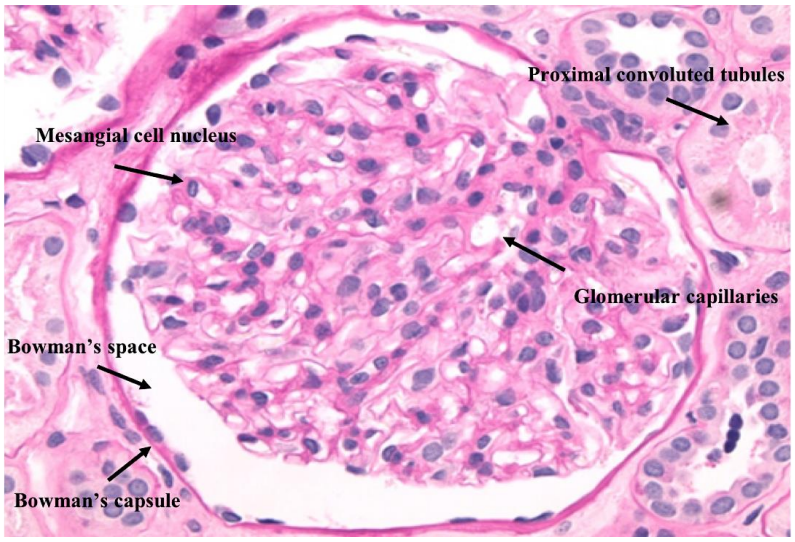

Figure 1.2: Normal glomerulus stained with periodic acid-Schiff stain. Original magnification x400 (Courtesy of Dr Radica Alicic) (5).

The main function of the glomerulus is to filter the blood with the assistance of three major components forming the glomerular filtration barrier (GFB): the fenestrated endothelium, the glomerular basement membrane (GBM) and the epithelial podocytes (6–8). The glomerulus is supplied with blood by the afferent arteriole which leaves the glomerulus via the efferent arteriole. The filtrate that has passed the three major components of the GFB enters Bowman's space and flows into the renal tubule (Figure 1.3). The structure of the GFB, allows plasma water and small solutes to flow freely from the

glomerulus. Proteins >60 kDa in size do not normally pass the GFB and if they do, are usually reabsorbed by the proximal tubule (9).

The tubule is divided into specialised segments that selectively reabsorb solutes (by passive diffusion, active transport and cotransport) and secrete molecules (by passive diffusion or active transport): (i) proximal convoluted tubule (PCT), (ii) the loop of Henle (descending and ascending loop), (iii) the distal convoluted tubule, and (iv) the collecting duct (10).

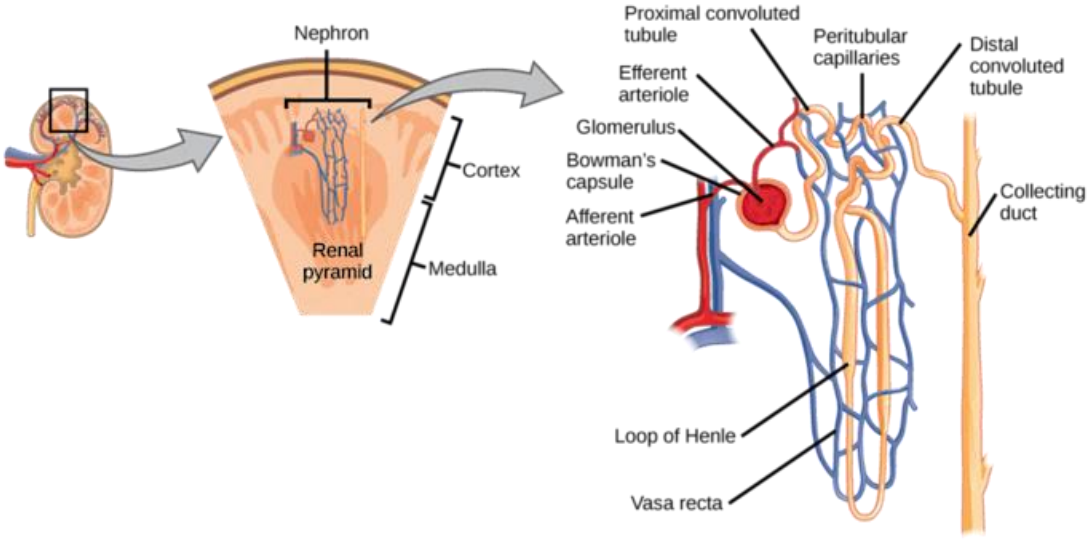

Figure 1.3: The anatomy of the nephron. Each kidney contains millions of nephrons. Each nephron consists of the glomerulus, the proximal convoluted tubule, the loop of Henle, the distal convoluted tubule and the collecting duct. (Creative Commons copyright of CC-BY) (11).

Reabsorption of water, sodium and potassium happen in different segments of the tubule. In health protein, glucose, amino acids, phosphate, calcium, sodium and water are reabsorbed by the proximal tubular epithelial cells (12–14). Further sodium, magnesium and remaining potassium and calcium that was not reabsorbed by the proximal tubules is absorbed by the ascending limb of the Loop of Henle and the distal convoluted tubule followed by fine tuning of sodium and water in the collecting duct. The amount of solutes, particularly sodium and water, reabsorbed in the various parts of the tubule varies to maintain homeostasis (15). Secreted molecules from the peritubular capillary network into urine include potassium, hydrogen ions, ammonium ions, creatinine, hormones and xenobiotics (16).

1.1.2 Markers of kidney dysfunction

As mentioned in the previous section, each segment of the nephron serves different excretory and reabsorption roles in filtering the urine and maintaining homeostasis of the body. Kidney dysfunction leads to increased levels of various markers in blood and urine depending on the severity of dysfunction (17). Serum and urinary markers together with histological changes (on kidney biopsy) can be used to assess the severity of kidney dysfunction. Common serum markers include creatinine (sCr), and cystatin-C (sCys-C). Creatinine is constantly produced as a by-product of creatinine phosphate in the muscles (18). Production varies depending on the muscle mass, but also on the diet and health status of the individual (19). Serum creatinine is freely filtered into the urine and in a healthy individual, creatinine clearance is 110-150 mL/min for men and 100-130 mL/min for women (20). For healthy men, the levels of creatinine in serum and urine are 60-110 µmol/L and 18-24 mg/kg/day, and for women 45-90 µmol/L and 15-20 mg/kg/day, respectively (21). Increased sCr levels are found in kidney dysfunction as a result of defective filtration competency of the kidneys (22).

Cystatin-C is another natural occurring by-product of cellular respiration to generate energy (Adenosine triphosphate (ATP)) and it is produced by all nucleated cells at a constant rate and is easily filtered by the glomeruli and excreted in the urine. Several studies suggest sCys-C is a more sensitive marker than creatinine to assess kidney function because it is not influenced by endogenous and exogenous factors which affect creatinine levels such as muscle mass, diet, exercise or dehydration (23–25). Kidney dysfunction will result in decreased filtration in the glomerulus, decreased excretion in the urine and accumulation of Cys-C in the serum.

The estimated glomerular filtration rate (eGFR) test is an online formula that is used to determine the kidney function and the stage of kidney disease of an individual (26). The eGFR formula requires information such the sex, ethnicity, age, sCr and/or sCys-C of the individual (e.g. CKD-EPI formula). CKD is typically identified using blood (sCr or sCys-C for the calculation of eGFR, in

moderate or advanced stages) or urine (albumin: creatinine ratio, in earlier stages) tests (27,28).

Proteins are essential for the normal function of the body and small proteins are filtered by the glomerulus and usually reabsorbed in the proximal tubules. Higher molecular proteins (>60 kDa) are not usually filtered and stay in the circulation.

Semi-quantitative dipstick urinalysis is used for spot urines to rapidly assess the presence of high levels of proteins in the urine and is an easy to use and low-cost test (17,29,30). In healthy individuals, albumin is not normally excreted (<30 mg/day) and albuminuria is another marker of kidney injury (31). This is usually quantified by the albumin to creatinine ratio (ACR) (32). Microalbuminuria refers to urine albumin levels of 30-300 mg/day and macroalbuminuria of >300 mg/day (33). The latter suggests significant glomerular dysfunction. Conversely, increased urinary excretion of low molecular weight proteins (<20 kDa) are a sign of tubular dysfunction and inability to reabsorb these proteins. However, these molecules are not routinely measured in clinical care.

1.1.3 Causes of Kidney Disease

There are numerous causes of kidney disease. Globally the most common causes are glomerulonephritis, diabetes, atheromatous vascular disease, polycystic kidney disease, congenital renal tract malformation and kidney stones (34). That is, as a result of auto-immune disease (glomerulonephritis), pre-existing metabolic conditions (diabetes) or inherited diseases (polycystic kidney disease and congenital malformations).

1.1.4 Chronic kidney disease (CKD) and its characteristics

When nephrons cease functioning there is initially a response in the remaining healthy nephrons with a compensatory increase in filtration (35). Independent of the initial cause of injury this compensatory response can lead to further nephron damage as a result of hyperfiltration. Hence, although the initial causes of kidney damage are diverse, including auto-immune and metabolic

disease, toxins and infections, if sustained any of these can result in a final common pathway including oxidative stress, inflammation, scarring and fibrosis and ultimately, kidney failure (36). Kidney diseases or their causes (such as diabetes mellitus) that are not managed will eventually lead to chronic kidney disease (CKD) (37). Long-term loss of kidney function can lead to the life-threatening condition of end-stage renal disease (ESRD) where patients need dialysis or kidney transplantation (renal replacement therapy-RRT).

Chronic Kidney Disease (CKD) is defined as persistent kidney dysfunction for at least 3 months as indicated by an eGFR of <60 mL/min/1.73 m^2 (38). CKD is divided into different stages depending on the filtration capability of the kidneys and the levels of albumin found in the urine. The Kidney Disease Improving Global Outcomes (KDIGO) organization has summarized the stages of CKD as shown in Table 1.1 (39). Stage 5 represents ESRD and patients require renal replacement therapy (RRT).

Prognosis of CKD by GFR and albuminuria categories: KDIGO 2012				Persistent albuminuria categories description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				≥30 mg/g <3 mg/mmol	30–300 mg/g 3–30 mg/mmol	>300 mg/g >30 mg/mmol
GFR categories (ml/min/1.73m²) description and range	G1	Normal or high	≥90			
	G2	Mildly decreased	60–89			
	G3a	Mildly to moderately decreased	45–59			
	G3b	Moderately to severely decreased	30–44			
	G4	Severely decreased	15–29			
	G5	Kidney failure	<15			

Table 1.1: The five stages of CKD as characterised by the decrease in the glomerular filtration rate (GFR). Stages 1 and 2, require additional criteria other than low eGFR to be considered, such as the level of albuminuria (28,39).

Worldwide, CKD is primarily a disease of the elderly (80% of the cases) with a prevalence of 5-10% of the total population (40,41). Figure 1.4 shows the worldwide prevalence of CKD for both men and women at all ages for the year 2017 (42). A recent report describing the disability-adjusted life years (DALYs) per 100,000 of all CKD cases between 2005 (29,488.3/100,000) and 2015 (35,259.7/100,000), showed a rise of 19.6% (43). CKD affects one in five men and one in four women between the ages of 65 and 74 (44). It is clear from Figure 1.4, that Central America has a high number of cases. CKD can exist on its own, or in combination with other clinical conditions such as obesity, diabetes mellitus and hypertension (9,10). More specifically, Figure 1.5 shows deaths by age group for men and women suffering from ESRD in the Americas as recorded for the year 2009 (last updated), illustrating that both genders are suffering from CKD leading to ESRD predominantly at ages over 60 years.

In England, CKD costs to the National Health Service (NHS) are more than breast, lung, skin and colon cancer combined (44). In the UK, around 5% of the population have CKD, although only 0.1% will progress to ESRD needing RRT, while the majority of the remainder will die prematurely from secondary complications of CKD such as cardiovascular disease (CVD) (6,7).

Early-stage CKD in primary care is commonly asymptomatic, and the exact pathology underlying its development can remain unknown. As eGFR decreases, the kidneys gradually fail to filter waste products, leading to accumulation in the circulation (45). Elevated levels of unfiltered waste contribute to a toxic milieu, “uraemia”. Symptoms of later stages include loss of appetite, swollen ankles, feet or hands and shortness of breath (46).

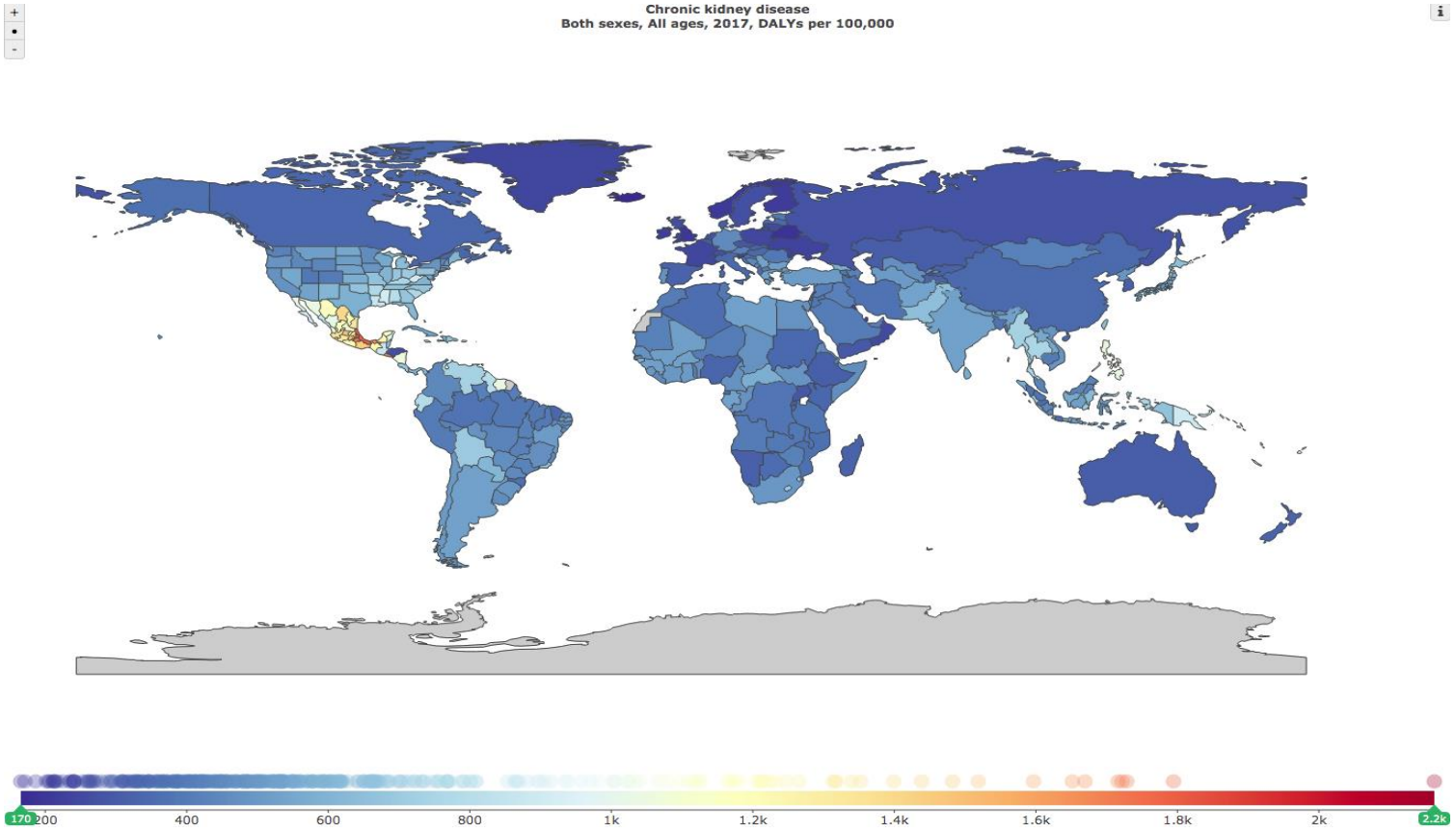

Figure 1.4: Global disability-adjusted life year (DALYs) per 100,000 from Chronic Kidney Disease (CKD) for men and women at all ages for the year 2017. The different colour shade represents the number in thousands of people.

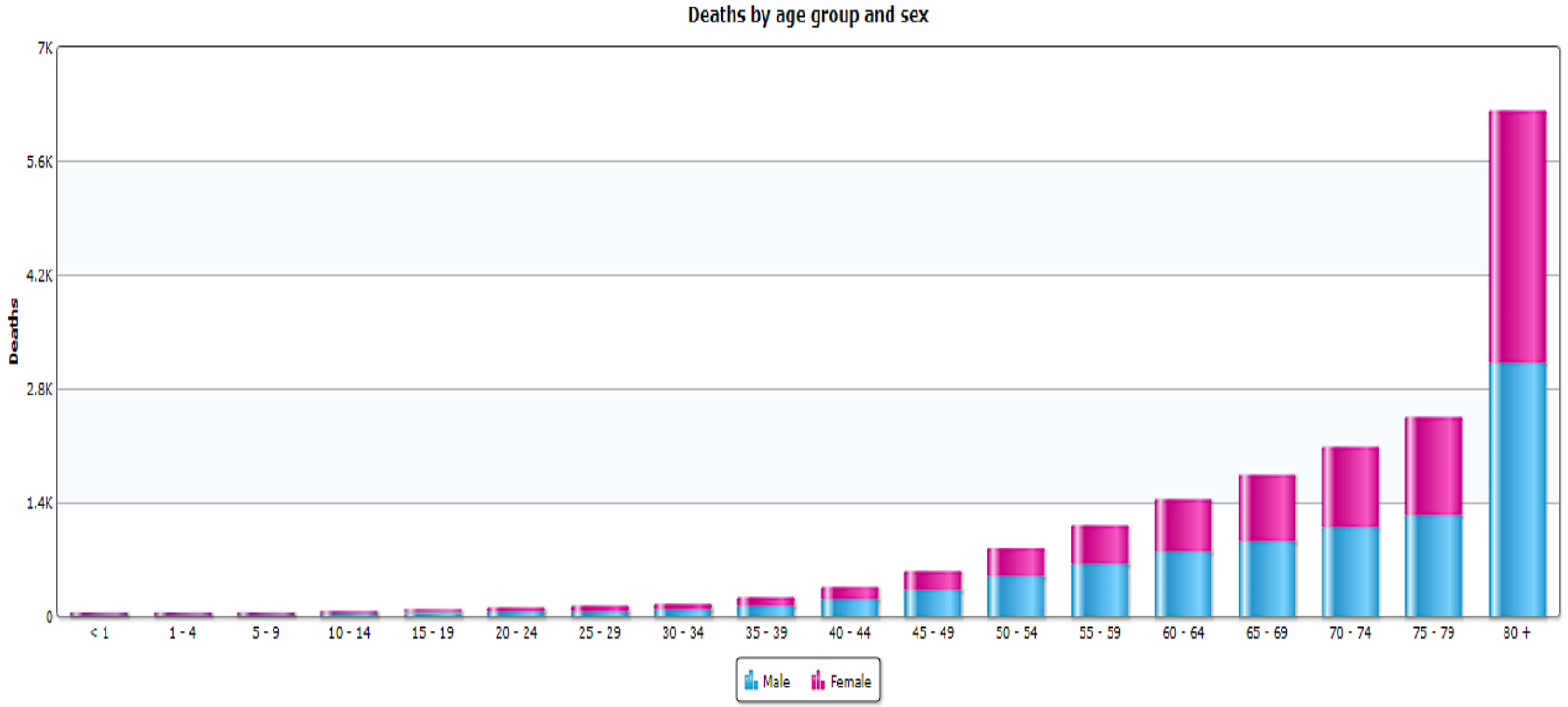

Figure 1.5: Pan American Health Organization: PAHO/WHO mortality in 2009 (ICD10 Code: N18 End-stage renal disease). Deaths by age group and sex in 24 countries including Grenada, Antigua and Barbuda, United-States of America, Puerto Rico, Virgin Islands (US), Nicaragua, Argentina, Cayman Islands, Saint Kitts and Nevis, Costa Rica, Guadeloupe, Martinique, Canada, Peru, Saint Vincent and the Grenadines, Venezuela, Panama, Ecuador, Colombia, French Guiana, Cuba, Paraguay, Mexico, Brazil and Paraguay. Blue: Male patients; pink: female patients.

1.2 CKD of Unknown aetiology (CKDu)

Over the last 20-30 years, a high prevalence of CKD has been noticed in various tropical regions around the world predominantly affecting young, male agricultural workers. Many different terms have been used to describe the disease of which CKD of unknown/uncertain aetiology (CKDu) and Mesoamerican Nephropathy (MeN) are the most widely used (others include CKD of non-traditional cause (CKDnt) and chronic interstitial nephritis in agricultural communities (CINAC)) depending on the geographical occurrence of the disease (47).

CKDu is defined as progressive kidney disease in which the usual causal factors that can lead to CKD, such as glomerulonephritis or diabetes mellitus, are absent (48). It is also known to predominantly affect young men working in physically intense manual labour, especially agriculture such as rice, sugarcane cultivation and sugar production in hot climates. To date, evidence suggests that the poor populations of Central America, Sri Lanka and India are primarily affected (37,38,40,41). Although, lack of evidence and studies from other countries means it remains unclear if there are other populations affected by CKDu (49). An international collaboration for the epidemiology of eGFR in low- and middle-income populations (Disadvantaged populations eGFR Epidemiology (DEGREE)) is collecting data in rural populations across different developing countries including India, Malawi, Peru and Mexico, to characterise the prevalence of low eGFR and assess the extent of CKDu in areas near the equator (50,51).

CKD in general is difficult to diagnose in the early stages as it is asymptomatic. In CKDu where comorbidities are absent and biopsies are generally not performed, it is difficult to give a clear definition of the disease which in turn hinders a better understanding of the causes that lead to kidney failure in young individuals. Many hypothesise that there is a common cause globally however the limited biopsy findings are nonspecific. Biopsies taken in different regions in relatively advanced stages of the disease do look similar (52,53). Although, these do not exclude the possibility of different exposures as the initial cause of injury that could have a similar histological presentation (54).

Other reasons why the theory of a common cause leading to CKDu is controversial, include differences in environmental exposures, such as temperature and diet, and also in the genetic background of the affected populations (55). Table 1.2 summarises the similarities and differences between the major sites where CKDu is observed.

	Central America	Sri-Lanka	India
Prevalence (age of onset and M:F ratio)	Third and fifth decade M:F=5:1 (56)	Fourth and fifth decade M:F=3:1 (57)	Fifth and sixth decade M:F=1:1 (58)
Clinical Presentation	Asymptomatic until ESRD	Asymptomatic until ESRD	Asymptomatic until ESRD
Renal Histology	Interstitial fibrosis Tubular atrophy Glomerulosclerosis Chronic glomerular ischaemia (52,59)	Interstitial fibrosis Tubular atrophy Glomerulosclerosis Glomerular collapse (60,61)	Interstitial fibrosis Tubular atrophy Normal glomeruli (62)
Endemic Areas	Nicaragua El Salvador Costa Rica (56)	North Central Province (63)	Uddanam coastal region (64,65)
Geographical Characteristics	Rural lowlands along Pacific coast (66)	Rural areas (66)	Rural coast (64)
Occupation	Agricultural workers more often sugarcane (67–70)	Agricultural workers more often vegetables and rice (63)	Agricultural workers more often coconut, rice, jackfruit and cashews (62)
Risk Factors	Sugarcane worker Heat stress Agrochemical use Heavy metal exposure Genetic predisposition Alcohol (63,71)	Agricultural worker Heat stress Agrochemical use Heavy metal exposure Genetic predisposition Alcohol, smoking (57,71)	Agricultural worker Heat stress Heavy metal exposure (62,71)
Socio-economic status	Low	Low	Low

Table 1.2: Clinical and epidemiological characteristics of CKDu in Central America, Sri-Lanka and India (55,72)**.**

1.2.1 The epidemic of CKDu in Central America

In the last two decades, CKD has emerged throughout Central America (73–75). A study published in 2002, first reported the unusual characteristics of patients receiving haemodialysis in San Salvador, El Salvador, which included

young age and the predominance of male agricultural workers without the usual CKD causal factors such as hypertension, diabetes mellitus, obesity or evidence of glomerulonephritis (76).

The term used in Central America to describe the disease is Mesoamerican Nephropathy (MeN) (74). The population suffering from MeN live in the lowlands of the Pacific coast of Central America (rather than the highlands) where factors such as humidity and temperature are higher (67,69,75). In Northwest Nicaragua, MeN mainly affects agricultural workers employed in sugarcane production (52,73). The high levels of poverty in the affected areas mean that ESRD patients rarely have access to treatment and thus many die through lack of access to RRT centres or lack of funds (77). In Central America over the last 20 years, it has been estimated that MeN has caused the premature death of ~20,000 mainly young male individuals (78). Over this time, the number of reported male deaths from ESRD of unspecified causes (ICD10 code: N19, Unspecified renal failure) in El Salvador and Nicaragua has almost quadrupled (Figure 1.6). The disease also has significant socioeconomic effects. Serum creatinine levels of workers are now being measured by the sugar-production industry at the beginning of each harvest season (79). If the results indicate increased levels of creatinine in the blood, workers are rejected for further work. As the sugar industry is the main source of income in these areas it makes it extremely hard for the affected individuals and their families to gain employment elsewhere.

Although the escalating death cases from unspecified renal failure (N19) found between 1997 to 2015 (Figure 1.6) could reflect inadequate surveillance in the past (a lot of cases have previously not been reported), the scale of the increase in combination with what affected communities are reporting, suggests that since the year 2000 many more people are suffering from CKDu (80,81). Recent studies investigating the presence of CKD before 1997 found the disease has been present from the 1970s although little attention was paid to the earlier incidence (82). It is clear that men are affected more than womenfrom ESRD of all aetiologies (N18) in Nicaragua as shown in Figure 1.7. Deaths often occur in men between the ages of 30 and 60 which contrasts

with CKD globally which occurs predominantly in the population >60 years old (Figure 1.5).

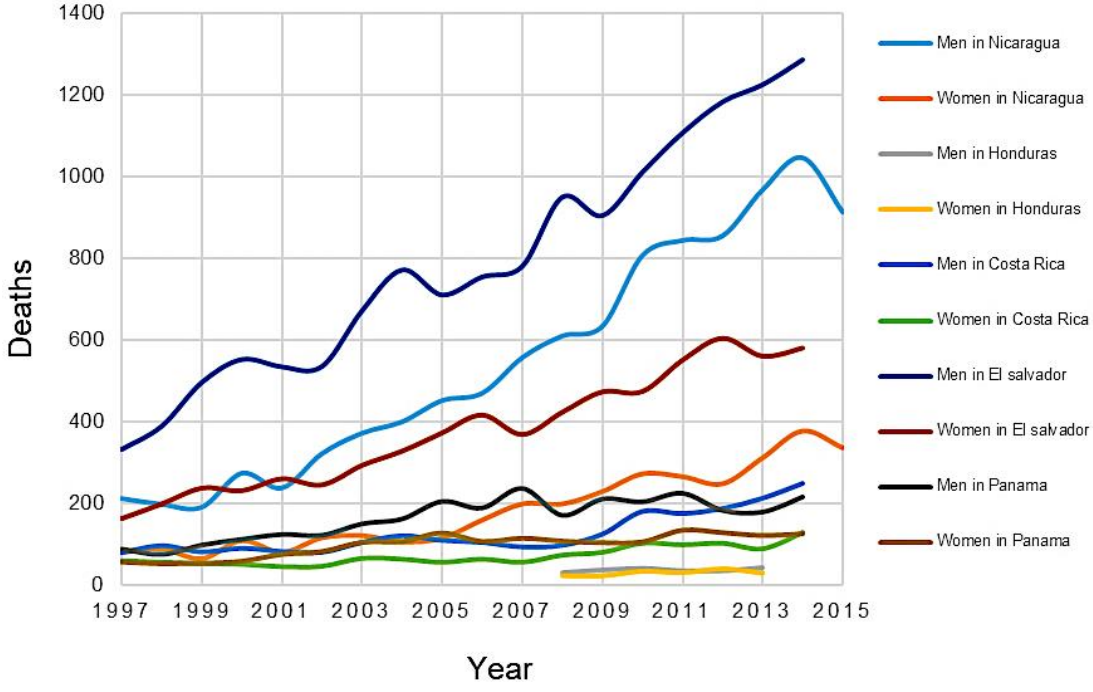

Figure 1.6: Mortality data for men and women in Nicaragua, Honduras, Costa Rica, El Salvador and Panama 1997-2015. Data retrieved from PAHO ICD10 code: N19 unspecified renal failure (80).

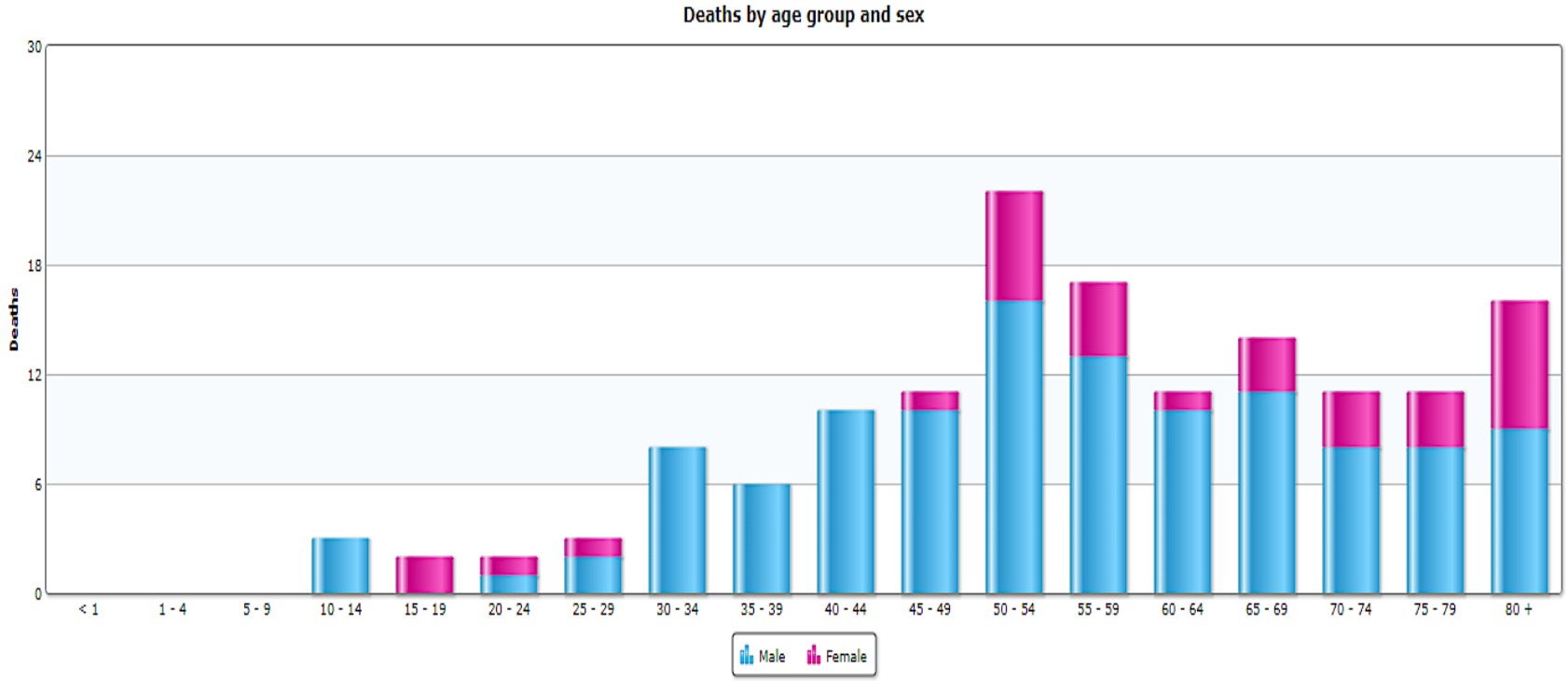

Figure 1.7: Certified deaths by age group and sex in Nicaragua from PAHO ICD-10 code: N18 (end-stage renal disease) for the year 2009 (80).

1.2.2 Nicaragua: A country of lakes and volcanoes

Nicaragua is located where north and south America meet (Central American isthmus; Figure 1.8) and has a population of ~6,546,000 (83). North of Nicaragua is Honduras and to the south is Costa Rica, to the east the Atlantic Ocean and to the west the Pacific Ocean.

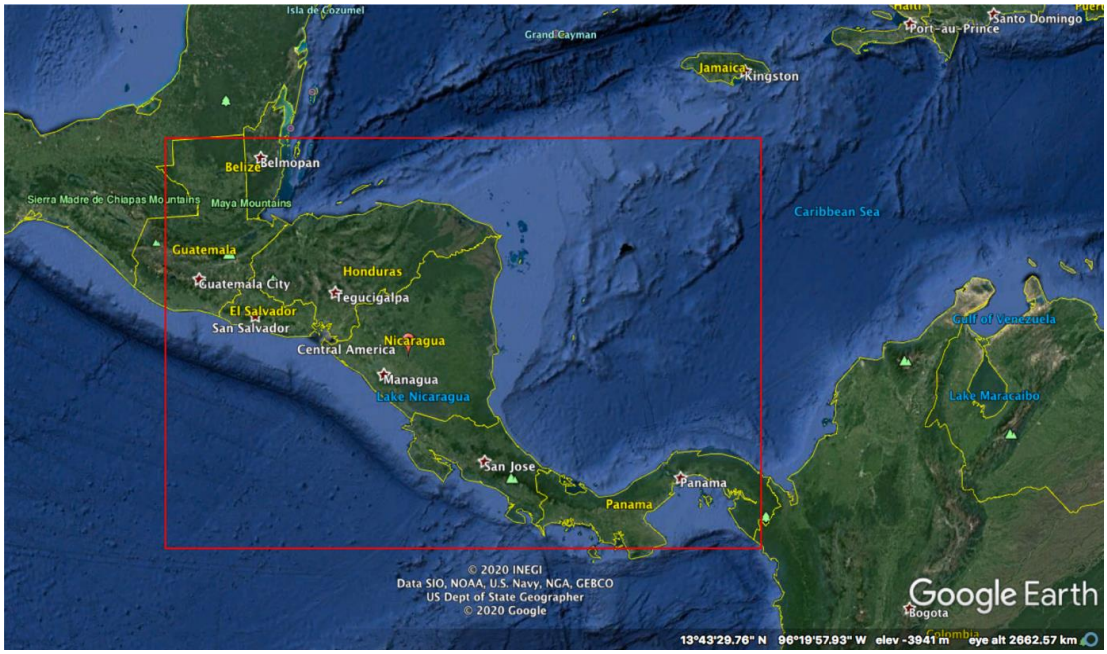

Figure 1.8: Map of the Central American Isthmus where North and South America meet. Central America includes the countries of Guatemala, Belize, Honduras, El Salvador, Nicaragua, Costa Rica and Panama (84)**.**

In Nicaragua, there are 23 volcanoes of which 19 are active, making it rich in elements derived from the volcanic activity and present in the air, water and soil (Figure 1.9) (85). The geothermal activity of the region also has an impact on the surface water such as volcanic lakes, and the groundwater leading to high concentrations of Arsenic (As), Boron (B), Iron (Fe) and manganese (Mn) as a result of mixing with variable proportions of saline geothermal sea water (86).

Additionally, Nicaragua has two lakes, Managua and Nicaragua, collecting an average precipitation of 1,694 mm/year and providing the water supply for the neighbouring cities. The two lakes are surrounded by several volcanoes increasing the risk of accumulative elements in potable water.

Figure 1.9: Nicaragua has 23 volcanoes of which 19 are active (green and red symbols) and 4 are inactive (grey symbols). In the last 20 years eruptions have occurred in the volcanoes: Cerro Negro, Department of León, 1999; Concepción, Department of Ricas, 2009; San Cristobal, Department of Chinandega, 2012; Momotombo, Department of León, 2015; San Cristobal, Department of Chinandega, 2016; and, Telica, Department of León, Masaya Volcano, Department of Masaya (red symbol) has a long history of eruptions with the biggest being in 2001 and the latest activity in March 2020. (Copyrights: Leaflet / OpenStreetMap contributors/ https://tinyurl.com/y6x6nbs6) (85).

The climate of Nicaragua is tropical, and the year is divided into two main seasons; the dry and the wet season. Between January to June (dry season) precipitation is rare whereas between June to December (wet season) it can rain almost every day especially in June, September and October (87,88). The country is split to three temperature zones: (i) the lowlands of the Pacific and Atlantic coasts where the temperature varies between 22 and 30˚C during the day, reaching 38˚C in May; (ii) the central region where the temperature varies between 17 and 25˚C; and (iii) the mountains where the temperature is between 12 and 20˚C. The daytime temperature in León and Chinandega fluctuates between 23 and 34˚C with high levels of humidity exposing the population to heat stress conditions, especially when workers do not take enough breaks or ensure adequate rehydration potentially exposing the workers in risk of acute kidney injury (AKI) (88,89).

Nicaragua is a developing country as classified by the United Nations Economic and Social Council based on human and economic vulnerability assets (90). The mortality rate of children under 5 years old is 17.2 per 1,000 live births. Based on data from UNICEF, around 16% of the population uses unimproved sanitation services. The population at risk of MeN drinks non-

piped water that usually comes from wells that have been reported as contaminated with faecal coliforms, and well water stored in barrels that were previously used to store pesticides (91,92).

1.2.3 Clinical and pathological characteristics of Mesoamerican Nephropathy (MeN)

As discussed above, MeN is asymptomatic in the preliminary stages with minimal proteinuria and normal blood pressure. The key laboratory finding in CKDu is increased serum creatinine (sCr) (reflecting a low eGFR) which is observed before symptoms develop, although at what is presumably, an already moderately advanced stage of disease (52,93). Even though both genders are affected, reports of a predominance of male agricultural workers suggests occupational risk factors may play a role in disease (94).

Although generally asymptomatic, anecdotal reports suggest that affected individuals report dysuria, a symptom known in Nicaragua as '*chistata*' (95). The absence of albuminuria until the later stages suggests the disease is not primarily of glomerular origin. Reports of elevated urinary biomarkers of tubular injury such as β-2microglobulin (B-2M), Neutrophil Gelatinase-Associated Lipocalin (NGAL) and N-acetyl-β-D-glucosaminidase (NAG), along with limited renal biopsies revealing glomerulosclerosis, fibrosis and tubulointerstitial damage, suggest involvement of the renal tubules (52,53,94,96). Histological studies by Wijkstrom et al. (2013 and 2017) of 8 and 19 male sugarcane workers with suspected MeN in El Salvador and Nicaragua, respectively, described the renal histology of the disease as chronic glomerular and tubulointerstitial damage (Figure 1.10) (50,51). Using light and electron microscopy to analyse biopsies they described non-specific glomerulosclerosis in combination with ischaemic changes, tubulointerstitial injury and mild vascular changes. The authors of these studies suggested their findings support the hypothesis that heat and cyclic dehydration with volume depletion are the underlying causes of the disease (97).

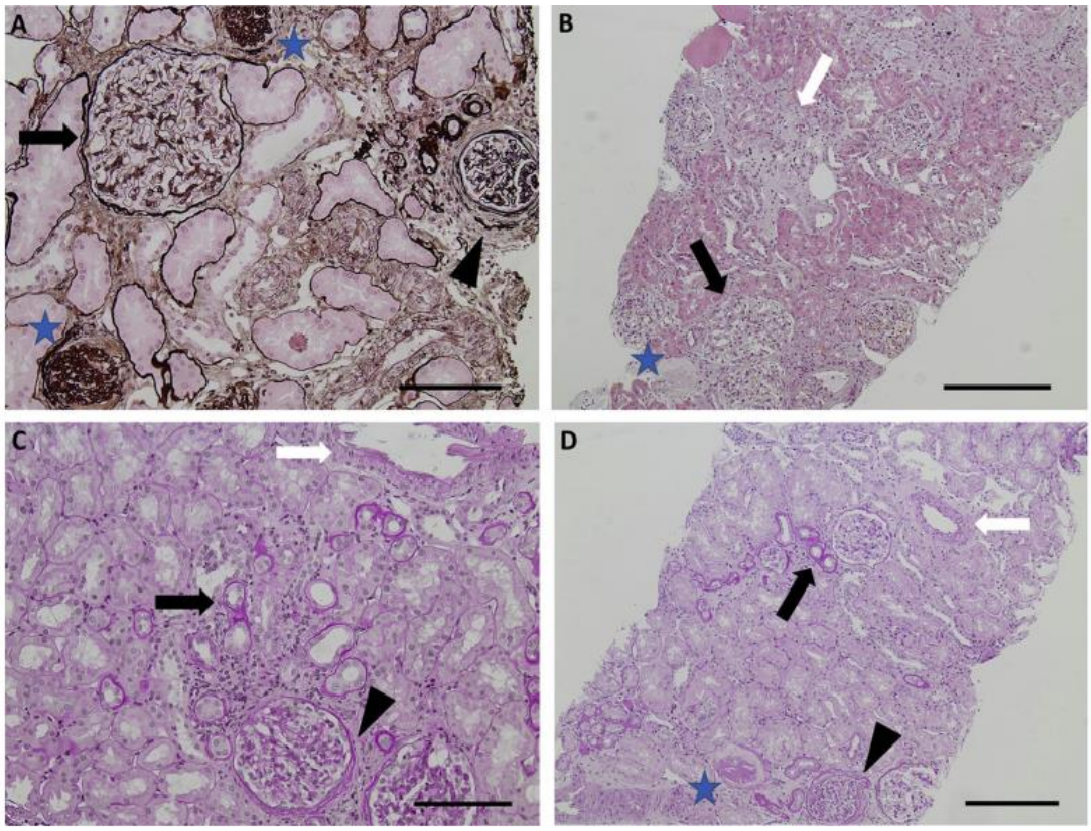

Figure 1.10: Light microscopy of biopsies from MeN patients in Nicaragua. **A.** Glomerulosclerosis (blue stars), glomerular hypertrophy (black arrow), glomerular ischemia with thickening of Bowman's capsule or wrinkling of capillary walls (arrowheads), Scale bar=100 µm. **B.** Glomerulosclerosis (blue star), moderate to severe glomerular hypertrophy (black arrow), mild to moderate interstitial fibrosis (white arrow), Scale bar=200 µm. **C.** Glomerular ischaemia with thickening of Bowman's capsule (arrowhead), interstitial fibrosis (white arrow), tubular atrophy (black arrow), Scale bar=100 µm. **D.** Glomerulosclerosis (blue star), glomerular ischemia with thickening of Bowman's capsule or wrinkling of capillary walls (arrowheads), tubular atrophy (black arrow) and mild to moderate interstitial fibrosis (white arrow), Scale bar=200 µm. A, C and D stained with periodic acid–Schiff (PAS)-methenamine; B stained with Ladewig stain (53).

1.2.3.1 Tubulointerstitial diseases

The tubular cells of the kidneys reabsorb the majority of the filtrate including small molecular proteins and glucose (15). Additionally, organic compounds can enter the tubule lumen via organic acid and organic base transporters at the surface of the proximal tubules. Tubular organic anion and cation transporters are responsible for the secretion of drugs into the renal tubules avoiding the GFB due to their size and their binding with other proteins (98,99). Impaired tubular secretion of drugs during kidney injury can lead to accumulation of drugs in the kidneys and nephrotoxicity (100). These mechanisms in combination with the concentrating ability of the kidney, can

result in increased renal concentrations of pharmacological agents and xenobiotics in their original nephrotoxic form or their metabolites (101).

In cases of dehydration, the concentration of the urine increases such that the luminal surfaces of the tubular cells are exposed to solute concentrations 300-1000 times higher than in the circulation (102). When urine is being concentrated everything that is filtered to be excreted increases in concentration. The result is that substances that normally pass without having an effect on the tubules will cause cell injury or even death in the different segments of the nephron (103).

Tubulointerstitial Diseases (TID) can be a result of a number of pathophysiological processes (104). Acute interstitial nephritis (AIN) is usually a result of an immune response to drugs such as antibiotics e.g. penicillin, and non-steroidal anti-inflammatory drugs (NSAIDs) as well as non-drug antigens or infection (105–107). Mononuclear and eosinophil cell infiltration of the interstitium is more likely to be responsible for this type of injury than the xenobiotic itself (108). Laboratory findings in TID can include mild proteinuria and haematuria and patients may have fever, skin rash and eosinophilia.

Acute tubular injury (ATI) occurs as a result of drugs and toxins acting directly on tubular cells resulting in cell death and if not managed, can cause renal failure, and it presents as acute kidney injury (AKI). Clinical characteristics of AKI include sickness, confusion and drowsiness (109). Renal function is acutely decreased leading to increased waste products in the blood stream (e.g. creatinine and urea) (110). Furthermore, recurrent AKI episodes have been reported as a risk factor for CKD. A retrospective study in patients with an AKI diagnosis in Hospital del Mar in Spain, found that after a 4-year follow-up, from 216 patients with recurrent AKI episodes 17.5% developed CKD stages 3A-4 (111). In 61.8% of those with recurrent AKI the occurrence of CKD was significantly higher than in those with only a single AKI episode.

Agents involved in acute tubular toxicity include NSAIDs, antibiotics, and chemotherapeutic agents (112). Laboratory findings include loss of glucose, amino acids, phosphate and sodium in the urine that normally would be

resorbed by the healthy tubular cells (13,113,114). However, these are rarely tested and more often an increased serum creatinine, urea and electrolytes are enough to give important diagnostic information (115).

Chronic interstitial Nephritis (CIN) is commonly a result of a wide range of persistent insults including nephrotoxic drugs, metals, and other environmental exposures and may develop as a result of AIN. Histologically, the majority of CIN presents with interstitial lymphocytic infiltrate and fibrosis (116).

Morphologically in MeN, interstitial fibrosis and tubular atrophy are the main characteristics, typically there is only a mild mononuclear cell infiltration (53). The progression of fibrosis results in a decrease in the number of functional nephrons and consequently decreased glomerular filtration ability ultimately leading to renal failure (117). Patients with CKDu across the world typically present with a histological picture consistent with CIN (or perhaps AIN/ATI with features of progressive CKD), raising questions about the type of the initial injury that results in CKDu. A wide variety of hypotheses have been described for CKDu and most of these proposed causes have also been suggested to be involved in the aetiology of MeN.

1.2.4 Potential causes of MeN

To date, no clear cause(s) of the disease have been identified. As discussed above, the urinary findings and kidney biopsies of patients with MeN suggest a tubular injury rather than a glomerular one, which supports a toxic or metabolic aetiology that eventually leads to kidney failure (74). Numerous hypotheses have been proposed to explain the development of the disease, including: (i) volume depletion as a result of occupational exposure to heat stress and dehydration; (ii) high fructose intake; (iii) environmental and/or occupational exposure to heavy metals; (iv) phyto- and mycotoxins; (v) agrichemicals; (vi) NSAIDs; (vii) infections; and, (viii) genetic susceptibility (118). In addition, the aetiology of MeN may be multifactorial, combining one or more of these factors, making drawing causal inferences even more challenging.

Subjectively those at highest risk appear at risk of a number of different exposures. Field workers experience severe heat stress and those employed to cut the sugarcane often do so with limited access to shade or water (119). The leaves of sugarcane are sharp and workers with no adequate personal protective equipment (PPE) experience injuries that may allow toxic substances to enter their bloodstream such as agrichemicals (insecticides and/or herbicides) commonly used to improve and protect crop yield. Sugarcane fields are commonly burned to remove the dense leafy matter of the cane, and then harvested by hand using machetes. The usage of machetes leads to sharp cane ends exposing workers to the possibility of skin injury and wounds (Figure 1.11) potentially providing another route of entry of toxic agents into the body.

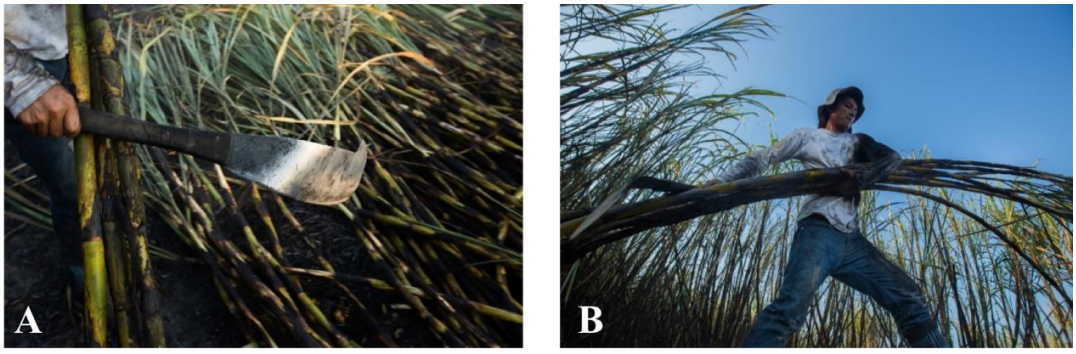

Figure 1.11: Working conditions and tools used during sugarcane harvesting. A. Manual harvesting of burnt sugarcane in El Salvador using machetes. **B.** Sugarcane worker during cut sugarcane collection (copyright to Tom Laffay/ La Isla Network).

These working conditions mean that workers are exposed to a wide range of potential environmental and occupational factors that could contribute to kidney damage. Moreover, workers may also expose other members of their family when, for example, they return to their houses with contaminated clothes and footwear, which can in turn, result in contaminated household items (120,121).

Intense, long working hours lead to musculoskeletal injuries and workers regularly take NSAIDs to alleviate their pain. Additionally, in a study by Butler-Dawson et al*,* it was showed that occurrence of AKI events depend on the dehydration status of the individual (122). Additionally, agricultural workers are more likely to come in contact with rodent-infected crops. Urine or faeces from

animals infected with e.g. *Leptospira interrogans*, can be deposited in the fields and workers can accidentally come in contact by direct contact with open wounds, inhalation or digestion of food contaminated with faeces (123,124).

1.2.4.1 Heat stress, dehydration and excessive workload

A leading hypothesis around the different parts of the world behind CKDu, involves heat stress and repeated dehydration to which workers are exposed to during long working hours without adequate rest. Exposure to high temperatures has been associated with an increased rate of AKI in agricultural workers in California after a single shift (125). Another study in a CKDu hotspot in Nicaragua (MeN), examining 194 male workers of three occupations, concluded that heat stress, dehydration and kidney dysfunction were more common in sugarcane workers compared to construction or small-scale farmers (119). More specifically, being a sugarcane cutter was associated with increased serum creatinine and low eGFR (16% of the cases).

Heavy exertion can also lead to breakdown of skeletal muscle cells, a condition known as rhabdomyolysis, leading to increased, nephrotoxic, free myoglobin and increased serum creatinine levels (as a result of reduced eGFR measured immediately after a working shift) (126). Additionally, in animal models, dehydration has been found to activate the aldose reductase pathway leading to increased osmolality, local oxidative stress in tubular cells and fibrosis (127).

A study conducted in Rhode Island, USA, investigating the effect of heat and heat-related Emergency Department (ED) visits, found an increase in the maximum daily temperature from 24˚C in 2005 to 30˚C in 2012 was associated with an increase in heat-related ED visits from 1.3% to 23.9%, respectively, although, the study noted the prevalence to be higher in vulnerable, at risk populations (<18 or ≥65 years old) (128). It should be noted that this analysis excluded patients if they had previous episodes of AKI, CKD or any other comorbidities such as hypertension meaning that the true values are likely to be higher.

In the rural areas of Nicaragua, sugarcane workers lose ~3-4 L of water each day depending on the humidity and the weather conditions (129). The US Occupational Safety and Health Administration (OSHA) advises that rest is required 75% of the time when the Wet Bulb Globe Temperature (WBGT) exceeds 30˚C. The WBGT is a guide to manage workload, which is calculated after taking into consideration the temperature of the specific area, the humidity, the wind, the sun angle and the cloud coverage. However, Nicaraguan agricultural workers do not take the required rest even when the WBGT in the fields reaches 35˚C (130,131).

In El Salvador, workers in the lowlands seem to be more affected than those working in the highlands, suggesting temperature may play a role in the development of MeN (74). In addition, Trabanino et al. (2015) identified a high prevalence of reduced eGFR with high dehydration frequency in 189 sugarcane workers in El Salvador (129). Cross-shift changes in urine specific gravity, osmolality and creatine (that were increased) and in serum creatinine, uric acid and urea nitrogen (that were increased) and chloride and potassium (that were decreased) were analysed.

In a recent study by Pryor et al. (2020), 20 healthy American men were exposed for 4 days to 2-hour bouts of high intensity exercise in either hot (n=12, 40˚C, 40% relative humidity) or mild (n=8, 24˚C, 20% relative humidity) environmental conditions with full access to water (132). Baseline sCr were similar between the two groups. Heat acclimation for 6 days prior to the excessive exercise, was not found to be protective against the increase in AKI biomarkers. Both groups had an increase in sCr and a lower eGFR, with the group exposed to high temperature and humidity having the highest increase and reduction, respectively.

Another recent study tested the effect of hyperthermia and dehydration during physical work in 12 healthy individuals (n=9 men and n=3 females), exposing them to: (i) access to water; (ii) access to cooling; (iii) access to a combination of water and cooling conditions; and, (iv) control (no access to water/hydration and cooling) conditions during a 2 hour period of exercise in an environment of 39.7±0.6˚C, 32±3% relative humidity environment (133). The highest levels

of hyperthermia (assessed by the increase in core temperature) and dehydration (assessed by the loss of body mass) occurred in the control group. Various kidney injury biomarkers in the urine such as albumin, NGAL and insulin-like growth factor binding protein 7 (IGFBP7) were also tested and were found to be elevated in the control group. Additionally, IGFBP7 remained elevated in the control group after correcting for urinary concentration. No differences in Tissue inhibitor of metalloproteinase 2 (TIMP-2), used as a marker of AKI, were observed across the groups. Overall, the investigators suggested the proximal tubules to be the primary location of kidney injury.

There are many countries around the world with higher average yearly temperatures (~27˚C) than Nicaragua (~24˚C), for example Nigeria in Africa and Thailand in Asia (134). In Thailand, the prevalence of CKD in the northeastern region has been reported to be only 1.9% higher than the national prevalence (135). In Nigeria on the other hand, a study reporting the primary renal disease that led to ESRD between 1990 and 2003, in 1001 male and 537 women, found that 51.6% of the cases had no known etiological factors such as hypertension and glomerulonephritis (136). Saudi Arabia and Haiti have a similar average yearly temperature as Nicaragua. In Saudi Arabia, CKD is prevalent in people with diabetes mellitus, hypertension and obesity (137,138). In Haiti, CKD has been shown to be associated with traditional risk factors such as hypertension (49.2%) and diabetes mellitus (36.3%) in 608 patients (139). These data suggest that heat stress cannot itself explain the epidemic of CKDu.

Many occupations can lead to excessive body heat where the core temperature will rise, the heart rate increases and sweat will evaporate depending on the clothing (140). Such occupations include mining, working in compressed air tunnels, boiler rooms, bakeries and catering kitchens as well as in brick-firing and ceramics plants. Athletes are also known to be exposed to repetitive renal injury (AKI) although to date this has been suggested to be a temporary condition rather a cumulative one that can lead to CKD (141).

In summary, given the wide ranging occupations and where high temperatures occur where CKDu has not yet been reported, and regions with tropical

climates without reported disease, the above suggest that heat stress itself is unlikely to be a sole factor that leads to MeN. That is, heat stress is not sufficient alone to cause CKDu but may of course exacerbate a pre-existing subclinical kidney dysfunction.

Interestingly, a recent study in a mouse model of heat stress testing the effect of the hyperosmolarity mediator, vasopressin, which can cause renal injury, found increased activation of the polyol pathway in the renal cortex with glomerular and tubulointerstitial injury and inflammation (127,142). The polyol pathway converts glucose into fructose and leads to high fructose production. Fructose eventually will cause an increase in intra- and extra-cellular sorbitol, reactive oxygen species (ROS) and low concentrations of nitric oxide (NO) and glutathione. In the next section the importance of fructose and its potential role in MeN will be discussed.

1.2.4.2 High fructose intake

In addition to heat and dehydration, consumption of high sugar drinks and the chewing of sugarcane during work, could increase the intake of fructose which has been proposed to aggravate kidney injury (78,118). Fructose is a monosaccharide available in various food sources including fruit. Sucrose is a disaccharide composed of fructose and glucose and is the main source of fructose in most countries (143,144). High-fructose corn syrup (HFCS) is widely used in soft drinks containing around 45% glucose and 55% fructose. It has been suggested that the population worldwide has been exposed to increased levels of sucrose over recent decades which in turn explains the increase in obesity, diabetes mellitus, metabolic syndrome, hypertension and CVD (145,146).

Metabolic syndrome has been demonstrated as an important risk factor for CKD, suggesting that fructose could have a causative role in the development of MeN due to increased consumption of sugary beverages across Nicaragua. The US National Health and Nutrition Examination Survey (NHANES) for the years 1999-2004 found that consumption of two or more high sugar beverages per day, was linked to an increased risk of albuminuria (147).

Fructose is absorbed by the intestine in the jejunum by glucose transporter-5 (GLUT-5) and is metabolised mainly by the liver (148,149). Breakdown of fructose leads to high uric acid levels (Figure 1.12) (150). In the circulation, fructose is taken by various cells including the cells of the S3 segment of the proximal tubule, via the same transporter, GLUT5, resulting in oxidative stress, inflammation and kidney injury by either the production of uric acid as described above or direct toxicity as described in *in vitro* and *in vivo* models (151,152). Increased serum uric acid can also occur as a result of other conditions such as rhabdomyolysis or hereditary hypouricemia (153).

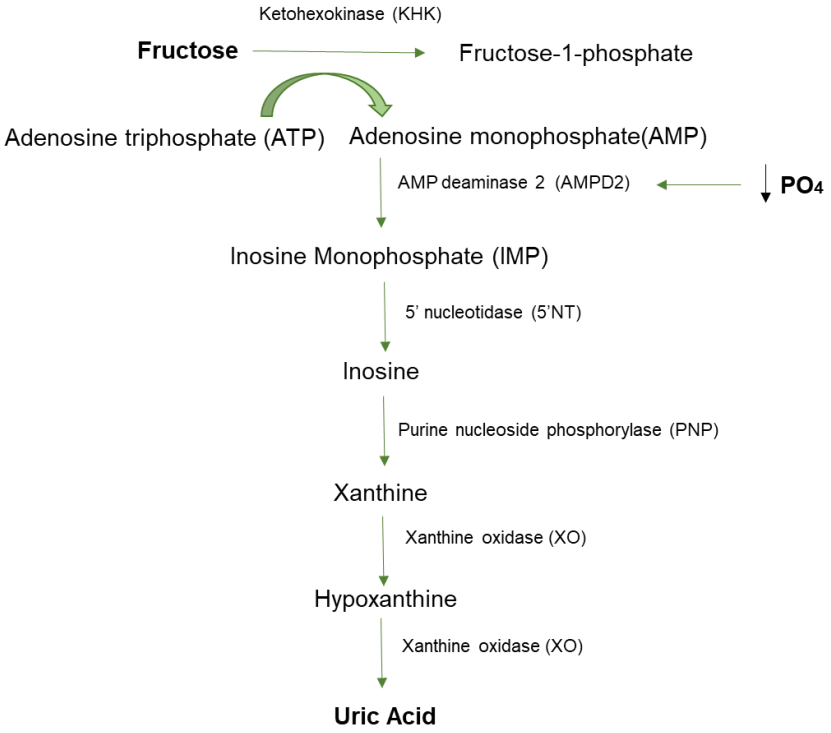

Figure 1.12: Uric acid production from fructose. Fructose is phosphorylated in the liver by ketohexokinase (KHK) producing fructose-1-phosphate (F-1-P) with the use of ATP. Depletion in phosphate (PO_4) levels triggers AMP deaminase 2 (AMPD2) activity which converts AMP to inosine monophosphate (IMP). IMP is then metabolized to inosine by 5′ nucleotidase (5′NT). Inosine is further degraded to xanthine by purine nucleoside phosphorylase (PNP) which in turn is converted to hypoxanthine by xanthine oxidase (XO). Finally, hypoxanthine is converted to uric acid.

In vivo, a study by García-Arroyo et al. (2017) in rats, showed that even small doses of fructose interacted with vasopressin and amplified the renal damage caused by dehydration (154). While in another study, high fructose was shown to increase the release of vasopressin which was not observed in fructokinase-

knockout mice (155). There is some experimental evidence in humans that demonstrates that high levels of fructose consumption can lead to high levels of fructose-1-phosphate through the activity of fructokinase, present in the proximal tubule of the kidneys (156). It has been proposed that, intracellular adenosine triphosphate (ATP) increases leading to intracellular phosphate depletion, activation of adenosine monophosphate deaminase (AMPD), generation of uric acid, oxidative stress, and production of chemokines (157). Uric acid is then released in the circulation, stimulating an inflammatory response in endothelial cells, the kidneys, the vasculature, and the pancreas (158).

Further evidence on fructose metabolism, has been presented using *in vivo* models where dehydration-associated hyperosmolarity was found to activate the aldose reductase pathway in the renal cortex, resulting in the generation of sorbitol that can be converted to fructose (127). In addition, in rats, a high fructose diet has been shown to accelerate the progression of renal disease with increased proteinuria, cardiac hypertrophy, glomerulosclerosis, tubulointerstitial fibrosis and increased expression of the inflammatory cytokine, MCP-1 (151,152,159). Finally, older *in vivo* studies in rats have also shown that fructose and sucrose can result in renal hypertrophy and tubulointerstitial disease (160,161). A similar histopathology to that found in MeN.

Twelve healthy men participated in a four-step experimental study including the consumption of 600 mL of distilled water with added glucose in concentrations 1, 2, 5 and 10% with each trial to be 7 days apart (162). Blood samples were taken every 10 minutes for an hour after consumption. The results showed that the consumption of 5 and 10% glucose solutions lead to decreased plasma volume and consequently increased osmolality resulting in increased viscosity of the blood after 10 minutes of ingestion. The effect lasted 10 minutes after the digestion of 5% glucose concentration and for all the time points after the digestion of 10% glucose concentration.

A study by Wesseling et al. (2016) involving 194 young male workers (sugarcane cutters, construction workers and small-scale farmers) in

Nicaragua found that an increase in serum uric acid was related to low eGFR, linking kidney impairment with the presence of increased levels of uric acid (119). This relationship was more obvious in sugarcane cutters than in the other groups. Finally, a recent case study revealed the consequences of hyperuricemia in a patient with MeN in El Salvador with the appearance of gouty arthritis and yellowish skin deposits in the ear lobes (163). However, the hypothesis that hyperuricemia can directly injure renal tubules and contribute to MeN has yet to be proved. To date, there is not strong evidence to suggest that increased uric acid has a causative role in the development of MeN since impaired kidney function in itself leads to increased uric acid in the bloodstream.

1.2.4.3 Pesticides and herbicides

Unfortunately, the use of agrichemicals to increase crop yield can result in adverse effects in humans. Typically, this is through inappropriate use, including; lack of protective equipment such as gloves, masks and eye protectors, application of inappropriate amounts, incorrect application, inappropriate storage, or inadequate training of personnel involved in application (164,165).

The use of agrochemicals such as insecticides and herbicides is widespread in the areas affected by CKDu (74). These pesticides vary widely in formulation and are composed of hundreds of active ingredients of various chemical structures and toxicities (118). There are limited data on the human nephrotoxicity of agrichemicals, however a recent retrospective cohort study from the USA that looked at the use of 39 pesticides in the states of Iowa and North Carolina, found a positive association between chronic exposure to herbicides, alachlor, atrazine, metalochlor, paraquat and pendimethalin, as well as the insecticide, permethrin, and the risk of ESRD. Of those chemicals cited, paraquat is commonly used in Central America (166).

Using data from the United States Renal Data System, Lebov et al. (2016), reported that amongst 55,580 male pesticide appliers, 320 had ESRD (166). They also reported a positive exposure-response association for the

herbicides alachlor, atrazine, metolachlor, paraquat, and pendimethalin and the insecticide, chlordane. A study in Delhi, India, recruited 300 individuals with healthy kidney function, CKDu and CKD from known causative factors, who were tested for blood organochlorine pesticides (OCPs) (167). The participants with CKDu were reported to have elevated levels of OCPs: alpha-Hexachlorocyclohexane (α-HCH), aldrin and β-endosulfan, compared to the other two groups whereas the higher the OCPs levels the lower the eGFR.

The mechanisms by which pesticides cause kidney injury and potentially lead to the development of CKD are not clear, although several different mechanisms have been proposed including lysosomal dysfunction, changes in the cell membrane, oxidative stress and the disruption of Ca^{2+} homeostasis leading to cell death (168–170). Pesticides and herbicides can also lead to oxidation of glutathione resulting in oxidation of cellular enzymes, ATP depletion and loss of mitochondrial function. Renal proximal tubules are vulnerable to toxic reagents and chemicals as a result of their high reabsorption and secretory activity (171).

Most herbicides such as paraquat are actively transported to the tubule lumen by organic cation transporters (OCTs). Renal biopsies in animals exposed to paraquat reveal vacuolation of the proximal convoluted tubules and proximal tubule necrosis (172). Other signs include mild histological changes in the glomeruli, proteinuria and haematuria. The mechanism of herbicide nephrotoxicity is not fully understood but it is assumed that the herbicide undergoes redox cycling in the presence of NADPH and oxygen, generating superoxide and the subsequent development of lipid peroxidation and membrane damage. The development of hydroxyl radicals results in oxidative damage to nucleic acids, proteins, and polysaccharides (173).

Different agrichemicals persist for different durations in the water and soil. For example, in the soil 2,4-Dichlorophenoxyacetic acid (2, 4-D) lasts 2-3 weeks; 2-methyl-4-chlorophenoxyacetic acid (MCPA), 6-8 weeks; and, 2,4,5-Trichlorophenoxyacetic acid (2, 4, 5-T), 2 months to one year, while their degradation in water is slower. 2, 4, 5-T is the most effective herbicide which acts on the broadest spectrum of woody species. In the human body there is

no evidence that 2, 4-D can have a cumulative effect however its contribution to toxicity remains unknown (174).

In Nicaragua, pesticides have been in use since the early 1950s. Unsafe conditions resulting in pesticide poisoning was reported by the Northwestern Nicaraguan Ministry of Health in 1993, demonstrating high levels of work-related poisoning in children under 16 years old (175). In a period of one month only in July 1987, 548 pesticide poisoning cases were detected mostly caused by carbofuran or methamidophos (77%). This study also reflects the exposure of a young population before the rise of MeN cases.

Direct contamination of water sources by pesticide run-off represents a major public health concern in Mesoamerica (176). In addition, the routine burning of sugarcane fields as part of the harvesting process could produce toxic particulate matter that can enter the body through inhalation, ingestion of contaminated food and water or cuts sustained from manual harvesting. The importance of these substances in the aetiology of CKDu remains to be elucidated.

1.2.4.4 Metals and metalloids

Environmental exposures to metals and metalloids such as arsenic (As), cadmium (Cd) and mercury (Hg), have been characterised as potential risk factors in CKDu (177). Although CKD caused by exposure to metals has been described among industrial workers, it is possible that the presence of metals in water, soil, air and food from natural or human sources could also expose the population to high levels through inhalation, ingestion and transcutaneous routes (178).

Heavy metals present in plasma are carried either in protein bound (non-diffusible) form or in complex/ionized (diffusible) free form. Following exposure, metals are cleared from the bloodstream and accumulate in various tissues including the brain and the kidneys (179). The kidney is a target organ in metal toxicity (180). Even in the resting state, environmental or occupational toxins may lead to renal damage due to the substantial proportion of cardiac output

that passes through the kidneys (181). The severity of kidney damage depends, as with most toxicants, on the type of metal, the metal species, the levels and the exposure time (acute or chronic). However, volume depletion in combination with the concentrating function of the kidney, as seen in dehydration, may mean higher concentrations of solutes are present. This could lead to increased toxicity from agents that in lower concentrations would not have the same detrimental effect. Various metals have nephrotoxic effects and are associated with the development of CKD (Table 1.3).

Metal	Source of exposure	Clinical Presentation	Pathophysiology	Environmental Data	Epidemiological Data	Evidence for a role in CKDu in Central America
Inorganic Arsenic (As^{3+} or As^{5+})	Water Seafood Medication Inhalation Pesticides (182)	Tubulointerstitial nephritis which associates with elevated urinary concentrations that provoke the disease. Extensive interstitial fibrosis with lymphocytic infiltration (183) Adverse effects of As (urine) exposure on the risk of proteinuria modifiable by changes in As exposure (184) Damage in the kidney capillaries, tubules, and glomeruli (185,186) Biopsies in dogs revealed glomerular sclerosis and tubular necrosis (187) Glomerular sclerosis, tubular necrosis, and increase in urine NAG concentrations (experiments in mice) (188)	Oxidative stress and DNA oxidative damage in kidney tissue (experiments in mice) (189) Inflammation (190,191) and oxidative stress (*in vitro* experiments) (192,193)	Groundwater containing As in Taiwan, Argentina, Mexico, and Chile, Bangladesh and areas with private wells in the United States is the main cause of death (194–196)	**Michigan**: Cerebrovascular diseases and cancer (serum levels) (197) **Utah**: Significantly elevated levels associated with nephritis and nephrosis (serum levels) (198) **Taiwan**. Positive dose - response relationship between urine Total As and CKD status based on eGFR (urinary levels) (199) **Sri Lanka**. In agricultural workers with CKD the individuals who excrete more than 35µg/L As in urine are 5.58 times more likely to develop CKDu (biopsy examination) (61) Urine As concentrations at levels that cause CKD (63) **Japan**: Inorganic As seems to have higher affinity for tissues and organs than methylated As. No statistically significant difference in the	Biological data from workers in Nicaragua demonstrate moderate levels in urine and blood (201)

					chemical species of As in the tissues and organs between humans who had died suddenly (of cerebral bleeding) and those who had died of chronic diseases such as cancer and pneumonia (biopsy examination) (200)	
Cadmium (Cd)	Food Cigarettes Water Inhalation (202)	Necrosis of proximal tubules, interstitial renal fibrosis (*in vivo*) (203) Other effects: Effects on bone (Loss of calcium from bones and ↑ kidney excretion) generally arise only after kidney damage has occurred and are likely to be secondary, resulting changes in calcium phosphate and vitamin D metabolism (204) Kidney stones (205) Urinary NAG and glucosuria Proteinuria and a decrease in GFR (human study) (206–208) Decrease in GFR in chronic exposure (209) Renal tubular functional abnormalities, including reduced reabsorption of B-2M, retinol binding protein (RBP), Ca, and P (human study) (210)	Free Cd in the cytosol causes generation of ROS (mitochondrial accumulation of Cd blocks the respiratory chain in complex III) and activates cell death pathways ((mitochondria of pig brain, heart, liver) (211,212) Cd inhibits endothelial NO synthase in blood vessels, which suppresses acetylcholine-induced vascular relaxation, and ultimately induces hypertension (blood pressure of rats) (213) Formation of anti-metallothionein (MT) antibodies. Once MT's capacity for Cd storage has been exceeded, free Cd induces the formation of antibodies against MT, which are also toxic to proximal tubular cells (immuno-toxicity) (experiments in mice) (214)		**Japan**: Proximal tubular dysfunction (PTD) has been found among the inhabitants of the Jinzu River basin due to environmental Cd exposure (215) **Belgium**: Exposure to low Cd levels can result in nephrotoxicity and up to 7% of exposed population suffer kidney injury (whole blood and urine) (216,217) **Sweden**: Cd-induced kidney toxicity can proceed concurrently through renal tubular and glomerular damage, even with a low exposure (blood and urine) (218) **Thailand**: Cd exposure levels are associated with increases in prevalence of high blood pressure and signs of renal tubular damage (urine and serum) (219) **Korea:** Association of environmental Cd	Nephrotoxic substances of Cd can originate from the burning of sugarcane (221) Among Ingenio San Antonio (ISA) field workers concentrations of Cd in urine did not increase during the harvesting period. In fact, were significantly lower in the post-harvest compared to pre-harvest period (p=0.04). Cd concentrations were not significantly different by job (p=0.2) and were generally consistent with concentrations in the US general population (201)

					exposure with CKD in adults with diabetes or hypertension (blood) (220) **Sri Lanka:** CKDu cases have concentrations in urine significantly higher compared to the controls, in both the endemic and the non-endemic areas. Among CKDu cases, the concentration of Cd in urine was positively correlated with Pb (63)	
Lead (Pb)	Contaminated air (222) Water Soil Food Consumer products	Acute Pb nephropathy is characterized by a generalized deficit of tubular transport mechanisms (Fanconi syndrome) and morphologically by the appearance of degenerative changes in the tubular epithelium and nuclear inclusion bodies containing Pb-protein complexes (223) Glomerular and tubulointerstitial changes, resulting in chronic renal failure, hypertension and hyperuricemia (224) Aminosiduria, glycosuria and hyperphosaturia. Inhibits excretion of waste products (case study) (225) Chronic tubulointerstitial nephritis and progressive deterioration of renal function (patients). Difficult to diagnose in the early stages (226)	Mitochondrial damage, formation of ROS, intracellular depletion of GSH and apoptosis (cultures of rat proximal tubular cells) (228) Affects enzymatic reactions in which Ca plays a role (228) (cell culture) (229) Activation of NFk-B, activation of the renin-angiotensin system and attraction of macrophages (inflammatory process in the renal interstitium tubulointerstitial damage and high blood pressure) (experiments in rats) (230) Decreases NO production and the expression of the enzyme guanylate cyclase, hypertension (*in vivo* and *in vitro*) (229–233)	In Sri Lanka, Pb is high in dry zone soils (235)	**United States**: Higher blood Pb levels were associated with a lower eGFR in people with hypertension (serum levels) (236) **Taiwan:** Individuals without diabetes with chronic nephritis and high levels of Pb in the body experience faster deterioration of renal function (blood levels) (237,238) **Shanghai**: Occupational exposure to Pb could cause a decrease in bone mineral density, leading to osteoporosis, and may affect bone metabolism (Pb-exposed workers) (blood and urine samples) (239) **Idaho:** In female smelter workers changes were	No evidence that Pb is associated with biomarkers of kidney injury or kidney function (201)

		Chronic interstitial nephritis by continued or repetitive exposure that causes toxic stress to the kidney (227)	Stimulates the activity of NADP(H) oxidase by increasing production of hydrogen superoxide and hydrogen peroxide, oxidative stress and intracellular redox potential (human endothelial and vascular smooth muscle cells) (234)	found in blood and bone Pb levels over time and were associated with increased bone resorption, especially among post-menopausal women (240)	
Mercury (Hg)	Seafood Water Dental amalgam Fuel emissions Batteries	Acute tubular necrosis. Glomerulosclerosis. Interaction with proximal tubules, proteinuria, glomerulonephritis and Nephrotic Syndrome (biopsy) (241–243)	Hg interferes with mitochondria (yeast cells). Increases ROS production, alteration in Ca homeostasis and increased lipid peroxidation. Alters membrane permeability and macromolecular structure due to its affinity for sulfhydryl and thiol groups, also causes DNA damage (244–246)	**Minamata** and **Iraq:** Poisoning (no access to publication)	Multinational chemical and pharmaceutical firm based in the United States, operates a ***chloralkali plant*** in Managua which utilizes elemental Hg in the production of chlorine and caustic soda for markets throughout Central America. The plant was found to be contaminating the waters of Lake Managua (on which the plant is located) (1981) (247)

Table 1.3: Elements with nephrotoxic effects. Route of toxicity, clinical features, pathophysiology of toxicity, environmental data from around the world and from Central America and biological data (248).

In acute metal exposure, the main sites of reabsorption are the proximal tubules (S1 segment), the loop of Henle and the terminal nephron segments. A number of metals are ionized intracellularly resulting in toxicity, membrane damage, disruption of mitochondrial respiration and expression of cytokines and ROS (249). Chronic metal exposure leads to conjugation with metallothionein and glutathione and release into the bloodstream. In the kidneys the conjugates will be reabsorbed through endocytosis in the proximal tubules leading to chronic inflammation, fibrosis and renal failure (249,250).

The most nephrotoxic elements include lead (Pb), cadmium (Cd), mercury (Hg) and arsenic (As). Specific biomarkers of metal exposure are rare and direct measure of the metals and metalloids the most widely used way of testing exposure.

1.2.4.4.1 Arsenic (As)

Arsenic is known for its toxic effects and humans can be exposed through various routes including the skin, consumption of contaminated water from natural sources and through agricultural use of pesticides and fertilisers (251). Arsenic also exists as different species and exposure from food sources such as rice and seafood can be a major source of exposure (252). There are two toxic inorganic forms of arsenic, trivalent (As^{3+}) and pentavalent (As^{5+}) arsenic and less toxic are methylated metabolites and also organic arsenic, (e.g. arsenobetaine from seafood) which is not toxic (253). Inorganic As exposure occurs from environmental sources such as rocks and water as well as from industrial processes. The biological half-life of ingested fish sources of As is estimated to be around 20 hours (254). In humans, As is eliminated from the circulation into the urine with a half-life of 10 hours (254).

The majority of inorganic trivalent or pentavalent arsenic, As^{3+} and As^{5+}, is easily biomethylated in the liver to toxic forms with tissue-binding properties, monomethylarsonic acid (MMAV) and dimethylarsinic acid (DMA), and around 50% of As excreted in the urine is in the DMA form (255–258). The 40-60% of the arsenic trioxide (airborne As^{3+}) that will be absorbed by the lungs (depends on the particle size and solubility) will be normally excreted through the urine

(258). Urinary excretion of As involves predominantly a mixture of MMA, DMA and some unmethylated inorganic species.

During chronic As exposure through ingestion, As accumulates in the liver, kidneys and other organ systems. A number of animal studies have demonstrated that the accumulation of As in the kidneys leads to endogenous depletion of antioxidant activity, causing oxidative stress and tissue damage (259–262). Additionally, exposure to pentavalent arsenic (As^{5+}) as arsenate and sodium arsenate (Na_3AsO_4), has been reported to lead to decreased antioxidant activity, changes in the fatty acid composition, renal DNA damage, [Ca^{2+}]-mediated cell death and downregulation of Nef-2 expression (263,264). Na_3AsO_4 has also been reported to increase serum creatinine, urea nitrogen levels and alter the activity of superoxide dismutase, GSH-peroxidase and catalase as well as increasing lipid peroxidation in renal tissue (265,266). In dogs, high doses of Na_3AsO_4 were found to cause glomerulosclerosis and severe ATN (187). In the killifish (Fundulus heteroclitus) experimental model, acute Na_3AsO_4 toxicity decreased mitochondrial function and up-regulated the multidrug resistance-associated protein 2 (MRP2) while chronic exposure had no effect on mitochondrial function suggesting a potential protective effect of increased MRP2 levels (267). Similarly, as seen in Pb exposure, toxic levels of As, result in high levels of NAG in the urine. Chronic exposure is characterised by increased urinary excretion of other low molecular weight proteins, aminoaciduria, glycosuria and phosphaturia (268).

Finally, experiments in two human cell lines: SY-5Y neuroblastoma and 293 human embryonic kidney (HEK293), showed reduced cell viability, increased apoptosis and DNA damage after exposure to arsenic trioxide (As_2O_3) (269). $NaAsO_2$ coupled with increased H3 histone acetylation and CBP/P300 recruitment has also been found to modulate CpG sites at a site -168 bases upstream of the transcription start site in the IL-8 gene promoter leading to increased production of the chemokine and causing changes in HEK-293 cell function and viability (270). The latter two studies suggest that arsenic can induce DNA damage that could affect the transcription of a variety of genes,

including those potentially involved in and important in the elimination of xenobiotics.

In a cross-sectional study of 374 Bangladeshi participants exposed to a wide range of As concentrations in contaminated well-water, low levels of plasma glutathione and a high oxidative stress (as a result of reduced GSH as an antioxidant) were associated with an increased risk of As-induced inflammation (271). Additionally, a 10% increase in urinary and blood As was associated with a reduction in eGFR in the same study. In the proximal tubules, As toxicity leads to depletion of intracellular GSH stores, activation of apoptotic proteins such as caspase 3 and caspase 9, and activation of the p53 apoptotic pathway. The increase in ROS causes inflammation and cell death (268). Lastly, animal and human studies have reported synergistic toxic effects of As and Cd with increased renal toxicity following exposure to the combination compared to either one individually (188,272).

1.2.4.4.2 Cadmium (Cd)

Populations can be exposed to cadmium (Cd) though occupational and environmental routes. Cd has a long biological half-life which means it can be detected in humans up to 30 years after exposure (273). Cd is absorbed and transported via the hepatic portal system to the liver, and accumulates in a number of organs including the kidneys (274).

It has been shown *in vivo* that Cd can be transported bound to albumin but also complexed with metallothionein (MT). Additionally, MT-Cd can be freely filtered by the glomerulus and reabsorbed via the zinc transporters, ZIP8 and ZIP14 by the proximal and distal tubules through endocytosis (275,276). Increase of intracellular Ca^{2+} and mitochondria depolarization, after Cd exposure, activates caspase leading to apoptosis through the ERK pathway and through glycogen synthase kinase (GSK-3β) activation in mesangial cells (277).

Studies have focused on identifying biomarkers of Cd nephrotoxicity. Early signs of Cd nephrotoxicity include low molecular weight proteinuria, specifically proteins that have not been reabsorbed by the injured tubules including B-2M,

RBP, and urinary α-1-microglobin (A1M) (278). RBP has been found to be a more sensitive marker than beta-N-acetyl-D-glucosaminidase and to be more stable in acidic urine following Cd toxicity (279). In the later stages of Cd toxicity, clinical characteristics include aminoaciduria, glucosuria, loss of calcium, phosphorus, uric acid and the inability of the kidneys to concentrate urine. The loss of calcium and phosphorus can lead to bone demineralization resulting in kidney stones (280).

In Japan around 1912, in the Jinzu river basin in Toyama, a Cd-polluted area, a rise in people suffering from kidney failure (Itai-Itai disease) was observed with increased excretion of B-2M which was related to the levels of Cd, decreased creatinine clearance and decreased renal phosphorus reabsorption leading to phosphorus depletion (281). Cd contaminated rice was found to be related with renal dysfunction in the Itai-Itai disease patients that resulted in renal disturbances in vitamin D and parathyroid hormone metabolism, and increased calcium loss in the urine (282–284).

A complicating factor when investigating the relationship between Cd and kidney injury was highlighted by a recent study in which measurement of urinary Cd levels at different time-points after cardiac surgery suggested Cd as a good biomarker for AKI but not a mediator of injury in itself (285). It was found that chronic accumulation of Cd in the kidneys can be released in the urine after tubular injury making Cd a good potential biomarker of acute injury in contrast to serum creatinine which can be influenced by multiple factors and may take many hours to rise after an injury. The evidence suggest that high levels of Cd exposure can cause kidney disease, but care should therefore be taken interpreting any associations between urinary Cd and measures of kidney function due to the possibility of reverse causation. Potential associations should be followed by further investigation of other biological materials (e.g., blood or hair) to verify its plausibility.

1.2.4.4.3 Lead (Pb)

Lead (Pb) is known for its effects in high doses including ATN and acute and progressive chronic renal failure. Pb has a biological half-life in the blood up to

35 days after exposure (254,286,287). Occupational exposures to Pb in battery and smelter factories where the exposure is high, has been shown to be associated with increased mortality due to renal disease (288–291). Importantly the onset of renal dysfunction is likely to be delayed from the time of exposure which makes it difficult to detect the source of exposure (292). In chronic and overt Pb toxicity, aminoaciduria is the most consistent nephropathic finding (293). In an Australian study, childhood exposure to Pb was found to be a contributory factor in chronic nephritis (294).

Pb is bound to albumin following absorption by the intestines or the lungs (295). Pb also binds to low-molecular weight proteins allowing it to be filtered freely by the glomeruli (295). Pb enters cells through endocytosis and erythrophagocytosis causing oxidative damage and cell death by uncoupling the respiratory chain of mitochondria (296). It is also proposed that Pb antagonizes Ca (replacing it) resulting in high levels of Ca excretion from the mitochondria that cause mitochondrial damage, induce oxidative stress and alter lipid metabolism (297).

It is hard to detect all the possible sources of Pb exposure when signs and symptoms of poisoning do not appear until life-threatening accumulative levels have been reached. Sensitive tests for assessing changes in kidney function caused by Pb have been proposed which include changes in urinary excretion of high and low molecular weight proteins such as albumin, transferrin, immunoglobin G, RBP, B-2M, A1M and urinary enzymes including acetylglucosaminidase (NAG), alkaline phosphatase (ALP) and gamma-glutamyl transferase (γGT) (223). NAG has been reported as the most sensitive marker of injury as it is highly expressed in the mitochondria of the proximal tubules and its excretion is a result of cell damage, necrosis and exfoliation following Pb toxicity (298,299).

In battery industry workers exposed to Pb, decreased urinary excretion of 6-keto-prostaglandin F1α (6-keto-PCFα) and increased excretion of thromboxane (TxB2) was noted (223,300). Additionally, a decrease in prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α) represent early renal changes (223). In primary cultures of rat proximal tubular cells, even low-

concentrations of Pb led to cell death and apoptosis as a result of oxidative stress (228). In the late stages of chronic Pb exposure glomeruli become sclerotic, similar to what is found in MeN patients.

1.2.4.4.4 Mercury (Hg)

Mercury and its forms are used for industrial purposes such as in fungicides. High levels of chronic industrial exposure to mercury (Hg) have been reported to be associated with proteinuria and glomerulonephritis (301,302). Mercury can be found in three forms: (i) elemental mercury; (ii) inorganic mercury; and, (iii) organic mercury compounds (303,304). Worldwide, the general population is exposed mainly though seafood to methylmercury (MeHg), inorganic mercury (I-Hg) (such as mercury chloride and mercury sulphide) from food, and mercury vapor (Hg^0) from environmental and industrial sources (305). Depending on its form, Hg can have different toxic properties. For example, mercuric chloride causes ATN through fragmentation of the plasma membrane, vesiculation and disruption of the endoplasmic reticulum, dissociation of the polysomes and mitochondrial swelling (306). The estimated biological half-life of I-Hg is around 60 days (307). Organic methyl MeHg can cross the blood brain barrier (BBB) and is a known neurotoxin. *In vitro*, in human tubular cells, there was a direct toxic effect of mercuric chloride when cells were exposed to high doses (100 µM) (308). In humans proteinuric kidney injury due to mercury toxicity is thought to involve development of immune-complex glomerulonephritis (309).

Workers exposed to mercury vapor in a chloralkali plant in Managua, Nicaragua, have been shown to have neurological symptoms including tremors and paraesthesia (247). Studies in the early 1980s showed that exposure to mercury vapor can cause subclinical renal symptoms including urinary excretion of high molecular weight proteins such as RBP, albumin and lysosomal enzymes (310,311). As a volcanic country, Nicaragua is frequently exposed to volcanic gases. A study by Witt et al. (2008), measuring the volcanic emissions of Masaya and Telica, reported these were enriched in Hg (312). Thus, people at risk of MeN are exposed to Hg. The role of Hg or its role as a contributory factor in the development of the MeN is still not known.

1.2.4.4.5 Silica (SiO_2)

Sugarcane leaves owe their rigidity and sharp edges to the presence of phytoliths of amorphous Silicon dioxide (SiO_2) in the tissue. Upon sugarcane burning, amorphous SiO_2 is converted to crystalline SiO_2 (specifically the polymorphs, cristobalite and quartz), airborne particles of which can be inhaled by field workers (313). Other potential routes of amorphous and crystalline SiO_2 entry into the body include cane chewing, direct contact with the skin and contamination of food or water. It is therefore possible that accumulation of crystalline SiO_2 in the kidneys might be a contributory factor in MeN (313–315). Respirable crystalline SiO_2 is a human lung carcinogen but there is little reported evidence of respiratory health problems among sugarcane workers, although these would be expected to develop only after 40-45 years of exposure (316). Occupational exposure to crystalline SiO_2 and the development of chronic nephropathy have been suggested as a cause of MeN, but the data are scarce (317).

Epidemiological data point to a link between occupational SiO_2 exposure and CKD, although the underlying mechanisms are unknown (317). Patients and controls from North Carolina, USA between 1980 and 1982, were interviewed providing information about their work history (exposure to SiO_2) and the association between exposure and development of CKD was established (317). A dose-dependent positive association was found between occupational exposure to SiO_2 and CKD risk.

The most common and well described consequence of SiO_2 exposure is lung fibrosis leading to silicosis. Some patients with silicosis have been reported to have high levels of protein in their urine (318). Further, animal studies show dose-dependent tubular cell degeneration with interstitial inflammation and fibrosis following SiO_2 exposure (319). These data suggest that SiO_2 can have direct effects on the glomeruli and tubules.

The proposed mechanism of SiO_2 toxicity is through either direct (silica particles in kidney) or indirect effects (320). Indirect toxicity when lungs are exposed to crystalline SiO_2 can lead to a systemic immune response, and

macrophage activation can result in glomerulonephritis (321). To date, no studies have focused on detecting SiO_2 particles in patients with MeN.

1.2.4.5 Phytotoxins and mycotoxins

Some previously unexplained cases of CKD are thought to be caused by exposure to plant (phyto-) and fungal (myco-) toxins (322,323). Balkan endemic nephropathy (BEN), first characterised in the 1950s, is now known to be caused by the consumption of aristolochic acid (AA), a phytotoxin found in plants of the birthwort family (Aristolochiaceae), with Aristolochia clematitis being the putative source of AA in BEN (324). BEN is a form of chronic interstitial nephritis affecting rural communities in the Balkans in areas of Bulgaria, Romania, and the area formerly known as Yugoslavia (325). Progressive renal damage and renal fibrosis resulting from AA administration have been demonstrated in animal models (326). Although, Aristolochia species are widespread throughout Mesoamerica, the strong male predilection seen in CKDu (assuming no occupational exposure), as well as the lack of evidence for increased incidence of renal tract malignancies, do not support an AA nephropathy although, further research is needed to definitively exclude a causal relationship (327).

Mycotoxins are secondary metabolites produced by many fungi (328). Ochratoxin A (OTA) and citrinin (CIT), are nephrotoxins produced by fungi of the genera Aspergillus and Penicillium, under conditions of high temperature and humidity. These mycotoxins are commonly found in food, well water and animal feed in tropical areas (329–332). OTA has been implicated in chronic interstitial nephropathy in Tunisia and other North African countries (333,334). OTA appears to inhibit factors that reduce ROS in proximal tubular epithelial cells, ultimately resulting in depletion of intracellular glutathione and cell death (335).

The mycotoxin CIT has been proposed to act synergistically with OTA (329,331). The synergistic effect of OTA and CIT has been demonstrated in animal models and in environmental data where CIT was present in 19 out of 21 food samples contaminated with OTA (336–338). Eighty-five to ninety

percent of CIT is eliminated in the urine (338). CIT produces acute tubular and necrosis of the S1 section of the proximal tubule (323). Recent studies have shown that during cooking, CIT in contaminated products leads to CIT decomposition to a more cytotoxic compound, CIT H1 (339,340).

OTA and CIT, are two of the most abundant food-contaminating mycotoxins and are found in foodstuffs such as in cereal products and coffee, making significant human exposure and resultant nephrotoxicity a distinct possibility. However, to date, CKDu patients have not been tested for exposure to phyto- or myco-toxins that could play a role in the development of the disease.

1.2.4.6 Infectious diseases

Central America is a tropical region with a wide range of zoonotic infectious diseases; caused by viruses for which animals (e.g. mosquitos and dogs) are the main host but which can transfer to humans through infected stings, aerosols, animal urine or faeces (341,342). Viral infections in the region include dengue fever, hantavirus, West Nile virus (in Mexico) and more recently, chikungunya virus. Rabies is also common across the region. Additionally, a Zika outbreak is currently affecting both south and central America. Common bacterial infections include typhoid fever, leptospirosis, listeriosis and rickettsia, and high rates of tuberculosis have been reported in Honduras and Belize (341). In contrast, government eradication programmes mean Malaria (parasitic) is now almost completely eliminated from the region (341).

1.2.4.6.1 Hantavirus

Hantaviruses are a group of more than 20 species of rodent-borne viruses. Human transmission occurs through aerosolisation of rodent secretions or direct contact (343). Hantaviruses, enveloped RNA viruses, are known nephrotoxic viruses and are endemic in tropical and subtropical regions of Central America where MeN occurs. Hantaviruses cause two main forms of disease: (i) haemorrhagic fever with renal syndrome (HFRS) (predominantly found in Europe and Asia); and (ii) hantavirus cardiopulmonary syndrome (HCPS) (predominantly found in the Americas). HFRS can present clinically in

different ways depending on the causative species, and symptoms can vary (344). Hantaan virus causes the severe form and the strain Puumala causes the mild form of HFRS. Severe forms of HFRS infections lead to increased vascular permeability and decline in renal blood flow which subsequently leads to a decrease in GFR and a decline in renal function (345). HCPS blood analysis reveals haemoconcentration and thrombocytopenia and remains important laboratory findings for its diagnosis (346). Additionally, these infections can cause bleeding and toxic shock syndrome. Symptoms of mild forms of hantavirus infections include fever, loin pain, abdominal pain, nausea, vomiting, renal dysfunction with low levels of haematuria and proteinuria (347). Early renal morphological changes caused by hantaviruses present with interstitial oedema, extravasated of red blood cells in the outer medulla, capillary congestion and interstitial inflammation (predominantly $CD8^+$ lymphocytes) (348). HFRS nephropathy shows tubulointerstitial nephritis with medullary haemorrhage, acute tubular necrosis is frequent but the glomeruli are usually normal (349,350).

Unfortunately, studies regarding hantavirus circulation in humans in Central America (except Panama) are scarce (351). However, a recent cross-sectional study in Sri Lanka investigating the relationship between past exposure to hantaviruses and the development of CKDu, suggested a potential link, where the odds of exposure to hantavirus were higher for the kidney disease patients than for controls (352).

1.2.4.6.2 Leptospirosis

Leptospirosis is a zoonotic disease that infects humans through contact with water, wet soil or food contaminated by the urine of infected animals and is known to be persistent in the soil and water for prolonged periods (353). In central America there is a spread of Leptospirosis and outbreaks have been observed in Costa Rica and Panama (341). In 1998, there was an outbreak of Leptospirosis and increased hospital admissions in Nicaragua which was reported to peak in 2001. Leptospirosis is often a mild disease but when severe can lead to AKI (354).

The major antigens of species of Leptospirosis are present on the outer membrane of the bacteria and are responsible for the renal dysfunction (355). In animal models, LipL32 in the bacterial membrane has been found to cause tubulointerstitial nephritis (356). LipL32 also led to increased expression of proinflammatory cytokines including MCP1 and Tumor Necrosis Factor-alpha (TNFα), which in turn lead to inflammatory infiltration and cell injury.

The bacteria Leptospira is present in the fields during the sugarcane cutting season potentially exposing the workers. A study by Riefhohl et al. (2017) in Nicaraguan sugarcane cutters showed that seropositivity for Leptospira was associated with high urinary NGAL and IL18 concentrations (357). Seropositivity was associated with lower mean eGFR when compared with those who tested negative. Furthermore, a positive test for Leptospira was more common in field workers than in non-field workers.

There are reports from Taiwan suggesting a potential susceptibility to CKDu development in those affected by Leptospira (358,359). There is the suggestion that, even in asymptomatic infected individuals, bacteria can colonise the kidney and increase the risk of renal fibrosis. A cross-sectional study by Ganoza et al. (2010) in Peru involving 314 participants reported asymptomatic renal colonisation of leptospires even in people without serological or clinical evidence of a recent infection (360). The chronic colonisation of Leptospira and the development of CKD has not been characterised (361). The symptoms of severe acute Leptospira infection typically involve non-oliguric acute renal failure, hypokalaemia, sodium wasting, interstitial oedema and mononuclear cellular infiltration however whether there are chronic forms of disease remains unclear (362).

1.2.4.7 Genetic predisposition

A number of rare genetic disorders resulting in CKD are known to cause similar histopathological characteristics to those of MeN (363–366). Genetic mutations in UMOD, HNF1B, REN and MUC1 and of the Nephronophthisis gene family have been found to cause autosomal dominant tubulointerstitial kidney diseases (ADTKD) (367). Patients develop ESRD between the third

and the sixth decade of life with absence of proteinuria and haematuria (368). These conditions present with different clinical characteristics and progression (Table 1.4) but share histological similarities to MeN.

There are examples of common genetic risk alleles in CKD, for example, Apolipoprotein L1 (APOL1) variants among African Americans in the USA (369). Fourteen percent of African Americans carry the APOL1 risk alleles (APOL1 G1 and G2) compared with 2% of Hispanics and Latin Americans and 0.05% of European Americans. APOL1 risk haplotypes are significantly associated with CKD, lower eGFR and ESRD (365,370). The proposed mechanism by which APOL1 G1 and G2 lead to kidney injury is by impairing the reverse cholesterol transport thereby promoting macrophage foam cell formation leading to inflammation in the glomeruli and in the renal interstitium (371).

A study of 200 Nicaraguan adolescents showed increased urinary levels of NGAL and NAG in children living near areas of high risk of MeN supporting a potential pre-existing predisposition or early environmental exposures in childhood that could be initiators of kidney injury (372). Although some studies have reported familial clustering of MeN, there is no evidence that CKDu is inherited as a Mendelian trait (49). For instance, clustering of exposures within family units with common exposures may explain the family history in many of those with MeN.

In common with most kidney diseases, a role for genetics in the development and progression of MeN/CKDu is likely. Even where not the primary cause of disease, identification of potential risk alleles that could impact on the handling of environmental exposures, may implicate a metal or agrichemical in the cause of MeN.

	UMOD	REN	Hnf1B	Nephronophthisis (resulting from different mutations in different genes)	MUC 1	MeN
Clinical Characteristics	Hyperuricemia and gout, minimal proteinuria and haematuria	Hypo-proliferative anaemia, low haemoglobin concentrations, hyperuricemia and gout, mildly elevated serum creatinine, low plasma renin levels, mildly elevated serum potassium	Congenital anomalies of the kidney and the urinary tract	Reduced renal concentrating ability, cystic renal disease, salt wasting resulting, chronic anaemia	Hyperuricemia and gout	Asymptomatic until the later stages, normal or mildly elevated blood pressure
Diagnosis	Elevated serum creatinine, elevated serum urate levels and reduced fractional excretion of uric acid, bland urinary sediment	Hypo-proliferative anaemia, serum uric acid >6mg/dL			Elevated serum creatinine, bland urinary sediment	Elevated serum creatinine
Kidney size	Normal	Normal to small	Multicystic and hypo- or dysplastic kidneys	Normal to small kidney size with cysts in 50% of the affected population	Normal to small with occasionally presence of cysts	
Age of onset	Teenage years	1st year of life		Juvenile	Teenage years	2nd-3rd decade of life
ESRD	4th-7th decade of life	4th-6th decade of life		Before the age of 30 (Median=13years)	2nd-7th decade of life	3rd-5th decade of life
Biopsy histology	Chronic interstitial fibrosis with focal tubular atrophy and interstitial fibrosis			Tubulointerstitial fibrosis, thickened and disrupted tubular basement membrane		Tubular atrophy, glomerulosclerosis, glomerular hypertrophy, ischaemia

Table 1.4: Comparison of the characteristics of the different autosomal dominant tubulointerstitial kidney diseases (ADTKD) caused by different genetic mutations in the genes UMOD, REN, Hnf1B, MUC 1 with MeN (363–366).

1.2.4.8 Non-steroidal anti-inflammatory drugs

The kidneys are the main organ for the excretion of drugs (373,374). NSAIDs are used commonly by workers in Nicaragua to alleviate muscle pain (95). NSAIDs reduce renal plasma flow by decreasing vasodilatory prostaglandins, potentially leading to AKI (375,376). In addition, exposure to NSAIDs can cause immunological responses leading to acute tubulointerstitial nephritis (AIN) or minimal-change glomerulonephritis that manifests as nephrotic proteinuria (377,378). Intermittent use of NSAIDs is not thought to be harmful, however dose-dependent effects should be considered in chronic use (379). Although NSAIDs have been suggested to be a major concern, no studies to date have reported positive associations between drug use and the decline in kidney function in the populations at risk of MeN (49).

In summary, there are a wide range of potential candidates as a cause of MeN. There is an active debate within the MeN/CKDu research community in which people argue strongly that one or other cause is responsible for the disease. Therefore, there remains a need for robust evidence either for or against any of the potential causes of CKDu. It is only once potential causes have been eliminated or validated that preventative measures can be pursued.

1.3 Aims and objectives

CKDu is a public health problem affecting the lives of thousands of young agricultural workers in many developing countries (72). Poverty in these areas makes it extremely difficult for the affected individuals to obtain treatment meaning the disease often leads to death at a young age. Numerous hypotheses have been suggested but no robust evidence as to the cause has yet been presented.

The overall aim of the current project was to identify factors that lead to MeN. Tests were performed on biological samples collected as outlined by González-Quiroz et al. (Figure 1.13), taken before and after the harvesting period for the first year (two visits 6 months apart) of the follow-up period (across 9 different communities of northwest Nicaragua) (51). We hypothesise

that causal agents (or markers of causal agents) can be detected in the biofluids of study participants who go on to lose kidney function during the subsequent follow-up.

The design of the study involved the testing of biosamples (serum and urine) taken at two different time-points and examining the association between: (i) different urinary kidney injury biomarkers; (ii) the presence of heavy metals and metalloids; (iii) the presence of pesticides; and (iv) the presence of OTA and CIT and the outcome of loss of eGFR (as a measure of kidney dysfunction). In addition, we hoped to use genetic variants to provide further insights into disease aetiology. Exploring risk variants present in genes important in modifying any individual's environmental exposure, may give valuable information as to the type of exposure responsible for the development of the disease. Finally, the study tested the heat stress and fructose hypotheses *in vitro* using the human kidney proximal tubular epithelial cell line, HKC-8. The use of an *in vitro* model allowed variables to be modified and tested individually and in combination.

Chapter 2: Materials and Methods

2.1 Study population and study design

In 2014, González-Quiroz et al., initiated a longitudinal cohort study of MeN and recruited 350 healthy young men and women (18-30 years old) without CKD, from 9 different communities in north-western Nicaragua (51). Participants have been followed up for 2 years with 6 monthly visits where questionnaires were completed, and blood and urine samples collected. The purpose and the importance of the study rests on the fact that participants were initially healthy and were then followed up which increases the chances of being able to establish associations between exposure and development of disease with each individual acting as their own control. Figure 2.1 shows the study design of the cohort (70).

The overall design of this study has many advantages over other studies conducted in the area including: (i) the participants at baseline are young and healthy; (ii) the follow-up period allows detection of decline in eGFR, a surrogate for progression to CKD; and (iii) samples collected at baseline can be associated with the outcome without being prone to reverse causation.

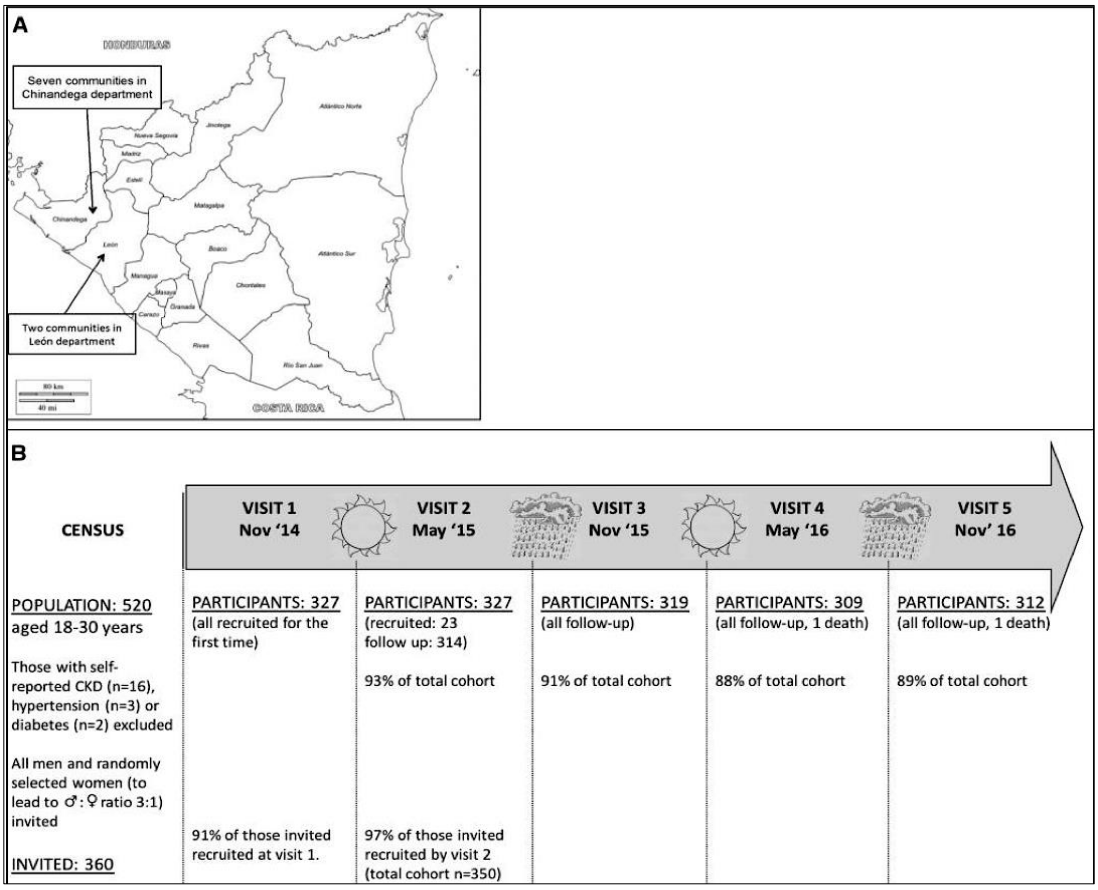

Figure 2.1: Cohort design of the study by González-Quiroz et al. A. Map of Nicaragua and the Departments from which participants were recruited. **B.** The overall plan of the study. Visits took place before and at the end of the sugarcane harvesting period. At visit 2, 23 more participants were recruited to meet initial recruitment targets and samples collected at this time point used for baseline analyses (51).

Samples from the on-going Colt Foundation-funded cohort study conducted by UCL, The London School of Hygiene and Tropical Medicine (LSHTM) and Universidad Nacional Autónoma de Nicaragua (UNAN)-León were used for this work (49). The recruited population included 350 young (18-30 years old) and apparently healthy subjects at risk of developing MeN of both genders (in a male: female ratio of 3:1) from 9 different communities in northwest Nicaragua (Figure 2.2) (51). The selection of a young population was based on the main aim of the study to capture individuals prior to a decline in kidney function (to reduce the risk of reverse-causality) and to follow these individuals over time.

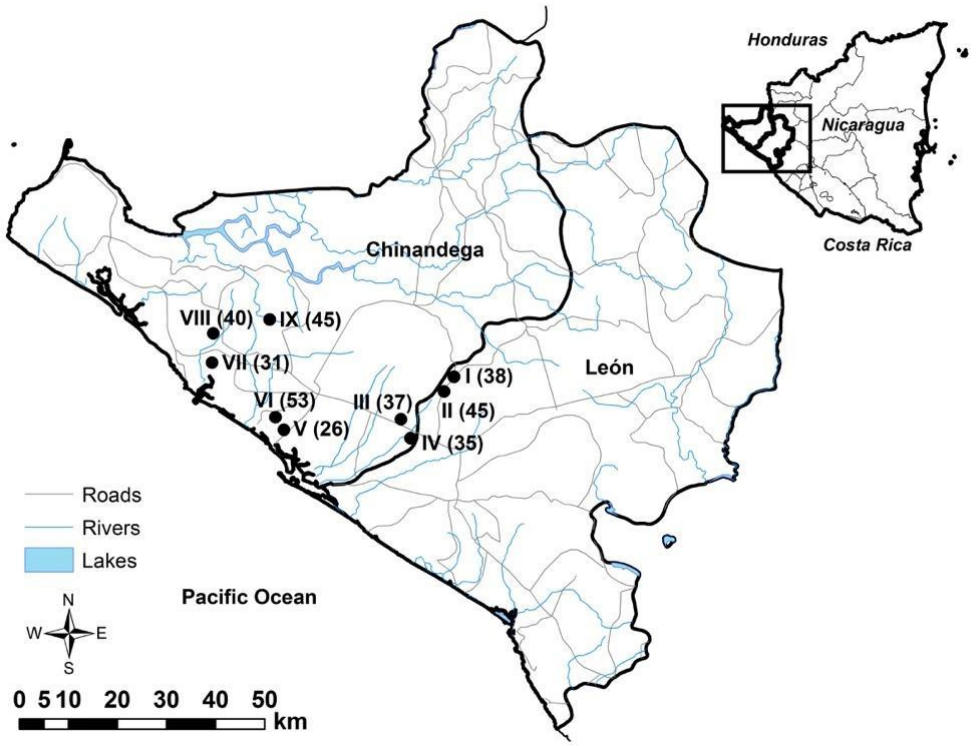

Figure 2.2: The 9 different communities in the department of León and Chinandega. The image at the top right shows a map of Nicaragua illustrating the north-western location of the 9 communities studied (I-IX); numbers in brackets indicate the number of participants in each community (total of 350 participants at baseline). Picture created by Dr Marvin González-Quiroz, National Autonomous University of Nicaragua at León (UNAN-León).

The association between baseline occupational and environmental exposures with the decline in kidney function was achieved by measuring the eGFR before and after the sugarcane harvesting season over a period of 2 years (2014-2016). The initial analyses of the questionnaires, led by Dr Marvin González-Quiroz, have been published (51). In addition, the study team in Nicaragua collected blood and urine samples. At baseline, one blood sample (4 mL) was collected in tubes containing anticoagulant (BD Vacutainer® EDTA tubes) and one without anticoagulant for serum separation. At all follow-up visits urine and blood samples for serum analysis were collected (BD Vacutainer®). Samples were kept at 4˚C for up to 6 hours until they were transferred to the laboratory for further processing. Blood samples collected without anticoagulant were centrifuged at 3,774 x g for 5 minutes at room temperature, serum was aliquoted (1 mL/vial; 4 vials in total) and stored at -20˚C for up to 2 weeks. Urine samples were aliquoted in tubes with boric acid

(BD Vacutainer®; 2.63 mg/mL boric acid (BA), 3.95 mg/mL sodium borate and 1.65 mg/mL sodium formate) and without (BD Vacutainer®) boric acid (used as a preservative to avoid bacteria growth). For analysis, samples were shipped in dry ice to the UCL Department of Renal Medicine, UCL Medical School, Royal Free Campus, London. In the UK, blood and urine samples were aliquoted and stored at -80˚C for 2 years (Table 2.1).

	Quantity	Number of visits
Blood Sample in EDTA	4 mL	Baseline visit
Serum Samples	4 mL	5 visits
Urine Samples in Boric Acid	4 mL	5 visits
Urine Samples without Boric Acid	14 mL	5 visits

Table 2.1: Total amounts of biofluids collected per visit / participant over a period of 2 years. Samples were stored for up to 2 weeks and then transferred to UCL Department of Renal Medicine for further analysis.

For each analysis different number of samples were available as shown in Figure 2.1. The decrease in samples is a result of loss to follow up. Table 2.2 summarises the number of samples that were analysed and were valid (enough sample was available to be quantified) for further analysis.

Analytes	Type of sample	Visit 1	Visit 2**
Strip-test analytes	urine	350	291
Creatinine	urine	350	291
NGAL	urine	55*	-
uRBP	urine	344	276
Heavy Metals	urine	350	291
Pesticides	urine	57*	46
OTA and CIT	urine	57*	46
Liver Function Tests	serum	349	291
Uric Acid	serum	349	291
Phosphate	serum	349	291

Table 2.2: Total number and type of samples analysed for its different analyte. All visit 1 measures included 327 participants recruited in November 2014 and a further 23 participants recruited in May 2015. * Samples were analysed only for a subgroup of those recruited in November 2014. ** All visit 2 samples analysed were from the group recruited in November 2014 only.

2.2 Chemicals and Reagents

Unless otherwise stated all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, United States).

2.3 Analysis of serum and urine samples from the cohort study

2.3.1 Automated clinical biochemistry system

The automated clinical biochemistry system, Cobas Integra plus 400, was used to analyse serum uric acid (UA), phosphorus, alanine aminotransferase (ALT), alkaline phosphatase (AST), gamma-glutamyl transferase (γGT), and serum and urine total bilirubin (BIL), in collaboration with Dr Helen Jones, UCL Institute of Liver and Digestive Health. Two hundred microlitres of each sample were placed in Cobas tubes (Sample cups; Roche Diagnostics, Burgess Hill, UK; Cat. no: 22-045-835) for analysis. The internal water reservoir of the machine was replaced every morning and standard curves, as well as a quality control (QC) analysis, were run before every serum and urine run. The QCs, provided by the company (Roche Diagnostics), were within a certified normal range and were measured daily to check both the precision and accuracy of the method.

2.3.1.1 Detection of uric acid, phosphorus, alanine aminotransferase, alkaline phosphatase and gamma-glutamyl transferase in serum samples

Serum samples from the whole cohort at visit 1 (n=347) and visit 2 (n=291) stored at -80˚C were tested using a Cobas Integra 400 Plus automated clinical biochemistry system. Standard curves and QCs were run for the analyte of interest and each sample 200 µL serum was transferred into Cobas Cup blue tubes (Reaction tube ‘Cobas cup’ 0.7 mL; Greiner, Cat. no: 742270).

Analytes tested included uric acid substrate (UA; Roche Diagnostics, Cat. no: 03183807 190), phosphate substrate (Phosphate; Roche Diagnostics, Cat. no: 03183793 122), the enzymes alanine aminotransferase (ALT; Roche Diagnostics, Cat. no: 20764957 322), aspartate transaminase (AST; Roche Diagnostics, Cat. no: 20762929), gamma-glutamyl transferase (γGT; Roche Diagnostics, Cat. no: 03002721 122) and total bilirubin (TOTAL BIL; Roche

Diagnostics, Cat. no: 05795397190). The principal of measuring each analyte is according to the International Federation of Clinical Chemistry (IFCC) (380).

The principle of quantifying alanine aminotransferase (ALT) is based on the fact that ALT catalyses the reaction between L-alanine and 2-oxoglutarate forming a pyruvate which is then reduced in the presence of NADH and catalysed by lactate dehydrogenase (LDH) forming L-lactate and NAD^+ (Figure 2.3A). The rate of NADH oxidation is directly proportional to the catalytic activity of ALT and determined by measuring the decrease in absorbance at 340 nm.

Aspartate aminotransferase (AST) on the other hand, catalyses the reaction between L-aspartate and 2-oxaloacetate to form l-glutamate and oxaloacetate. The oxaloacetate then reacts with NADH in the presence of malate dehydrogenase (MDH) to form NAD^+ (Figure 2.3B). The rate of NADH oxidation is directly proportional to the catalytic activity of AST and it is determined by measuring the decrease in the absorbance at 340 nm.

Gamma-glutamyltransferase (γGT) detection is based on a colorimetric assay in which γGT transfers the g-glutamyl groups of L-gamma-glutamyl-3-carboxy-4-nitroanilide to glycylglycine. The last reaction, in the presence of γGT, results in L-gamma-glutamyl-glycylglycine and 5-amino-2-nitrobenzoate (Figure 2.3C). The amount of 5-amino-2-nitrobenzoate is proportional to the γGT and it is determined by measuring the increase in the absorbance at 409 nm.

The determination of uric acid is based on a colorimetric enzymatic test in which uricase cleaves uric acid to form allantoin and hydrogen peroxide (Figure 2.3D). The intensity of the colour of the quionone-dimine formed is directly proportional to the uric acid concentration and it is determined by measuring the increase in absorbance at 552 nm.

Inorganic phosphate forms an ammonium phosphomolybdate complex with ammonium molybdate in the presence of sulfuric acid (Figure 2.3E). The concentration of phosphomolybdate formed is directly proportional to the

inorganic phosphate concentration and is determined by measuring the increase in absorbance at 340 nm.

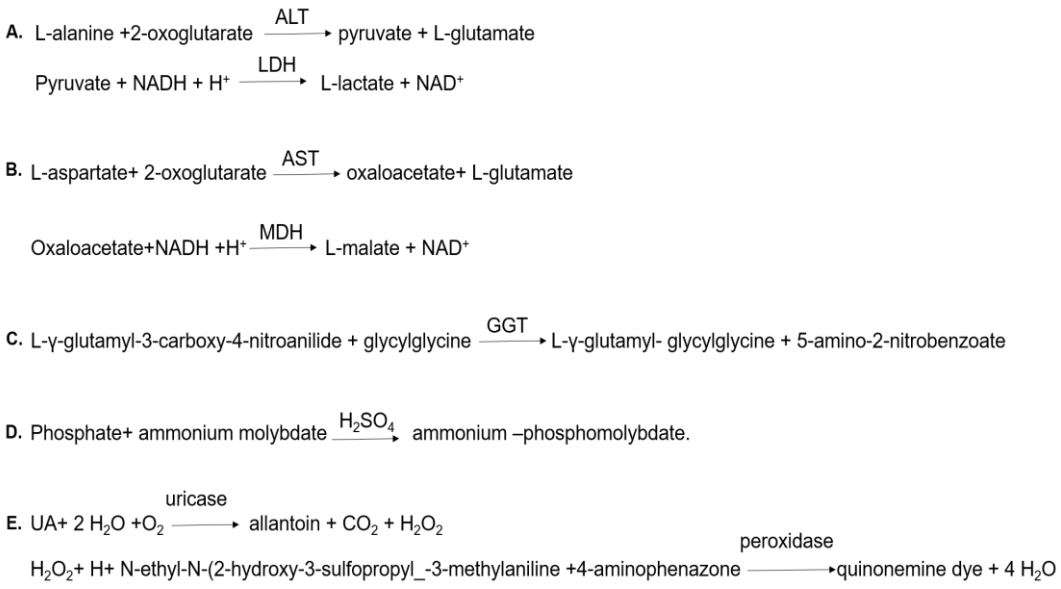

Figure 2.3: Reactions for each analyte tested. A. Alanine aminotransferase (ALT). **B.** Alanine aminotransferase (ALT). **C.** γ-glutamyltransferase (γGT). **D.** Phosphate. **E.** Uric Acid (UA).

Lastly, total bilirubin couples with diazonium ions when both are present in an acidic medium creating azobilirubin and a red azo-dye that can be detected at 552 and 629 nm.

Table 2.3 shows the quality reference levels for the healthy and pathological controls for each of the reagents and the expected levels for both men and women (Roche Diagnostics).

	Precicontrol Clin Chem Multi 1 Reference Range (QC)	SD	Precicontrol Clin Chem Multi 2 Pathological Range (QC)	SD	Limit of detection (LOD)	Expected references values (up to)		Units
						Men	Women	
Alanine aminotransferase (ALT)	37.1-53.3	2.7	102-144	7	2	41	33	U/L
Aspartate Aminotransferase (AST)	36.7-52.9	2.7	117.0-165.0	8	2	40	32	U/L
Total bilirubin (BIL)	14.1-20.1	1.0	52.4-75.2	3.8	2.5			µmol/L
gamma-glutamyl transferase (γGT)	39.6-57.0	2.9	183.0-261.0	13	3	10-71	6-42	U/L
Phosphate	1.1-1.5	0.06	1.8-2.5	0.1	0.1 (mmol/L)	0.8-1.5		µmol/L
Uric acid (UA)	245.0-336.0	15	569.0-773.0	34	11.9	202.3-416.5	142.8-339.2	µmol/L

Table 2.3: Certified Quality Control (QC) ranges for healthy and pathological controls. Standard deviation (SD), limit of detection (LOD) for each of the reagents and expected upper references values for each analyte provided by Roche Diagnostics.

2.4 Urine Analysis

Urine samples were collected in Vacutainer tubes with or without BA preservative. Urine analysis of the first and second visit was performed on samples containing preservative.

2.4.1 Strip-test analysis

Strip-test urinalysis is a first-line clinical test for the early detection of urinary abnormalities (381). Urine samples were initially examined macroscopically to assess the colour and the presence of particulates followed by strip-test analysis in conjuction with a BSc Applied Biomedical Sciences student and questionable resuls were discussed. The strips used (Siemens Multistix 10 SG) contain 10 different reagents to detect specific analytes resulting in a change in the colour of the pad (Figure 2.4). Colours are visually compared against the manufacturer's colour chart relative to the suggested incubation time. As the amount of urine was limited, 500 µL of each sample were pipetted along the strip instead of the dipping method suggested by the manufacturer.

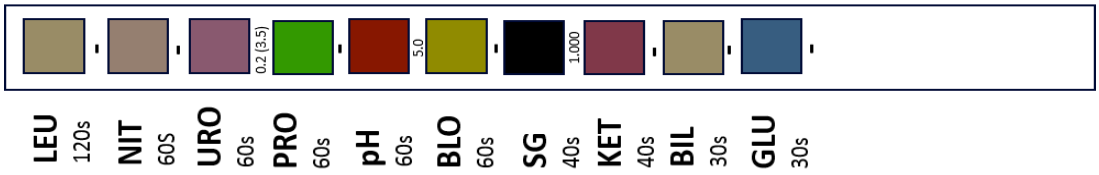

Figure 2.4: Representation of a strip and the different analytes. The reactions present on the strip-test can detect leukocytes (LEU), nitrogen (NIT), urobilinogen (URO), protein (PRO), pH, blood (BLO), specific gravity (SG), ketones (KET), bilirubin (BIL) and glucose (GLU) levels in the urine. The incubation time required before each reaction is read is shown in seconds (s).

2.4.2 Colorimetric assays for urine creatinine and albumin

Biochemical assays are widely used to quantify analytes in biofluids (382). To quantify an analyte in an unknown sample it is necessary to generate a standard curve with known concentrations of the analyte. Creatinine and albumin were measured in urine samples using the Jaffe and the bromocresol green reactions, respectively (383,384). Samples were read at specific wavelengths using the Biochrom EZ Read 400 Microplate Reader and Galapagos software.

2.4.2.1 Creatinine assay

Individuals' hydration state may vary so solute concentrations of interest must be corrected for urinary concentration. Ninety percent of the creatinine found in urine results from the breakdown of creatine-phosphate (CP) in the muscles (385). Although, creatinine concentration can also be affected by muscle breakdown and diet, assuming these are constant, total daily urinary creatinine excretion should be the same within an individual (with stable kidney function) and hence the creatinine concentration found in urine reflects the overall urinary concentration. Creatinine was measured and used as a correction factor for the variation in urinary concentration in the spot urine samples from each participant (386).

Creatinine in urine samples from visits 1 and 2 was measured using the Jaffe reaction. A stock of 100 mg/dL creatinine was used to produce a series of dilutions. Fifty microlitres of each standard (0-20 mg/dL) and samples (20-fold dilution) were placed in individual wells of a 96-well plate (96-well flat-bottom plates; Corning, Cat. no: 10695951) in duplicate. A creatinine quality control sample (HSE; 03-12 certified range 5.88-6.48 mmol/L), provided by the HSE (participation in EQA scheme RIQAS, Northern Ireland), was run in duplicate each time to verify the accuracy of the method. Subsequently, 100 µL alkaline picrate reagent (0.13% picric acid, 1M sodium hydroxide in a 5:1 ratio) were added to each well. The alkaline picrate solution reacts with creatinine forming an orange-red complex. Plates were incubated for 30 minutes at room temperature and optical density (OD) was measured (Biochrom EZ Read 400 Microplate Reader) at 492 nm. The concentration of each sample was corrected by the dilution factor and then converted from mg/dL to mmol/L by multiplying by the conversion factor of creatinine (0.08842).

2.4.2.2 Albumin assay

Normally the major plasma protein albumin is not filtered and remains in the blood. However, when the GFB is damaged, albumin enters the urine and thus has diagnostic value in various kidney diseases (387). For the determination of albumin, a colorimetric method using the Bromocresol Green (BCG)

Albumin Assay Kit (Cat. no: MAK124), including BCG reagent and Albumin Standard (5 g/dL), was performed according to the manufacturer's instructions with modification of the volumes used in each step to minimize the cost. Two microlitres of each standard (0-0.5 g/dL) and undiluted urine sample (visits 1 and 2) were transferred to a 96-well flat-bottomed plate in duplicate. BCG reagent was brought to room temperature and 50 µL were added to each well. Plates were incubated for 5 minutes at room temperature and the absorbance read at 620 nm (Biochrom EZ Read 400 Microplate Reader).

2.4.2.3 Enzyme-linked immunosorbent assays (ELISA)

The ELISA utilises an antibody with specificity for a certain antigen (a similar principle to the Western blot immunoassay Section 2.6.7.2). Many ELISAs are available commercially. Antibodies are immobilised in each well of a polystyrene microlitre plate. Samples with an unknown amount of the antigen under investigation are added to the wells and react with the antibodies. Next, an antibody specific to the protein of interest is added to form a sandwich antibody-antigen-antibody complex. The use of an enzyme-linked secondary antibody which binds to the Fc region of the detecting antibody in combination with a chemical substrate allows the enzymatic conversion into a colour or fluorescent signal which absorbs at specific wavelengths. The intensity of the signal, the optical density (OD), will be proportional to the concentration of the protein of interest in a sample over the dynamic range of the assay. Finally, using a series of standards with known concentrations of the protein of interest, a standard curve is drawn and used to determine the absolute concentration in samples of unknown concentration.

2.4.2.3.1 Retinol Binding protein (RBP)

RBP is a low molecular weight protein that is freely filtered by the glomeruli and reabsorbed by the proximal tubules (279,388,389). When glomerular filtration is normal or only marginally impaired, urinary RBP (uRBP) reflects the proximal tubule reabsorption capacity. Urine samples of the whole cohort at visit 1 and visit 2 were analysed by Dr Howard Mason, Health and Safety Executive (HSE), Buxton, UK using a Tecan robotic immunoassay platform.

Briefly, Nunc Immuno MaxiSorp 96 well plates (Cat. no: M9410-1CS) were coated with 100 µL of the coating antibody solution (1 µg/ml RBP coating antisera in carbonate coating buffer; 45 mM carbonate buffer; pH 9.6-10 in 1 litre ultrapure water) in each well, sealed and left overnight at 4˚C. The next day, plates were washed with 1x washing buffer (from 20x stock: 2.75 M NaCl, 0.2 M Phosphate buffer, 1% Tween-20; pH 7.4) and 200 µL of blocking reagent (5x PBS tablets (Cat. no: P4417-100TAB; 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer) with 10 mL fish gelatin, 20 mL sucrose and 2 mL Bronidox stock made up in 1 litre ultrapure water) were added to each well for 1-2 hours at room temperature. Plates were inverted to discard excess buffer.

Standards (0-76 ng/dL) were prepared by serial dilution of the standard (76 ng/mL) in assay buffer. One hundred microlitres of each sample and standard in duplicate, were placed into the wells of the 96-well plates and incubated for 60 minutes at room temperature. Samples were discarded and wells were washed using t times with 350 µL 1x washing buffer. One hundred microliters of 1/1000 RBP biotinylated antibody diluted in Assay Buffer were added and incubated for 60 minutes at room temperature. Antibody was discarded and wells were washed 3 times with 1x washing buffer. One hundred microliters of 1/5000 diluted horseradish peroxidase streptavidin (Vector Laboratories, Cat. no: SA-S004) were added and incubated for 30 minutes at room temperature. Horseradish peroxidase streptavidin was discarded, and wells were washed 3 times with 1x washing buffer. One hundred microliters of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution (KPL diagnostics, Cat. no: 5420-0025) were added and incubated for 30 minutes at room temperature. Finally, 100 µL of stop solution (0.5 M sulphuric acid) were added and the absorbance was measured at 450 nm using a microplate reader (Tecan Group Ltd.).

2.4.2.3.2 NGAL

Baseline urine samples from men previously frozen and stored at -80˚C for 2.5 years were analysed for NGAL. Only samples from men were analysed due to limited resources and the fact the disease occurs predominantly in the male population. NGAL has been described to increase in ischaemic, septic and

nephrotoxic conditions (390,391). It is also used to detect early pathophysiologic processes and has been identified as a specific, sensitive and highly predictive biomarker of AKI (392). Quantification of NGAL was performed according to the manufacturer's instructions (Ray Biotech, Cat. no: ELH-Lipocalin2).

A 1:50 or a 1:25 dilution of each urine sample was used. Dilutions were performed with the assay diluent provided. Standards (0-1,000 pg/mL) were prepared by serial dilution of the protein standard (50 ng/mL) provided by the manufacturer. One hundred microlitres of each sample and standards were added in duplicate to the plate provided in the kit and the plate incubated for 60 minutes at room temperature with gentle shaking. The plate was washed 4 times with the washing buffer provided to remove any remaining sample. Biotinylated antibody (100 µL/well) was added to each well and the plate was incubated for 60 minutes at room temperature with gentle shaking. The washing step was repeated and then 100 µL streptavidin were added to each well. The plate was incubated for 45 minutes at room temperature with gentle shaking. The streptavidin was discarded, and washes repeated. TMB substrate reagent (100 µL/well) was added, the plate covered with foil and incubated for 30 minutes at room temperature with gentle shaking. Finally, 50 µL stop solution was added to each well and the optical density measured immediately at 450 nm (Biochrom EZ Read 400 Microplate Reader).

2.4.2.3.3 Glucose

Glucose is reabsorbed in the proximal tubules (393). Excess glucose levels in the blood and tubular injury can result in pathological levels of glucose in the urine. A selection of samples from visit 1 and visit 2, from men in the different kidney function groups (Chapter 3 Section 3.2) (Stable kidney function = 4, Established kidney dysfunction = 7, and Declining kidney function = 8) were tested for urinary glucose levels.

For the detection of glucose in urine a colorimetric assay was used according to the manufacturer's instructions (Invitrogen, Cat. no: EIAGLUC). Samples with high levels of creatinine (urine creatinine > 20 mmol/L) were diluted 1:2

with the assay diluent. Standards (0-32 mg/dL) were prepared by serial dilution of the standard (320 mg/dL) provided by the manufacturer. Twenty microlitres of each sample and standard were put into the wells of the 96-well flat-bottomed plate in duplicate. Twenty-five microlitres of 1x Horseradish Peroxidase Concentrate (HRP) were added to each sample plus 25 µL substrate and 25 µL 1x glucose oxidase. The plate was incubated for 30 minutes at room temperature and the optical density measured at 560 nm (Biochrom EZ Read 400 Microplate Reader). The analytical sensitivity of the assay is 0.423 mg/dL.

2.4.3 Assessment of urinary proteins by SDS-polyacrylamide gel electrophoresis

The electrophoresis of proteins relies on the fact that proteins migrate under the application of electric field from the negative to the positive pole. The proteins separate based on their molecular weight when denatured with β-mercaptoethanol (to disrupt disulphide bonds) and high temperature (100˚C for 5 minutes). The concentration of polyacrylamide in the gel used depends on the range of molecular weights of the proteins of interest. Proteins which have low molecular weight require a higher % acrylamide gel to achieve good separation while high molecular weight proteins require a lower % acrylamide gel to allow the proteins to migrate into and separate in the gel.

Samples were run on a 5% polyacrylamide stacking gel (for 8 mL of gel: 5.5 mL dH_2O, 1.3 mL 30% a bis-acrylamide (Protogel, Cat. no: A2-0072), 1 mL 1.5 M Tris, pH 8.8, 80 µL 10% SDS, 80 µL ammonium persulfate, 8 µL TEMED) and a 20% polyacrylamide separating gel (for 15 mL of gel: 963 µL dH_2O, 10 mL 30% bis-acrylamide, 3.72 mL 1.5 M Tris, pH 8.8, 150 µL 10% SDS, 150 µL ammonium persulfate and 8 µL TEMED).

Five urinary samples (positive in protein strip test) were initially diluted 1:3 or 1:15 in PBS. Thirty microlitres of diluted samples were mixed with one quarter volume of sample buffer (4x Laemmli Sample buffer; Bio-Rad, Hercules, California, United States, Cat. no: 1610791). Proteins were denatured by incubating for 5 minutes at 100˚C and cooling on ice prior gel electrophoresis. In the presence of SDS in the gel and the sample buffer, the denatured proteins

are being negatively charged. Samples were run on 20% polyacrylamide gels to maximise the resolution of low molecular weight proteins (10-20 kDa) that could indicate proximal tubule injury (400). A known molecular weight marker was also loaded on the gel (4 µL; 2-250 kDa; Bio-Rad, Cat. no: 161-0374). Gels were run for ~75 minutes at 150-170 Volts in electrophoresis running buffer (250 mM Tris, 1.92 M Glycine, 35 mM SDS).

2.4.3.1 Silver nitrate stain

Silver staining was used to detect proteins present in samples after SDS-PAGE; and is commonly used when increased sensitivity to detect small amounts of protein (<1 ng) is needed compared to for example, Coomassie Blue staining (394).

After electrophoresis, gels were incubated for 35 minutes in fixer (40% ethanol, 10% acetic acid, 50% dH_2O) and then washed with dH_2O for 2 hours at room temperature with constant agitation to remove excess acetic acid. Gels were incubated in 0.02% sodium thiosulfate for 1 minute followed by 2 x 1 minute washes with dH_2O, then incubated with cold 0.1% fresh silver nitrate solution at 4˚C for 20 minutes. Gels were washed with dH_2O (2 x 1 minute) to remove silver nitrate and developed in 3% sodium carbonate, 0.17% formaldehyde until proteins bands were visualised. Gels were washed for 20 seconds with dH_2O, and staining was terminated by incubation in 5% acetic acid for 5 minutes. For long-term storage gels were stored at 4˚C in 1% acetic acid. Bands detected at a low molecular weight (25-10 kDa) from 2 samples, were excised from the gel using a surgical scalpel, placed in 1.5 mL microfuge tubes and shipped to the Advanced Mass Spectrometry Facility in the School of Biosciences, University of Birmingham for proteomic analysis.

2.4.4 Inductively-Coupled Plasma-Mass Spectrometry (ICP-MS)

The detection of metals and metalloids in all urine samples from visit 1 and visit 2 was performed with Inductively-Coupled Plasma-Mass Spectrometry (ICP-MS) in collaboration with Dr Jackie Morton, HSE, Buxton, UK. The urine samples analysed from visits 1 and 2 were initially collected in tubes with BA. Table 2.4 shows the metals and metalloids investigated.

Aluminium (Al)	Chromium (Cr)	Lead (Pb)	Selenium (Se)*
Arsenic (As)*	Cobalt (Co)	Manganese (Mn)	Strontium (Sr)
Cadmium (Cd)	Copper (Cu)	Mercury (Hg)	Silicon (Si)*

Table 2.4: Metals and metalloids measured in urine using ICP-MS. *indicates the metalloids.

2.4.4.1 Principle of ICP-MS

The principle of ICP-MS is based on the fact that each element of the periodic table has a different mass to charge ratio (m/z). An ICP-MS converts an aspirated solution (in this case urine) into ions, confers positive charge and detects them depending on their unique m/z. The method is sensitive, accurate and can analyse multiple elements simultaneously (395).

As illustrated in Figure 2.5, the sample is first transformed into an aerosol in a nebulizer with argon gas, and through the ICP is converted into gaseous atoms that are positively ionised. Ions are then focused through the ion lenses (region of high atmospheric pressure) and introduced to the MS (vacuum chamber of low pressure). The MS is a quadrupole mass filter which separates ions depending on their mass-to-charge ratio. This is achieved by the application of a radio frequency field that permits only ions of a specific m/z ratio to pass. Finally, a detector receives the electrical signal of ions proportional to their concentration, amplifies this and then the software will use this response with those obtained with calibration standards to calculate concentration of elements at known m/z ratios.

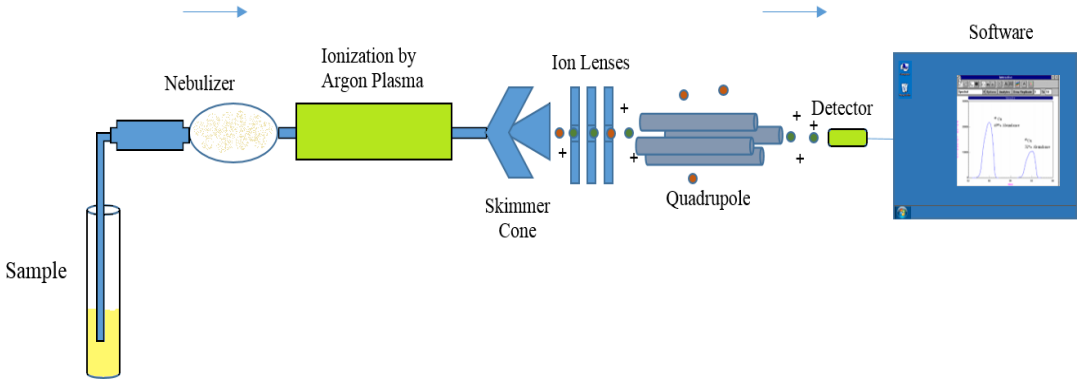

Figure 2.5: Schematic of the main components of the ICP-MS.

2.4.4.2 ICP-MS protocol for the detection of metals and metalloids in urine samples

2.4.4.2.1 Multi-element analysis in urine

Typically, ICP-MS (Thermo IcapQ ICP/MS) conditions were as follows: the sample was introduced to the spray chamber at a flow of 1 mL/min, with a radio frequency power of 1500W, nebuliser flow was 1.05 L/min and the extraction voltage was 130 V. Daily, the instrument was tuned, optimised and rinsed with 2% nitric acid for up to 60 minutes before the analysis.

The instrument was run in normal standard mode (STD) and in collision cell mode with kinetic energy discrimination (KED) using an in-sample switching capability (this allows the instrument to acquire data from each sample first in normal mode and then adding a collision cell gas and acquiring in KED mode). It was necessary to analyse those elements with known isotopic and isobaric interferences in cell gas mode to remove the interferences, e.g., for chromium m/z 52 in a urine sample there are significant interferences from $^{35}Cl^{16}O^{1}H^{+}$, $^{40}Ar^{12}C^{+}$ and by introducing a low flow of helium gas these interferences are removed. Each signal for the elemental m/z ratio is acquired for a time that builds up the mass spectrum for that element, the dwell time is a fraction of a millisecond, generally the longer the dwell the better the precision of the data. In a multi-elemental analysis, the dwell time is optimised depending on the abundance and sensitivity for each element. Table 2.5 shows the dwell time and the measurement mode of each element. Each isotope is sequentially determined.

Each day, a fresh 1 mg/L multi-element standard of Al, As, Cd, Co, Cu, Cr, Mn, Pb, Se was prepared from individual ICP-MS standards (BDH, Poole, UK) (0.1 mL 1,000 mg/L ICP-MS standard in 100 mL dH_2O) and calibration standards were made with a series of dilutions to obtain concentrations 0.01-10 µg/L. Mercury (Hg) was analysed separately (Section 2.4.4.2.2) due to its instability in solutions with other elements (396).

QC materials were also analysed: urine certified reference Bio-Rad level 1 (Liquichek urine chemistry control; Bio-Rad Laboratories, Cat. no: 397) and

ClinChek (CC) levels 1 and 2 (ClinChek Level 1, Recipe, Cat. no: 8847 and ClinChek Level 2, Recipe, Cat. no: 8848). QC samples were analysed at the start and end of a run, ClinCheck1 was analysed after every 10 samples and ClinCheck1 and ClinCheck2 were analysed after every 20 samples. A 1 µg/L standard was also analysed at the start and end of the run.

Identifier	Dwell time (s)	Limit of Detection (µg/L)	Measurement mode
^{27}Al	0.1	0.0205	STD
^{75}As	0.1	0.0008	KED
^{111}Cd	0.2	0.0001	STD
^{59}Co	0.1	0.0003	KED
^{52}Cr	0.2	0.0005	KED
^{65}Cu	0.1	0.0009	KED
^{202}Hg	0.2	0.0156	STD
^{208}Pb	0.01	0.0009	STD
^{55}Mn	0.1	0.0004	KED
^{78}Se	0.2	0.0183	KED
^{28}Si	0.2	5.2736	KED
^{88}Sr	0.1	0.0062	KED

Table 2.5: Typical instrument modes for each element with their relative atomic mass. Elements were run either in normal standard mode (STD) or kinetic energy discrimination mode (KED). The dwell time for each ion to reach the detector, in seconds (s); and the limit of detection (LOD) of each element in µg/L are given.

Samples, standards and QC material were diluted 1:20. For each sample/standard/QC a 100 µL aliquot was added to 1.9 mL diluent solution (0.5% m/v EDTA, 2% v/v nitric acid (Thermo Fisher Scientific, Hampton, New Hampshire, United States, Cat. no: A200-612GAL), and 0.25 µg/L of internal standards (Platinum (Pt), Rhodium (Rh), Indium (In), Germanium (Ge), Yttrium (Y)). The samples were vortexed and placed in the ICP-MS sample tray. The autosampler washing/rinse solution was 1 L 2% v/v nitric acid.

2.4.4.2.2 Mercury analysis in urine

Typically, ICP-MS (Thermo X series II ICP/MS) conditions were as follows: the sample was introduced to the spray chamber at a flow of 1 mL/min, with a radio frequency power of 1400 W, nebuliser flow was 0.82 L/min, the extraction voltage was 105 Volts. Daily, the instrument was tuned, optimised and rinsed

with 1% hydrochloric acid with gold (Au) (to stabilise the mercury rather than nitric acid) for up to 60 minutes before analysis. The instrument was run in normal standard mode (STD). Hg and Au are thought to form an amalgam, and, in this way, Hg can be measured.

Each day, a fresh 1 mg/L solution of standard inorganic mercury (0.1 mL 1,000 mg/L ICP-MS standard (BDH, Poole, UK) in 100 mL dH_2O) was made. Finally, 1 mL nitric acid and 10 µL Au were added to this standard. It is important to note that samples lose Hg upon freezing and every time they are exposed to the atmosphere (396). The Au used during the preparation of each solution and sample minimises this loss. Calibration standards of 1:20 dilutions (0.05-10 µg/L) were made fresh every day with the addition of 1% nitric acid and 0.01% Au standard (1,000 mg/L) to each 100 mL volumetric flask.

QC materials were also analysed: urine certified reference Bio-Rad level 1 and ClinChek1 and ClinkChek2. QC samples were analysed at the start and end of a run, ClinkCheck1 was run after every 10 samples, ClinChek1 and ClinkChek2 were also run after every 20 samples. A 2.5 µg/L standard was also analysed at the start and end of the analysis.

Samples, standards and QC material were diluted 1:20. One hundred microlitres of each sample/standard/QC were added to 1.9 mL of diluent solution. The sample was vortexed and placed in the ICP-MS autosampler tray. The autosampler washing/rinse solution was 1 L 1% hydrochloric acid containing 10 µg/L (0.001% v/v) Au standard.

2.4.5 Detection of agrichemicals in urine

Pesticides and metabolites of pesticides (selected on the basis evidence of local use and the available analytical capability), were measured in all the samples from men with rapidly declining kidney function and a number of age-, community- and occupation-matched individuals from the stable group, in collaboration with Dr Christian Lindh, Division of Occupational and Environmental Medicine, Lund University, Sweden. Pre-analytical steps and data analyses were conducted in the UK but actual measurements were conducted by Dr Lindh's team.

In addition, samples from visit 1 (n=57) and where available, from the same individuals from visit 2 (n=46) were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for: (i) the phenoxyacid herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxy acetic acid (MCPA), and the herbicide N-(phosphonomethyl)glycine (glyphosate); (ii) metabolites of insecticides; chloro-3,3,3-trifluoro-1-propen-1-yl-2,2-dimethylcyclopropanecarboxylic acid (CFCA); 3,5,6-trichloro-2-pyridinol (TCP); 4-fluoro-3-phenoxybenzoic acid (4F3PBA), 3-phenoxybenzoic acid (3-PBA); cis/trans 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA), which are all pyrethroid metabolites except TCP which is a metabolite of the organophosphate chlorpyrifos; and, (iii) metabolites of conazole fungicides; hydroxy-tebuconazole (TEB-OH) and 5-hydroxy-tiabendazole (5-OH-TBZ), and metabolite of pyrimidine fungicides, 3-hydroxy-pyrimetanil (OH-PYR), and of dithiocarbamate fungicides, ethylenethiourea (ETU) (397–399).

2.4.5.1 Principle of LC-MS/MS

Liquid chromatography (LC) is a separation process used to isolate the individual components of a mixture. This process involves mass transfer of a sample through a polar mobile phase and non-polar stationary phase. The combined technique of MS and High Performance Liquid Chromatography (HPLC) is commonly known as LC-MS. Combining the two analytical methods reduces experimental error and improves accuracy (400).

LC-MS/MS has two (tandem) quadrupole (Q1 and Q2) mass spectrometers, in the middle a hexapole mass spectrometer, and one detector allowing high specificity analysis. The ions produced (precursor ion) are analysed in the first MS (Q1) at a mass and then the second MS (Q2) at a different mass, this ‘mass shift’ from Q1 and Q2 can be natural so one ion fragments (the parent ion) further into smaller products (the daughter ions), or forced so a gas reacts to form other compounds (401). The process involves the fragmentation of the sample with various methods as detailed below.

2.4.5.2 LC-MS/MS protocol for the detection of pesticides and pesticide metabolites in urine samples

Three different methods were used for the preparation of the samples for the different pesticides and metabolites. Fifty-seven samples (50 males and 7 females from the stable and the declining kidney function groups) from visit 1 and 46 samples (40 males and 6 females from the stable and the declining kidney function groups) from visit 2 were tested for the presence of pesticide and pesticide metabolites.

2.4.5.2.1 De-conjugation of urine samples prior to LC-MS/MS

Urine samples were analysed according to a modified method described by Ekman et al. (397). Briefly, 500 µL of each sample were de-conjugated using 25 µL internal standard (IS) solution, 150 µL 1 M ammonium acetate (pH 6.5) and 10 µL β-glucuronidase/arylsulphatase. Quantitative analysis was conducted using liquid chromatography-triple quadrupole linear ion trap mass spectrometer (QTRAP 5500; AB Sciex, Foster City, CA, USA).

This de-conjugation method was used for the detection of: (i) 3,5,6-trichloro-2-pyridinol, a metabolite of the organophosphate insecticide chlorpyrifos (TCP); (ii) the phenoxy acid herbicides 2,4-dichlorophenoxyacetic acid (2,4-D); (iii) 4-chloro-2-methylphenoxy acetic acid (MCPA); (iv) 3-phenoxybenzoic acid (3-PBA); and (v) sum of cis/trans 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA).

It was also used for the detection of metabolites of pyrethroid insecticides (e.g. permethrin, cypermethrin, deltamethrin, cyfluthrin); (i) 4-fluoro-3-phenoxybenzoic acid (4F3PBA), a metabolite of the pyrethroid insecticide cyfluthrin; (ii) chloro-3,3,3-trifluoro-1-propen-1-yl-2,2-dimethylcyclopropanecarboxylic acid (CFCA), a metabolite of the pyrethroid insecticide bifenthrin; (iii) 3-hydroxy-pyrimetanil (OH-PYR), a metabolite of the fungicide pyrimethanil; hydroxy-tebuconazole, related to metabolism of the fungicide tebuconazole (TEB-OH); and, (iv) 5-hydroxy-tiabendazole (5-OH-TBZ) which is related to metabolism of the fungicide thiabendazole.

2.4.5.2.2 Hydrolysis of urine samples prior to LC-MS/MS

Urine samples were analysed according to Ekman et al. 2013 (398). Briefly, the samples were hydrolysed with 20 µL 2.5 M NaOH at 100˚C for 60 minutes. Quantitative analysis was conducted using a two-dimensional LC system connected to the MS/MS as above. Hydrolysis was performed for the analysis of the metabolite of dithiocarbamate fungicides, ethylenethiourea (ETU).

2.4.5.2.3 Acidification of urine prior to LC-MS/MS

Urine samples were analysed according to a modified method by Jensen et al. (399). Briefly 600 µL of sample were acidified with 0.1% formic acid and quantified using the LC-MS/MS system as above.

The pesticide N-(phosphonomethyl) glycine (glyphosate), a systemic herbicide known by the trade name 'Roundup', was prepared with acidification. All pesticides and metabolites detected were normalised to the urinary creatinine levels. The limits of detection (LOD) for each pesticide and metabolite of pesticides are presented Table 2.6.

Compound	LOD (ng/mL)
ETU	0.1
TCP	0.1
CFCA	0.1
3PBA	0.1
2,4-D	0.1
4F3PBA	0.1
DCCA	0.1
MCPA	0.1
5-OH-TBZ	0.05
OH-PYR	0.1
TEB-OH	0.1

Table 2.6: Limits of detection (LOD) for each pesticide and metabolite of pesticides.

2.4.6 Detection of Ochratoxin A (OTA) and Citrinin (CIT) in urine

Fifty-seven samples (50 males and 7 females from the stable and the declining kidney function groups) from visit 1 and 46 samples (40 males and 6 females from the stable and the declining kidney function groups) from visit 2 were

tested for the presence of OTA and CIT in the urine. Preparation and extraction of urine samples for OTA and CIT measurement was done in collaboration with Kate Jones at HSE, Buxton, UK. The analysis of the extracted samples was performed by Carla Martins, Department of Food and Nutrition, National Institute of Health Dr Ricardo Jorge (INSA), Lisbon, Portugal. A procedure based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) and previously described by Vidal et al. was used for sample preparation (402,403).

Briefly, 2 mL urine were aliquoted into a 50 mL centrifuge tube, OTA-13C20 (Ochratoxin A 10 µg/mL; Cat. no: 33416) was added at 20 µg/L and mixed with 18 mL acetonitrile (ACN)/water/formic acid (53:44:3, v/v/v; LC-MS grade; Merck). Four grams of $MgSO_4$ and 1 g NaCl were added and vigorously mixed by hand for 10 seconds. The pressure was released by opening the lid and after re-sealing tubes were shaken for further 10 seconds. Samples were placed on a rotary shaker (Stuart Roller mixer SRT9) for 10 minutes and then centrifuged at 4,000 x g for 6 minutes at room temperature. Five millilitres of the non-polar fraction were transferred to a glass test-tube (Chromatographydirect, Cheshire, UK, Cat. no: STT-03-16100). Finally, samples were evaporated to dryness under N_2 at 40˚C (Pierce location if not already given Reacti-VapIII). Samples were then shipped to the Department of Food and Nutrition, National Institute of Health Dr Ricardo Jorge (INSA), Lisbon, Portugal and further processed for the detection of OTA and CIT (402,404).

For the analysis, the residue was dissolved in 0.5 mL of the injection solvent (dH_2O/ACN, 85:15, v/v), filtered (Syringe Filter unit 0.22 µm; PVDF 33 mm, gamma sterilised; Millipore, Burlington, Massachusetts, United States, Cat. no: SLGV033RS), then transferred to UHPLC vials (Thermo Fisher, Waltham, Massachusetts, United States, Cat. no: 60180-508) for LC-MS/MS analysis, and 10 µL of each sample was then injected into the UHPLC system (404).

Samples were analysed in an Acquity UHPLC system coupled to a Triple Quadrupole (TQD) mass spectrometer (Waters®, Milford, MA, USA). MassLynx™ version 4.1 and QuanLynx® version 4.1 (Waters®) were used for

data acquisition and processing. Quantification was carried out with an internal matrix-matched calibration with spiked blank urine samples. LOD and limit of quantification (LOQ) were considered as the signal-to-noise ratio (S/N), which should be more than 3 and 10, respectively.

2.5 DNA isolation from whole blood

Part of this work was to assess associations between genetic variants important for the metabolism of xenobiotics and any loss of kidney function that might provide insights into pathways that might increase the risk associated with the environmental exposures under investigation.

2.5.1 Blood isolation

DNA was isolated from whole blood samples from 350 subjects collected at visit 1. DNA was isolated using the DNA Blood Midi Kit (Qiagen, Hilden, Germany, Cat. no: 51185) according to the manufacturer's instructions. A Genome Wide Association Study (GWAS) was performed as part of a separate St Peter's Trust-funded project and data were used to perform hypothesis-driven Mendelian randomisation analysis.

Whole blood samples (4 mL) collected at baseline in tubes containing anticoagulant/EDTA (Section 2.1) and stored at -80˚C were thawed on ice. Briefly, 1-2 mL of whole blood, were aliquoted to a sterile 15 mL tube (Corning, Cat. no: CLS43079). Where necessary (i.e., the blood sample was less than 2 mL), the volume was brought to 2 mL with phosphate-buffered saline (PBS; VWR Life Sciences, Cat. no: 97062-730). Proteinase K (200 µL; Qiagen, Cat. no: 19133) was added followed by 2.4 mL AL-buffer (Qiagen, Cat. no: 19075) to digest cellular proteins. Samples were mixed by inverting the tubes 15 times and incubated at 70˚C for 10 minutes. At the end of the incubation, all the samples were simultaneously inverted for at least 1 minute then 2 mL 96-100% ethanol was added and the tubes inverted 10 times to precipitate DNA. The mixture was carefully transferred to the membrane filter unit and left to stand for 5 minutes to ensure binding of DNA to the filter.

The filter units were centrifuged for 3 minutes at 1,850 x g to remove the liquid. DNA remained on the filter as it is insoluble in ethanol. The DNA on the filter was then washed to remove any remaining ethanol. Washing buffer AW1 (2 mL) was added carefully to the filter, left to stand for 1 minute, and then centrifuged for 1 minute at 4,500 x g at room temperature. Washing buffer AW2 (2 mL) was added and samples centrifuged for 15 minutes at 4,500 x g at room temperature. Filters were transferred to elution tubes (Qiagen). DNase/RNase-free water (300 µL) was added and the filters were incubated for 5-6 minutes before being centrifuged at 4,500 x g at room temperature for 2 minutes. Genomic DNA was collected in the elution tube and transferred to a well of a 96-well plate for long-term storage at -20˚C.

To quantify the concentration and assess the purity of the DNA, the absorbance of the sample at A230, 260 and 280 nm was measured using a NanoDrop1000 (Thermo Scientific). The purity of DNA is given by the A260:A280 ratio with ratios > ~1.8 considered as pure. A ratio lower than 1.8 indicates contamination of the sample with proteins or other contaminants that absorb at or near 280 nm. A low A260:A230 ratio would also indicate the presence of organic compounds or chaotropic salts in the sample. The concentration of DNA in each sample is calculated based on the Beer-Lambert Law ($A=\varepsilon C\lambda$ where ε: wavelength dependent extinction coefficient; λ: the length of the sample through which the light passes and C: the concentration of the sample) (405).

DNA integrity was determined by agarose gel electrophoresis. Agarose (1% w/v) was dissolved in 1x TBE Buffer (0.7 mM Tris-HCl, 0.9 mM boric acid, 0.02 M EDTA, pH 8.0) by heating in a microwave oven. Once the solution had cooled, ethidium bromide (0.15 µg/mL) was added. Ethidium bromide intercalates into DNA and allows DNA to be visualised under UV light.

Samples were prepared containing 2 µL genomic DNA, 2 µL 1x Tris-EDTA Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and 1 µL 1x DNA gel loading dye (New England BioLabs, Massachusetts, United States, Cat. no: B7021S). Five microlitres of each sample and a 100 bp DNA ladder (New England BioLabs, Cat. no: N3271S) were loaded on to the agarose gel in 1x TBE and

run for 90 minutes at 120 V. Gels were examined under UV light (UVITECH UVIdoc Gel Documentation System) to check for DNA degradation (visible as a smear). Finally, DNA samples (200 ng) were aliquoted and air-dried in Abgene storage 96 well-plates (Abgene, New Hampshire, United States, Cat. no: AB0859) and shipped to the Medical Center Rotterdam, Erasmus MC University, Netherlands, for GWAS analysis using the Infinium® Global Screening Arrary-24 v1.0.

2.5.2 Identification of risk loci in genes associated with xenobiotic metabolism

A systematic literature search was conducted in PubMed and eBooks, of risk alleles in genes responsible for the elimination of metals and pesticides. Search terms included a combination of text words and headings for 'cytochrome 450', 'metal metabolism', 'pesticide metabolism', 'xenobiotic metabolism', 'enzymes', 'genes', 'metabolism exposure', pesticide exposure', 'elimination', 'polymorphisms', 'SNPs', 'genetic variation', and each of the element in this study. The results of the search are presented on the following Tables (Table 2.7 and Table 2.8 for metals and pesticides, respectively).

From the below SNPs, rs11191439, rs1695, rs11643815, rs4872479, rs2257082, rs3745274, rs28399499, rs1058930, rs11572080, rs12769205, rs749292, rs2502805, rs3003596 and rs662 were present in the array and were further analysed and compared with the loss of eGFR in mL/min/year by Dr Ben Caplin.

Gene	SNP (risk allele)	Mechanism	Ref.
ABCC1	rs11075290 (C)	Accumulation of MeHg in early development	(406)
ABCC2/ MRP2	rs1885301 (G) rs717620 (A)	Altered transcriptional activity and protein structure leading to deficient Hg transport and elimination	(407,408)
ABCB1/ MDR	rs2273697 (A) rs2032582 (G)	Defective promoter site activity and altered protein resulting in defective Hg elimination and higher cord blood MeHg concentrations	(406)
AS3MT	rs11191439 (CT/ CC) rs3740393	Low catalytic activity leading to decreased affinity for arsenic compounds, lower catalytic rate and lower stability influencing As methylation capacity	(409)
GSTP1	rs1695 (G)	Decreased enzymatic activity and, consequently, an increase in the accumulation of As and Al in the body	(410–412)
MT1A	rs8044719 (T) rs11076161 (G)	Low urinary Cd excretion and increased blood concentrations	(413,414)
T2A	rs28366003 (G)	Low urinary Cd, Cu and Zn excretion and an increase in the blood	(413,415,416)
MT4	rs10636 (C) rs11643815 (A)	Altered transcriptional activity and protein structure Defective promoter site activity and altered protein Altering Pb and Hg elimination	(417–419)
MTF1	rs4653329 (T)	Low urinary Cd excretion	(413)
SLC7A5/ LAT1	rs33916661 (G)	Altered transcriptional activity leading to increased urinary Hg levels	(420)
SLC22A6 / OAT1	rs4149170 (T)	Altered transcriptional activity leading to lower urinary Hg levels	(420)
SLC22A8 / OAT3	rs4149182 (C)	Altered transcriptional activity leading to lower urinary Hg levels	(420,421)
SLC39A8	rs4872479 (T)	Increased expression in kidney proximal tubules leading to increased re-uptake of Cd and reduced excretion in the urine	(422,423)
SLC39A14	rs870215 (AG/AA)	Increased expression in kidney proximal tubules leading to increased re-uptake of Cd and reduced excretion in the urine	(422)
XPO5	rs2257082 (C)	Affects mRNA structure and translation rate leading to increased levels of lead in the blood	(424–426)

Table 2.7: Genetic variants associated with the activity of proteins responsible for the elimination and homeostasis of heavy metals.

Gene	SNP (risk allele)	Mechanism	Ref.
CYP2B6	rs3745274 (T) 15582C>T 1459C>T rs28399499 (T) rs3211371 (T)	Affects enzyme activity. Low expression leads to decreased drug clearance and disrupted expression	(427–429)
CYP2C8	rs1058930 (G) 556C>T 556C>G rs11572080 (T)	Leads to reduced enzymatic activity	(430,431)
CYP2C19	rs12769205 (G)	Premature stop codon altering the size and function of the enzyme	(432,433)
CYP3A4	rs35599367 (T)	Leads to reduced enzymatic activity	(431)
CYP19A1	rs749292 (A)	Altered enzyme structure and function	(434,435)
NR1I3	rs2502805 (T) rs79769623 (A)? rs75114882 (C) rs75090438 (A) rs3003596 (C)	Disruption of different transcription factor sites altering glyphosate elimination	(436)
PON1	rs854560 (A) rs662 (G)	Low activity of the protein, low catalytic efficiency on some organophosphates (OP)	(437,438)

Table 2.8: Genetic variants associated with the activity of proteins responsible for the elimination of pesticides.

2.6 Cell Culture

The immortalised normal adult human proximal tubule cell line HKC-8, was used in the *in vitro* experiments (439).

2.6.1 Recovery of cells from cryogenic stocks

Cells were plated from cryogenic stocks. Dulbecco's-modified Eagle's medium containing 1 g/L D-glucose (DMEM; Thermo Fisher, Cat. no: 21885-025), 10% v/v Foetal Bovine Serum (FBS; Life Technologies, Carlsbad, California, United States, Cat. no: 10500064) and 1% antibiotics (Thermo Fisher, Cat. no: 15140122) from stock (containing 10,000 units penicillin and 10 mg streptomycin/mL in 100 mL proprietary citrate buffer) were added to a T-75 cell culture flask (final volume 15 mL) (Corning; Thermo Fisher, Cat. no: 14190144) and placed a humidified incubator at 37˚C with 5% CO_2 to equilibrate. Cells were thawed at 37˚C for 1-2 minutes. To minimise cell death, it is important to dilute/remove the cryoprotectant (10% dimethyl sulfoxide (DMSO) as quickly as possible. Thawed cells were immediately transferred to a 15 mL tube, with 6 mL medium, mixed and centrifuged at 201 x g at 4˚C for 10 minutes. The supernatant was discarded and the cell pellet gently resuspended in 1 mL culture medium. Cells were then added to the flask containing pre-equilibrated medium, gently mixed and placed in the incubator.

2.6.2 Cell maintenance and passaging

Cells were grown in DMEM with 10% FBS and 1% antibiotics (complete culture medium) at 37˚C in a humidified atmosphere of 5% CO_2. The medium was changed every 2-3 days. Cells were passaged at 80-90% confluence. The medium was aspirated and cells were washed with 5 mL PBS/T75 flask to remove residual medium and cell debris. Trypsin/EDTA (TrypLE™ Express Enzyme; Thermo Fisher, Cat. no: 12604013) 2 mL/flask, were added and the cells incubated for 2-4 minutes at 37˚C until the cells detached. Trypsin activity was neutralised by the addition of 8 mL complete culture medium and cells pipetted to mix and ensure a single cell suspension. Cells were passaged 1:4.

2.6.3 Cryopreservation of cells

To maintain cell stocks, surplus cells (not needed for experiments) were frozen down. Cells were trypsinised as described in Section 2.6.2 and then centrifuged at 201 x g for 5 minutes at room temperature. The supernatant was discarded and the pellet resuspended in cold freezing medium (FBS with 10% cell culture-grade DMSO). The cell suspension was aliquoted 1 mL/vial in cryovials (Cat. no: V7384). The vials were placed in a Mr Frosty container (Thermo Fisher, Cat. no: 5100-0001) containing isopropanol overnight at -80˚C; to allow the lowering of the temperature by -1˚C/min to -80˚C. The following day, the cryovials were transferred to liquid nitrogen (-196˚C) for long-term storage.

2.6.4 Exposure of HKC-8 cells to heat stress

One aetiological hypothesis pertaining to MeN is a combination of heat stress and dehydration, as workers work in extreme temperatures for long hours without adequate rest-time and hydration (47,96,119). A recent study in Californian agricultural workers showed an increase in the core temperature of 1˚C associated with the incident of AKI during a working shift, inducing a systemic inflammatory state (125). Therefore, the effect of elevated temperature on renal tubular epithelial cells experimentally was explored using either heat alone or heat in combination with osmotic stress to mimic dehydration.

Cells were plated in 6-well plates (Corning, Cat. no: CLS3506) at 0.8 x 10-6 cells/well in DMEM with 10% FBS, 1% antibiotics and grown to 80% confluence. Cells were incubated overnight with DMEM containing 0.5% FBS to synchronise the cell cycle in G0. The next day, the medium was replaced with fresh DMEM containing 0.5% FBS, 1% antibiotics or DMEM with 0.5% FBS, 1% antibiotics and 25 mM mannitol. Mannitol is a monosaccharide and was used to induce osmotic stress to replicate dehydration conditions. Plates of cells to be exposed to a higher temperature (39˚C) were placed in a separate incubator with a humidified atmosphere of 5% CO_2. Control plates were placed at 37˚C with a humidified atmosphere of 5% CO_2. Cells were incubated at 37˚C

(control) or 39˚C (heat stress), 37˚C plus 25 mM mannitol (osmotic stress) or 39˚C plus 25 mM mannitol (heat and osmotic stress) for 8, 24 or 48 hours.

2.6.5 Exposure of HKC-8 cells to fructose

As mentioned in Chapter 1, workers with MeN consume increased amounts of sugary beverages and chew sugarcane which can lead to high fructose levels in the circulation (119). Hence the effect of high concentrations of fructose on renal tubular epithelial cells was tested *in vitro*.

Cells were plated in 6-well plates (0.8 x 10-6 cells/well) in DMEM with 10% FBS, 1% antibiotics and grown to 80% confluence. Cells were incubated overnight with DMEM containing 0.5% FBS to synchronise the cell cycle in G0. The next day, the medium was replaced with fresh DMEM with 0.5% FBS, 1% antibiotics with or without 45 mM (as studied before in a diabetic *in vitro* model) glucose, fructose or L-glucose (osmolality control) for 8, 24 and 48 hours (the basal/control glucose concentration of the medium was 5 mM) (440–442).

Finally, the effect of the combination of heat stress and high fructose, sugar concentrations were tested. Confluent, quiescent cells were exposed to 10 mM fructose (previously published data showed to induce a proinflammatory response in proximal tubule cells *in vitro*), glucose or L-glucose at 37 and 39˚C for 8 and 72 hours (151).

2.6.6 Gene expression analysis

2.6.6.1 RNA isolation

Cells were harvested and lysed for mRNA following the manufacturer's instructions (RNeasy Mini Kit; Qiagen, Cat. no: 74106). The principle of the method relies on the fact that the spin column contains a silica-based membrane filter that selectively binds RNA (up to 100 µg RNA longer than 200 bases).

At the end of the experiments, the medium was aspirated from each well of the 6-well plates placed on ice and the cells were washed once with 1x cold PBS. PBS was aspirated and 350 µL RLT (Qiagen) lysis buffer/well were added.

RLT buffer breaks intramolecular protein disulphide bonds, lyses the cells and inactivates RNAses to ensure purification of intact RNA. Cell scrapers (Thermo Fisher, Cat. no: 11597692) were used to detach the cells and promote cell lysis. The lysate was transferred to a 1.5 mL microfuge tube (Cat. no: Z334006), then 350 µL 70% ethanol was added and the solutions mixed to ensure appropriate binding conditions. A total of 700 µL of each sample were placed in individual RNeasy spin columns seated in collection tubes and centrifuged at ≥ 8,000 x g for 15 seconds. The columns were washed by centrifugation with 700 µL RW1 followed by two washes with 500 µL RPE with the flow-through discarded each time. After the final RPE wash, columns were centrifuged at 14,000 x g for 1 minute to eliminate any RPE carryover. Columns were placed in clean collection tubes, 30-50 µL RNAse-free water were added to elute the RNA and columns centrifuged at ≥ 8,000 x g for 1 minute. The eluted RNA was added back to the column and centrifuged again. The extracted RNA was stored at -80˚C.

The concentration and purity of the isolated RNA were measured in a 1 µL sample using the NanoDrop1000 and concentrations were calculated as described in Section 2.5.1. Samples with an A260:280 ratio of ~2.0 were used for further analysis.

2.6.6.2 mRNA reverse transcription

The reverse transcription of mRNA to cDNA was performed with a 1-step method based on the High-Capacity cDNA Reverse Transcription Kit, following the manufacturer's instructions (Applied Biosystems, Foster City, California, United States, Cat. no: 4368814). Ten microlitre reaction volumes were prepared in duplicate for each sample, each containing a total of 1 µg RNA. A 2x reverse transcription master mix (containing dNTPs, primers, reverse transcriptase and buffer) was prepared and 10 µL added to 10 µL of each RNA sample. Reverse transcription was performed in a thermal cycler (G-Storm GS1 thermal cycler) as shown in Table 2.9.

Cycling Conditions	Temperature (˚C)	Time (min)
Step 1	25	10
Step 2	37	120
Step 3	85	5
Step 4	4	∞

Table 2.9: Cycle conditions for the reverse transcription of RNA to cDNA.

2.6.6.3 Primer design

Primers, for which a published sequence was not identified in the literature, were designed using Primer-Blast (NCBI). Criteria for choosing primers included: primers should span exon-intron boundaries, GC content should be at least 55%, and the melting temperature of each primer should be comparable (~60˚C). At least 2 sets of primers were designed and tested for each gene with the best set used for further analysis.

The optimisation of the primers was conducted using a range of primer concentrations with serial dilutions of the RNA. RT-qPCR products were electrophoresed in a 1.5% agarose gel in 1x TBE buffer to determine amplicon size and the presence/absence of multiple products and primer/dimer formation.

2.6.6.4 Genes expression

Expression of various genes associated with kidney injury, inflammation and fibrosis such as Kidney Injury Molecule-1 (KIM-1), Monocyte Chemoattractant Protein-1 (MCP-1), Transforming Growth Factor-β (TGF-β) and Collagen type I, α1 (COL1A1) was examined (443,444).

KIM-1 is a type 1 transmembrane protein expressed, expressed in multiple tissues and cell types including the proximal tubules and is up-regulated in the urine after kidney injury (445,446). In response to injury, the extracellular domain of the protein is cleaved resulting in increased levels in the urine indicating increased release of the protein from injured cells (447). MCP-1 is a small heparin-binding chemokine that acts as a chemotactic factor for monocytes resulting in inflammation in kidney injury (448,449).

Numerous pathways can lead to renal fibrosis, and progressive scarring characteristic of CKD. The pre-eminent fibrogenic factor implicated in renal fibrosis is TGF-β (450). TGF-β1 is the most abundant isoform and is produced by a variety of resident renal cells types in response to injury. TGF-β1 stimulates production of extracellular matrix components including collagens and fibronectin which accumulate in the progressive fibrosis associated with CKD (451,452). COL1A1 is the most abundant collagen isoform in humans and is increased in CKD (453,454).

Two constitutively expressed (housekeeping) genes, Hypoxanthine Phosphoribosyltransferase 1 (HPRT1) and Succinate dehydrogenase complex, subunit A (SDHA), whose expression levels are not influenced by cell injury were used for normalisation. HPRT1 is an enzyme that allows cells to recycle purines for DNA and RNA production (455). SDHA is a component of SDH, an enzyme that plays a vital role in the citric acid cycle and oxidative phosphorylation (456).

2.6.6.5 Real-time quantitative polymerase chain reaction (qPCR)

Real-time qPCR was performed to test the number of copies of a specific amplified target sequence in real time. Table 2.10 shows the sequence of the forward and reverse primers for the genes of interest as well as the annealing temperature which was the same for all the primer sets.

Human Gene	Forward Primer	Reverse Primer	Annealing Temperature
KIM-1	TCCGTGGCCCTTTTTGCTTA	GAGCCTGCTGTTCAGATCCA	60 ˚C
MCP-1	TCCCAAAGAAGCTGTGATCTTCA	TCTGGGGAAAGCTAGGGGAA	60 ˚C
TGFβ	CTAATGGTGGAAACCCACAACG	TATCGCCAGGAATTGTTGCTG	60 ˚C
COL1A1	CCCGTGGACCTGTTCTTTGT	CACAGCGGTACCTGTCC	60 ˚C
HPRT1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	60 ˚C
SDHA	AGAAGCCCTTTGAGGAGCA	CGATCACGGGTCTATATTCCAGA	60 ˚C

Table 2.10: Forward and reverse primers for the target genes.

Thirteen microlitres of SYBR green mix (Qiagen, Cat. no: 204054) were added to 2 µL cDNA (10 ng/µL) and samples were placed in the Thermocycler (Corbett Research PCR Rotorgene 6000) for a total of 30 cycles (Table 2.11). During each cycle, fluorescent signal, proportional to the amplified target DNA

product, is detected. The results of each target gene of interest were normalized against the two housekeeping genes (HPRT1 and SDHA).

A cycler threshold (CT) was set for the number of cycles required to detect a signal of each amplified gene product (Rotorgene ScreenClust HRM Software 2.3.1.49). The CTs were then transformed to relative gene expression levels using the following equation: 2^ ((Average CT of the house keeping genes SDHA and HPRT1) - (CT of the sample)).

		Two-step cycling	
	PCR initial activation	**Denaturation**	**Annealing/extension**
Time	5 min	10 sec	30 sec
Temperature (˚C)	95	95	60

Table 2.11: qPCR conditions for amplification of cDNA. Two-step cycling was performed for 30 cycles.

2.6.7 Protein isolation

Total cell proteins were extracted from cultured cells. At the end of the experiment 6-well plates were transferred onto ice. The media from the cells was collected in 1.5 mL microcentrifuge tubes, centrifuged at 90 x g for 3 minutes to remove debris and dead cells, transferred to clean tubes and stored at -80˚C until further analysis. Cells were washed twice with cold PBS (1 mL/well) to remove residual medium. The final PBS wash was aspirated, another 1 mL cold PBS was added, and cells scraped off using a cell scraper. The cells were transferred to a 1.5 mL microfuge tube and centrifuged at 735 x g at 4˚C for 4 minutes. The supernatant was discarded and 80-100 µL (depending on the size of the pellet) RIPA buffer containing protease inhibitors (Cat. no: P8340) in a 1:100 ratio, were added. The pellet was resuspended by pipetting up and down several times. Samples were incubated for 30 minutes on ice, followed by centrifugation for 10 minutes at 14,000 x g at 4˚C. The supernatant (containing proteins) was transferred to a clean 0.5 mL microfuge tube and the pellet containing insoluble cell components and debris, was discarded.

2.6.7.1 Protein quantification

For the colorimetric determination of the protein concentration of the samples, the microplate Pierce bicinchoninic acid (BCA) Protein Assay (Thermo Scientific, Cat. no: 23225) was used. The principle of the method is based on the fact that proteins in an alkaline medium reduce Cu^{+2} to Cu^{+1}. When BCA is added to the reduced Cu^{+1} complex, it produces a purple colour that can be detected at 562 nm. Protein quantification in unknown samples requires the preparation of a series of dilutions with known protein concentrations (standard curve) from which the concentration in the samples can be determined.

Serial dilution standards and samples were prepared according to the manufacturer's instructions. Samples and standards (0-2 mg/mL bovine serum albumin (BSA)) were added to individual wells of a 96-well plate in duplicate and then mixed with WR reagent in a 1:8 ratio. Plates were shaken on a plate-shaker at room temperature for 30 seconds and incubated in the dark for 30 minutes at 37˚C. Plates were cooled to room temperature and the absorbance was measured at 562 nm using a plate reader (Biochrom EZ Read 400 Microplate Reader) with Galapagos software. The average of each standard and sample was then calculated. The OD of the blank was subtracted from each standard and sample. The standard curve was plotted and the linearity assessed by the determination of the R^2 which should be close to 1. The protein concentration of the samples was calculated from the standard curve.

2.6.7.2 SDS-PAGE and Western Blotting

Western blotting was used to detect specific proteins in the cell lysates. The technique involves the separation of the proteins based on their molecular weight in a gel when an electrical field is applied (electrophoresis), transfer of proteins to a membrane, incubation with primary antibodies against the proteins of interest, the detection of the primary antibody binding using an enzyme-linked secondary antibody, incubation with a chemiluminescent substrate and detection of the signal using a chemiluminescence imager.

2.6.7.2.1 Polyacrylamide gel electrophoresis (PAGE) of proteins

From the protein quantification (Section 2.6.7.1), the amount of sample required to run the same amount of protein was calculated. Where the volume of samples varied, all samples were made to the same volume with lysis buffer. Laemmli buffer (4x Laemmli Sample buffer; Bio-Rad Laboratories, Cat. no: 1610791) containing β-mercaptoethanol was added to give a final concentration of 1x. Proteins were denatured by incubating for 5 minutes at 100˚C and cooling on ice prior gel electrophoresis. On each gel, 4 µL molecular weight marker (1-250 kDa dual colour MW ladder; Bio-Rad Laboratories, Cat. no: 161-0374) was also run. Samples were run on a 5% polyacrylamide stacking gel and a 12% polyacrylamide separating gel (Section 2.4.3).

Gels were run in electrophoresis buffer (25 mM Tris, 192 mM Glycine and 0.1% v/v SDS) for ~75 minutes at 150-170 Volts, until the sample buffer dye reached the bottom of the gel.

2.6.7.2.2 Transfer of proteins to nitrocellulose membrane

After electrophoresis, it is essential to transfer the proteins from the gel to a material that is easier to handle. Proteins were transferred to a nitrocellulose membrane using a wet transfer apparatus (Bio-Rad Laboratories, Cat. no. 1703930). In the electric field negatively charged proteins in the gel (due to SDS) migrate towards the positive pole of the device and so are transferred to the membrane.

The nitrocellulose membrane (Amersham Protran 0.2 µm transfer membrane, Cat. no. GE10600053) was pre-equilibrated in transfer buffer (25 mM Tris, 192 mM Glycine and 20% v/v methanol) for 5 minutes. The gel and membrane were then assembled as follows: 2 sponges, 4 sheets Whatman filter paper (Whatman® filter paper, Cat. no: WHA5201911) followed by the nitrocellulose membrane, the gel, 4 sheets Whatman filter paper and 2 sponges. The assembly was gently compressed with a pipette rolled across the surface to extrude air bubbles which would interfere with protein transfer, the ‘sandwich’ was placed in a Bio-Rad Transfer device containing transfer buffer and ran at 100 Volts for 60 minutes (current at 10-15 mA) on ice.

The efficiency of transfer was checked by staining the membrane with Ponceau S containing 5% acetic acid (Cat. no: P7170) for 5 minutes to visualise the proteins on the membrane. The membrane was de-stained with water prior to immunoblotting.

2.6.7.2.3 Western blot immunoassay

Membranes were blocked for 60 minutes in TBS-0.1% Tween 20 (TBS-T) containing 5% w/v dried skimmed milk (Marvel, Premier Foods, St Albans, United Kingdom) at room temperature on a rocker. Membranes were incubated with primary antibody (Table 2.12) in TBST-5% Marvel overnight at 4˚C on a roller, then washed with TBS-T (4 x 5 minutes) and incubated for 60 minutes at room temperature with horseradish peroxidase (HRP)-linked secondary antibody in 2% w/v Marvel in TBS-T. Membranes were drained to remove excess solution and placed on a sheet of Saran Wrap.

Antibodies	Species	Dilution	Company	Cat. Number
β-actin	Rabbit	1:10,000	Sigma-Aldrich	A2066
COL1A1	Mouse	1:100	Novus Biologicals	NB600-450
TGFβ	Mouse	1:100	R & D	MAB2401-SP
KIM-1	Mouse	1:1,000	R & D	MAB1750-SP
MCP-1	Rabbit	1:1,000	Abcam	ab9669
Anti-mouse-HRP	Goat	1:1,000	Abcam	ab6789
Anti-rabbit-HRP	Goat	1:1,000	Abcam	ab191866

Table 2.12: Antibodies used for Western Blotting.

The primary-secondary antibody complex was detected using Enhanced Chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Cat. no: WBKLS0100). ECL reagents (A and B) were mixed 1:1. The solution was pipetted evenly over the membrane and incubated for 1 minute, transferred to UVP Biospectrum 810 Imaging System and images were taken.

Primary antibodies were stripped from the membranes using striping buffer (Restore Western Blot Stripping Buffer, Cat. no: 21059) for 5-10 minutes at 37˚C, washed, re-blocked and re-probed for β-actin. Densitometry was performed using Image J software (457). From the three proteins tested (MCP-1, KIM-1 and TGF-β), only MCP-1 gave specific bands and it was further

explored for each of the experiments performed. Results from the densitometry of MCP-1 were normalised to β-actin (protein of interest/ β-actin).

2.6.8 ELISA to detect chemokines in cell supernatants

Conditioned media samples (Section 2.6.7) were thawed at room temperature and protein concentrations were quantified using the BCA Protein Assay (Section 2.6.7.1). Commonly, FBS proteins present in the media are a limitation in secretome analysis as being present in relatively high concentrations, they tend to mask cell derived proteins. Although methods to avoid the background of serum proteins particularly albumin are available, there were not performed within this present study (458). All media contained the same percentage of FBS (0.5%) so any changes due to FBS present in the media should be proportional and changes should occur by the other proteins excreted from the cells.

2.6.8.1 MCP-1

As mentioned earlier, MCP-1 is a monocyte chemoattractant which means it needs to be secreted from cells to perform its function. The secretion of MCP-1 by HKC-8 cells was measured using an ELISA kit according to the manufacturer's instructions (ELISA MAX, Deluxe Set Human MCP-1/CCL1; BioLegend, Kentish Town, London, Cat. no: 438804). Plates (96-well flat-bottom plates; Corning, Cat. no: 10695951) were coated the day before the assay by incubation at 4˚C with the anti-MCP-1 antibody. The next day, plates were washed 4 times with washing buffer (BioLegend, Cat. no: 421601), 200 µL 1 x Assay Diluent A were added and the plate shaken for 60 minutes on a plate shaker, to block the wells. Following the blocking step, the wells were washed 4 times with 100 µL washing buffer per well. One hundred microlitres of each sample and standards (7.8-500 pg/mL) were added in duplicate to the appropriate labelled wells and incubated at room temperature for 2 hours with shaking on a plate shaker. Plates were then washed 4 times with 100 µL washing buffer per well. One hundred microlitres of the detection antibody (human MCP-1) were added to each well, incubated for 60 minutes and the wash steps repeated. Avidin-HRP (100 µL) solution was added to each well and incubated for 30 minutes and the plates washed again. One hundred

microlitres of freshly prepared TMB substrate solution were added to each well and incubated for 20 minutes. Finally, 100 µL stop solution were added to terminate the reaction and the colour production. The absorbance was measured at 450 nm and 570 nm on plate reader (Biochrom EZ Read 400 Microplate Reader) within 15 minutes of terminating the reaction. The absorbance at 570 nm of each sample was subtracted from the absorbance at 450 nm to correct for background.

2.6.8.2 KIM-1

KIM-1, is a transmembrane protein the outer domain of which is cleaved in response to injury increasing the concentration in the cell supernatant (445). The presence of KIM-1 in cell media in the different experimental conditions was measured using an ELISA kit according to the manufacturer's instructions (Human KIM1 PicoKine ELISA Kit; BosterBio, Cat. no: EK0883). The ELISA kit provided a pre-coated 96-well plate. One hundred microlitres of each sample and standards (0-2,000 pg/mL) were added in duplicate to the appropriately labelled wells and incubated at 37˚C for 90 minutes. The solution was discarded by inversion, 100 µL Biotinylated Anti-human KIM-1 were added to each well and incubated at 37˚C for 60 minutes. Wells were washed 3 times with 300 µL washing buffer per well, 100 µL Avidin-Biotin-Peroxidase Complex were added and the plates incubated at 37˚C for 30 minutes. Wells were washed with 300 µL washing buffer. Ninety microlitres of Colour Developing Reagent were added to each well and incubated in the dark for 30 minutes, 100 µL of stop solution were then added and the absorbance measured at 450 nm within 30 minutes (Biochrom EZ Read 400 Microplate Reader).

2.6.8.3 IL-1β

IL-1β is a proinflammatory cytokine that activates an immune response (459). In an *in vitro* experiment in the human proximal tubular cell line HK-2, IL-1β was found to be highly expressed in response to aristolochic acid-induced injury (460). IL-1β in cell supernatants was measured using an ELISA kit according to the manufacturer's instructions (ELISA MAX, Deluxe Set Human IL-1β; BioLegend, Cat. no: 437004). Plates (96-well flat-bottom) were coated

with the capture antibody the day before use and incubated overnight at 4˚C. The next day, plates were washed 4 times with washing buffer (BioLegend, Cat. no: 421601) and 200 µL Assay Diluent A were added for 60 minutes with shaking on a plate shaker, to block the wells. Wells were washed 4 times with 100 µL washing buffer per well. Fifty microlitres of each sample and standards (1.95-125 pg/mL) were added in duplicate in the appropriately labelled wells and incubated at room temperature for 2 hours shaking on a plate shaker. Plates were then washed 4 times with 100 µL washing buffer per well. One hundred microlitres of the detection antibody (human IL-1β) were added to each well, incubated for 60 minutes and the plates washed again. Avidin-HRP (100 µL) solution was added to each well, incubated for 30 minutes and washed again. One hundred microlitres of freshly prepared TMB substrate solution were added to each well and incubated for 20 minutes. Finally, 100 µL stop solution were added to terminate the reaction and colour production. The absorbance was measured at both 450 nm and 570 nm on a Biochrom EZ Read 400 Microplate Reader within 15 minutes of terminating the reaction. For each sample the absorbance at 570 nm was subtracted from the absorbance at 450 nm to correct for background.

2.7 Statistical Analyses

For the biological sample association studies, study participants were divided into kidney function status groups (stable, declining and established) that were identified using a growth mixture modelling technique by Richard Silverwood, LSHTM (70). This approach allowed the identification of subpopulations with differing trajectories in a continuous outcome over time (eGFR). These groups were generated separately for men and women and all the analyses reported here are based on whether participants were members of each of these groups.

Data analysis was performed before and after creatinine correction and log transformation of the values of each urine sample to achieve a normal distribution across the data when distribution was skewed as determined visually with a histogram of the raw data. Data were analysed using GraphPad Prism V.8.4.1 with the significance threshold set at p=0.05. Descriptive

analyses were undertaken separately for men and women. Unpaired Student's t-test was used to test the null hypothesis that there were no differences in the parameter of interest between the kidney function trajectory groups in men only. Comparisons of frequencies were performed using chi-squared or Fisher's exact test (where expected counts in a cell were <5).

Calculation of the effect size for the assessment of the power of the effect of the different kidney groups was performed using G*power 3.0.10. A post hoc power calculation was performed assuming an 80% power and α error probability of 0.05 to detect an effect of >0.8 Standard Deviation (SD) for pesticide metabolites (sample size of 25 from the stable group versus 25 from the declining group) and an effect of >0.59 SD for the metal and metalloid urine analysis (sample size of 213 from the stable group versus 25 from the declining group). GraphPad Prism V.8.4.1. was used for graph preparation.

For the results of *in vitro* experiments, Microsoft Excel was used to import and prepare results prior to analysis. Gene expression quantification was followed by correcting for two housekeeping genes. GraphPad Prism V.8.4.2. was used to perform unpaired student's t-test and two-way ANOVA. Results from qPCR and ELISA analyses were examined with two-way ANOVA (Tukey's and Dunnett's multiple comparisons tests) to assess associations between each experimental group.

Chapter 3: Urinary and serum biomarkers

3.1 Introduction

Clinical findings in the early stages of MeN development report absence of albuminuria and haematuria suggesting a potential tubular site of injury rather than a primary glomerular disease is responsible for the development of the disease. A number of urinary low molecular weight proteins have been proposed as markers of tubular injury due to failure of reabsorption by the tubules, including B-2M and RBP (461). Moreover, serum uric acid (UA) has been proposed as a potential mediator in the development of MeN, and phosphate can be an early indicator of tubular dysfunction (38,163). Finally, liver function tests (LFTs) (such as ALT and AST) can provide information about the function of the liver; the main organ of xenobiotic elimination (462).

In this chapter strip-tests, ELISAs, proteomic and biochemical analyses were performed on urine and serum samples (from visits 1 and 2) to explore any associations between these and the decline in kidney function in a population at risk of MeN. Any associations found would be indicative of a potential early biomarker(s) that could be utilised to assess the function of the kidneys well before serum creatinine levels are increased and proactive measurements can be taken to prevent further damage.

3.2 Outcome definition

The study population consisted of 263 men and 87 women recruited from nine communities. Sixty-six percent of the participants (n=198) worked in agriculture including sugarcane and banana plantations at the beginning of the study (visit 1) (Table 3.1). The majority of the participants were <25-year-old (63.9% of men and 58.6% of women) at visit 1. The number of men consuming alcohol was much higher (53.6%) compared to women (9.2%) at visit 1.

After a growth mixture modelling analysis, performed by Dr Richard Silverwood (LSHTM), the study population was divided into groups of kidney function trajectories for men and women (68). Amongst men, three groups were

identified: (i) stable kidney function (n=213; mean eGFRcr-cys at baseline: 116±14 mL/min/1.73 m^2; slope: -0.6 mL/min/year) referred to as the 'stable group'; (ii) rapid decline in kidney function (n=25; mean eGFRcr-cys at baseline: 112±19 mL/min/1.73 m^2; slope: -18.2 mL/min/year) referred to as the 'declining group'; and, (iii) renal dysfunction at baseline (n=25; mean eGFRcr-cys at baseline: 58±17 mL/min/1.73 m^2; slope: -3.8 mL/min/year) referred to as the 'established group'. Amongst women, two groups were identified: (i) stable kidney function (n=84; mean eGFRcr-cys at baseline: 121±11 mL/min/1.73 m^2; slope: -0.6 mL/min/year); and, (ii) rapid decline in kidney function (n=3; mean eGFRcr-cys at baseline: 132±8 mL/min/1.73 m^2; slope: -14.6 mL/min/year). Given the low numbers of women experiencing rapid decline and the absence of any women with established kidney dysfunction, comparisons of analytes between kidney function trajectory groups was restricted to men only.

Variables		Gender							
		Male				Female			
	Categories	Visit 1 (n=263)		Visit 2 (n=213)		Visit 1 (n=87)		Visit 2 (n=78)	
		n	%	n	%	n	%	n	%
Age (years)	≤25	168	63.9	128	60.1	51	58.6	37	47.4
	>25	95	36.1	85	39.9	36	41.4	41	52.6
Current occupation	Sugarcane work	50	19.0	104	48.8	10	11.5	9	11.5
	Agriculture work	118	44.9	54	25.4	6	6.9	6	7.7
	Banana work	13	4.9	15	7.0	1	1.2	3	3.9
	Commerce-related jobs	5	1.9	14	6.6	9	10.3	7	9.0
	Construction	10	3.8	6	2.8	0	0.0	-	-
	Other occupations	14	5.3	10	4.7	3	3.4	4	5.1
	Economically inactive population	53	20.2	10	4.7	58	66.7	49	62.8
Water supply	Tap water	139	52.9	-	-	47	54.0	-	-
	Dug well	98	37.3	-	-	28	32.2	-	-
	Drilled well	26	9.9	-	-	12	13.8	-	-
Smoking status	Light smoker	103	39.1	98	46.0	1	1.2	1	1.3
	Non-smoker	160	60.8	115	54.0	86	98.9	77	98.7
Alcohol consumption	Any	141	53.6	114	53.5	8	9.2	6	7.7
	None	122	46.3	99	46.5	79	90.8	72	92.3
Involved in pesticide application	Yes	135	51.3	63	29.6	12	13.8	12	15.4
	No	128	48.7	150	70.4	75	86.2	66	84.6

Table 3.1: Selected sociodemographic variables of the cohort at visit 1 (pre-harvest) and visit 2 (post-harvest) stratified by gender. In total, 350 participants were recruited of whom 16.9% were lost to follow-up at visit 2 due to emigration.

3.3 Urine analysis

3.3.1 Strip-test analysis and albumin: creatinine ratio estimation

The ACR and a variety of analytes (leukocytes, nitrates, protein, blood, urobilinogen, ketones, bilirubin, glucose, pH and specific gravity (SG)) were measured using Multistix 10SG Reagent Strips (Siemens, Cat. no: PMC0353) with the help of Brenda La Rosa García, UCL Applied Medical Sciences BSc student, as part of her final year project.

3.3.1.1 Strip-test analytes in men and women at visit 1 and visit 2

Before testing the original samples, the effect of BA and freezing (4˚C, -20˚C and -80˚C) on the accuracy of the strip-test method was tested in fresh urine samples. Leucocytes demonstrated false negatives whereas blood showed false positives when stored at -20˚C and below. SG was found to decrease with the presence of BA. False positives for bilirubin were found when the storage temperature was below -20˚C. The pH values determined by the strip-test were not analysed further since BA keeps the pH below 7 (maintain an acidic pH) to prevent disruption of pus cells containing dead tissue, cells and bacteria (463). The rest of the analytes did not seem to be affected by the presence of BA in the different temperatures and they were found negative.

The different analytes were compared between men and women at visit 1 and visit 2 (Table 3.2). A small number of men and women at visit 1 (6.5% and 3.4%, respectively) had traces or positive for leukocytes strip-test. In the contrary, at visit 2 only women were found to have traces and positive for leucocytes strip test (12.9%). On the contrary, men were reportd negative for the majority of strip-tests (96.7%), and none with positive results (traces accounted to 3.3%). From our preliminary analysis on the effect BA and freezing-thawing has on strip-test results, leukocytes were demonstrating false negative results and the cohort data cannot be assesed. The incidence of ACR≥30 was higher at visit 2 (9.9%) for men compared to visit 1 (0.8%). For both sexes at visit 2 more samples were found to have SG>1020. Traces of protein and a positive protein strip-test were detected in men and women, respectively, 14.8% (n=39) and 19.5% (n=17) at visit 1 and 17.8% (n=38) and

19.2% (n=15) at visit 2. On the other hand, ACR≥30 in men and women, respectively, was 0.8% (n=2) and 4.6% (n=4) at visit 1, and 9.9% (n=21) and 3.8% (n=3) at visit 2, respectively. Urobilinogen was normal for all cases at visit 1 and visit 2. Bilirubin strip-test for both men and women was negative for ~50% of the samples and detectable traces and positive samples were ~50%.

Characteristic	Gender		Visit 1 (n=350) (n, %)	Visit 2 (n=291) (n, %)
Leukocytes	Male	Negative	246 (93.5)	206 (96.7)
		Trace	5 (1.9)	7 (3.3)
		Positive	12 (4.6)	0 (0.0)
	Female	Negative	84 (96.6)	68 (87.2)
		Trace	2 (2.3)	2 (2.6)
		Positive	1 (1.1)	8 (10.3)
Nitrates	Male	Negative	259 (98.5)	211 (99.1)
		Trace	0 (0.0)	0 (0.0)
		Positive	4 (1.5)	2 (0.9)
	Female	Negative	87 (100.0)	74 (94.9)
		Trace	0 (0.0)	0 (0.0)
		Positive	0 (0.0)	4 (5.1)
Protein	Male	Negative	224 (85.2)	175 (82.2)
		Trace	25 (9.5)	26 (12.2)
		Positive	14 (5.3)	12 (5.6)
	Female	Negative	70 (80.5)	63 (80.8)
		Trace	13 (14.9)	11 (14.1)
		Positive	4 (4.6)	4 (5.1)
Blood	Male	Negative	214 (81.4)	186 (87.3)
		Trace	30 (11.4)	20 (9.4)
		Positive	19 (7.2)	7 (3.3)
	Female	Negative	70 (80.5)	35 (44.9)
		Trace	8 (9.2)	14 (17.9)
		Positive	9 (10.3)	29 (37.2)
Ketones	Male	Negative	262 (99.6)	213 (100.0)
		Trace	1 (0.4)	0 (0.0)
		Positive	0 (0.0)	0 (0.0)
	Female	Negative	87 (100.0)	78 (100.0)
		Trace	0 (0.0)	0 (0.0)
		Positive	0 (0.0)	0 (0.0)
Bilirubin	Male	Negative	140 (53.2)	100 (46.9)
		Trace	85 (32.3)	113 (53.1)
		Positive	38 (14.4)	0 (0.0)
	Female	Negative	36 (41.4)	38 (48.7)
		Trace	34 (39.1)	23 (29.5)
		Positive	17 (19.5)	17 (21.8)
Glucose	Male	Negative	263 (100.0)	213 (100.0)
		Trace	0 (0.0)	0 (0.0)
		Positive	0 (0.0)	0 (0.0)
	Female	Negative	87 (100.0)	78 (100.0)
		Trace	0 (0.0)	0 (0.0)
		Positive	0 (0.0)	0 (0.0)
SG	Male	≤1020	256 (97.3)	194 (91.1)
		>1020	7 (2.7)	19 (8.9)
	Female	≤1020	81 (93.1)	69 (88.5)
		>1020	6 (6.9)	9 (11.5)
ACR	Male	<30 mg/g	261 (99.2)	192 (90.1)
		≥30 mg/g	2 (0.8)	21 (9.9)
	Female	<30 mg/g	83 (95.4)	75 (96.2)
		≥30 mg/g	4 (4.6)	3 (3.8)

Table 3.2: Frequencies of the different strip-test analytes in males and females at visit 1 and visit 2.

3.3.1.1 Association of strip-test analytes and kidney function

At visit 1, for both men and women, no differences were observed in any of the characteristics in the different kidney function groups (stable, declining or established for men) (Table 3.3). In contrast, after performing a Fisher's exact test, results from visit 2 showed that, despite overall low prevalence of proteinuria, more men in the declining and established groups had elevated ACR (≥30; p=0.014). Furthermore, less men with established kidney dysfunction had trace levels of bilirubin (p=0.004). No statistical analysis was performed for women as the number of women in the declining function group was only three. Appendix 1, Table A1.1 and A1.2 illustrate the results of each strip-test analyte between the different age groups, genders, residential community, and quality of well water participants were consuming based on analysed well water samples at visit 1 and visit 2.

Characteristic	Visit 1				Visit 2			
	Stable (n=213)	Declining (n=25)	Established (n=25)	Sig. between groups	Stable (n=176)	Declining (n=18)	Established (n=19)	Sig. between groups
Leukocytes (n, %)								
Negative	200 (93.9)	24 (96.0)	22 (88.0)	0.834	171 (97.2)	17 (94.4)	18 (94.7)	1.0
Trace	4 (1.9)	0 (0.0)	1 (4.0)		5 (2.8)	1 (5.6)	1 (5.3)	
Positive	9 (4.2)	1 (4.0)	2 (8.0)		0 (0.0)	0 (0.0)	0 (0.0)	
Nitrates (n, %)								
Negative	209 (98.1)	25 (100.0)	25 (100.0)	1.0	174 (98.9)	18 (100.0)	19 (100.0)	1.0
Trace	0 (0.0)	0 (0.0)	0 (0.0)		2 (1.1)	0 (0.0)	0 (0.0)	
Positive	4 (1.9)	0 (0.0)	0 (0.0)					
Protein (n, %)								
Negative	181 (85.0)	22 (88.0)	21 (84.0)	0.601	149 (84.7)	13 (72.2)	13 (68.4)	0.141
Trace	19 (8.9)	2 (8.0)	4 (16.0)		19 (10.8)	4 (22.2)	3 (15.8)	
Positive	13 (6.1)	1(4.0)	0 (0.0)		8 (4.5)	1 (5.6)	3 (15.8)	
Blood (n, %)								
Negative	175(82.2)	19 (76.0)	20 (80.0)	0.972	155 (88.1)	16 (88.8)	15 (79.0)	0.358
Trace	23 (10.8)	4 (16.0)	3 (12.0)		17 (9.7)	1 (5.6)	2 (10.5)	
Positive	15 (7.0)	2 (8.0)	2 (8.0)		4 (2.3)	1 (5.6)	2 (10.5)	
Ketones (n, %)								
Negative	212 (99.5)	25 (100.0)	25 (100.0)	1.0	176 (100.0)	18 (100.0)	19 (100.0)	1.0
Trace	1 (0.5)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	0 (0.0)	
Positive	0 (0.0)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	0 (0.0)	
Bilirubin (n, %)								
Negative	111 (52.1)	12 (48.0)	17 (68.0)	0.628	76 (43.2)	8 (44.4)	16 (84.2)	0.004
Trace	70 (32.9)	9 (36.0)	6 (24.0)		100 (56.8)	10 (55.6)	3 (15.8)	
Positive	32 (15.0)	4 (8.0)	2 (8.0)		0 (0.0)	0 (0.0)	0 (0.0)	

Glucose (n, %)								
Negative	213 (100.0)	25 (100.0)	25 (100.0)	1.0	176 (100.0)	18 (100.0)	19 (100.0)	1.0
Trace	0 (0.0)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	0 (0.0)	
Positive	0 (0.0)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	0 (0.0)	
SG (n, %)								
≤1020	207 (97.2)	24 (96.0)	25 (100.0)		158 (90.0)	18 (100.0)	18 (94.7)	
>1020	6 (2.8)	1 (4.0)	0 (0.0)	0.863	18 (10.0)	0 (0.0)	1 (5.3)	0.332
ACR (n, %)								
<30 mg/g	211 (99.1)	25 (100.0)	25 (100.0)	1.0	163 (92.6)	14 (77.8)	15 (78.9)	0.014
≥30 mg/g	2 (0.9)	0 (0.0)	0 (0.0)		13 (7.4)	4 (22.2)	4 (21.1)	

Table 3.3: Frequencies of strip-test characteristics in men at visit 1 and visit 2, between the stable, declining and established kidney function groups. Sig: significance tested by Fisher's exact test; SG: specific gravity; ACR: Albumin to creatinine ratio; n: number of participants.

3.3.2 Urinary Protein Analysis

Samples which were positive for protein on the strip-test but negative for albuminuria, were investigated to determine what proteins were contributing to strip-test positivity (as described in Section 3.3.1.1) as low-molecular weight proteinuria is common in tubular disease. A number of urine samples with positive strip-test protein and positive albumin by the (BCG) Albumin Assay Kit (+/+), positive strip-test protein and negative albumin (+/-), and negative for both (-/-), were electrophoresed in a polyacrylamide gel. A positive control (Positive Ct) containing 1 mg/mL Bovine Serum Albumin (BSA) and a negative control (Negative Ct) were also included. Silver staining of the gel (Figure 3.1) showed that a sample with a positive strip-test and positive albumin contained a 66.5 kDa protein characteristic of albumin (black arrow; Figure 3.1), as well as some smaller molecular weight proteins (25-66 kDa) which are also present in the positive control (possibly a combination of degraded albumin and other small molecular weight proteins). In the sample with a positive strip-test and negative albumin, small molecular weight proteins (<25 kDa) were detected (Red circle; Figure 3.1).

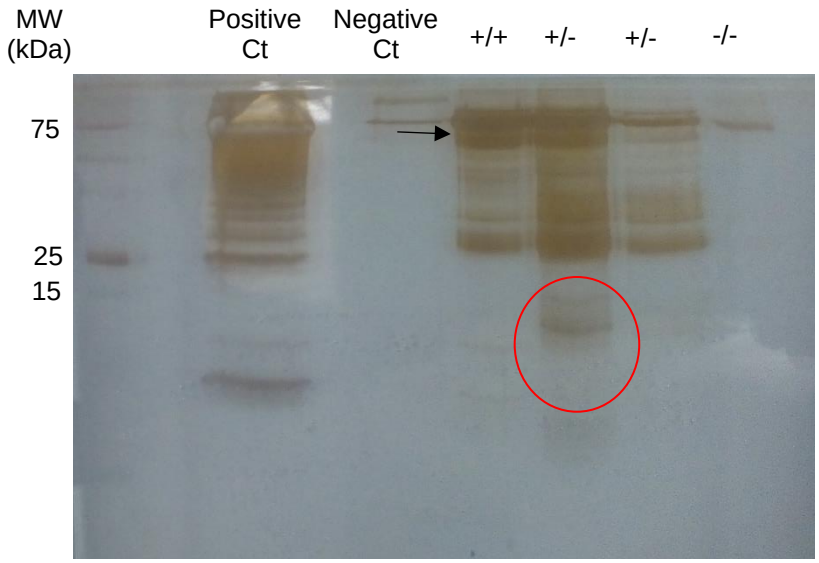

Figure 3.1: Silver staining of urinary proteins of selected samples. Urine samples were diluted 1:3 with PBS, electrophoresed on a 20% polyacrylamide gel and silver stained. MW: 10-200 kDa molecular weight marker; Positive Ct: Positive control; 1 mg/mL BSA in PBS; Negative Ct: Negative control, PBS. +/+: Urine sample with a positive strip-test and positive albumin.; +/-: Urine samples with positive strip-test and negative albumin; -/-: Urine sample which was negative for both analyses.

Two urine samples that tested positive for protein in the strip-test but negative for albumin were subjected to further protein analysis to determine whether the low molecular proteins present in the samples were potentially of tubular origin and indicative of tubular injury. The low molecular proteins detected by silver staining were excised from the gel and sent to the Scientific Facilities of Life and Environmental Sciences (LES), School of Biosciences, University of Birmingham, for proteomic analysis.

The results (Appendix 1, A1.3 and A1.4), showed in both samples, the presence of B-2M, MAC-inhibitory protein (MAC-IP or CD59), Cys-C, and SH3 domain binding glutamic acid-rich-like protein 3 (SH3BGRL3).

3.3.3 Biomarkers of tubular injury

Retinol Binding protein (RBP) and Neutrophil gelatinase-associated lipocalin (NGAL) were measured in urine samples at visit 1 and visit 2.

3.3.3.1 Retinol-binding protein (RBP)

RBP is produced by the liver and its function is to transport retinol (vitamin A) (464). The low molecular weight (21 kDa) means that the protein freely passes the GFB. In the healthy kidney, RBP is reabsorbed and degraded by the proximal tubules (465). In disease or injury, uRBP increases as proximal tubules cannot reabsorb the RBP.

Urinary RBP (uRBP) was measured at HSE in collaboration with Dr Jackie Morton as part of the screen for metals, to assess dysfunctional tubular reabsorption as a marker of tubular injury.

3.3.3.1.1 uRBP concentrations in men and women at visit 1 and 2

Urine samples from visit 1 and visit 2 were analysed using an ELISA kit by Dr Jason Howard (HSE, Buxton). Table 3.4 shows the median uRBP levels and the interquartile range (IQR) for each gender at both visits. Between total men and women at the same visit, no significant associations were noted. Between men at visit 1 and visit 2, a significant difference (p=0.0415) was found where uRBP levels were higher at visit 1 than at visit 2.

		Visit 1 (n=344)			Visit 2 (n=276)		
	Gender	**Range**	**Median**	**IQR (25^{th}-75^{th})**	**Range**	**Median**	**IQR (25^{th}-75^{th})**
uRBP (µg/mmol creatinine)	Male	0.0-318.0	7.5	4.0-14.0	0.0-187.0	5.0	3.0-9.0
	Female	0.0-415.0	6.0	4.0-9.0	0.0-18.0	4.0	0.0-7.0

Table 3.4: Urinary Retinol-binding Protein (RBP) in men and women at visit 1 and visit 2.

3.3.3.1.2 uRBP concentrations in the different kidney trajectory groups

Following, the levels of uRBP between men and women in the stable, declining and established kidney trajectory groups were compared (Table 3.5).

After correction with creatinine levels and log transformation to achieve normal distribution of the measurements, the levels of uRBP were compared among the different kidney trajectory groups from visit 1 and 2 using one-way ANOVA (Figure 3.2). In men the levels of uRBP were increased in the established kidney dysfunction group at both visit 1 (p=0.0018) and visit 2 (p=0.0320) compared to both the stable and declining groups (Figure 3.2).

Test	Visit 1 (n=344)						Visit 2 (n=276)					
	Stable kidney function		Declining kidney function		Established kidney function		Stable kidney function		Declining kidney function		Established kidney function	
	Male (n=211)	Female (n=81)	Male (n=24)	Female (n=3)	Male (n=25)	Female (n=0)	Male (n=170)	Female (n=67)	Male (n=18)	Female (n=3)	Male (n=18)	Female (n=0)
	Median (IQR 25th – 75th)											
uRBP (µg/mmol creatinine)	7.0 (4.0-13.0)	6.0 (4.0-9.0)	8.0 (4.3-13.5)	9.0 (3.0-26.0)	14.0 (4.0-57.0)	-	5.0 (3.0-8.0)	4.0 (3.0-7.0)	7.5 (3.0-17.5)	3.0 (3.0-7.0)	8.0 (2.8-23.0)	-

Table 3.5: Urinary RBP of the stable, declining and established trajectory groups of men and women at visit 1 and visit 2.

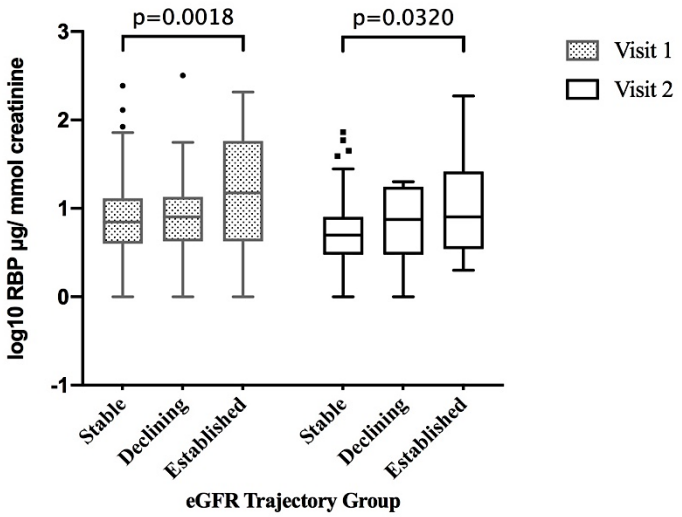

Figure 3.2: Association of log levels of uRBP at visits 1 and 2 stratified by kidney function trajectory in men. Total valid values for men at visit 1: n=211 for the stable group, 24 were in the declining and 25 in the established kidney dysfunction groups; and, at visit 2: n=170 in the stable group with 18 in each of the declining and established dysfunction group.

3.3.3.2 NGAL concentrations in the different kidney trajectory groups

NGAL is expressed by various cell types in response to injury and acts to protect cells by reducing apoptosis and increasing proliferation (466,467). NGAL levels increase rapidly in serum and urine after renal tubular injury (468).

Levels of NGAL were assessed in 105 urine samples from men at visit 1 in collaboration with Dr Amin Oomatia (UCL Department of Renal Medicine). After correction to creatinine, and log transformation of the NGAL levels and analysis by one-way ANOVA, a significant association was found between the stable and the established kidney dysfunction groups (p=0.003) with an increase in the established dysfunction group, as shown in Figure 3.3. There was also a slight increase in the declining group compared to the stable group however this did not achieve statistical significance (p=0.0548).

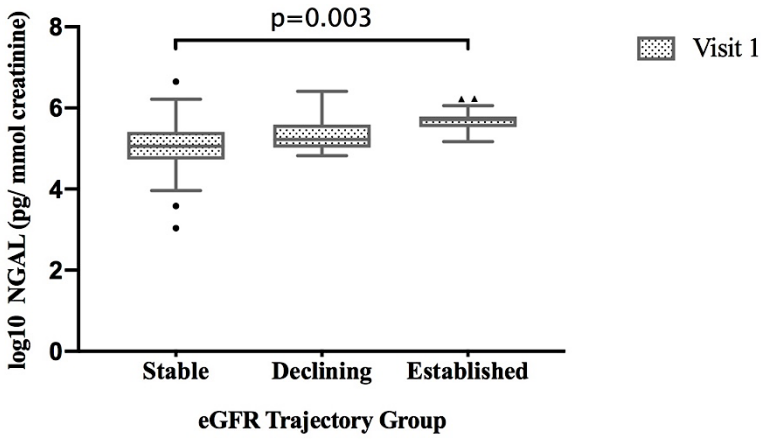

Figure 3.3: Comparison of excreted urinary NGAL levels in men at visit 1 stratified by kidney function trajectory. Total valid values for men at visit 1 n=55 from the stable group, n=25 from the declining and n=25 from the established kidney dysfunction group.

3.3.3.3 Glucose concentrations in the different kidney trajectory groups

Glucose passes the GFB and is reabsorbed by the proximal tubules. In proximal tubule injury and when reabsorption reaches the tubular capacity, glucose starts leaking into the urine (469). In the initial validation experiment checking the effect of BA and freezing on the different analytes of the strip-test, glucose did not report any false positive. False negative strip-tests by addition of glucose to the samples was not assessed at the beginning of the study (not reported glucosuria in MeN patients before). For that reason, to ensure that the glucose measurement on the strip-test reported were not false negatives, we tested a number of different samples with ELISA.

Urinary glucose levels in men (Stable kidney function=4, Established kidney dysfunction=7 and Declining kidney function=8) at visit 1 and visit 2 were measured using an ELISA kit with standard values ranging between 0-15 mg/dL. All samples were between 0-1.65 mg/dL. No further analysis was performed.

3.4 Serum analysis

3.4.1 Kidney function tests

Uric acid (UA) is a bi-product of normal metabolic breakdown of purines and in health is excreted in the urine (470). In impaired kidney function, UA accumulates in the circulation and it is a good indicator of kidney function (471,472). It has been suggested that UA which is endogenously produced due to high fructose breakdown, can lead to MeN (163). Additionally, kidneys are responsible for the elimination of excess phosphate from the serum. Advanced CKD is associated with increased serum phosphate due to reduced filtration capacity (473). Additionally, renal tubules are responsible for reabsorption of phosphorus (474). Thus, isolated tubular dysfunction can lead to increased urinary phosphorus and hypophosphatemia.

To test if UA and phosphate are associated with the decline in kidney function, serum samples from visit 1 (n=350) and visit 2 (n=291) were analysed using the Cobas Integra 400 plus analytical system (Roche Diagnostics). For each analysis, the total of valid values of each test are presented in the results.

3.4.1.1 Serum biomarker concentrations in men and women at visit 1 and 2

Initially, the concentration of each analyte was compared between men and women, as shown in Table 3.6. Between all men and women, men had significantly higher levels of UA at visit 1 ($p<0.0001$) and visit 2 ($p<0.0001$). The levels of UA and phosphate in serum were significantly increased in both men and women at visit 2 compared to visit 1 ($p<0.0001$).

Test (Units)	Healthy Range	Visit 1 (n=349)				Visit 2 (n=291)		
		Gender	Range	Median	IQR (25^{th}-75^{th})	Range	Median	IQR (25^{th}-75^{th})
Uric Acid (µmol/L)	200-430	Male	149.0-462.0	295.0	262.5-340.0	181.0-615.0	334.5	295.0-393.4
	140-360	Female	107.0-409.0	221.0	177.0-263.0	134.0-500.0	247.0	218.0-279.0
Phosphate (mmol/L)	0.81-1.45	Male	0.7-1.4	1.0	0.9-1.2	0.7-1.8	1.2	1.1-1.3
	0.81-1.45	Female	0.8-1.5	1.1	1.0-1.2	0.9-1.9	1.3	1.1-1.4

Table 3.6: Serum uric acid and phosphate concentrations in men and women at visit 1 and visit 2. Total of 264 men and 85 women a visit 1 and 217 men and 74 women at visit 2.

3.4.1.2 Serum kidney biomarker concentrations in the different kidney trajectory groups

Initially, the different levels of serum UA and phosphate were compared between men and women in the stable and the declining trajectory groups (Table 3.7). The established group of men was not included as there was no equivalent group for comparison for women. Between men and women in the stable kidney function group, increased serum levels of UA were evident in men at visit 1 ($p<0.0001$) and visit 2 ($p<0.0001$) compared to women. Women in the stable group had elevated phosphate levels at visit 2 compared to the stable group of men ($p=0.0002$). Overall, men had higher median levels of UA at both visits. The levels of phosphorus were consistent in men and women between visits1 and 2.

Multiple comparisons using one-way ANOVA was performed between the different kidney function trajectory groups at visit 1 and visit 2 for serum UA and phosphate concentrations in men (Figure 3.4).

In the different kidney trajectory groups, there was a significant difference in the levels of UA between men with stable and men with established kidney dysfunction at visit 1 and visit 2 (Figure 3.4A). The serum phosphate levels showed no differences between the different trajectory groups at visit 1 and visit 2 (Figure 3.4B).

	Visit 1 (n=349)						Visit 2 (n=291)					
	Stable kidney function		Declining kidney function		Established kidney function		Stable kidney function		Declining kidney function		Established kidney function	
Test (Units)	**Male (n=214)**	**Female (n=82)**	**Male (n=25)**	**Female (n=3)**	**Male (n=25)**	**Female (n=0)**	**Male (n=180)**	**Female (n=71)**	**Male (n=18)**	**Female (n=3)**	**Male (n=19)**	**Female (n=0)**
	Median (IQR 25th – 75th)											
Uric Acid (µmol/L)	289.5 (258.0-324.8)	222.5 (177.0-263.0)	280.0 (243.5-326.5)	150.0 (142.0-236.0)	401.0 (350.6-547.0)	-	329.0 (285.3-373.8)	247.0 (218.0-279.0)	319.0 (285.5-396.5)	225.0 (221.0-347.0)	473.0 (400.0-632.0)	-
Phosphate (mmol/L)	1.1 (0.9-1.2)	1.1 (1.0-1.2)	1.1 (1.0-1.2)	1.1 (1.0-1.1)	1.1 (1.0-1.3)	-	1.1 (1.1-1.3)	1.3 (1.1-1.9)	1.2 (1.1-1.4)	1.3 (1.1-1.4)	1.2 (1.1-1.5)	-

Table 3.7: Serum uric acid and phosphate concentrations in the stable and the declining trajectory groups of men and women at visit 1 and visit 2.

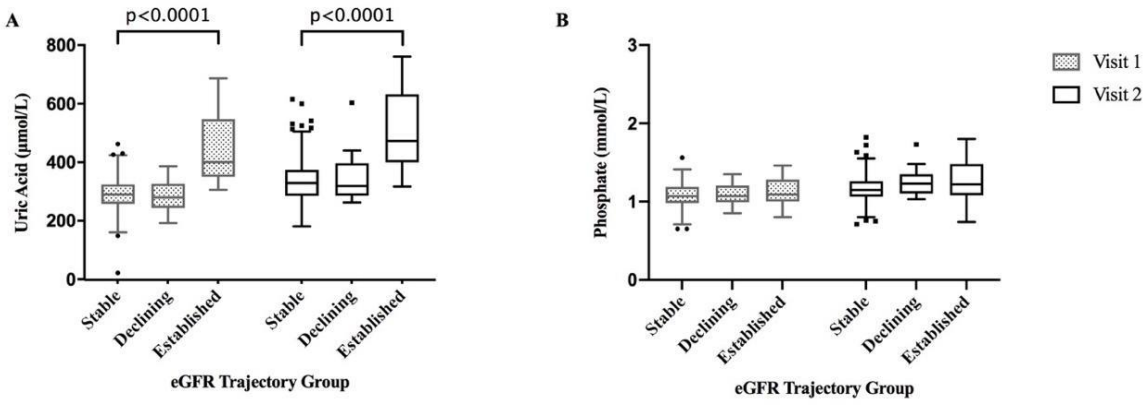

Figure 3.4: Serum kidney biomarkers in men at visits 1 and 2 stratified by kidney function trajectory. A. Uric acid concentrations in men at visit 1 and visit **2. B.** Phosphate concentrations in men at visit 1 and visit 2.

3.4.2 Liver function tests

During strip-test analysis of urine samples at visit 1 and visit 2, a high number of bilirubin positive tests were documented. Our validation experiment reported high false positivity in the samples with and without BA stored below -20˚C. While a large amount of urine samples were found positive for bilirubin (~50%) at each visit (but not all as we would expect due to storage conditions), it was considered important to assess serum bilirubin. Additionally, in our participants we measured liver enzymes that could indicate liver dysfunction making them susceptible to xenobiotic exposure since liver is the main organ of xenobiotic metabolism and elimination (475). Liver damage can lead to decreased elimination of xenobiotics causing high toxic levels of substances that can affect other organs, including the kidneys which is the main organ of elimination. To evaluate liver function, the liver enzymes alanine transaminase (ALT or SGPT), aspartate transaminase (AST), gamma-glutamyl transferase (γGT), and total bilirubin were measured (462).

ALT and AST are important enzymes in amino acid metabolism. AST is found in the liver but also in heart, skeletal muscle, kidneys, brain, and red blood cells while ALT is mostly found in the liver. The AST/ALT ratio is commonly used in the clinic as a biomarker for liver function and the diagnosis and differentiation of medical conditions (476,477). Liver injury results in higher levels of ALT than

AST. A ≥2:1 ratio can indicate alcoholic liver disease or non-alcoholic steatohepatitis (478).

γGT, is an enzyme which plays a major role in the synthesis and degradation of glutathione and drug and xenobiotic detoxification (479). It is mostly found in the liver and plays an important diagnostic role in diseases of the liver, pancreas and biliary system (480).

Bilirubin is the final catabolic product of the breakdown of red blood cells and is excreted in bile and urine. In the liver, bilirubin is conjugated with glucuronic acid to form a water-soluble form of bilirubin (direct bilirubin) which is more easily excreted. In the colon, bilirubin is oxidised to urobilin and stercobilin. A small amount of urobilin is normally excreted in the urine. In liver or in biliary system diseases, conjugated bilirubin leaks out from hepatocytes leading to high urinary excretion. In haemolytic anaemia, where red blood cell breakdown is high, bilirubin is not detected in the urine due to its lipophilicity but there is increased urinary urobilinogen excretion (481).

3.4.2.1 Serum liver test concentrations in men and women at visit 1 and 2

Serum samples from visit 1 and visit 2 were analysed using the Cobas Integra 400 plus analytical system (Roche Diagnostics). Table 3.8 shows the median levels and the interquartile range (IQR) for each gender at both visits.

Of all the samples tested, 347 out of 350 were valid for visit 1 and 291 out of 330 for visit 2. The levels of AST in men were significantly higher ($p<0.0001$) than the levels in women at visit 1. The same was not observed at visit 2 where the median levels for both men and women were comparable.

			Visit 1 (n=349)			Visit 2 (n=291)		
Test (Units)	**Healthy Range**	**Gender**	**Range**	**Median**	**IQR (25^{th}-75^{th})**	**Range**	**Median**	**IQR (25^{th}-75^{th})**
ALT	7-55	Male	2.0-62.3	9.4	6.8-12.7	1.8-62.3	9.9	7.0-12.7
(U/L)		Female	2.5-71.6	10.3	7.0-15.0	3.2-151.0	10.35	7.5-16.8
AST	10-40	Male	11.1-108.5	19.8	17.3-23.2	11.3-168.0	20.9	17.4-26.3
(U/L)		Female	2.5-71.6	10.6	7.1-13.8	11.4-88.7	17.6	14.9-88.7
AST/ALT ratio	~0.8	Male	0.8-2.7	2.2	1.7-2.7	0.0-8.9	2.1	1.6-2.8
		Female	0.6-4.8	1.8	1.3-4.8	0.6-4.7	1.6	1.2-4.7
γGT	9-48	Male	0.9-105.6	17.2	13.4-26.0	4.4-137.0	18.9	14.6-27.0
(U/L)		Female	1.1-77.1	16.6	12.0-24.8	5.3-113.4	19.4	11.6-29.1
Total bilirubin	1.71-20.5	Male	0.0-18.8	0.0	0.0-4.5	0.0-18.8	3.0	0.0-5.7
(μmol/L)		Female	0.0-13.6	2.4	0.0-5.6	0.0-17.6	2.6	0.0-5.6

Table 3.8: Serum ALT, AST, γGT and total bilirubin levels in men and women at visit 1 and visit 2. Total of 264 men and 85 women a visit 1 and 217 men and 74 women at visit 2.

The levels of ALT were the same across the visits for both men and women, but with the median in women slightly higher than in men. The AST/ALT ratio was significantly higher in men at visit 1 (p=0.0232) and visit 2 (p=0.0124) compared with women.

The median levels of γGT were the same across the visits for both men and women with a few cases to have very high concentrations when compared to the normal values. Total bilirubin was in the normal range for both men and women at visit 1 and visit 2. However, the levels of total bilirubin in men were significantly higher (p=0.0037) at visit 2 compared to visit 1.

3.4.2.2 Serum liver test concentrations in the different kidney function trajectory groups

Initially, we compared the different levels of serum ALT, AST, γGT and total bilirubin in men and women in the stable and the declining trajectory groups (Table 3.9).

At both visits no significant associations were found between men in each kidney function group for any of the parameter although, higher median levels of AST/ALT ratio were noted in the established kidney trajectory group in men at both visit 1 and visit 2 compared to the other groups.

Multiple comparison one-way ANOVA was performed between the different kidney function trajectory groups at visit 1 and visit 2 for serum ALT, AST and γGT concentrations of each gender (Figure 3.5). Log transformation of the data was performed to acquire normal distribution across the data values. No differences were observed in men at visit 1 and visit 2. The levels of all liver tests for both men and women remained similar across the groups between the two visits.

No differences were observed in men at visit 1 and visit 2. The levels of all liver tests for both men and women remained similar across the groups.

	Visit 1 (n=324)						Visit 2 (n=272)					
	Stable kidney function		Declining kidney function		Established kidney function		Stable kidney function		Declining kidney function		Established kidney function	
Test (Units)	**Male (n=214)**	**Female (n=82)**	**Male (n=25)**	**Female (n=3)**	**Male (n=25)**	**Female (n=0)**	**Male (n=184)**	**Female (n=71)**	**Male (n=18)**	**Female (n=3)**	**Male (n=19)**	**Female (n=0)**
	Median (IQR 25th – 75th)											
ALT (U/L)	9.5 (7.0-13.2)	10.1 (7.0-13.3)	7.6 (6.6-10.3)	23.8 (5.4-46.3)	9.2 (5.6-12.1)	-	10.0 (7.1-15.6)	10.0 (7.5-16.7)	10.1 (7.7-11.9)	13.5 (7.5-17.1)	8.2 (6.4-15.0)	-
AST (U/L)	19.9 (17.3-23.0)	18.3 (15.5-20.8)	18.6 (15.6-22.7)	22.4 (13.8-53.7)	21.0 (18.0-25.4)	-	20.6 (17.4-26.3)	17.6 (14.9-22.0)	21.9 (17.6-24.2)	21.0 (16.0-22.2)	21.9 (17.2-28.7)	-
AST/ALT Ratio	2.2 (1.6-2.7)	1.8 (1.4-2.3)	2.2 (1.8-2.7)	1.2 (0.9-2.6)	2.6 (2.0-3.9)	-	2.0 (1.5-2.7)	1.6 (1.2-2.1)	2.2 (1.8-2.8)	1.6 (1.3-2.1)	2.3 (2.0-3.3)	-
γGT (U/L)	16.9 (13.1-25.1)	16.5 (12.0-23.1)	17.9 (14.5-24.7)	33.4 (11.6-39.2)	17.9 (14.0-25.0)	-	19.3 (14.7-27.5)	19.4 (11.6-29.1)	16.4 (13.9-25.4)	11.6 (11.5-22.2)	18.7 (15.0-28.0)	-
Total bilirubin (µmol/L)	0.0 (0.0-4.8)	2.4 (0.0-5.7)	0.0 (0.0-4.5)	0.0 (0.0-3.3)	0.0 (0.0-2.3)	-	3.5 (0.0-5.7)	2.7 (0.0-5.6)	3.9 (0.0-7.7)	0.0 (0.0-6.9)	0.0 (0.0-0.0)	-

Table 3.9: ALT, AST, γGT and total bilirubin levels in the serum of the stable, declining and established kidney function groups of men and women at visit 1 and visit 2.

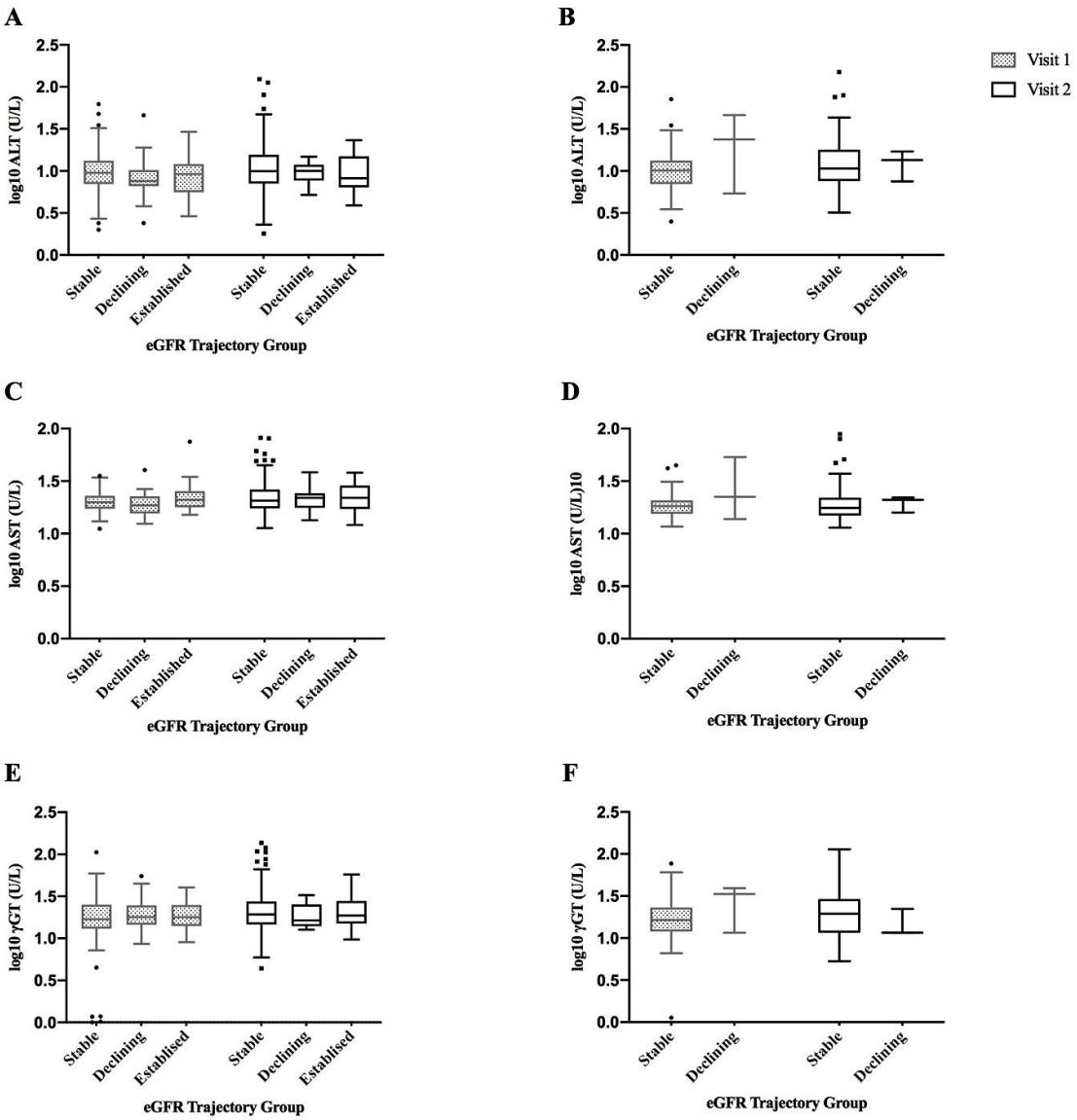

Figure 3.5: Liver function marker concentrations in serum in the whole cohort at visits 1 and 2 stratified by kidney function trajectory in men and women. Log transformed serum concentrations are shown. **A.** Alanine aminotransferase (ALT) concentrations in men at visit 1 and visit 2. **B.** Alanine aminotransferase (ALT) concentrations in women at visit 1 and visit 2. **C.** Aspartate transaminase (AST) concentrations in men at visit 1 and visit 2. **D.** Aspartate transaminase (AST) concentrations in women at visit 1 and visit 2. **E.** γ-glutamyl transferase (γGT) concentrations in men at visit 1 and visit 2. **F.** γ-glutamyl transferase (γGT) concentrations in women at visit 1 and visit 2.

3.5 Discussion

Across the globe, CKDu, has emerged in at least two different geographical areas (Central America and South Asia) (57,63). The histopathological characteristics of patients have suggested that these may be a similar disease (54). Hypotheses as to the cause are numerous. However, differences across

the areas, such as the differences in use of agrichemicals and/or dietary exposures, can complicate investigation. Conversely, similarities such as environmental and occupational conditions also need to be considered. The present longitudinal cohort study allowed us to follow the same population over time and characterise exposures at baseline (at the time of exposure, reducing recall bias) and prior to the onset of kidney dysfunction (reducing the possibility of associations due to reverse-causation).

The use of a strip-test, has been proven to be a quick and inexpensive tool to detect early CKD hence we investigated whether it would also be a useful tool to predict the decline in the kidney function of the study participants (482,483). However, urine strip-test analysis of samples at visit 1 and visit 2 did not reveal associations of any of the analytes and the progression of kidney dysfunction. The absence albuminuria (ACR<30 mg/g) is consistent with previous reports suggesting that CKDu is not a primary glomerular disorder (56).

Increased ACR was observed at visit 2 across the male participants (9.9% with ACR≥30 mg/g) which was even higher in the established and declining groups; while at visit 1 only 0.8% of total men had ACR≥30 mg/g. At visit 1 only two men had an ACR≥30 mg/g in the stable group and there were no individuals with this level of ACR in the established and declining groups. On the other hand, despite the majority of participants not showing remaining proteinuria, at visit 2 all groups had an increase in ACR with the most notable being in the established and declining kidney function groups. This suggests that during the harvesting period (which takes place between visit 1 and visit 2), participants might be exposed to factors that increase the excretion of albumin. Such factors could include heat stress or increased workload supporting the idea that occupational factors may be involved in the development of CKDu. An alternative explanation might be an increased intake of dietary protein by the workers to meet their musculoskeletal needs and the ability to purchase meat due to being employed (484).

Investigation of bilirubin levels, first by strip-test, showed measurements 1-6 x greater than the normal levels of bilirubin in urine (3 µmol/L) in around 50% of the samples at each visit in both men and women. In urine, only the water-

soluble conjugated form of bilirubin with glucuronic or sulfuric acid can be detected (485). In the absence of urobilinogen as indicated by the strip-test (normal in 100% of the cases), acute hepatitis, haemolytic or hepatocellular causes of bilirubinuria seem less likely. Considering the results of the initial validation experiment on the effect BA preservative and the subsequent freezing has on reporting false positives for bilirubin on the strip-test, serum analysis of liver function was considered important to rule out any possible liver dysfunction. In addition, liver function was tested to explore the possibility of coexistent liver injury as the liver is the main organ responsible for metabolism of xenobiotics, which in turn, could lead to kidney injury. The levels of all the liver function test markers remained low across the visits and among the different kidney function groups in men. Women of the declining group had higher median levels of ALT and γGT at visit 1.

A positive strip-test for protein (15% of men and 18% of women at visit 1 and 17% of men and 18% of women at visit 2) and the parallel absence of albumin (only 5.4% of both men and women at visit 1, and 13.7% of both men and women at visit 2 with ACR≥30 mg/g), prompted further investigation of the presence of small molecular proteins of tubular origin in the urine. Two samples with a positive protein strip-test and negative albumin were sent to the Scientific Facilities-LES, School of Biosciences, University of Birmingham, for proteomic analysis. Results from both samples showed the presence of B-2M, MAC-IP (also known as CD59), Cys-C, degradation products of collagen and SH3BGRL3, suggesting that these may be potential biomarkers of kidney injury. B-2M in urine is usually increased when tubular disease is present (486). Furthermore, Cys-C, is not normally detected in urine and increased urinary excretion of cystatin-c could be a valid biomarker of tubular injury. Both B-2M and Cys-C have been suggested as potential biomarkers of CKDu (487). More research is needed around other potential useful markers such as SH3BGRL3 and degradation products of collagen to assess their importance in the early diagnosis of MeN and/or CKDu in other parts of the world.

uRBP is one of the most commonly used biomarkers of tubular reabsorption and is commonly measured in parallel with analysis of metals in the urine

(487,488). At visit 1, in both men and women, there were no significant differences in uRBP levels between any groups. These results suggest uRBP is not useful for predicting the decline of kidney function in CKDu. The slight rise post-harvest in levels (visit 2) of uRBP in men with established kidney dysfunction could indicate an exacerbation during the exertion of the harvesting period in those with pre-existing kidney damage.

Selected samples from the stable group (n=36) and all the samples of men with declining (n=25) or established (n=25) kidney function were tested for urinary excretion of NGAL; a protein expressed by neutrophils, expression of which is upregulated in early post-ischaemic or nephrotoxic conditions when tubular injury has occurred (489). Significantly elevated levels of NGAL were detected at visit 1 in those with established renal dysfunction. Although the increased NGAL in the established group is unsurprising given the impaired eGFR. The small increase (but not significant) identified in the declining group is unlikely to be useful in detecting early disease.

Urine markers that can detect the early stages of the development of MeN in agricultural workers of Central America and throughout the world are crucial for disease diagnosis and management as well as efforts at identifying a cause. Currently MeN is being diagnosed when the kidney function has dramatically decreased at a time when prevention is impossible and any causal factor is likely to have occurred years earlier, making identifying the relevant exposure very challenging.

Increased levels of serum UA and phosphate are associated with established kidney disease (471). It has been suggested that UA can play a causative role in the development of MeN rather being a result of the disease (163). We tested whether levels of these serum analytes are altered prior to disease in a healthy population at risk of developing MeN. Our data show that between the stable and the declining groups of men at visit 1 and visit 2, there were no differences in the serum levels of UA or/and phosphate. However, the group with established kidney dysfunction had significantly higher levels of UA when compared to the stable group. These results suggest that increased serum UA are the result of kidney dysfunction rather than playing a causative role in the

development of the disease. Higher levels of UA and phosphate were observed at visit 2 compared to visit 1 for both men and women. This could be an effect of high serum osmolality resulting from dehydration as hyperuricemia has been associated with low water, or alternatively, higher protein consumption (490,491).

3.6 Conclusions

MeN is causing the death of tens of thousands rural workers in Central America. Although many hypotheses have been proposed, the exact cause(s) of the disease remains to be established.

Our strip-test data, confirm that the disease is not a primary glomerular disorder as there was absence of albuminuria and heamaturia in the majority of the samples in all kidney trajectory groups. The absence of associations between the different trajectory groups, suggest that a simple urine strip test is unlikely to be useful in identifying individuals in the early stages of the disease. In addition, levels of injury markers such as uRBP and NGAL between the stable and the declining kidney function trajectory groups did not differ making them poor markers for detecting early stages of the disease.

Finally, our data do not support the use of serum UA or phosphate as biomarkers of early renal damage in the development of MeN, or support UA as an important causal factor. Thus, there remains an urgent need to find more accurate early-stage biomarkers such as the ones reported after the proteomic analysis of low molecular proteins in the urine of participants (e.g. urinary Cys-C and B-2M).

Chapter 4: Investigation of xenobiotics in MeN

4.1 Introduction

MeN is a disease with late diagnosis when patients already have advanced stages of disease. Clinical findings involve elevated serum creatinine levels in the absence of hypertension, diabetes and/or a glomerular disease. Biopsies are not taken until the later stages of the disease (stage 3-4) (492). Diagnosis of late stages of MeN does not allow for early intervention to control and prevent further damage to the kidneys or to identify potential causal exposures.

The kidneys metabolise, concentrate and excrete a wide range of xenobiotics (38). A number of these e.g. NSAIDs, heavy metals and pesticides have been shown to cause kidney injury and/or been proposed to be causal factors in MeN (493). In this chapter, a range of metals and metalloids, pesticides and their metabolites, the mycotoxins, ochratoxin A (OTA) and citrinin (CIT), were measured in the urine and compared across people with stable kidney function and those who lose their kidney function over the 2 year study period to explore any associations between them and the decline in kidney function in a population at risk of developing MeN. Any associations found would be indicative of a potential causative relationship between the analyte and the future decline of kidney function and preventive measurements can be applied to minimise exposure.

Genetic predisposition and variation in genes involved in xenobiotic elimination of metals and pesticides, may play a role in the handling of toxic substances and the development of MeN. Therefore, we also tested whether a number of known genetic risk loci for xenobiotic elimination were associated with the decline in kidney function that could provide information of the type of exposure that could potentially play a contributory factor.

4.2 Results of elemental analysis

Various metals were measured in urine samples from visit 1 and 2, using ICP-MS, in collaboration with Dr Jackie Morton (HSE, Buxton).

4.2.1 Urinary metal concentrations in men and women at visit 1 and 2

Initially, the corrected for urinary creatinine concentration of each metal was compared between sexes as shown in Table 4.1. The levels of Al, total As and Si, were significantly higher than the suggested reference limits for both genders and at both visits (494–497). Women had overall higher median levels of excreted Al than men at visit 1 and visit 2. Levels of excreted Mn were notably higher in women compared to men at visit 2.

After log-transformation of each sample to achieve a normal distribution among the samples, the differences and associations between the two visits were tested. Men at visit 1 had significantly higher excreted levels of Al ($p=0.0008$), and Se ($p=0.0304$) when compared to men at visit 2. No other associations were found. Overall, no pattern was observed regarding the metal excretion. People with increased metal excretion at visit 1 did not necessarily had increased levels at visit 2 and vice versa.

The tables in Appendix 2 (Table A2.1 and A2.2) show the results of each element between the different age groups, gender, residential community, and quality of well water participants were consuming based on analysed well water samples at visit 1 and visit 2. Participants over 25 years old were found to have higher excreted levels of Al and Cd at both visits when compared to the participants below 25 years old. Across all communities, at visit 1 the levels of Al, As, Cu and Si were higher at visit 1 when compared to visit 2 with some communities having levels twice higher when compared to visit 2. No great differences were observed in the mean levels of metal excretion across the different occupations.

Metals and metalloids (µmol/mol creatinine)	Visit 1		Visit 2		
	Men (n=263)	Women (n=87)	Men (n=213)	Women (n=78)	Ref. upper values
	Median (25^{th} -75^{th} IQR)	Median (25^{th} -75^{th} IQR)	Median (25^{th} -75^{th} IQR)	Median (25^{th} -75^{th} IQR)	
Aluminium (Al)	360.6 (216.5-629.2)	384.9 (222.8-631.2)	232.7 (157.4-393.9)	330.3 (170.6-534.1)	55.6*
Total arsenic (As)	120.0 (74.7-186.2)	120.6 (81.3-168.3)	83.1 (60.1-135.5)	100.4 (62.7-148.0)	14.2**
Cadmium (Cd)	0.3 (0.2-0.5)	0.4 (0.2-0.6)	0.2 (0.2-0.4)	0.3 (0.2-0.5)	1.2***
Chromium (Cr)	0.8 (0.8-1.4)	0.9 (0.6-1.6)	0.7 (0.5-1.6)	0.9 (0.6-1.9)	1.1*
Cobalt (Co)	0.9 (0.7-1.4)	1.2 (0.8-1.8)	0.8 (0.6-1.7)	1.0 (0.7-2.0)	3.1*
Copper (Cu)	16.0 (12.3-21.8)	14.0 (12.2-22.5)	12.5 (10.0-16.4)	13.2 (9.8-18.2)	30.8*
Lead (Pb)	0.3 (0.2-0.4)	0.3 (0.2-0.4)	0.3 (0.2-0.5)	0.3 (0.2-0.5)	1.9*
Manganese (Mn)	3.3 (1.6-6.4)	3.9 (2.3-7.0)	2.5 (1.4-5.6)	4.1 (2.6-7.6)	1.4*
Mercury (Hg)	0.1 (0.05-0.14)	0.1 (0.05-0.23)	0.1 (0.03-0.14)	0.1 (0.05-0.2)	1.6***
Selenium (Se)	23.9 (18.8-32.7)	27.1 (21.1-35.1)	19.8 (15.5-26.8)	23.7 (18.5-29.2)	152.0***
Silicon (Si) (mmol/mol creatinine)	179.9 (110.2-276.2)	152.9 (77.4-207.0)	165.6 (118.1-234.1)	147.1 (108.2-219.1)	100.0****
Strontium (Sr)	282.8 (121.4-627.0)	200.1 (84.0-437.9)	191.7 (137.7-261.5)	153.8 (111.3-237.0)	560.0****

Table 4.1: Creatinine-adjusted urinary concentrations of metals and metalloids in men and women at visit 1 and visit 2. Data are presented as median and interquartile range (IQR). Ref upper values: Upper values for a variety of reference populations. *Belgian population suggested upper limit (494), **Mexican American population suggested upper limit (495), *** Canadian population suggested upper limit (496), ****UK population suggested upper limit (497).

4.2.2 Urinary metal concentrations in the different kidney trajectory groups

After log-transformation of the values of each sample to achieve a normal distribution across the data, comparison of each group of kidney function with each element was performed by one-way ANOVA. Data are presented as median and the interquartile ranges between the different kidney function trajectory groups at visit 1 and visit 2, respectively (Table 4.2 and 4.3).

In men the levels of Sr were significantly lower ($p=0.0016$ and $p<0.0001$ at visit 1 and visit 2, respectively) in the established dysfunction group compared to the stable group. The levels of Si were significantly higher ($p=0.0401$) in the established dysfunction group compared to the stable group of men at visit 2. No other differences were found.

Figure 4.1 shows the comparison of the log-transformed levels of As, Cd, Pb and Hg in each kidney function trajectory group, between the two visits in men and women, respectively, against the reported upper limits (dotted line) in different populations.

Between the two visits, the levels of As ($p=0.0424$), Cd ($p<0.0001$), Cu ($p<0.0001$) and Se ($p<0.0001$) were significantly lower in men in the stable group at visit 2 compared to men in the stable group at visit 1. However, when the log-transformed Cr-uncorrected concentrations of As, Cd, Cu and Se, were compared, these differences were no longer apparent (data not shown). No other associations were found.

Metals and metalloids (µmol/mol creatinine)	Visit 1 (n=350)					
	Stable kidney function		Declining kidney function		Established kidney function	
	Male (n=213)	Female (n=84)	Male (n=25)	Female (n=3)	Male (n=25)	Female (n=0)
	Median (IQR 25th – 75th)					
Aluminium (Al)	360.3 (212.2-616.0)	369.4 (222.3-624.0)	337.9 (201.5-711.1)	731.0 (407.8-731.0)	442.8 (334.8-738.9)	-
Total arsenic (As)	113.5 (70.9-185.5)	120.0 (80.0-167.5)	111.3 (75.5-187.0)	157.9 (147.2-157.9)	141.9 (90.2-198.2)	-
Cadmium (Cd)	0.3 (0.2-0.5)	0.4 (0.2-0.5)	0.3 (0.2-0.6)	0.4 (0.3-0.4)	0.3 (0.2-0.6)	-
Chromium (Cr)	0.9 (0.6-1.4)	0.9 (66.0-1.6)	0.7 (0.4-1.9)	1.5 (1.0-1.5)	1.0 (0.7-1.7)	-
Cobalt (Co)	1.0 (0.7-1.4)	1.2 (0.8-1.7)	0.9 (0.6-1.5)	1.6 (1.3-1.6)	0.8 (0.7-1.2)	-
Copper (Cu)	16.1 (12.4-21.3)	15.8 (12.0-22.0)	16.3 (12.6-23.8)	22.6 (19.1-22.6)	14.5 (11.9-27.6)	-
Lead (Pb)	0.3 (0.2-0.5)	0.3 (0.2-0.37)	0.2 (0.2-0.4)	0.24 (0.20-0.24)	0.3 (0.2-0.4)	-
Manganese (Mn)	3.1 (1.5-6.4)	3.8 (2.3-7.0)	0.8 (1.8-9.3)	5.3 (4.0-5.3)	4.6 (2.9-6.5)	-
Mercury (Hg)	0.1 (0.05-0.2)	0.1 (0.05-0.2)	0.1 (0.03-0.1)	0.05 (0.03-0.05)	0.1 (0.3 -0.1)	-
Selenium (Se)	24.4 (19.4-33.7)	27.2 (20.9-34.8)	21.0 (18.8-20.9)	25.9 (21.2-25.9)	21.0 (16.8-27.1)	-
Silicon (Si) (mmol/mol creatinine)	281.0 (182.0-411.1)	152.4 (83.0-203.6)	167.3 (88.4-289.0)	173.5 (137.3-320.4)	191.0 (131.0-359.0)	-
Strontium (Sr)	281.0 (182.0-411.1)	231.0 (151.0-372.0)	232.0 (137.0-434.0)	637.4 (134.0-661.0)	141.3 (81.3-277.0)	-

Table 4.2: Range of the various elements at visit 1 in men and women in the different kidney function groups.

Metals and metalloids (µmol/mol creatinine)	Visit 2 (n=291)					
	Stable kidney function		Declining kidney function		Established kidney function	
	Male (n=176)	Female (n=75)	Male (n=18)	Female (n=3)	Male (n=19)	Female (n=0)
	Median (IQR 25th – 75th)					
Aluminium (Al)	230 (157.2-372.3)	329.8 (169.5-521.9)	216.7 (118.1-523.9)	824.0 (257.0-824.0)	318.9 (178.6-454.2)	-
Total arsenic (As)	80.4 (59.0-131.7)	100.5 (62.1-147.5)	86.3 (52.0-156.0)	94.0 (66.3-94.0)	115.6 (67.2-175.7)	-
Cadmium (Cd)	0.2 (0.2-0.4)	0.3 (0.2-0.5)	0.3 (0.2-0.5)	0.2 (0.18-0.2)	0.3 (0.2-0.5)	-
Chromium (Cr)	0.7 (0.5-1.5)	1.0 (0.6-1.9)	1.2 (0.5-1.9)	0.7 (0.6-0.67)	1.1 (0.3-2.2)	-
Cobalt (Co)	0.8 (0.7-1.7)	1.0 (0.7-1.9)	1.0 (0.6-1.6)	1.8 (1.0-1.8)	0.8 (0.5-1.1)	-
Copper (Cu)	12.4 (9.9-16.4)	13.1 (9.6-18.2)	13.2 (10.5-16.9)	18.1 (17.0-18.1)	13.1 (11.5-16.5)	-
Lead (Pb)	0.3 (0.2-0.5)	0.3 (0.2-0.5)	0.3 (0.2-0.5)	1.0 (0.7-1.0)	0.3 (0.2-0.4)	-
Manganese (Mn)	2.4 (1.4-5.3)	3.9 (2.3-7.6)	3.4 (1.8-6.4)	5.1 (2.4-5.1)	3.8 (1.8-11.6)	-
Mercury (Hg)	0.1 (0.03-0.2)	0.1 (0.05-0.2)	0.1 (0.002-0.1)	0.04 (0.02-0.04)	0.05 (0.01-0.1)	-
Selenium (Se)	20.4 (15.8-26.9)	24.1 (18.2-29.9)	19.3 (13.5-27.5)	18.8 (18.6-18.8)	16.8 (14.0-25.0)	-
Silicon (Si) (mmol/mol creatinine)	158.0 (114.1-224.9)	145.4 (107.6-22.1)	177.3 (119.9-268.7)	155.0 (142.7-155.0)	226.1 (158.5-307.8)	-
Strontium (Sr)	205.5 (146.7-276.8)	153.1 (111.4-227.0)	168.9 (131.7-301.3)	272 (67.3-272.0)	127.3 (84.8-151.9)	-

Table 4.3: Range of the various elements at visit 2 in men and women in the different kidney function groups.

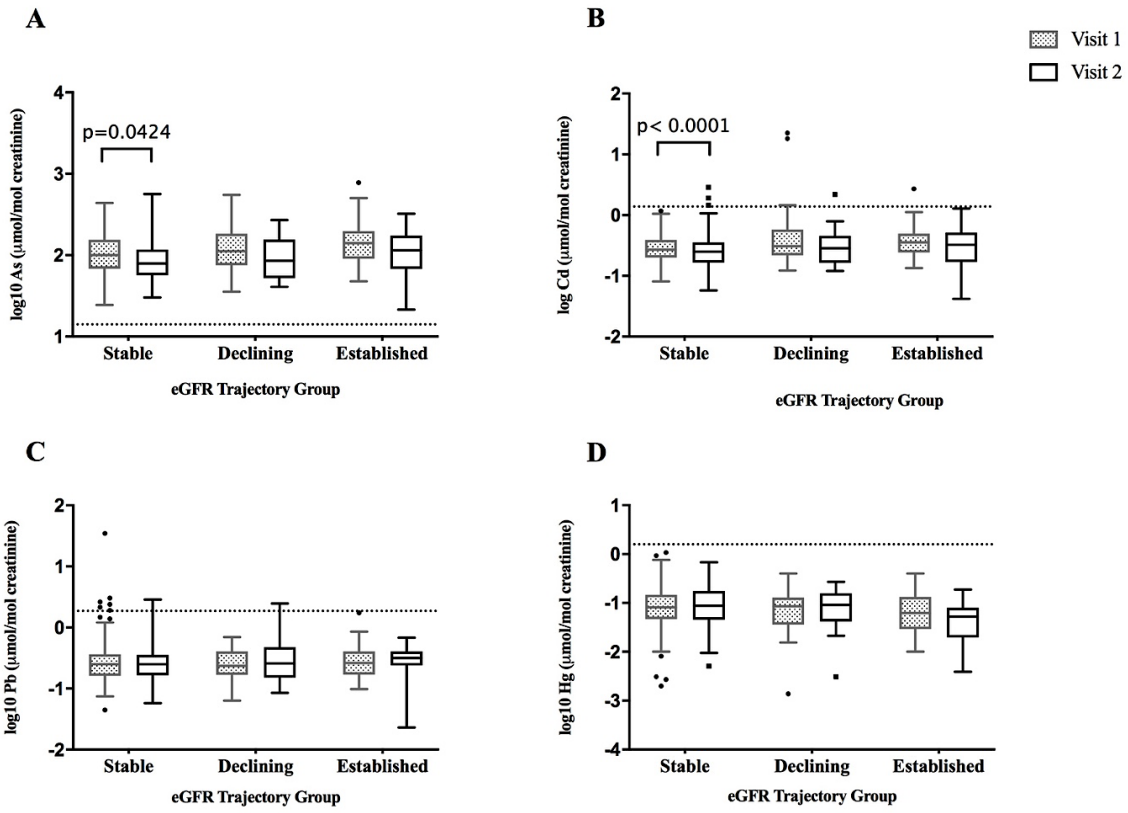

Figure 4.1: Selected log transformed creatinine-corrected urinary metal concentrations in men at visits 1 and 2 stratified by kidney function trajectory. A. Arsenic (As) levels. The dotted line represents the upper limits as characterised for a Mexican American population (495). **B.** Cadmium (Cd) levels. The dotted line represents the upper limits as characterised from Belgian and Canadian populations (494,496). **C.** Lead (Pb) levels. The dotted line represents the upper limits as characterised from a Belgian population (494). **D.** Mercury (Hg) levels. The dotted line represents the upper limits as characterised from a Belgian population (494).

4.3 Pesticides and pesticide metabolites

In the sugarcane industry, herbicides are the most widely used pesticides, and a mixture of 2,4-D, terbutryn and ametryn is commonly used in sugar production in the study area (498). Twelve pesticides or their metabolites (selected because of evidence of local use along with analytical capability) were analysed by LC-MS in collaboration with Dr Christian Lindh (Lund University, Lund, Sweden). Pesticides and metabolites tested included; (i) 2,4-D; (ii) 3-PBA; (iii) 4F3PBA; (iv) CFCA; (v) DCCA; (vi) ETU; (vii) glyphosate; (viii) MCPA; (ix) OH-PYM; (x) 5-OH-TBZ; (xi) TEB-OH; and, (xii) TCP.

Due to the high cost of analysis, a nested case-control set of samples were tested for urinary pesticides and metabolites comprising urine samples from 25 men in the stable group and 25 of the declining group, 4 women in the stable group and the 3 women in the declining group at visit 1 and visit 2.

4.3.1 Urinary pesticide and pesticide metabolite concentrations in men and women at visit 1 and 2

Table 4.4 shows the urinary concentrations of the different pesticides and metabolites of pesticides tested. The values of each pesticide and metabolite were corrected with the levels of urinary creatinine and log-transformed to achieve a normal distribution. There was no significant difference in the urinary levels of each analyte between the two visits for men and women. The majority of the pesticides were below the limit of detection (LOD). The highest median Cr-adjusted urinary concentrations were found for 2,4-D, 3-PBA, DCCA, ETU, TEB-OH and TCP in both men and women. Glyphosate urinary concentrations were below the LOD (0.1 ng/mL) for the majority of the samples (68% and 70% of all samples tested at visit 1 and visit 2, respectively). However, a higher proportion of samples were >LOD for 4F3PBA, CFCA and OH-PYM at visit 2 than visit 1. Overall, no pattern was observed regarding pesticide or metabolite excretion. People with increased pesticide or metabolite excretion at visit 1 did not necessarily had increased levels at visit 2 and vice versa.

Pesticides and metabolites (μmol/mol creatinine)	Gender				Samples ≥ LOD	
	Male		Female		Whole cohort	
	Visit 1 (n=50)	Visit 2 (n=40)	Visit 1 (n=7)	Visit 2 (n=6)	Visit 1 (n=57)	Visit 2 (n=46)
	Median (25^{th} -75^{th} IQR)	Median (25^{th} -75^{th} IQR)	Median (25^{th} -75^{th} IQR)	Median (25^{th} -75^{th} IQR)	%	%
2,4-D	1.0 (0.3-2.5)	0.3 (0.1-0.7)	0.1 (<LOD-1.1)	0.2 (<LOD-0.8)	91.2	91.3
3-PBA	0.9 (0.6-1.7)	0.7 (0.3-0.8)	2.0 (0.5-2.2)	1.0 (0.6-2.3)	100.0	100.0
4F3PBA	<LOD	<LOD	<LOD	<LOD	8.8	21.7
CFCA	<LOD	<LOD	<LOD	<LOD	5.3	15.2
DCCA	0.9 (0.4-1.6)	0.5 (0.3-0.9)	1.4 (0.5-1.7)	1.4 (0.5-2.9)	98.2	100.0
ETU	0.7 (0.2-1.6)	0.4 (0.2-0.7)	0.4 (0.2-1.7)	0.3 (0.2-2.6)	92.9	100.0
Glyphosate	0.0 (<LOD-0.1)	0.0 (<LOD-0.04)	<LOD	0.0 (<LOD-0.1)	31.6	30.4
MCPA	<LOD	<LOD	<LOD	<LOD	0.00	0.0
OH-PYM	<LOD	<LOD	<LOD	<LOD	1.7	17.4
5-OH-TBZ	<LOD	<LOD	<LOD	<LOD	0.00	0.0
TEB-OH	0.2 (0.1-0.3)	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.1 (<LOD-0.3)	91.2	89.1
TCP	0.5 (0.3-1.1)	0.1 (<LOD-0.2)	0.5 (0.2-0.7)	0.0 (<LOD-0.2)	98.2	93.5

Table 4.4: Creatinine-adjusted urinary concentrations of pesticides and pesticide metabolites from a nested case-control set of men and women at visit 1 and visit 2. Data are presented as median and interquartile range (IQR) alongside percentage of study participants with samples above the limit of detection (LOD). LOD of 2,4D, 3-PBA, 4F3PBA, CFCA, DCCA, ETU, glyphosate, MCPA, OH-PYM, TEB-OH and TCP: 0.1 ng/mL. LOD of 5-OH-TBZ: 0.5 ng/mL.

4.3.2 Urinary pesticide and pesticide metabolite concentrations in the different kidney trajectory groups

When comparing those with stable kidney function and those with declining kidney function, no differences were observed in the concentrations of any of the pesticides or metabolites in either men (Figure 4.2) or women. Between the two visits, there was the suggestion of a decrease in the log-transformed Cr-corrected concentration of 2,4-D, 3-PBA, DCCA, ETU, TEB-OH and TCP in both men and women although not significant.

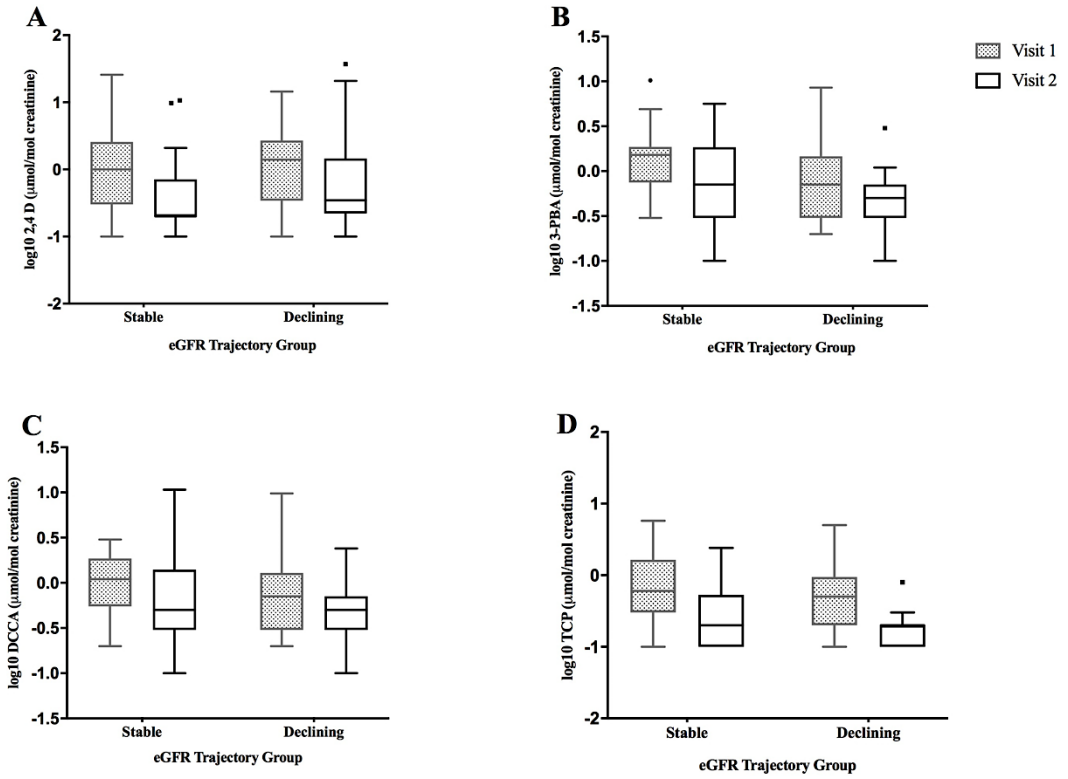

Figure 4.2: Selected log transformed creatinine-corrected urinary pesticide and metabolites of pesticides concentrations in men at visits 1 and 2 stratified by kidney function trajectory. A. 2,4 D. **B.** 3-PBA, metabolite of various pyrethroids. **C.** DCCA, metabolite of various pyrethroids. **D.** TCP, metabolite of organophosphates.

4.4 OTA and Citrinin

Ochratoxin A (OTA) is a toxin produced in food products (e.g. cereals, fruits, spices, rice and cheese) by *Aspergillus section Circumdati* and *Aspergillus section Nigri* in warm environments and by *Penicillium verrucosum* and *P. nordicum* in cooler environments (331). Citrinin (CIT) is produced in food products by *Monascus* species and *Penicillium*. Increased levels of OTA and CIT have been found to synergistically lead to nephropathic processes (499).

In the communities where MeN is prevalent, there is a lack of appropriate food storage equipment such as refrigerators due the lack of electricity. Consequently, people store food in cupboards at room temperature leading to potential contamination. Hence, we wanted to determine whether OTA and CIT can be found in the bio-fluids of our cohort. Due to the high cost of analysis, again a nested case-control set of samples (25 men in the stable group and 25 of the declining group, 4 women in the stable group and the 3 women in the declining group at visit 1 and visit 2), were analysed by liquid Chromatography-tandem mass spectrometry by Carla Martins, Department of Food and Nutrition, National Institute of Health Dr Ricardo Jorge (INSA), Lisbon, Portugal. OTA and CIT levels in all samples were below the LOD (≤0.82 nmol/L) therefore no further analysis was performed.

4.5 Genetic Analyses

Genetic predisposition plays an important role in the development of many diseases including diseases of the kidneys (368,370). There are no genetic studies connecting specific genes to the development of MeN, although the pattern of MeN running in members of the same family (based on family history data), gives rise to questions about possible genetic susceptibility of the population. However, even in the absence of Mendelian disease natural genetic variation can be used as a tool to investigate casual associations with environmental exposures. Mendelian randomisation is a technique where associations between outcomes and randomly occurring genetic risk alleles which are known to lead to a biological increase in exposure to a hypothesised cause of disease, can be examined. This approach overcomes many of the

issues around bias and reverse causation that occur with direct measurement of potential exposures.

DNA from blood samples of the whole cohort from visit 1 was extracted and analysed using a genome-wide array. We then examined the association between genetic risk loci in genes responsible for xenobiotic metabolism and the decline in kidney function in the population at risk of developing MeN.

4.5.1 Quality control analysis for DNA isolated for genome-wide array

DNA from 351 samples was sent for a global screening array (GSA) at Erasmus University, Rotterdam, Netherlands. Standard Quality Control (QC) analysis of the samples was reported by the Erasmus Medical Centre for 700,000 variants in the first instance.

Samples were filtered to check the quality metrics of the variants. Cluster separation measuring the separation between the genotype clusters, showed 1,577 variants to have cluster below 0.27, indicating poor quality calls according to the Illumina protocol, and should be excluded from analysis. The mean normalised intensity of the heterozygote cluster showed good intensity values for all the variants. Four samples were excluded from the dataset with call rate <97.5%. In total, 347 out of 351 study samples were analysed.

4.5.2 Relationships between genetic risk loci for metal and pesticide management and the decline in kidney function

Analyses were conducted by Dr Ben Caplin and used mixed modelling approaches to account for population stratification/cryptic relatedness. Associations between risk alleles present in the genome-wide array and the slope of eGFR as a quantative trait (calculated by ordinary least squares) in mL/min/1.7m^2/month are shown in Table 4.5. There were no associations of the majority of the loci and the decline in kidney function. The locus on chromosome 6, rs2257082 was found to have a borderline significant association with each C allele associated with an additional loss of 0.1 mL/min/1.7m^2/month of eGFR (p=0.03, uncorrected for multiple comparisons).

Chromosome	SNP	Allele 1	Allele 2	Frequency	beta	SE	p-value
			Metals				
10	rs11191439	C	T	0.1	-0.02	0.1	0.8
11	rs1695	G	A	0.5	-0.001	0.05	0.9
16	rs11643815	A	G	0.1	-0.08	0.1	0.4
8	rs4872479	T	G	0.1	-0.01	0.1	0.9
6	rs2257082	T	C	0.3	-0.1	0.05	**0.03**
			Pesticides				
19	rs3745274	T	G	0.3	0.002	0.05	0.9
19	rs28399499	C	T	0.01	0.2	0.2	0.3
10	rs1058930	G	C	0.01	0.04	0.2	0.8
10	rs11572080	A	G	0.04	-0.1	0.1	0.6
10	rs12769205	G	A	0.1	0.01	0.1	0.9
15	rs749292	A	G	0.3	0.1	0.05	0.2
1	GSA-rs2502805	T	C	0.3	-0.03	0.05	0.5
1	rs3003596	G	A	0.4	-0.004	0.05	0.9
7	rs662	G	A	0.5	0.03	0.05	0.5

Table 4.5: Genetic variants in genes responsible for the elimination and handling of metals and pesticides and their association with the decline in kidney function in our study. Presented are the SNPs measured in the GWAS, the chromosome location, the two alleles (Allele 1 and Allele 2), the frequency of the alleles, the effect size (beta), the standard error (SE) and the p-value or the association with slope of eGFR for an additive effect of each SNP in the mixed model.

4.6 Discussion

Exposure of agricultural workers in Central America to nephrotoxins has been well characterised and it has been hypothesised that this may have a causal or contributory role in the development of MeN. Environmental or occupational toxins may lead to renal damage due to the substantial proportion of cardiac output that passes through the kidneys (181). The severity of kidney damage depends, in most toxicants, on the type of toxicant, the type of species, the level and duration of exposure (acute or chronic).

Our analysis of various metals and metalloids suggest significant environmental exposure to Al and total As (although we were unable to speciate As to get an understanding of the source of exposure) (500). Median levels of Al and total As for both men and women at visit 1 and visit 2 were higher than the reference ranges reported for European and Mexican American populations, respectively, 4-7 times higher for Al and 6-9 times for total As. Although systemic symptoms of acute Al and As toxicity can be non-specific (e.g. headache and nausea), characteristic manifestations (e.g. altered mental state, hyperpigmentation, keratosis, melanosis or nail changes) were not reported at the clinic visits (501). High levels of metals (e.g. As or Cd) can induce acute kidney injury and have also been proposed as urinary biomarkers of kidney injury of other types (285,502). It is unclear if, or at what levels, these metals cause chronic kidney injury (503). Population-level urinary As is highly variable, and lack of speciation limits the inferences that can be drawn as we cannot identify the source of As (dietary or environmental). As is present in various pesticides and use and/or unsafe storage of these agrichemicals could lead to direct exposure of workers and their family members and/or to water and soil contamination. Fish is the main dietary source of organic As which is taken up from seawater. Furthermore, Nicaragua is a volcanic country potentially increasing the population's exposure to metals, through contaminated water, soil or air (504). Although one study measuring As in 19 workers with MeN showed As excretion in the normal range, other published data from 99 sugarcane workers in north-western Nicaragua reported that the eGFR of the group with the most elevated urinary

concentrations of As (above 90th percentile) was 9 mL/min/1.73 m^2 lower than in those with low urinary As levels (53,201).

In our study, a decrease in the median concentrations of Al, Cr, Co, Cu, Mn, Se and Sr was observed between visit 1 and visit 2 for both men and women. This may be due to changes in metal or chemical concentrations in water sources at different times of the year (dry versus wet season). It is also possible that this relates, at least in part, to creatinine correction as these differences were no longer apparent when uncorrected levels were examined as median levels of creatinine in the samples from visit 2 were relatively higher than visit 1. In turn, this increase in urinary creatinine might result from urinary concentration (due to hotter weather and more sweating due to manual labour), increased creatinine production (e.g. due to increased protein intake during the harvest season, and muscle breakdown) or both. Between men with stable and men with declining kidney function no differences were observed in the excretion of the different metals at visit 1 and visit 2 providing evidence against a causative role in the development of MeN. However, at visit 2, but not at visit 1, the levels of urinary Silica in men with established kidney dysfunction were found to be higher compared to the stable group. Si is known to increase excretion of Al from the body and that could explain the high urinary concentrations of Al found in our study (505). Men with established kidney dysfunction were similarly found to have higher levels of excreted Al when compared to the stable group. Even though urinary Al levels were similarly increased in the established group at visit 1, the same was not seen for Si, which could indicate lack of exposure of the population before the harvesting period and increased exposure to Si during the harvesting period. Silica particles are present in the ashes of sugarcane which is burnt at the beginning of the harvesting period. In this way workers are at risk of exposure through inhalation, digestion and/or open wounds on the skin.

Non-occupational exposure to low doses of pesticides occurs widely from residues in food and/or contaminated air and water (506). Pesticides are widely used in Nicaragua to increase crop quality and yield. Subsistence farmers use organophosphate and pyrethroid insecticides (included in this

study), as well as paraquat (119,507). Detectable levels of 2,4-D, 3-PBA, DCCA, ETU, TEB-OH and TCP were found in the majority of samples tested from both the stable and the declining kidney function groups in men and women. Overall, the measured concentrations of pesticide metabolites suggest exposure to 2,4-D, chlorpyrifos, of which TCP is a metabolite, and pyrethroids (such as cypermethrin of which 3-PBA and DCCA are metabolites). Although glyphosate has been proposed to play an important role in the development of CKDu the urinary levels of glyphosate in the present study were below the LOD in the majority of samples (508). However, between men with stable and men with declining kidney function no differences have been reported in the excretion of pesticides and their metabolites at visit 1 and visit 2 providing evidence against a causative role in the development of MeN.

The absence of detectable urinary OTA and CIT make it unlikely that contamination of food with these fungal toxins is a cause of MeN in Nicaragua. This contrasts with findings from other endemic areas with endemic CKDu, in particular Tunisia, where a high prevalence of OTA in serum has been reported in those with chronic interstitial kidney disease (334).

Finally, only one of the risk alleles for altered xenobiotic metabolism/excretion that was present on our gene-array, showed any association with decline in kidney function and this association was borderline and most likely a consequence of multiple testing. Furthermore, the levels of Pb in blood (the intermediate phenotype, where the minor allele at rs2257082 would be expected to lead to a Pb increase), have not been quantified. Testing associations between additional loci known to alter xenobiotic exposure and decline in kidney function might be useful in further characterising the role of metals or agrochemicals in disease.

4.7 Conclusions

Higher levels of the metals and pesticide metabolites tested were not associated with decline in renal function providing evidence against a causal role for metals and pesticides in the disease. However, high but intermittent exposure to the measured substances, and to other widely used pesticides

that were not measured in the present study as well as interactions between the exposures measured and other factors, remain potential causes of MeN. In addition, our findings suggest that exposure to OTA and/or CIT is not a cause of MeN in Nicaragua.

Chapter 5: Exploring the effect of heat and fructose *in vitro*

5.1 Introduction

A leading hypothesis of the cause of MeN is heat stress, as a consequence of the long hours spent by the workers under the sun. Additionally, fructose has been proposed due to workers' consumption of high sugar beverages during the day, in order to keep hydrated and to avoid water that is potentially hazardous during harvesting, and they might ingest fructose through chewing sugarcane (119,509). In addition to the serum and urine clinical data of the cohort (Chapter 3 and Chapter 4), we wanted to explore the direct effect of heat stress and/or fructose concentrations and their combination, at different time points on human proximal tubule cells *in vitro* to asses if the could play a role in MeN development and progression.

Cell lines play a valuable role in research allowing the investigation of cell function in health and in disease. Low molecular weight proteinuria, along with increased excretion of tubular markers (e.g. NGAL) and biopsies suggest that MeN patients suffer from proximal tubule injury and damage at the initial stages of the disease (47,510,511). Renal tubules are lined by metabolically-active epithelial cells that play an important part in regulating internal homeostasis and are more vulnerable to injury. Ischemia and exposure to nephrotoxins can cause injury to these cells leading to acute kidney dysfunction (512). Studies focusing on heat and fructose have generally used mouse models which have the benefit of investigating effects in a whole organism, but may not reflect the physiology of human cells (127,142). The human renal proximal tubular epithelial HKC-8 cell line (human proximal tubular cells immortalised using a hybrid Adeno 12-SV40 vector) was selected as a model system that is easily manipulated short-time in contrast to animal models, and it is a well characterised and widely used as an experimental cell line of kidney injury (513–516).

5.2 Response of proximal human tubular epithelial cells to heat and fructose

HKC-8 cells were grown in low-glucose DMEM of 310-360 mOsm/kg osmolality and exposed to heat (39˚C vs 37˚C) and fructose (15 and 45 mM) individually or in combination when cells reached 70-80% confluency, for up 72 hours. At each time-point, cells were harvested and lysed for RNA extraction. cDNA was synthesised and the expression ratio (R) of injury and fibrogenic markers (MCP-1, KIM-1, TGF-β and COL1A1) relative to two housekeeping genes (HPRT1 and SDHA), was measured by RT-qPCR. In addition, conditioned media were collected and the levels of secreted MCP-1, KIM-1 and IL-1β were measured using ELISA. Commercial antibodies for the detection of MCP-1, KIM-1 and TGF-β by Western blotting were also tested. Of the three tested (MCP-1, KIM-1 and TGF-β), only the antibody to MCP-1 gave specific bands and was used to examine the expression of this chemokine under the different experimental conditions.

5.2.1 Heat and high osmolality

5.2.1.1 Effect of heat and high osmolality on gene expression

The effect of heat and high osmolality on the mRNA levels of MCP-1, KIM-1, TGF-β, COL1A1, and lactate dehydrogenase A (LDHA) relative to the housekeeping genes HPRT1 and SDHA were assessed by RT-qPCR. Confluent HKC-8 cells were made quiescent (to achieve synchronisation of the culture and decrease variability in the response) by incubation in 0.5% FBS media for 24 hours and then exposed to four different conditions: control temperature (37˚C), heat (39˚C), control temperature plus high osmolality (37˚C + 25 mM Mannitol), heat plus high osmolality (39˚C + 25 mM Mannitol) for 8, 24 and 48 hours. As a positive control for renal cell injury, cells were treated with $CoCl_2$ (25 mM) at the control temperature for the same time period. Figure 5 shows the relative expression of the genes at 8 (Figure 5.1), 24 (Figure 5.2) and 48 (Figure 5.3) hours.

After 8 hours (Figure 5.1) at 39˚C there were no differences in expression of MCP-1, KIM-1, TGF-β and LDHA compared to the control group (37˚C). In

contrast, levels of COL1A1 mRNA were increased. Cells in high osmolality for 8 hours expressed higher levels of TGF-β while there were no differences in the expression of MCP-1, KIM-1, COL1A1 and LDHA. Exposure to high temperature and high osmolality led to increased expression of MCP-1, TGF-β and COL1A1 mRNAs compared to the control group but there were no differences in the expression of KIM-1 and LDHA. Cobalt chloride decreased expression of MCP-1, KIM-1 and COL1A1 mRNAs compared to the control group while expression of LDHA was elevated.

After 24 hours (Figure 5.2) at 39˚C, there was no change in expression of MCP-1, KIM-1, TGF-β and LDHA mRNAs compared to the control group. In contrast, levels of COL1A1 were increased. Exposure to high osmolality for 24 hours decreased expression of TGF-β mRNA while there were no differences in the expression of MCP-1, KIM-1, COL1A1 and LDHA. High temperature and high osmolality had no effect on the expression of any of the genes tested. Cobalt chloride decreased expression of COL1A1 mRNA compared to the control group while expression of LDHA was elevated. There were no differences in the expression of MCP-1, KIM-1 and TGF-β after exposure of cells to $CoCl_2$ for 24 hours.

After 48 hours (Figure 5.3) at 39˚C, there was no change in expression of KIM-1. MCP-1 gene expression was decreased compared to the control group, while levels of TGF-β and COL1A1 were elevated. High osmolality for 48 hours led to increased expression of MCP-1 mRNA but had no effect on any of the other genes examined. High temperature and high osmolality led to a similar increase in expression of TGF-β and COL1A1 compared to the control group as seen with exposure to high temperature alone. Cobalt chloride decreased the expression of COL1A1 mRNA compared to the control group while the expression of TGF-β, LDHA was elevated. There were no differences in the gene expression of MCP-1 and KIM-1after exposure of cells to $CoCl_2$.

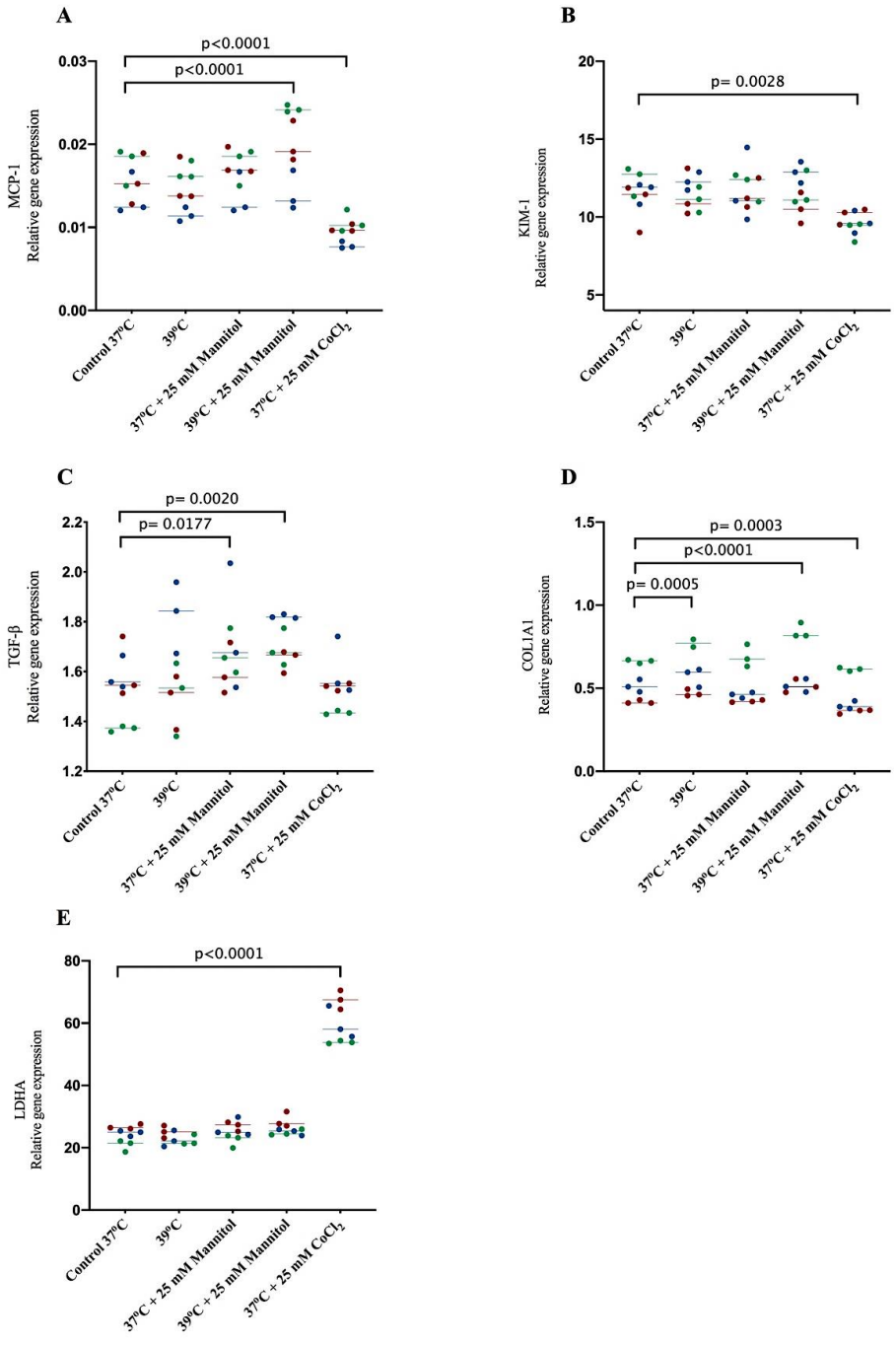

Figure 5.1: Effect of 8 hour heat and high osmolality on gene expression in HKC-8 cells. Cells were exposed to control temperature (37˚C), heat (39˚C), control temperature and high osmolality (37˚C + 25 mM Mannitol), heat and high osmolality (39˚C + 25 mM Mannitol) or $CoCl_2$ (25 mM) for 8 hours and mRNA expression assessed by RT-qPCR. Expression levels were normalised using the housekeeping genes, SDHA and HPRT1. **A.** MCP-1. **B.** KIM-1. **C.** TGF-β. **D.** COL1A1 and **E.** LDHA. Three independent experiments (each denoted by a different colour) were performed with three replicates per group. The horizontal line shows the median for each experiment. Two-way ANOVA examining effect of experiment and condition. Dunnett's multiple comparisons test was performed (comparing each condition with control) and $p<0.05$ was considered statistically significant.

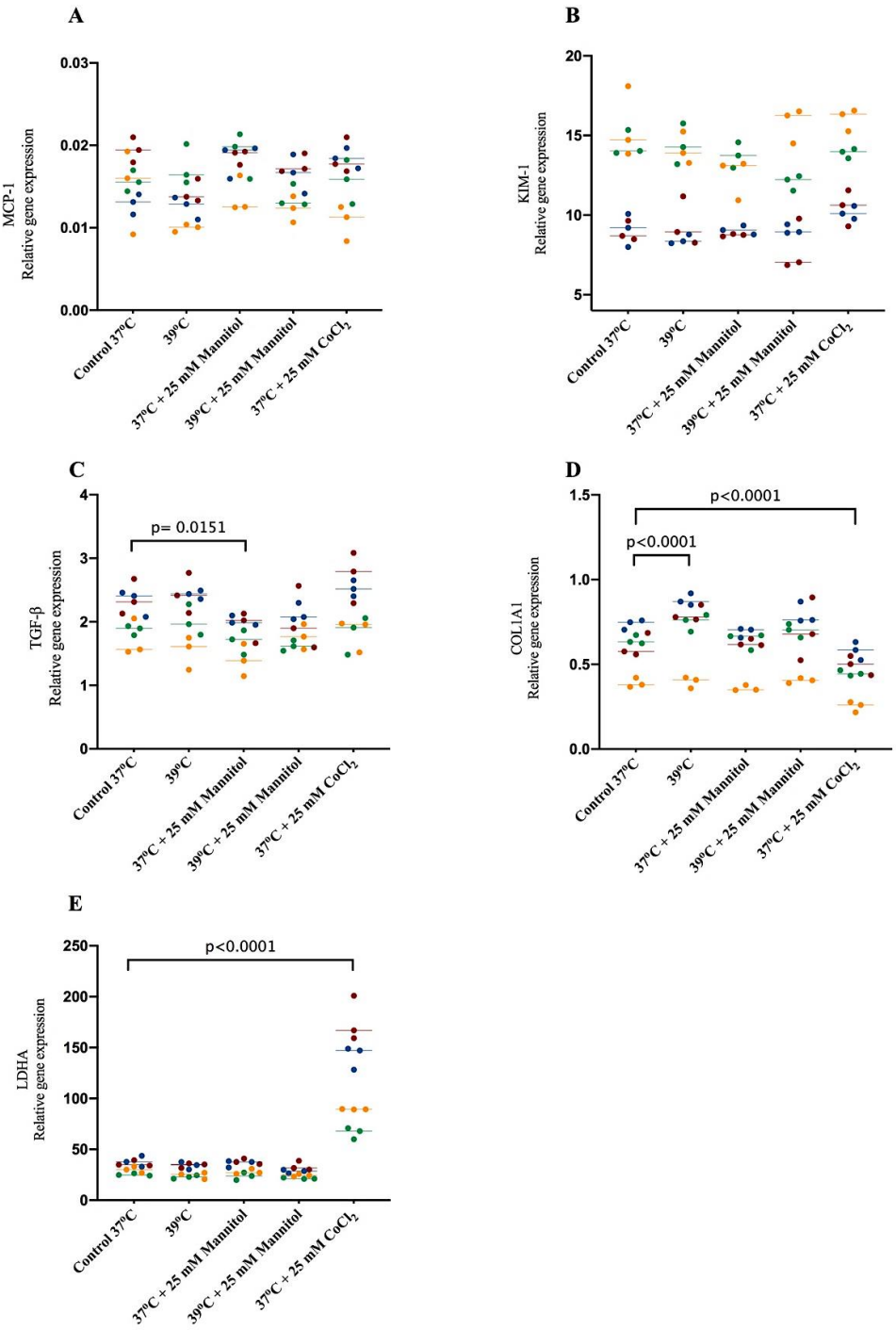

Figure 5.2: Effect of 24 hour heat and high osmolality on gene expression in HKC-8 cells. Cells were exposed to control temperature (37˚C), heat (39˚C), control temperature and high osmolality (37˚C + 25 mM Mannitol), heat and high osmolality (39˚C + 25 mM Mannitol) or $CoCl_2$ (25 mM) for 24 hours and mRNA expression assessed by RT-qPCR. Expression levels were normalised using the housekeeping genes, SDHA and HPRT1. **A.** MCP-1. **B.** KIM-1. **C.** TGF-β. **D.** COL1A1 and **E.** LDHA. Four independent experiments (each denoted by a different colour) were performed with three replicates per group. The horizontal line shows the median for each experiment. Two-way ANOVA examining effect of experiment and condition. Dunnett's multiple comparisons test was performed (comparing each condition with control) and $p<0.05$ was considered statistically significant.

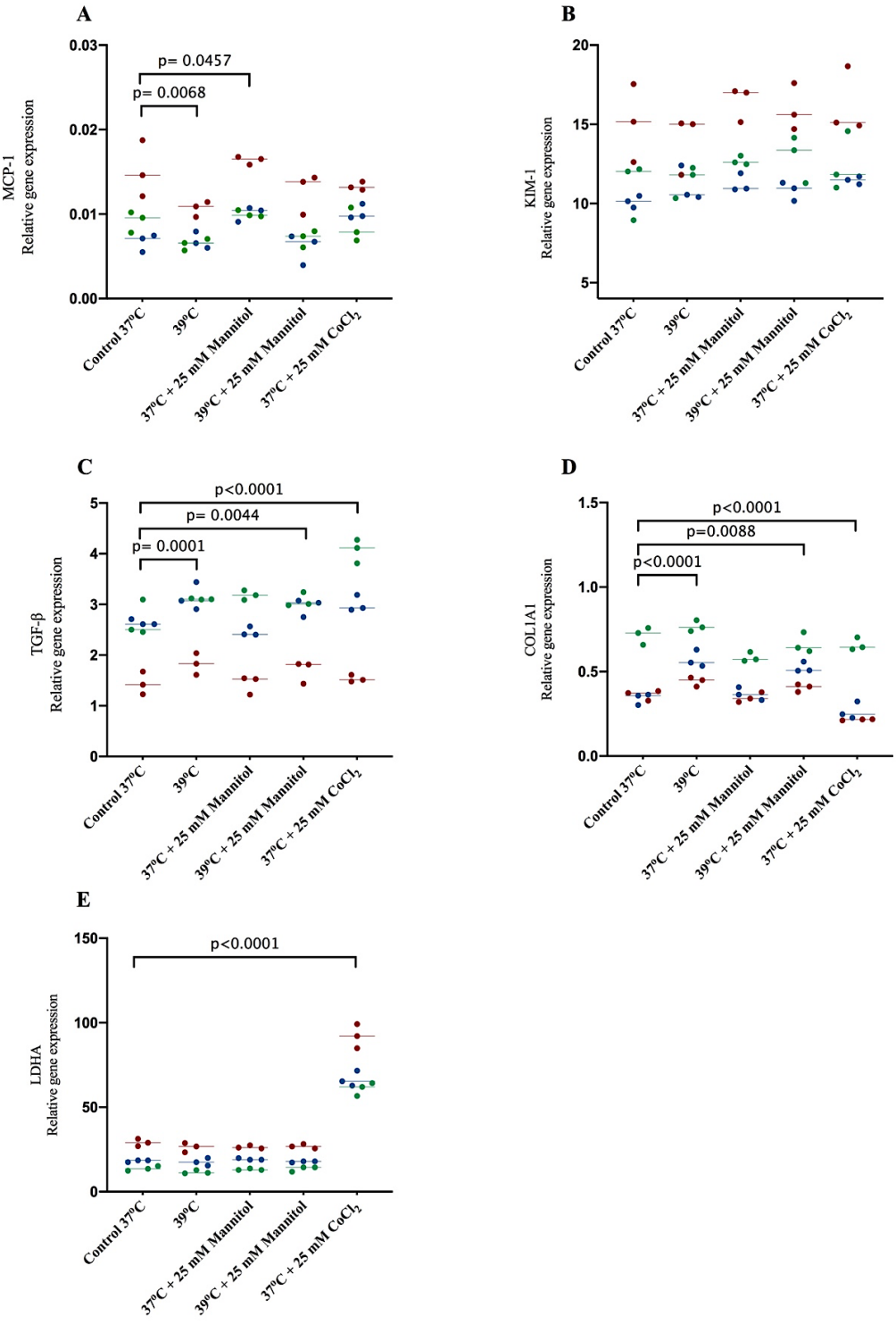

Figure 5.3: Effect of 48 hour heat and high osmolality on gene expression in HKC-8 cells. Cells were exposed to control temperature (37˚C), heat (39˚C), control temperature and high osmolality (37˚C + 25 mM Mannitol), heat and high osmolality (39˚C + 25 mM Mannitol) or $CoCl_2$ (25 mM) for 48 hours and mRNA expression assessed by RT-qPCR. Expression levels were normalised using the housekeeping genes, SDHA and HPRT1. **A.** MCP-1. **B.** KIM-1. **C.** TGF-β. **D.** COL1A1 and **E.** LDHA. Three independent experiments (each denoted by a different colour) were performed with three replicates per group. The horizontal line shows the median for each experiment. Two-way ANOVA examining effect of experiment and condition. Dunnett's multiple comparisons test was performed (comparing each condition with control) and $p<0.05$ was considered statistically significant.

5.2.1.2 The effect of heat and osmolality on chemokine secretion

Cell conditioned media were collected at each time-point and assayed for secreted MCP-1 (Figure 5.4), KIM-1 (Figure 5.5) and IL-1β (Figure 5.6). Due to the cost of ELISAs and limited resources, three samples (one of each independent experiment) for MCP-1 and IL-1β were analysed, while for KIM-1 only two samples (one replicate from two independent experiments) were analysed for each condition.

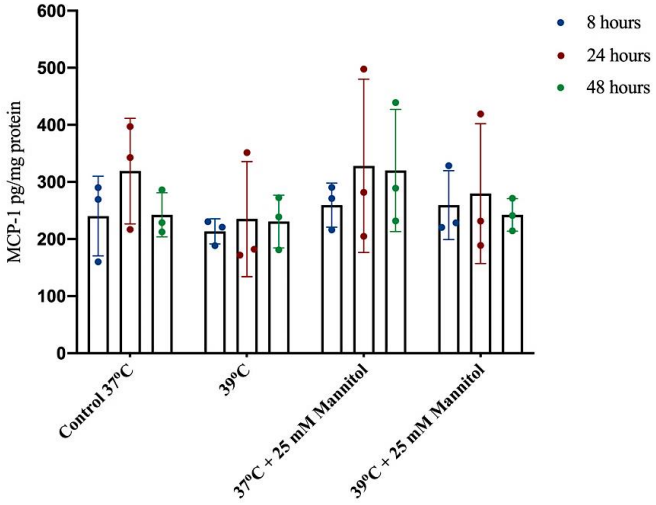

Figure 5.4: Effect of heat and high osmolality on MCP-1 secretion after 8, 24 and 48 hours. Cells were exposed to control temperature (37˚C), heat (39˚C), control temperature and high osmolality (37˚C + 25 mM Mannitol), and heat and high osmolality (39˚C + 25 mM Mannitol) for 8 (blue), 24 (red) or 48 hours (green). Secreted levels were normalised to the total cell protein. Three independent experiments with one sample per group. Two-way ANOVA, examining the effect of time and condition. Tukey's multiple comparisons test was used comparing each condition to each other condition. $p<0.05$ was considered statistically significant. No Significance (NS) for an effect of time or between conditions.

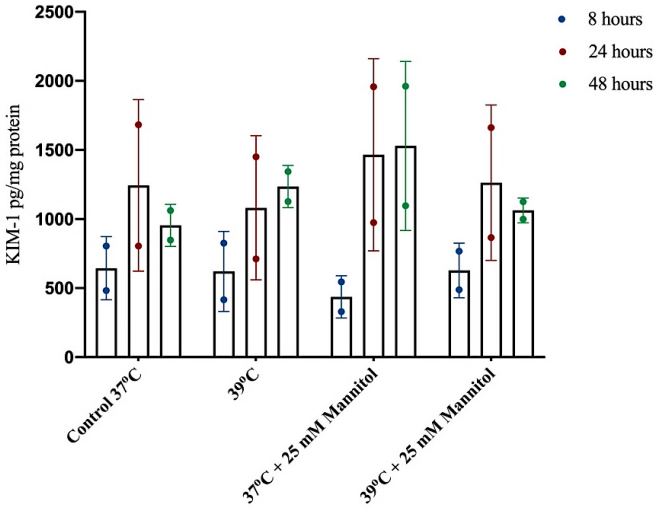

Figure 5.5: Effect of heat and high osmolality on KIM-1 secretion after 8, 24 and 48 hours. Cells were exposed to control temperature (37˚C), heat (39˚C), control temperature and high osmolality (37˚C + 25 mM Mannitol), and heat and high osmolality (39˚C + 25 mM Mannitol) for 8 (blue), 24 (red) or 48 hours (green). Secreted levels were normalised to the total cell proteins. Two independent experiments with one sample per group. Two-way ANOVA, examining the effect of time and condition. Tukey's multiple comparisons test was used comparing each condition to each other condition. $p<0.05$ was considered statistically significant. $p=0.01$ for an effect of time. No Significance (NS) between conditions.

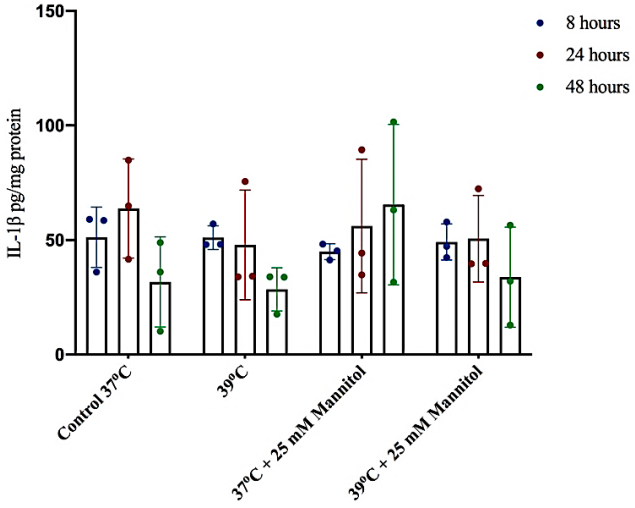

Figure 5.6: Effect of heat and high osmolality on IL-1β secretion after 8, 24 and 48 hours. Cells were exposed to control temperature (37˚C), heat (39˚C), control temperature and high osmolality (37˚C + 25 mM Mannitol), and heat and high osmolality (39˚C + 25 mM Mannitol) for 8 (blue), 24 (red) or 48 hours (green). Secreted levels were normalised to the total cell proteins. Three independent experiments with one sample per group. Two-way ANOVA, examining the effect of time and condition. Tukey's multiple comparisons test was used comparing each condition to each other condition. $p<0.05$ was considered statistically significant. No Significance (NS) for an effect of time or between conditions.

No significant differences were observed in the levels of secreted MCP-1, KIM-1 or IL-1β in HKC-8 cells exposed to the different experimental conditions for 8, 24 and 48 hours.

5.2.1.3 The effect of heat and osmolality on intracellular MCP-1 protein levels

Cellular MCP-1 protein levels were assessed by Western blotting. A representative experiment is shown in Figure 5.7. As it can be seen in Figure 5.7D, the protein levels at 8 hours seemed to be decreased after exposure to increased osmolality, i.e. with both high osmolality and heat and high osmolality compared to the control temperature with a similar effect at 48 hours although no differences were seen between groups at 24 hours. Exposure to cobalt chloride followed a similar pattern with the control group at 24- and 48-hour time points. On the other hand, at 8 hours the protein levels appeared to be lower when compared to its control.

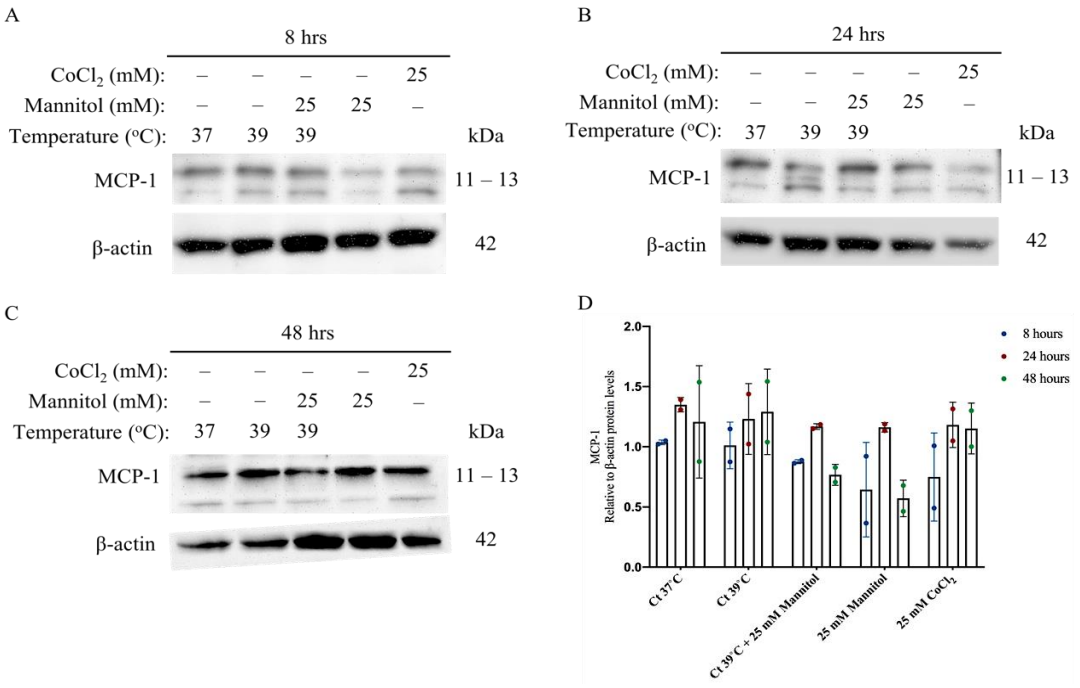

Figure 5.7: Effect of heat and high osmolality on cellular MCP-1 protein expression. Confluent quiescent HKC-8 cells were exposed to control temperature (37˚C), heat (39˚C), high osmolality (37˚C + 25 mM Mannitol), heat and high osmolality (39˚C + 25 mM Mannitol) or $CoCl_2$ (25 mM) for **A.** 8 hours, **B.** 24 hours, or **C.** 48 hours. Western blots of cellular proteins (25ug protein/sample) were probed with an antibody to MCP-1 and re-probed after striping of the membranes, with β-actin as a loading control. **D.** Densitometric analysis was performed using Image J and expression normalised to β-actin as the loading control. Each colour represents a different time point. Representative blots are shown from one of the two independent experiments performed. Two-way ANOVA, examining the effect of time and condition. Tukey's multiple comparisons test was used comparing each condition to each other condition. $p<0.05$ was considered statistically significant. No Significance (NS) for an effect of time or between conditions.

5.2.2 Elevated fructose levels

5.2.2.1 The effect of elevated fructose on gene expression

The expression of mRNAs for MCP-1, KIM-1, TGF-β, COL1A1 and LDHA (as a positive control for cellular injury in response to $CoCl_2$) was assessed in HKC-8 cells after incubation in normal (control) glucose (5 mM), elevated fructose (45 mM), elevated glucose (45 mM), elevated L-glucose (45 mM as an osmolality control), and $CoCl_2$ (25 mM) for 8 (Figure 5.8), 24 (Figure 5.9) or 48 (Figure 5.10) hours. Workers are exposed to fructose from various sources (e.g. beverages and sugarcane chewing) (359). Although, there are no studies suggesting or quantifying the actual levels of fructose the population is exposed to, we initially tried to exacerbate and test if there is any effect.

After 8 hours in 45 mM fructose, HKC-8 cell expression of MCP-1, KIM-1, TGF-β and LDHA mRNAs was increased compared to the control group. The levels of COL1A1 did not alter across the groups. Elevated glucose levels also increased expression of TGF-β but had no effect on MCP-1, KIM-1, COL1A1 or LDHA gene expression. Interestingly, 45 mM L-glucose, as a control for increased osmolality up-regulated expression of MCP-1, KIM-1, TGF-β and COL1A1 but LDHA did not seem to change. Cobalt chloride reduced expression of MCP-1 when compared to the control group while the expression of TGF-β and LDHA was elevated compared to the control group. Exposing the cells to $CoCl_2$ for 8 hours had no effect on the mRNA levels of KIM-1 and COL1A1.

After 24 hours in 45 mM fructose, HKC-8 cell expression of MCP-1, KIM-1, TGF-β and LDHA mRNAs did not alter when compared to the control group. On the other hand, the levels of COL1A1 decreased for both the elevated fructose and the L-glucose groups. Interestingly, 45 mM L-glucose, as a control for increased osmolality up-regulated expression of KIM-1, but TGF-β, MCP-1 and LDHA did not seem to change. Cobalt chloride reduced expression of MCP-1 when compared to the control group while the expression of LDHA was elevated compared to the control group.

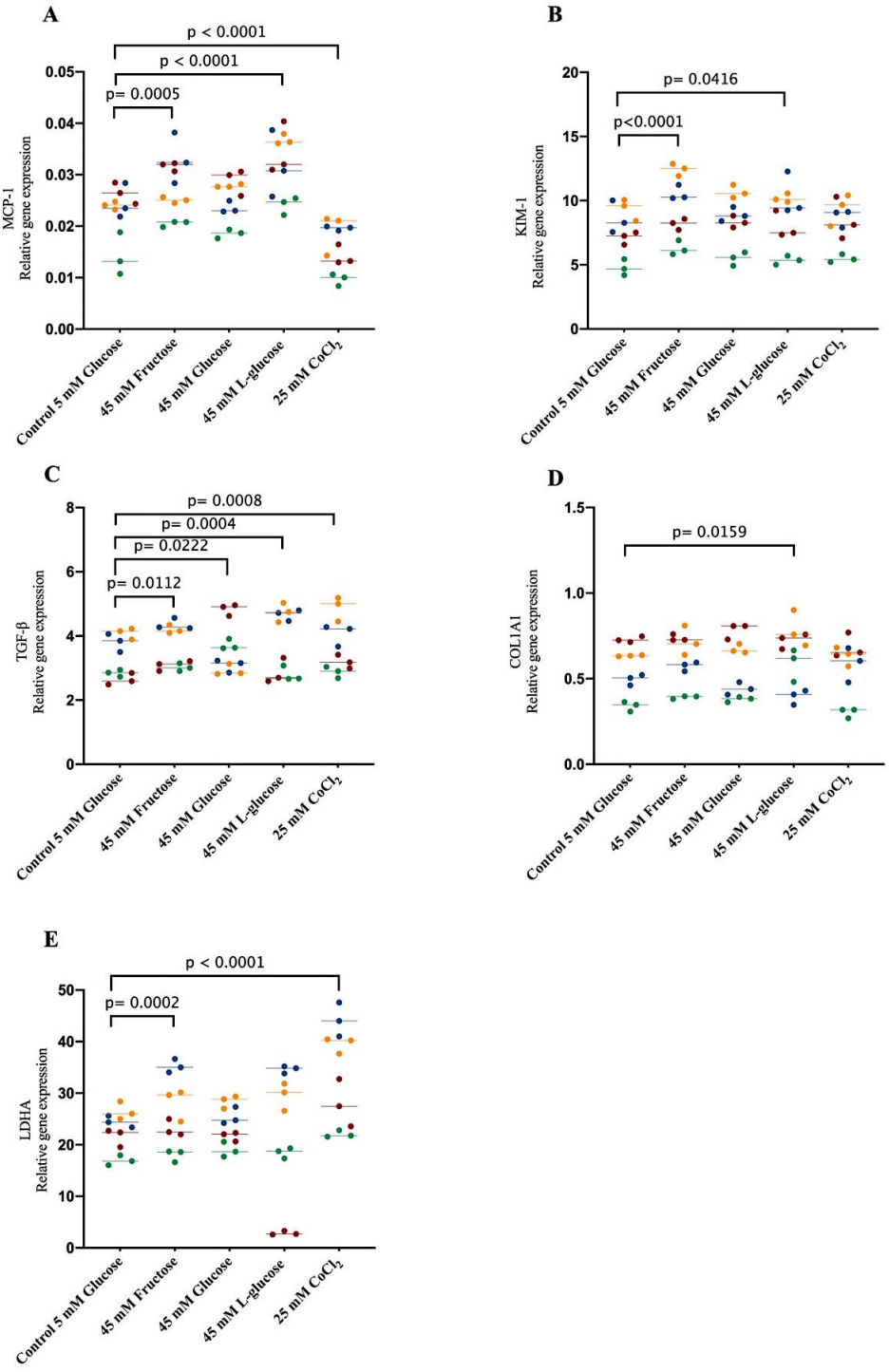

Figure 5.8: Effect of 8 hour exposure to elevated fructose and glucose on gene expression in HKC-8 cells. Cells were exposed to control (5 mM Glucose), elevated fructose (45 mM Fructose), elevated glucose (45 mM Glucose), control of osmolality (45 mM L-glucose), or $CoCl_2$ (25 mM) for 8 hours and mRNA expression assessed by RT-qPCR. Expression levels were normalised using the housekeeping genes SDHA and HPRT1. **A.** MCP-1. **B.** KIM-1. **C.** TGF-β. **D.** COL1A1 and **E.** LDHA. Four independent experiments (each denoted by a different colour) were performed with three replicates per group. The horizontal line shows the median for each experiment. Two-way ANOVA examining effect of experiment and condition. Dunnett's multiple comparisons test was performed (comparing each condition with control) and $p<0.05$ was considered statistically significant.

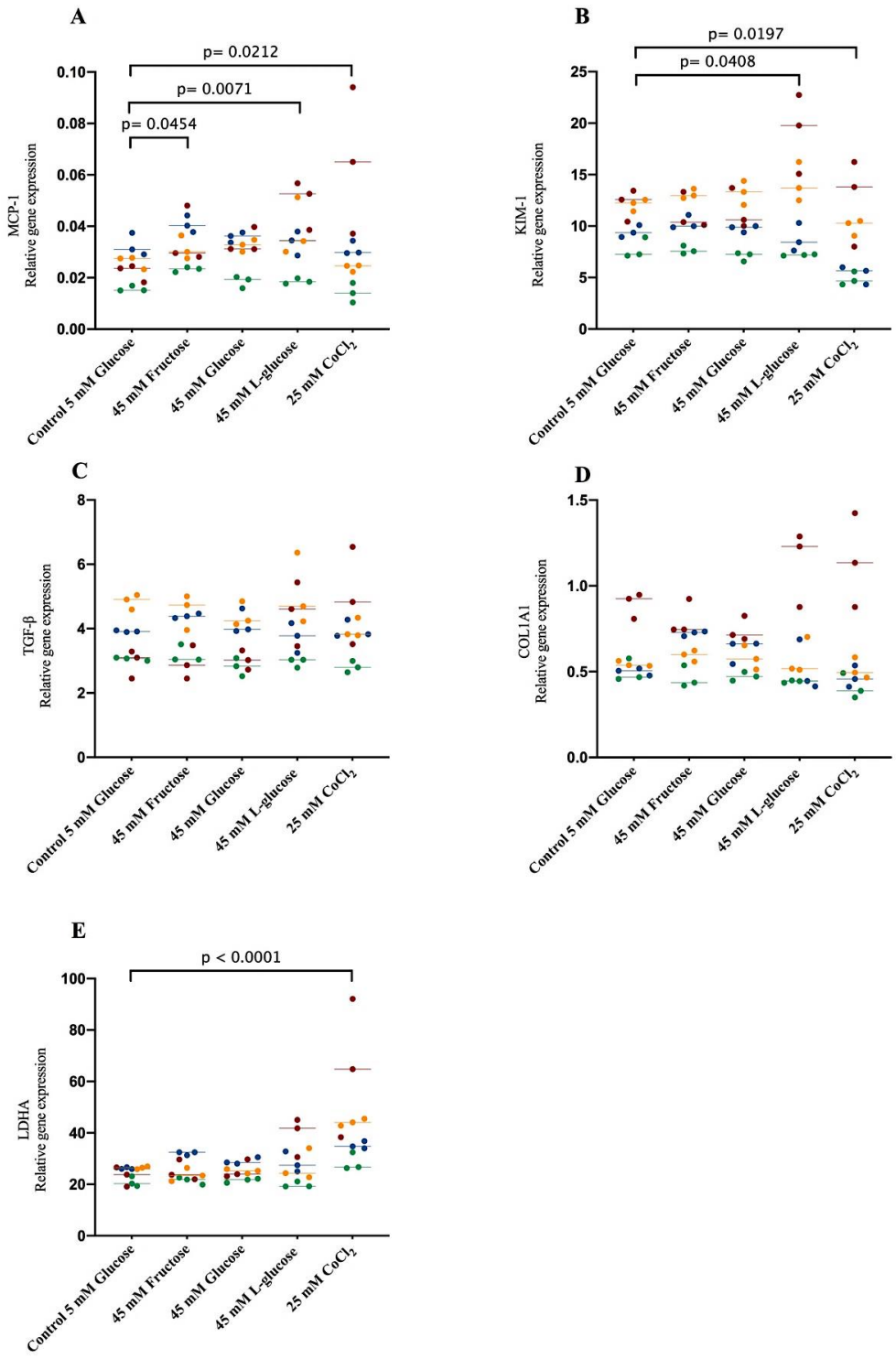

Figure 5.9: Effect of 24 hour exposure to elevated fructose and glucose on gene expression in HKC-8 cells. Cells were exposed to control (5 mM Glucose), elevated fructose (45 mM Fructose), elevated glucose (45 mM Glucose), control of osmolality (45 mM L-glucose), or $CoCl_2$ (25 mM) for 8 hours and mRNA expression assessed by RT-qPCR. Expression levels were normalised using the housekeeping genes SDHA and HPRT1. **A.** MCP-1. **B.** KIM-1. **C.** TGF-β. **D.** COL1A1 and **E.** LDHA. Four independent experiments (each denoted by a different colour) were performed with three replicates per group. The horizontal line shows the median for each experiment. Two-way ANOVA examining effect of experiment and condition. Dunnett's multiple comparisons test was performed (comparing each condition with control) and $p<0.05$ was considered statistically significant.

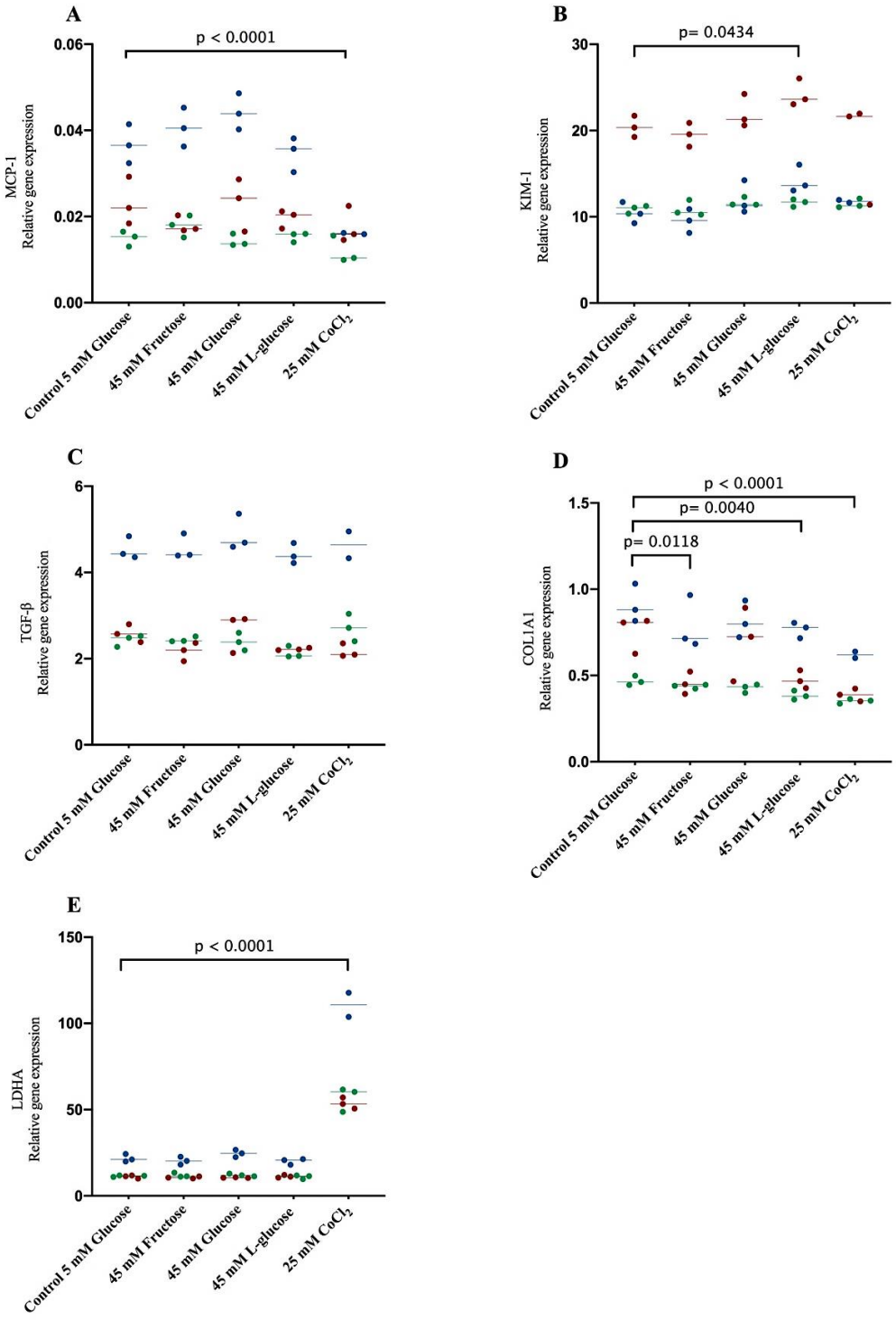

Figure 5.10: Effect of 48 hour exposure to elevated fructose and glucose on gene expression in HKC-8 cells. Cells were exposed to control (5 mM Glucose), elevated fructose (45 mM Fructose), elevated glucose (45 mM Glucose), control of osmolality (45 mM L-glucose), or $CoCl_2$ (25 mM) for 8 hours and mRNA expression assessed by RT-qPCR. Expression levels were normalised using the housekeeping genes SDHA and HPRT1. **A.** MCP-1. **B.** KIM-1. **C.** TGF-β. **D.** COL1A1 and **E.** LDHA. Four independent experiments (each denoted by a different colour) were performed with three replicates per group. The horizontal line shows the median for each experiment. Two-way ANOVA examining effect of experiment and condition. Dunnett's multiple comparisons test was performed (comparing each condition with control) and $p<0.05$ was considered statistically significant.

After 48 hours in 45 mM of fructose, expression of COL1A1 mRNA was reduced compared to the control group (5 mM Glucose) while there was no change in any of the other genes studied. Elevated glucose levels also had no effect on expression of any of the genes studied. In contrast, elevated L-glucose (control for osmolality) increased expression of KIM-1 mRNA and decreased COL1A1 gene expression compared to the control group. Cobalt chloride reduced expression of MCP-1 and COL1A1 mRNAs but increased LDHA gene expression compared to the control group. KIM-1 and levels remained to control levels.

5.2.2.2 The effect of elevated fructose on chemokine secretion

Cell conditioned media were collected at each time-point and assayed for secreted MCP-1 (Figure 5.11), KIM-1 (Figure 5.12) and IL-1β (Figure 5.13). Due to the cost of ELISAs and limited resources three samples (one from each independent experiment) for MCP-1 and IL-1β were analysed, while for KIM-1 only two samples (one replicate from two independent experiments) were analysed for each condition at each time-point.

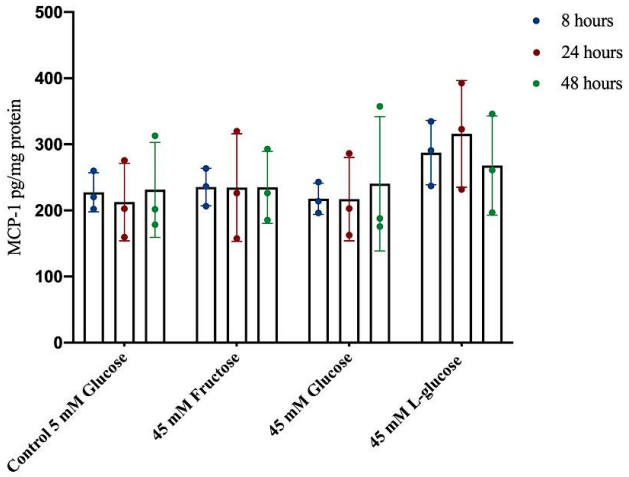

Figure 5.11: Effect of elevated fructose and glucose on MCP-1 secretion after 8, 24 and 48 hours. Cells were exposed to control (5 mM Glucose), elevated fructose (45 mM Fructose), elevated glucose (45 mM Glucose) or a control of osmolality (45 mM L-glucose) for 8 (blue), 24 (red) or 48 hours (green). Secreted levels were normalised to the total cell proteins. Three independent experiments with one sample per group. Two-way ANOVA, examining the effect of time and condition. Tukey's multiple comparisons test was used comparing each condition to each other condition. $p<0.05$ was considered statistically significant. No Significance (NS) for an effect of time or between conditions.

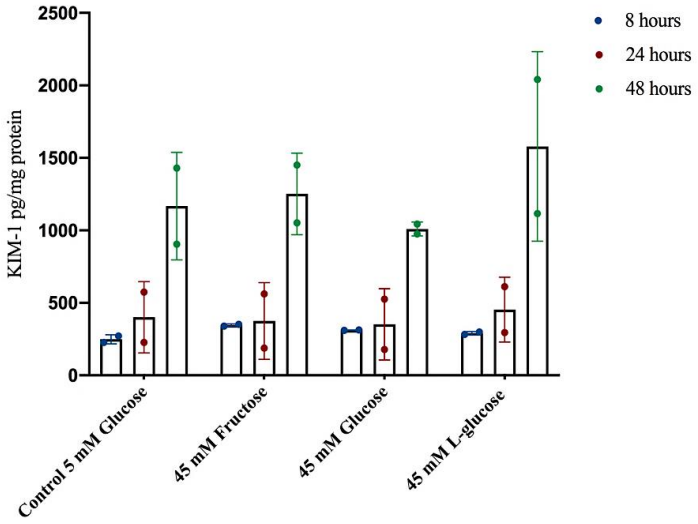

Figure 5.12: Effect of elevated fructose and glucose on secreted KIM-1 after 8, 24 and 48 hours. Cells were exposed to control (5 mM Glucose), elevated fructose (45 mM Fructose), elevated glucose (45 mM Glucose) or a control of osmolality (45 mM L-glucose) for 8 (blue), 24 (red) or 48 hours (green). Secreted levels were normalised to the total cell proteins. Two independent experiments with one sample per group. Two-way ANOVA, examining the effect of time and condition. Tukey's multiple comparisons test was used comparing each condition to each other condition. $p<0.05$ was considered statistically significant. $p<0.0001$ for an effect of time. No Significance (NS) between conditions.

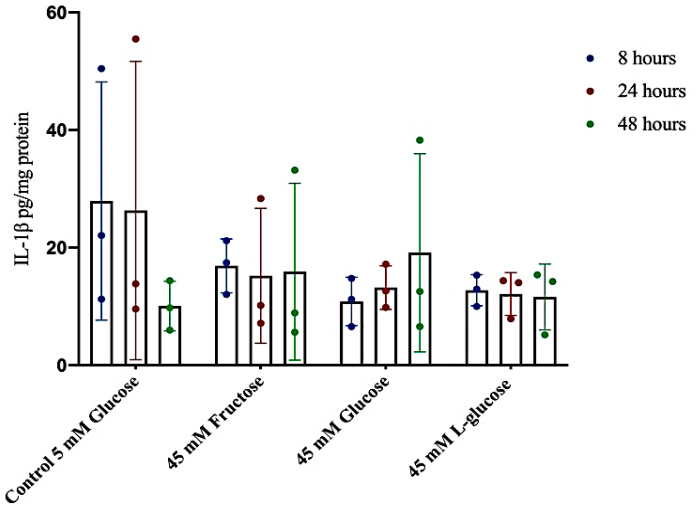

Figure 5.13: Effect of elevated fructose and glucose on secreted IL-1β after 8, 24 and 48 hours. Cells were exposed to control (5 mM Glucose), elevated fructose (45 mM Fructose), elevated glucose (45 mM Glucose) or a control of osmolality (45 mM L-glucose) for 8 (blue), 24 (red) or 48 hours (green). Secreted levels were normalised to the total cell proteins. Three independent experiments with one sample per group. Two-way ANOVA, examining the effect of time and condition. Tukey's multiple comparisons test was used comparing each condition to each other condition. $p<0.05$ was considered statistically significant. No Significance (NS) for an effect of time or between conditions.

In cells exposed for 8, 24 and 48 hours, there was a trend toward increased levels of secreted MCP-1 in the L-glucose group compared to the control,

although not statistically significant. After 48 hours of exposure, secreted KIM-1 was found to be increased in all groups including the controls, when compared with the same exposures after 8 and 24 hours. Although, there were no differences between the different groups of the same time-points. Levels of secreted IL-1β tended to decrease over time in all the groups compared to controls at 8 and 24 hours. The secretion of IL-1β at the control group showed a drop after 48 hours when compared to levels at 8 and 24 hours.

5.2.2.3 The effect of elevated fructose on MCP-1 protein levels

Western blots for MCP-1 protein expression from one experiment in which cells were exposed to the different experimental conditions for 8 or 48 hours are shown in Figure 5.14. No signals were detected on the blot of the 24 hour samples.

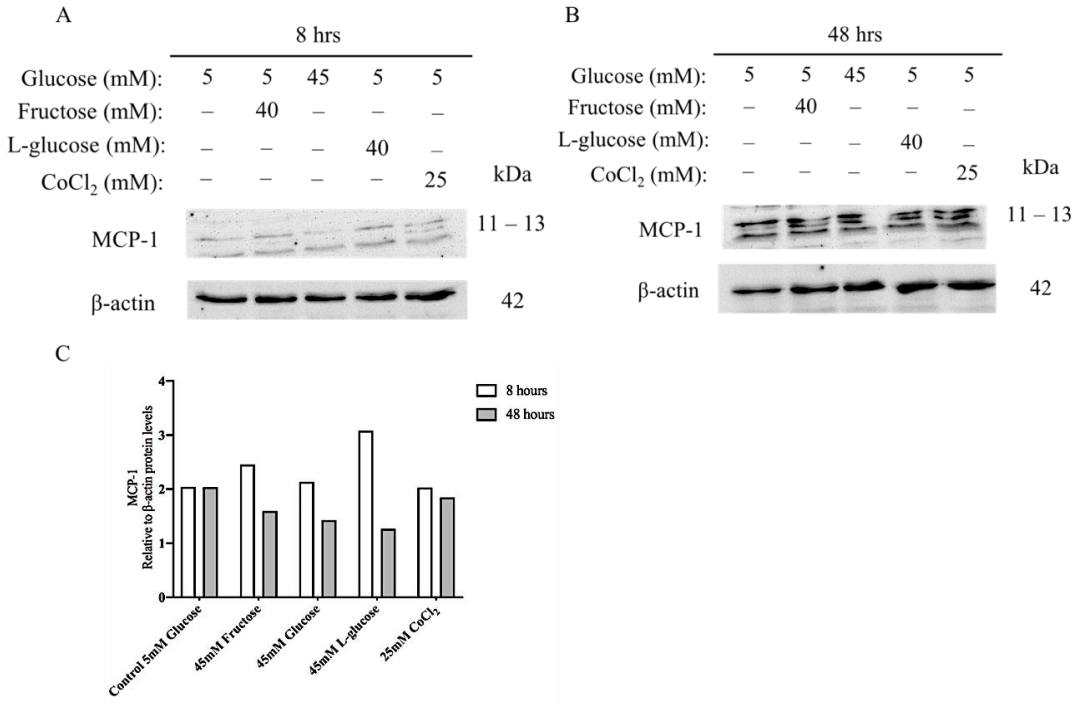

Figure 5.14: Effect of elevated fructose and high osmolality on cellular MCP-1 protein expression. Confluent quiescent HKC-8 cells were exposed to control (5 mM Glucose), elevated fructose (45 mM Fructose), elevated glucose (45 mM Glucose) or controls for osmolality (45 mM L-glucose) and cell injury ($CoCl_2$) for: **A.** 8 hours, or **B.** 48 hours. Western blots of cellular proteins (25ug protein/sample) were probed with an antibody to MCP-1 and re-probed after striping of the membranes, with β-actin as a loading control (n= 1 experiment with 1 experimental replicate per group). **C.** Densitometric analysis was performed using Image J and expression normalised to β-actin as the loading control. Blot is from one experiment performed. No statistical analysis was performed.

The data suggest that levels of secreted MCP-1 increased after 8 hours exposure to 45 mM fructose with a marked increase after exposure to 45 mM L-glucose. In contrast, secreted MCP-1 decreased after 48 hours in 45mM fructose, 45 mM glucose and 45 mM L-glucose but were unaffected by $CoCl_2$. However as only one sample was able to be quantified, it was not possible to perform statistical analysis or to draw any definitive conclusions.

5.2.3 Combined heat and fructose

5.2.3.1 The effect of heat and fructose on gene expression

According to the literature, even low levels (10 mM) of fructose can have an effect on MCP-1 secretion levels in HK-2 cells (another immortalised human proximal tubular epithelial cell line) after 72 hours (151). To examine if this effect was conserved across the two different cell lines, we initially replicated the findings of that study in an mRNA level. Additionally, the combination of heat and rehydration with high in fructose beverages have been proposed to be implicated in kidney injury (442). For that reason, the combined effect of heat and fructose for 8 (acute response) and 72 hours (chronic response) was tested in HKC-8 cells. The experimental groups examined are shown in Table 5.1.

	Temperature (˚C)	**Designation**
5 mM Glucose (Control)	37	Control 37˚C
5 mM Glucose	39	Control 39˚C
15 mM Fructose	37	Fructose 15 mM 37˚C
15 mM Fructose	39	Fructose 15 mM 39˚C
15 mM Glucose	37	Glucose 15 mM 37˚C
15 mM Glucose	39	Glucose 15 mM 39˚C
15 mM L-Glucose (Osmolality control)	37	L-glucose 15 mM 37˚C
15 mM L-Glucose	39	L-glucose 15 mM 39˚C

Table 5.1: Experimental groups of heat in combination with fructose, glucose or L-glucose.

The expression of mRNAs for MCP-1 (Figure 5.15), KIM-1 (Figure 5.16), TGF-β (Figure 5.17) and COL1A1 (Figure 5.18) was assessed by RT-qPCR.

Associations were tested with two-way ANOVA and compared between the same condition at the two different temperatures and between the different conditions at the same temperature.

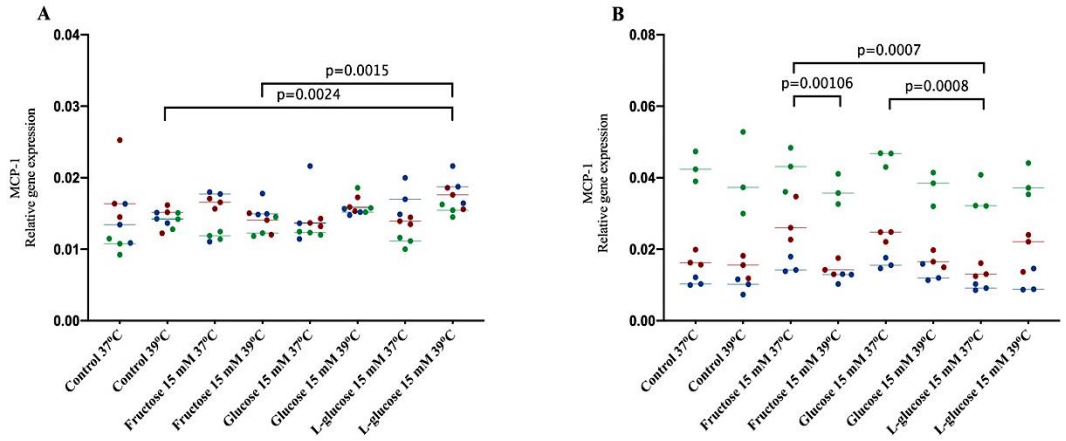

Figure 5.15: Effect of heat and fructose and glucose on MCP-1 gene expression. Cells were exposed to control 5 mM Glucose at 37˚C (Control 37˚C), control 5 mM Glucose at 39˚C (Control 39˚C), 15 mM Fructose at 37˚C (Fructose 15 mM 37˚C), 15 mM Fructose at 39˚C (Fructose 15 mM 39˚C), 15 mM Glucose at 37 ˚C (Glucose 15 mM 37˚C), 15 mM Glucose at 39˚C (Glucose 15 mM 39˚C), 15 mM L-glucose at 37˚C (L-glucose 15 mM 37˚C) and 15 mM L-glucose at 39˚C (L-glucose 15 mM 39˚C) for **A.** 8 and **B.** 72 hours and mRNA expression assessed by RT-qPCR. Expression levels were normalised using the housekeeping genes SDHA and HPRT1. Three independent experiments (each shown as a different colour in the graph with medians) were performed with three replicates in each experimental group. Two-way ANOVA examining the effect of condition and experiment. Tukey's multiple comparisons test was performed comparing each condition with each other condition and $p<0.05$ was considered statistically significant.

After 8 hours (Figure 5.15A) in increased temperature and 15 mM fructose or glucose, there was no change in the expression of MCP-1 mRNA levels compared to the heat control. The levels of MCP-1 were elevated in response to heat and L-glucose when compared to the heat control and to the heat and elevated fructose group. After 72 hours (Figure 5.15B), the levels of MCP-1 mRNA remained stable across each condition at 37˚C and 39˚C separately. Although, there was a trend of the cells exposed to 39˚C, having lower levels when compared to the control temperature (37˚C) across all conditions. Additionally, MCP-1 mRNA expression was increased compared to the group in 15 mM L-glucose at 37˚C. A similar increase was observed in the 15 mM glucose at 37˚C group compared to cells in 15 mM L-glucose at 37˚C.

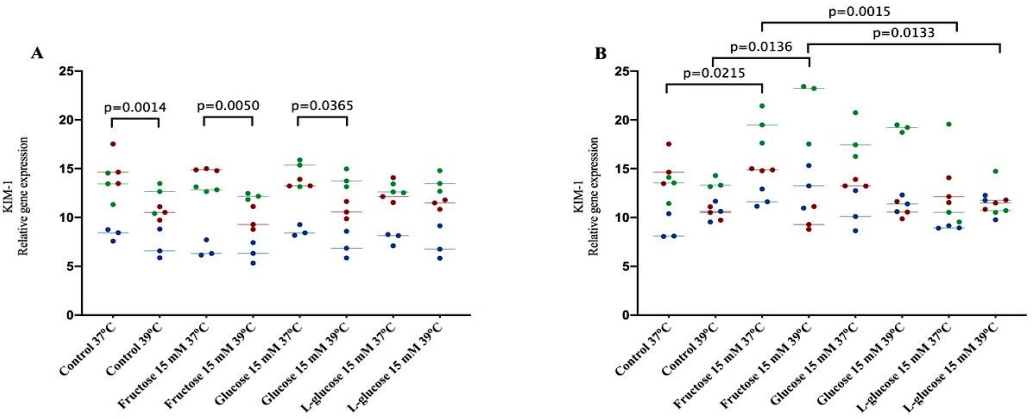

Figure 5.16: Effect of heat and fructose and glucose on KIM-1 gene expression. Cells were exposed to control 5 mM Glucose at 37˚C (Control 37˚C), control 5 mM Glucose at 39˚C (Control 39˚C), 15 mM Fructose at 37˚C (Fructose 15 mM 37˚C), 15 mM Fructose at 39˚C (Fructose 15 mM 39˚C), 15 mM Glucose at 37 ˚C (Glucose 15 mM 37˚C), 15 mM Glucose at 39˚C (Glucose 15 mM 39˚C), 15 mM L-glucose at 37˚C (L-glucose 15 mM 37˚C) and 15 mM L-glucose at 39˚C (L-glucose 15 mM 39˚C) for **A.** 8 and **B.** 72 hours and mRNA expression assessed by RT-qPCR. Expression levels were normalised using the housekeeping genes SDHA and HPRT1. Three independent experiments (each shown as a different colour in the graph with medians) were performed with three replicates in each experimental group. Two-way ANOVA examining the effect of condition and experiment. Tukey's multiple comparisons test was performed comparing each condition with each other condition and $p<0.05$ was considered statistically significant.

After 8 hours (Figure 5.16A) in heat and 15 mM of fructose and glucose, the expression of KIM-1 mRNA levels were decreased when compared to their control condition at 37˚C. After 72 hours (Figure 5.16B) the levels of KIM-1 mRNA were increased in the 15 mM fructose at 37˚C, 15 mM and 15 mM Fructose at 39˚C when compared to their untreated controls at 37˚C and 39˚C respectively. Elevated fructose at 37˚C and 39˚C led to increased KIM-1 mRNA levels, even when compared to the L-glucose at both control and heat conditions.

After 8 hours (Figure 5.17A) in heat the expression of TGF-β mRNA was decreased compared to the control temperature. Elevated fructose, glucose and L-glucose in control (37˚C) and heat (39˚C) conditions did not have any effect on the expression of TGF-β mRNA. On the other hand, after 72 hours (Figure 5.17B), the levels of TGF-β mRNA were decreased in the fructose and glucose groups when compared to their untreated controls. The expression of TGF-β mRNA, was lower in the group combining heat with fructose and

glucose, when compared to the same groups at the control temperature (37˚C). L-glucose did not demonstrate these effects.

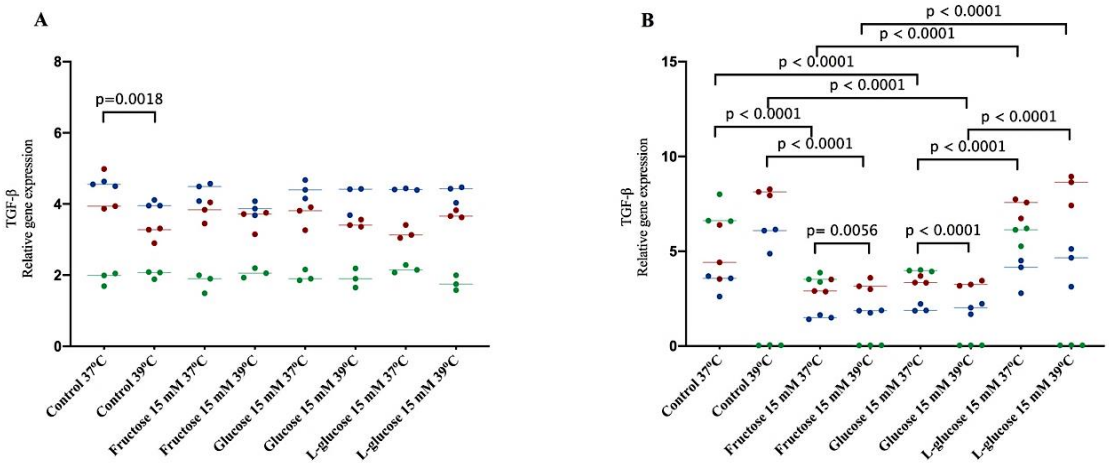

Figure 5.17: Effect of heat and fructose and glucose on TGF-β gene expression. Cells were exposed to control 5 mM Glucose at 37˚C (Control 37˚C), control 5 mM Glucose at 39˚C (Control 39˚C), 15 mM Fructose at 37˚C (Fructose 15 mM 37˚C), 15 mM Fructose at 39˚C (Fructose 15 mM 39˚C), 15 mM Glucose at 37 ˚C (Glucose 15 mM 37˚C), 15 mM Glucose at 39˚C (Glucose 15 mM 39˚C), 15 mM L-glucose at 37˚C (L-glucose 15 mM 37˚C) and 15 mM L-glucose at 39˚C (L-glucose 15 mM 39˚C) for **A.** 8 and **B.** 72 hours and mRNA expression assessed by RT-qPCR. Expression levels were normalised using the housekeeping genes SDHA and HPRT1. Three independent experiments (each shown as a different colour in the graph with medians) were performed with three replicates in each experimental group. Two-way ANOVA examining the effect of condition and experiment. Tukey's multiple comparisons test was performed comparing each condition with each other condition and $p<0.05$ was considered statistically significant.

After 8 hours (Figure 5.18A) in heat in the different experimental conditions, the expression of COL1A1 remained the same in all groups. In contrast, after 72 hours (Figure 5.18B) in heat alone, COL1A1 mRNA levels were higher compared to the control (Control 37˚C). Exposure to fructose or glucose, both in control and heat conditions, led to decreased COL1A1 mRNA expression compared to their untreated controls. COL1A1 gene expression in the L-glucose at 37˚C and L-glucose at 39˚C remained at the same levels to their controls.

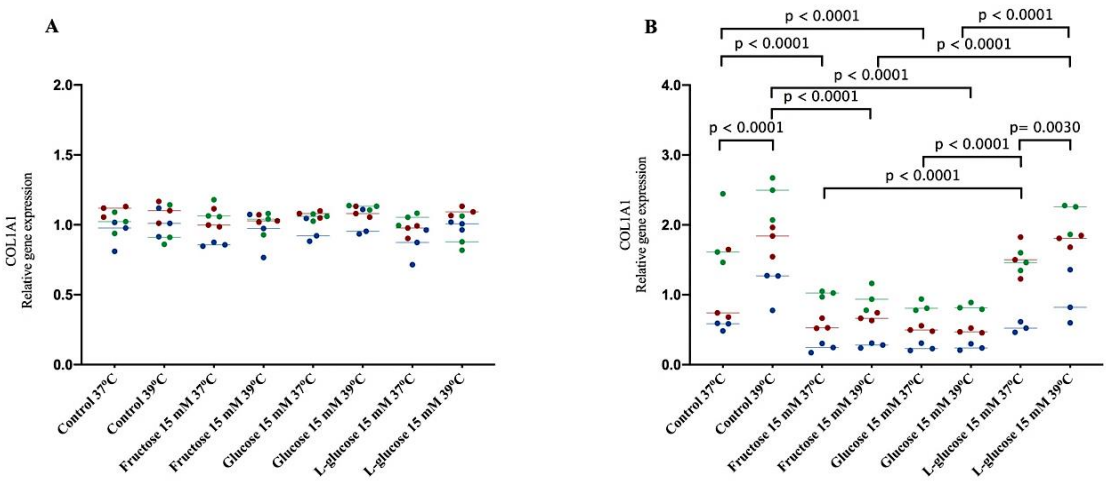

Figure 5.18: Effect of heat and fructose and glucose on COL1A1 gene expression. Cells were exposed to control 5 mM Glucose at 37˚C (Control 37˚C), control 5 mM Glucose at 39˚C (Control 39˚C), 15 mM Fructose at 37˚C (Fructose 15 mM 37˚C), 15 mM Fructose at 39˚C (Fructose 15 mM 39˚C), 15 mM Glucose at 37 ˚C (Glucose 15 mM 37˚C), 15 mM Glucose at 39˚C (Glucose 15 mM 39˚C), 15 mM L-glucose at 37˚C (L-glucose 15 mM 37˚C) and 15 mM L-glucose at 39˚C (L-glucose 15 mM 39˚C) for **A.** 8 and **B.** 72 hours and mRNA expression assessed by RT-qPCR. Expression levels were normalised using the housekeeping genes SDHA and HPRT1. Three independent experiments (each shown as a different colour in the graph with medians) were performed with three replicates in each experimental group. Two-way ANOVA examining the effect of condition and experiment. Tukey's multiple comparisons test was performed comparing each condition with each other condition and $p<0.05$ was considered statistically significant.

5.2.3.2 The effect of heat and elevated fructose on chemokine secretion

Cell-conditioned media were collected at each time-point and assayed for secreted MCP-1 (Figure 5.19), KIM-1 (Figure 5.20) and IL-1β (Figure 5.21). Due to the high cost of ELISAs, three samples for MCP-1 and IL-1β were analysed, while for KIM-1 only two samples (one replicate from two independent experiments) were analysed for each condition.

In cells exposed to the different experimental conditions for 8 hours, levels of secreted MCP-1 (Figure 5.19A), KIM-1 (Figure 5.20A) and IL-1β (Figure 5.21A) were not different across the groups, although, a trend of decreased MCP-1 secretion was noted in the glucose groups in both control and heat conditions when compared to their untreated controls. Additionally, levels of secreted IL-1β tended to decrease after exposure of all experimental groups to heat.

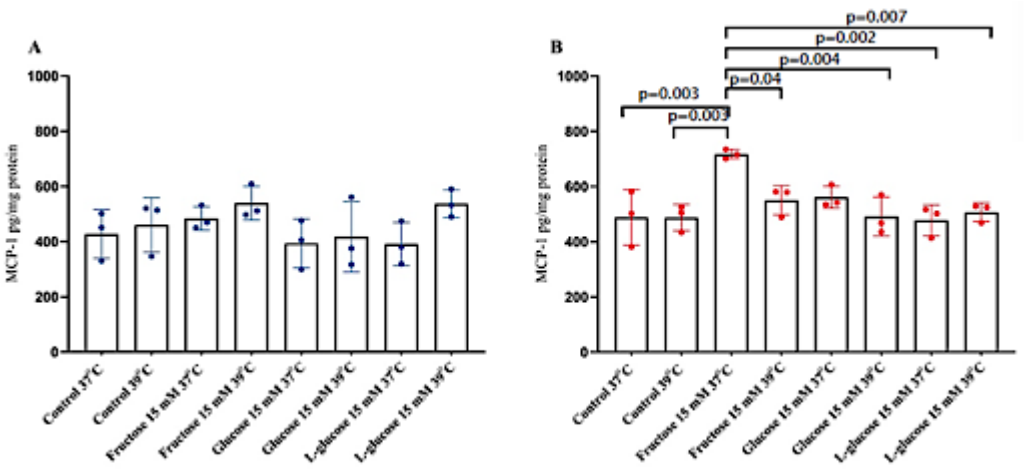

Figure 5.19: Effect of heat and elevated fructose and glucose on secreted MCP-1. Cells were exposed to control 5 mM Glucose at 37˚C (Control 37˚C), control 5 mM Glucose at 39˚C (Control 39˚C), 15 mM Fructose at 37˚C (Fructose 15 mM 37˚C), 15 mM Fructose at 39˚C (Fructose 15 mM 39˚C), 15 mM Glucose at 37 ˚C (Glucose 15 mM 37˚C), 15 mM Glucose at 39˚C (Glucose 15 mM 39˚C), 15 mM L-glucose at 37˚C (L-glucose 15 mM 37˚C) and 15 mM L-glucose at 39˚C (L-glucose 15 mM 39˚C) for **A.** 8 and **B.** 72 hours. Secretion levels were normalised to total cell protein. Three independent experiments with one sample per group. Two-way ANOVA, examining the effect of condition. Tukey's multiple comparisons test was used comparing each condition to each other condition. $p<0.05$ was considered statistically significant.

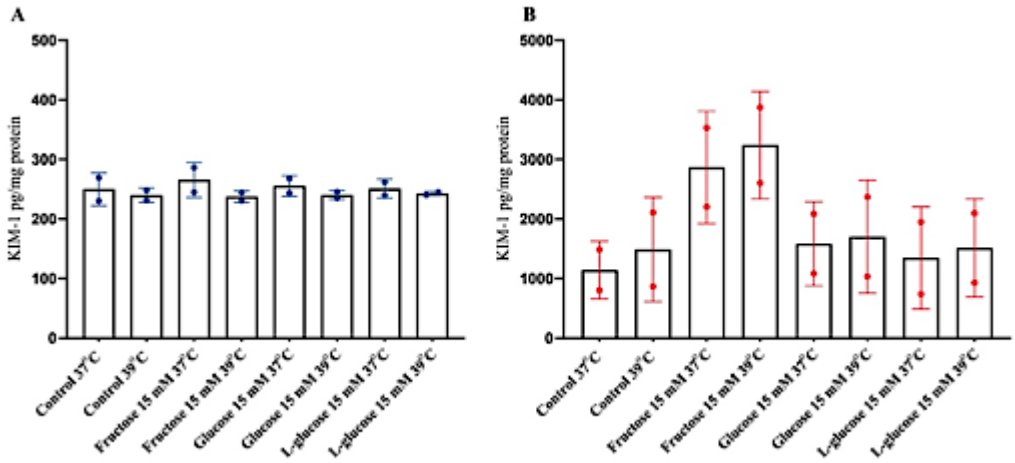

Figure 5.20: Effect of heat and elevated fructose and glucose on secreted KIM-1. Cells were exposed to control 5 mM Glucose at 37˚C (Control 37˚C), control 5 mM Glucose at 39˚C (Control 39˚C), 15 mM Fructose at 37˚C (Fructose 15 mM 37˚C), 15 mM Fructose at 39˚C (Fructose 15 mM 39˚C), 15 mM Glucose at 37 ˚C (Glucose 15 mM 37˚C), 15 mM Glucose at 39˚C (Glucose 15 mM 39˚C), 15 mM L-glucose at 37˚C (L-glucose 15 mM 37˚C) and 15 mM L-glucose at 39˚C (L-glucose 15 mM 39˚C) for **A.** 8 and **B.** 72 hours. Secretion levels were normalised to total cell protein. Two independent experiments with one sample per group. No statistical analysis was performed.

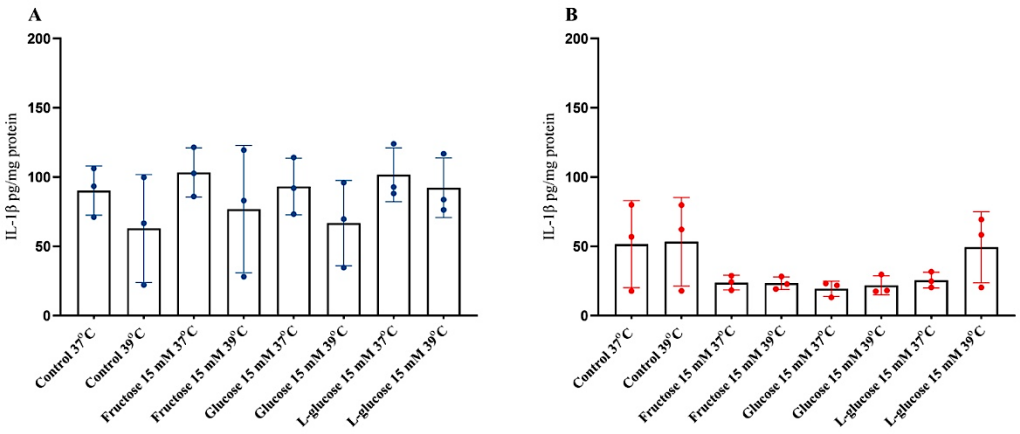

Figure 5.21: Effect of heat and elevated fructose and glucose on secreted IL-1β. Cells were exposed to control 5 mM Glucose at 37˚C (Control 37˚C), control 5 mM Glucose at 39˚C (Control 39˚C), 15 mM Fructose at 37˚C (Fructose 15 mM 37˚C), 15 mM Fructose at 39˚C (Fructose 15 mM 39˚C), 15 mM Glucose at 37 ˚C (Glucose 15 mM 37˚C), 15 mM Glucose at 39˚C (Glucose 15 mM 39˚C), 15 mM L-glucose at 37˚C (L-glucose 15 mM 37˚C) and 15 mM L-glucose at 39˚C (L-glucose 15 mM 39˚C) for **A.** 8 and **B.** 72 hours. Secretion levels were normalised to total cell proteins. Three independent experiments with one sample per group. Two-way ANOVA, examining the effect of condition. Tukey's multiple comparisons test was used comparing each condition to each other condition. $p<0.05$ was considered statistically significant. No Significance (NS) between conditions.

At 72 hours, levels of secreted MCP-1 (Figure 5.19B) did not change after combined exposure to heat and elevated fructose or glucose. Secreted levels of MCP-1 in the 15 mM fructose at 37˚C group, were higher than in the group exposed to both heat and 15 mM fructose. In addition, after 72 hours exposure to the different experimental treatments, secreted KIM-1 (Figure 5.20B) showed a trend to increase in the fructose groups at both temperatures. Overall, heat appeared to increase the rate of secreted KIM-1 in all the different experimental conditions. Finally, the levels of secreted IL-1β (Figure 5.21B) tended to decrease at both temperatures with fructose, glucose and L-glucose with the exception of 15 mM L-glucose at 39˚C that remained similar to its control levels.

5.2.3.3 The effect of heat and fructose on intracellular MCP-1protein expression

MCP-1 protein levels from one experiment at 72 hours, are shown in Figure 5.22. No statistical analysis was performed as only two independent experiments were technically successful and more than two are needed to perform statistical analysis.

Although no conclusions can be drawn from analysis of a single set of samples the data suggest that in the control, glucose and L-glucose groups, heat led to lower levels of MCP-1 protein compared to the same treatment at 37˚C but not in the fructose group where levels of expression looked similar.

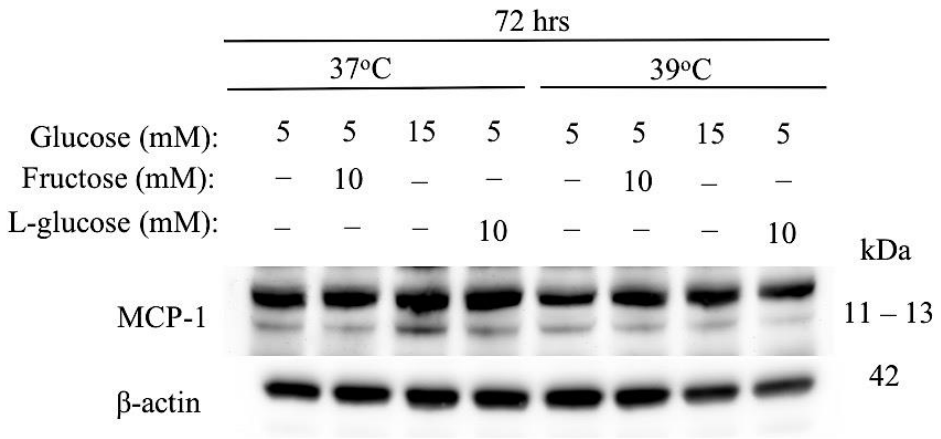

Figure 5.22: Effect of 72-hour heat and elevated fructose and glucose on cellular MCP-1 protein expression. Confluent quiescent HKC-8 cells were exposed to control 5 mM Glucose at 37˚C (Control 37˚C), control 5 mM Glucose at 39˚C (Control 39˚C), 15 mM Fructose at 37˚C (Fructose 15 mM 37˚C), 15 mM Fructose at 39˚C (Fructose 15 mM 39˚C), 15 mM Glucose at 37˚C (Glucose 15 mM 37˚C), 15 mM Glucose at 39˚C (Glucose 15 mM 39˚C), 15 mM L-glucose at 37˚C (L-glucose 15 mM 37˚C) and 15 mM L-glucose at 39˚C (L-glucose 15 mM 39˚C) for 72 hours. Western blots of cellular proteins (25ug protein/sample) were probed with an antibody to MCP-1 and re-probed after stripping of the membranes, with β-actin as a loading control.

5.3 Discussion

We conducted a range of experiments examining the effect of temperature, fructose/glucose (at varying concentrations) and increased osmolarity on HKC-8 cells for up to 72 hours. Taken together the results suggest: (i) exposure to heat and combined heat and high osmolality, increased MCP-1 mRNA expression with no changes in KIM-1. This is associated with an early pro-fibrotic response at 8 with increases in both TGF-β and COL1A1 mRNA. (ii) Exposure of cells to high levels of fructose (45 mM) increased expression of proinflammatory genes early on (8 hours) and increased expression of the profibrotic gene TGF-β, that resolves by 24 and remains low at 48 hours. However, the latter effect was seen with both D- and L-glucose suggesting this might be mediated by increased osmolarity rather than the increased fructose *per se*. (iii) Combined heat and fructose (15 mM) levels led to late KIM-1

mediated inflammatory response (72 hours) but reduced expression of profibrotic genes (Table 5.2). Increased secretion of MCP-1 was noticed when cells were exposed to fructose at control but not high temperatures.

By using $CoCl_2$ we demonstrated the cells maintain the capacity to respond to injurious stimuli for the duration of the experiments to ensure that in a case of injury we would be able to report it. Cobalt chloride induces cell hypoxia and upregulates the transcription factor hypoxia-inducible factor 1α (HIF1α) which binds within the promoter regions of its target genes to drive their expression (517). HIF1α has been shown to induce the expression of glycolytic enzymes such as LDHA consistent with this, increased LDHA was observed in our experiments where $CoCl_2$ was used as a positive control for cell injury (517). Interestingly, levels of MCP-1, KIM-1 and COL1A1 were dramatically decreased in the presence of $CoCl_2$ in our experiments. Hypoxia has been shown to reduce MCP-1 expression in rheumatoid arthritis synovial fibroblasts (RASF) suggesting chemokine expression is oxygen-dependent and that could explain the low expression of MCP-1 we observed in cells after exposure to $CoCl_2$ (518).

Comparing our results to published data examining the impact of heat in other experimental models we see some similarities but also contrasting results. Evidence from animal models demonstrate conflicting results. For example, when young pigs were exposed to heat stress (37˚C versus 25˚C control temperature) for 12 hours this resulted in increased expression of the nuclear factor Kappa-B (NF-κB) in skeletal muscle suggesting an inflammatory

Condition	MCP-1 mRNA Levels	MCP-1 Protein levels	MCP-1 Secreted	KIM-1 mRNA levels	KIM-1 Secreted	TGF-β mRNA levels	COL1A1 mRNA levels	IL-1β Secreted
8 hours								
39˚C	-	-	⇣	-	-	-	↑	-
39˚C + 25 mM Mannitol	↑	⇣	-	-	-	↑	↑	-
45 mM Fructose	↑	⇡	-	↑	⇡	↑	-	⇣
45 mM L-glucose	↑	⇡	↑	↑	⇡	↑	↑	⇣
39˚C +Fructose 15 mM	-	x	-	⇣	-	-	-	⇣
37˚C + L-glucose 15 mM	-	x	-	-	-	-	-	-
39˚C + L-glucose 15 mM	↑	x	-	-	-	-	-	-
24 hours								
39˚C	⇣	⇣	⇣	-	-	-	↑	⇣
39˚C + 25 mM Mannitol	⇣	⇣	⇣	-	-	-	-	⇣
45 mM Fructose	↑	x	-	-	-	-	⇣	⇣
45 mM L-glucose	↑	x	⇡	↑	⇡	-	⇣	⇣
48 hours								
39˚C	↓	-	-	-	⇡	↑	↑	-
39˚C + 25 mM Mannitol	-	-	-	-	-	↑	↑	-
45 mM Fructose	-	⇣	-	-	-	-	↓	⇡
45 mM L-glucose	-	⇣	↑	↑	⇡	-	↓	-
72 hours								
39˚C +Fructose 15 mM	⇣	⇣	↑	↑	⇡	↓	↓	⇣
37˚C + L-glucose 15 mM	-	⇡	-	-	-	-	-	⇣
39˚C + L-glucose 15 mM	-	⇣	-	-	-	-	-	-

Table 5.2: Summary of *in vitro* results. HKC-8 cells were exposed to heat (39˚C), high osmolality (39˚C + 25 mM Mannitol, 45 mM L-glucose and 39˚C + L-glucose 15 mM), elevated fructose (45 mM Fructose) and a combination of heat and fructose (39˚C +Fructose 15 mM) when compared with the control for each experimental procedure over time. MCP-1, KIM-1, TGF-β and COL1A1 mRNA levels, MCP-1, KIM-1 and IL-1β secreted protein levels. MCP-1 cellular protein expression was measured by RT-qPCR, ELISA and Western blotting, respectively. Significant increase (↑), significant decrease (↓) and a trend (2-fold changes or above were observed but not meeting formal statistical significance) of increase (⇡) and a trend (2-fold changes or above were observed but not meeting formal statistical significance) of decrease (⇣) compared to the control groups. No differences are indicated as (-), where levels were not measured this is indicated by (x).

response. NF-κB is a transcription factor which drives expression of various inflammatory and immunological genes including MCP-1 (519). However, in our study, exposure to heat (39˚C) did not seem to increase MCP-1 mRNA levels (although the combination of heat and mannitol did at 8 hours) in human proximal tubular epithelial cells. In the experiments on heat, mannitol alone was found to increase the expression of TGF-β after the first 8 hours. Heat stress has also been shown to induce the activity of a number of heat shock proteins (HSP) such as HSP70 (520). The expression of HSP72, responsible for the adaptation of medullary cells to osmotic stress by stabilising intracellular proteins, was not quantified in our study (521). Increased expression of HSPs might be expected to stabilise MCP-1 mRNA levels however we observed reduced expression of MCP-1 in response to heat with and without high osmolality at 24 hours (following an increase with heat and mannitol at 8 hours) (522). These differences and the differences observed over time, could be a result of the different cell type, cell death and/or absence of systemic responses. However, our results are consistent with experiments performed in other species. For example, cows in second lactation exposed to heat (28˚C) for 4 days after adaptation to thermoneutral conditions (15˚C) for 6 days, where intestinal inflammation and high expression of TGF-β was reported into the mucosa and submucosa of the jejunum (523).

In a recent study, PBMCs from healthy volunteers were isolated and converted to immature dendritic cells (DCs) and exposure of these DCs to fructose (5-25 mM for 24-72 hours) lead to increased secretion of IL-1β, IL-6 and TNF-α (524). IL-1β secretion increased at 24, 48 and 72 hours when exposed to 15 mM fructose. In contrast, in our study there was a trend to decrease levels of secreted IL-1β in response to 15 mM of fructose in both control and heat conditions. Reasons for the differences in findings include the storage of the samples prior to analysis and the possibility of differences in the differences between the different cell types. Studies in rats and the HK-2 cell line response to high fructose alone have found increased expression of MCP-1 mRNA levels (151,152,159). In our experiments, fructose (15 mM) at 37˚C led to increased MCP-1 mRNA expression and secretion at 72 hours of exposure, whereas fructose and heat together, held the expression of MCP-1 to control

levels. Conversely, exposure of HKC-8 cells to 45 mM fructose increased mRNA expression of MCP-1 at 8 and 24 hours. However, a similar increase was observed in cells exposed to 45 mM L-glucose, suggesting that the effect is potentially mediated by increased osmolality.

The structural similarity of L-glucose and glucose but not mannitol, could explain the differences in the responses seen between the osmolality control between the heat (Mannitol as an osmotic control) and elevated fructose (L-glucose as an osmotic control) experiments. L- glucose and mannitol do not interact with glucose carriers but their osmotic effect on the cells may well be different and is still not well understood (525). The increase of MCP-1 after a short exposure to mannitol might be predicted since it has been shown that in mesothelial cells high osmolarities induced with exposure to isosmotic medium (300 mOsm/kg H_2O), activate the transcription factor NFAT5 leading to increased MCP-1 mRNA levels at 4, 8 and 16 hours as well as increased secretion noted at 16 and 24 hours (526).

The expression of COL1A1 mRNA after exposure of cells to 45 mM fructose was unexpectedly decreased. The expression of COL1A1 is regulated by TGF-β1 and TGF-β mRNA expression might be expected to mirror that of COL1A1 mRNA (527). Consistent with this we observed lower TGF-β and COL1A1 after 72 hours in the fructose (15 mM) and glucose groups for both control and heat temperatures. There was a reduction in TGF-β and COL1A1 with high concentrations fructose and glucose after 72 hours at either 37˚C or 39˚C.

In mice models, rehydration with fructose under heat exposure (39.5˚C) induced high renal expression of IL-18 and NLRP3, leading to inflammation and renal injury suggesting that fructose can accelerate injury and the inflammatory response (528). Fructose (10% fructose-containing drinking water) rehydration was reported to enhance vasopressin levels activating the aldose reductase-fructokinase pathway in the kidneys and can exacerbate the effect that heat stress has on the cells with an increase in secreted MCP-1 (154). In our study, long-term (72 hours) heat in an environment of high fructose (15 mM) led to increased KIM-1 expression, and decreased TGF-β and COL1A1 mRNA whereas there were no changes 8 hours at either 37˚C or

39˚C. Despite the decrease in TGF-β and COL1A1 gene expression in response to fructose (72 hours) we did identify an increase in secreted MCP-1 with fructose at 37˚C but not at 39˚C. Suggesting high temperature ameliorated the inflammatory impact of fructose at 72 hours. The reasons for these divergent results are unclear. As mentioned earlier, exposure of HK-2 cells at fructose for 72 hours, led to increased secretion of MCP-1 in the cell culture media (151). Similar effect was observed in our study when HKC-8 cells were exposed to fructose in control temperature. The secreted and protein levels, but not the mRNA, of MCP-1, were increased in the fructose group at 37˚C. The low expression levels of MCP-1 could be explained through to high abundance of the expressed protein which leads to negative feedback on gene expression (529).

Our *in vitro* experiments have some limitations that should be discussed. Primarily, the absence of interactions with other cell types, even though it allows us to examine the individual cellular response to different conditions, it does not represent what will happen in the whole organism setting since the body will respond as a whole to external changes of the environment in cellular, hormonal and inflammatory levels. Fructose is primarily metabolised by the liver and the amount that eventually reaches the kidneys may vary between individuals. Primary cell lines could be an important tool in contrast with the immortalized cell line HKC-8 used in the present study, since they retain the morphological and functional characteristics of their tissue of origin and have high relevance *in vivo*. However, they are delicate to handle and have short life-span. The number of replicates we used for testing the secretion of cytokines and protein levels of MCP-1 were very low (through to low protein yield) and would need further experiments so more robust data can be generated. Investigation of a broader range of molecules involved in different pathways (such as caspase pathway and expression of heat shock proteins) would give a better understanding of cell response in the different experimental conditions. Similarly, the time-points studied (8, 24, 48 and 72 hours) might have missed chronic (e.g. between an hour or days of exposure) responses. On the other hand, the longer cells are grown the higher the chances are of changes in phenotype to occur through to hyper-confluency and accumulation

of metabolic products making it impossible to test exposure for more than 3 days since the initiation of an experiment requires a level of confluency to generate enough cells that can respond from early on in the new conditions.

These studies focused on the response of human proximal tubular epithelial cells to heat stress, high osmolality and high sugar individually or in combination however it is likely that the pathogenesis of MeN is multifactorial and it would be of interest to examine the effect of proposed exposures such as metals and pesticides in our cell model. More experiments are needed to verify the hypothesis of the effect that repetitive heat shock has in human cells line injury and fibrogenic response. A combined experiment of toxicants, heat and fructose would replicate very closely the spectrum of exposure that the population at risk of MeN may encounter (118).

5.4 Summary

Heat stress and exposure to high levels of fructose have been suggested to be mediators in the development of MeN (129,528,530). From our *in vitro* experiments, it appears that an increase in osmolality due to dehydration but not necessarily heat stress, may have an effect in fibrosis in a short time frame (acute). Prolonged exposure to heat at the cellular level, seems to increase inflammatory response but increased osmolality decreased the inflammatory response in the tubular cells. The effect of osmolality *in vivo* will vary from apical to basal surface so a different experimental model would be essential to fully understand these effects. Lastly, from our preliminary experiments, long term exposure to fructose and glucose appeared to downregulate fibrotic pathways suggesting that they play a limited role in the progression of kidney fibrosis. A more complex experimental model is needed to address systemic interactions.

Chapter 6: Discussion and future perspectives

6.1 Summary of findings

Across the globe, CKDu has emerged in at least two different geographical areas (Central America and South Asia) (63,531–533). The histopathological characteristics of the patients suggest that these may be a similar disease (59). Hypotheses are numerous, however differences between the areas, such as the differences in the use of pesticides and/or dietary exposures, can complicate investigations. Similarities such as environmental and occupational conditions also need to be considered. To date, aetiological studies have been mainly cross-sectional, a design which is prone to recall-bias and reverse-causation (57,58,119,129,440). The samples from the longitudinal cohort study used in this thesis allows us to follow the same population over time and characterise exposures at baseline (reducing recall-bias) and prior to the onset of kidney dysfunction (reducing the possibility of associations due to reverse-causation).

We looked for urine biomarkers of early disease (Chapter 3). The urine strip-test, is a quick and inexpensive tool to detect, along with other analytes, protein and blood in the urine indicating a possible glomerular dysfunction or injury. In our study, urine strip-test analysis of samples from visit 1 and visit 2 did not reveal associations of any of the analytes with the progression of kidney dysfunction. The absence of blood (haematuria) and albuminuria (<30 ACR) in our study is consistent with previous reports suggesting that CKDu is not a primary glomerular disorder (56). Simple urine strip-tests are therefore unlikely to be useful in identifying individuals in the early stages of the disease.

A positive strip-test for protein (15% of men and 18% of women at visit 1 and 17% of men and 18% of women at visit 2) and the parallel absence of albumin (only 5.4% of both men and women at visit 1, and 13% of both men and women at visit 2 had an ACR above 30 mg/g), prompted further investigation of the presence of small molecular weight proteins of tubular origin. Results from urine samples sent for proteomic analysis, showed the presence of B-2M, CD59, and Cys-C, suggesting that these may be potential biomarkers of

kidney injury. B-2M in urine is usually increased when tubular disease is present (388). CD59 is a major regulator of complement activity and is expressed in most human cells including kidney cells (534). Its main function is to bind to the C5-8 complex and inhibit the assembly of C9 monomers into the terminal complement thereby protecting cells from complement-mediated injury. CD59 has been reported to be associated with reduced kidney injury and atherosclerotic endothelial injury in experimental diabetes and it has been suggested that high urinary levels of CD59 lead to lower risk of ESRD by its binding to the C5–8 complex inhibiting in this way the assembly of C9 monomers into the terminal complement complex, protecting the cell from injury mediated from the complement (534–537). Cys-C, normally filtered by the glomerulus, is reabsorbed by the proximal tubules and increased urinary Cys-C has been shown a marker of renal tubular damage (538–541). Collagen degradation during tissue repair and remodelling and presence of collagen fragments in the urine have been shown to be mainly driven by metalloproteinase (MMP) activity and to provide a tool to assess kidney fibrosis in mice (542). Based on animal experiments, urinary collagen markers have been described as good diagnostic and predictive markers for renal fibrosis (543). To summarise, in a population at risk of MeN where biopsies are rarely taken particularly in the early stages, novel urinary biomarkers for the assessment of renal injury would be valuable. We identified low-molecular weight proteins in urine samples including Cys-C and B-2M (Appendices Table A1.3 and A1.4). These and other molecules need to be further explored on a population level to assess their sensitivity and specificity for predicting CKDu.

We also measured two low-molecular weight urinary proteins across the study population: NGAL and uRBP. NGAL is predominantly secreted by activated neutrophils but also by epithelial cells including kidney tubular cells in response to epithelial injury, inflammation or bacterial infection (544). NGAL is an early biomarker of AKI as a response to renal tubular damage and plays a protective role (545,546). NGAL has been used to access renal injury in patients at risk of developing kidney dysfunction (547). In our study, selected samples from men in the stable group (n=36) and all the samples of those with declining (n=25) or established (n=25) kidney function at visit 1 were tested for urinary

NGAL. Significantly elevated levels were detected at visit 1 in those with established kidney dysfunction, perhaps not unexpectedly given the impaired eGFR. The small increase in NGAL identified in the declining group means it is unlikely to have useful predictive power at the population level.

uRBP is one of the most commonly used biomarkers of tubular reabsorption and it is commonly measured in parallel with metal analysis in the urine (488). At visit 1, in both men and women there were no significant differences in uRBP between any of the groups. These results suggest uRBP is not useful for predicting the decline of kidney function. The slight rise in post-harvest levels (visit 2) of uRBP in men with established kidney dysfunction could indicate a sub-acute injury during the harvesting period for men with already damaged kidneys providing evidence that injury occurs during harvesting season.

Increased ACR (≥30 mg/g) was observed at visit 2 across a large percentage of the male participants (9.9%) and an even higher proportion in the established and declining groups. In women, ACR (≥30 mg/g) remained similar at visit 1 (4.6%) and visit 2 (3.8%). This suggests that during the harvest period (between visit 1 and visit 2), male but not female participants, might be exposed to factors that can increase the excretion of albumin in those with an underlying damage. Such factors could be heat stress or an increased workload. An alternative explanation might be an increased intake of dietary protein that workers consume to meet their energy needs and/or a change in diet to include more meat due to an increase in income as a result of employment.

Bilirubin levels were first tested by strip-test and then verified by an automated clinical biochemical analysis which showed levels 1-6 times higher than the normal levels of bilirubin in urine (3 µmol/L) in ~50% of all cases at each visit (most individuals positive at visit 1 were also positive at visit 2). In the absence of urobilinogen (which was normal for all cases at both visits), haemolytic or hepatocellular causes of bilirubinuria seem less likely (485). In urine, only the conjugated (water-soluble) forms of bilirubin with glucuronic or sulfuric acid can be detected (485). Increased delivery of conjugated bilirubin to the bowel

(biliary obstruction) or haemolytic anaemia could lead to increased urinary excretion which could be the case for our cohort because of gallstones, parasites and/ or a viral infection.

Non-alcoholic fatty liver disease (NAFLD) through dyslipidaemia, type II diabetes or insulin resistance is thought to be associated with the development of renal disease, metabolic syndrome and cardiovascular-related mortality (548–550). Due to the high positivity in urine bilirubin, liver function tests (ALT, AST, γ-GT and total bilirubin) were performed on serum samples of all participants at visit 1 and visit 2. Serum ALT, AST and γ-GT median levels were in normal ranges, although there were some exceptions of participants with levels even 4-fold higher than the upper reference levels (potentially reflecting high alcohol consumption (Table 3.1), NAFLD or infective hepatitis which was not quantified during this study). When each test was compared between the different kidney function groups in men no differences were observed. On the other hand, women in the declining group had higher serum ALT and AST levels when compared to the control at visit 1 which was not apparent at visit 2. Since the number of women in the declining group was very low (n=3), conclusions cannot be made and increase of serum liver function markers could be due to chance.

In this study we also tested the main proposed hypotheses that exposures to metals and metalloids, pesticides and mycotoxins could play a primary causative role in the development of MeN (357). Although urinary levels of Al, As, Mn and Si were above published reference values, there were no associations with decline of kidney function for either men or women (494–497,551). Reference values were acquired from UK, Belgian and North American populations and their relevance to central American populations is not clear. For our population (residing in a high activity volcanic area) these high levels of metals may be ‘normal’ and thus no resultant adverse effects may occur. These data also demonstrate higher levels of Al, As and Si at visit 1 (pre-harvest) versus visit 2 (post-harvest) in both men and women, suggesting individuals are being exposed to these metals from environmental rather than occupational sources. Reduced environmental exposure during the

harvesting period (dry season) could be explained due to decreased rain and water drainage coming from the highlands and the volcanic areas (water rich in these elements) to the lowlands (where people at risk live and work). Alternatively, there may be other sources (e.g. contaminated crops, volcanic ash) of exposure to elements and exposure through different routes such as inhalation and/or ingestion might lead to elevated urinary concentrations. Studies, investigating the contamination of food and water with these elements in the study areas, could give a clearer understanding regarding the source of exposure to these elements. In our study the group of men with the lower eGFR (established kidney dysfunction group), had higher mean As levels when compared to the stable and declining kidney function groups.

Studies have shown that accumulated metals in cortical and distal tubules (e.g. Cd) can be released and excreted in the urine after kidney injury (552). A similar mechanism could explain the increased levels of As in the urine in the established kidney dysfunction group since it has been shown that As accumulates in the proximal convoluted tubules, and any superimposed episodes of injury could potentially lead to its secretion (553). However, in health, arsenic is biomethylated, resulting in forms that are easily eliminating (such as dimethylarsinic acid (DDA) and methylarsonic acid (MMA)) but these were not quantified in our study. Negative control samples stored in the same tubes were tested to ensure there were no interfering substances, but As was not detected, suggesting that contamination during the collection procedure is unlikely. Another potential explanation could be overall decreased methylation capacity of As of the population. Similar to our study in Sri Lanka, high urinary levels of As have also been reported in endemic areas with obvious dermatological manifestations in CKDu patients (532). However, high levels of As in urine were found in both CKDu-endemic and non-endemic regions, suggesting a small role or no role in the development of CKDu (554).

Eight different polymorphisms in the human As (III) S-adenosylmethionine (SAM) methyltransferase (hAS3MT) gene have been found to lead to decreased catalytic activity thought to low affinity to arsenite or SAM and a lower stability, although only rs11191439 was included to our gene-array, it did

not show any association with the decline of kidney function (555). Together the above, the presence of heavy metals in the environment could play a contributory role in exacerbating already damaged kidneys but one should also consider the possibility that increased urinary metal concentrations could be a consequence of kidney injury from other causes as a result of release of accumulated metals from the kidney cells.

We also tested biosamples for agrochemicals. Non-occupational exposure to low doses of pesticides occurs widely from residues in food and/or contaminated air and water (506). Pesticides are widely used in Nicaragua to increase crop quality and yield and the country doubled its pesticide use between 2000 and 2004 (556). Unfortunately, there are no published quantitative data regarding the use of pesticides after that date to provide information more relevant to the study period. In the sugarcane industry, herbicides are the most widely used pesticides, and a mixture of 2,4-D, terbutryn, and ametryn is commonly used on the crops in the study area (498). Subsistence and plantation farmers use organophosphate and pyrethroid insecticides (included in this study), as well as paraquat (119,507). Detectable levels of 2,4-D, 3-PBA, DCCA, ETU, TEB-OH and TCP were found in the majority of samples tested from both the stable and the declining kidney function groups for men and women. Overall, the measured concentrations of urinary pesticide metabolites, suggest exposure to 2,4-D, chlorpyrifos of which TCP is a metabolite; and pyrethroids (such as cypermethrin of which 3-PBA and DCCA are metabolites). Although glyphosate has been proposed to play a role in the development of CKD, the urinary levels of excreted glyphosate, along with the rest of pesticides (4F3PBA, CFCA, MCPA, OH-PYM and 5-OH-TBZ) tested in the present study, were below the LOD in the majority of the samples and not different across the different kidney function groups making it unlikely to have an etiological role in the development of CKDu (508). In 1993, carbofuran and methmidophos poisoning was reported in farmers and children in Nicaragua (175). However, no further investigation was performed to assess a possible kidney injury. Neither carbofuran nor methamidophos is in use nowadays, though other pesticides and herbicides (e.g. paraquat and

terbufos) that are in use, were not measured in the present study so it is possible that we might have missed a potential causative agent.

Mycotoxins have also been implicated in CKD so we also performed assays to explore this exposure (335). Field workers live in the low-income communities of Nicaragua and lack many essentials including equipment to hygienically store food products (329–332). Additionally, Nicaragua is a tropical country with a mean temperature during the year of 20˚C and high humidity in the lowlands. High temperature and humidity are the ideal environment for fungi to develop. Fungi produce various mycotoxins as a secondary metabolite of biosynthetic pathways, of which some are pathogenic. Ochratoxin A (OTA) and citrinin (CIT), are nephrotoxins produced by fungi of the genera *Aspergillus* and *Penicillium* under conditions of high temperature and humidity. OTA has been implicated in chronic interstitial nephropathy in Tunisia and other North African countries (333,334). Our study is the first to test individuals at risk of MeN for mycotoxins. Results for both OTA and CIT show levels below the LOD. The absence of urinary OTA and CIT make it unlikely that these fungal toxins are a cause of MeN in Nicaragua. A study in Tunisia, found high prevalence of OTA in serum which were related to the development of CKD (334). Although, we did not measure levels of OTA and CIT in the serum of our study participants to directly compare our results, a study measuring blood and urine levels of OTA in a Bulgarian population found that from 100% of the blood samples containing OTA, 98% of these individuals excreted OTA in the urine (322). This suggests that if there were significantly high OTA and CIT serum levels in our population, we would have been able to detect it.

Genetic polymorphisms can play an important role in how the body responds to the environment and the potential toxicants (176). We tested the relationship between number of variants known to be implicated in metal and pesticide handling and decline in kidney function. We found little evidence using a Mendelian randomization method that the loci affecting xenobiotic levels impacted on outcomes in our population. This adds to the evidence against the tested xenobiotics being the cause of disease. However, there are a number of other variants (Table 2.6 and 2.7) that were not included in the initial

gene-array that require further investigation to comprehensively examine the effect of any potential genetic susceptibility to xenobiotic exposure.

Along with the data from the biosamples of the cohort study, the hypotheses that heat stress and elevated fructose or their combination causes kidney injury was explored using human kidney tubular epithelial cells (HKC-8). Workers are being exposed to extreme heat conditions for prolonged periods of time without adequate shade and water (129,131,132). Excessive exercise in high temperatures leads to dehydration, muscle breakdown and increased risk of AKI (126,133). Additionally, rehydration with high fructose beverages is common in the fields (as observed and reported during the cohort visits). Soft drinks containing high amounts of fructose increase the production of uric acid from fructose metabolism, which can stimulate an inflammatory response and lead to kidney injury (153).

In our study, exposure of HKC-8 cells to heat (39˚C) increased expression of mRNA of the fibrosis-related genes TGF-β and COL1A1. In contrast, experiments performed in male Wistar rats after muscular injury, have shown that in heat stress (42˚C for 20 minutes) levels of collagen deposition as measured by Masson's trichrome staining were maintained at control levels, whereas in cold stress (0˚C for 20 minutes) collagen deposition was increased (557). The differences could be explained by the fact that a considerably higher temperature was used, and/or the animal model has a complete physiological response to heat that can interact and mediate the effects of heat in the body. On the other hand, in mice, dehydration has been reported to increase the production of another type of collagen (collagen III) in the kidneys suggesting that dehydration through elevated temperature can result in fibrosis (127). Moreover, experiments in transgenic mice demonstrated that overexpression of heat shock protein 27 (HSP27) is thought to inhibit fibrotic pathways while HSP70, HSP90 and HSPB1 genes play an important role in regulating the proliferation of peripheral blood mononuclear cells (PBMCs) enhancing a protective response to the harmful effects of heat by reducing inflammation (558–560). Unfortunately, we did not explore the expression levels of HSPs.

Exposure of HKC-8 cells to high levels of fructose increased expression of mRNAs of the injury and inflammatory related genes KIM-1 and MCP-1. This observation is consistent with a number of studies. *In vitro*, exposure of HK-2 cells to fructose has been found to lead to increased secretion of MCP-1 from cells after 72 hours of exposure (159). Additionally, *in vivo* experiments on male C57BL/6J mice fed with elevated glucose and fructose showed increased hepatic inflammation with high levels of MCP-1, TLR4, IL-1β and TNF-α protein expression (561). In addition, hamsters fed a high fat–high fructose diet have increased expression of KIM-1 in renal tissue although high fat and high fructose were not tested separately (562).

Finally, long-term exposure (72 hours) of HKC-8 cells to heat and/or fructose (15 mM) levels (but not glucose) increased expression of KIM-1 (in both control and heat conditions) and suppressed expression of TGF-β and COL1A1 mRNA. This was not observed when cells were exposed for 48 hours to heat and fructose (45 mM) alone. Long-term (72 hours) exposure of human proximal tubular epithelial cells (PTEC) to glycated albumin (but not with high glucose) has been found to increase expression of KIM-1 (563). Expression of COL1A1 was dramatically decreased in both the fructose and glucose groups (at both temperatures) but the decrease was not so dramatic in the controls of osmolality. Similar were the findings after 48 hours of exposure to fructose and L-glucose alone. From the above, it can be seen that the longer the exposure to fructose lasts, the greater the decrease in COL1A1 expression levels suggesting an effect of high fructose and glucose.

To summarise, our *in vitro* experiments suggest an early injury response to fructose and a late unexpected downregulation of fibrotic pathways. This could potentially be a result either of adaptation, where the cell saves energy to spend in the production of other essential proteins to mediate the injury which occurs (as seen in increased expression of KIM-1) by gene silencing through DNA methylation in the promoter region of the TGF-β gene or the use of the wrong type of cells (interstitial fibroblasts) that would normally respond (564–566).

6.2 Strengths and limitations of the study

This study is the first to systematically analyse urine samples for various potential nephrotoxic factors before and after the sugarcane harvest using longitudinal outcome data in a Nicaraguan population at risk of developing MeN. Previous studies have generally used a cross-sectional design which is prone to reverse causation and makes it more challenging to draw conclusions regarding causation. Furthermore, the community-based nature of this study means the exposures in the entire at-risk population are assessed with lower risk of selection bias.

In addition, although our study is only moderately sized, the nature of the disease and its high prevalence in this population, means that the power to detect associations with the outcome (rapid decline in eGFR) is higher than many studies with a similar number of participants. Calculations of the effect size (Post-hoc) suggested it would be possible to detect associations of clinical importance (Chapter 2 Section 2.7). Clinically-relevant increases in urinary pesticide metabolites and OTA or CIT concentrations have not been defined, but it seems likely we would have detected important differences between the kidney function trajectory groups if there was a true association.

However, some further limitations of the present study should be noted. The small number of women did not allow us to associate strong findings in the declining group with the development of MeN. Additionally, difficulties were noted in the analysis of the urine samples that have been stored for a prolonged period at -80˚C in BA preservative. Storage in BA made it impossible to measure the pH in these samples as the function of BA is to maintain an acidic pH to avoid bacteria growth.

The analysis of only spot urine samples (when for some analytes, e.g. metals, whole blood may better reflect long term exposures) may mean a degree of misclassification of exposures through to differences in the dehydrated state of the person and the time passed since the latest uresis. This approach is likely to capture those with highest overall exposures, although it is possible that those with intermittent exposure may also be subject to misclassification.

A comprehensive assessment of time-accumulated metal/ pesticide exposure would require a biomonitoring over extended periods to assess retrospective exposures which were beyond the scope of this study. Additionally, it is possible that biosamples other than those examined might be better for some metals, for example hair or nails have been suggested as a biomatrix to assess chronic exposure (567). However, a recent study in an Asian population of various ages, found no correlation between metal (including Pb, As and Cd) intake and the corresponding levels in hair (568).

Additionally, the limited sample size of the cohort meant we were underpowered to investigate interactions between the measured exposures and other risk factors (e.g. xenobiotics and heat/dehydration) that might underlie the evolution of disease. Finally, budgetary constraints meant that the study could not investigate a wider panel of biomarkers in the urine of the whole cohort at both visit 1 and visit 2 that could potentially provide additional clues as to which biomarkers might be useful in detecting early disease. We also did not investigate all the possible pesticides used in the area and their metabolites missing in this was important causative data.

In vitro models allow us to examine closely the response of individual cell types to different exposures. The protein levels of COL1A1 were not quantified and it would be of high importance to be explored to understand a potential negative feedback that could result in its downregulation. However, the absence of interactions with other cell types as well as systemic responses may be crucial in determining response to injury thus the responses of tubular cells that we reported cannot necessarily be extrapolated.

6.3 Future work

The present study was limited in time and resources, meaning that not all potential metal, agrichemical or phytotoxin exposures could be investigated. Thus, future research to further explore the present tested hypotheses and others (e.g. the role infectious diseases play in AKI) would nonetheless add to current understanding.

By the end of this study, we hoped that we would be able to associate causal factors and the decline of kidney function. Additionally, we hoped to be able to identify biomarkers of early kidney injury related to the decline of kidney function which could be used as early diagnostic tools in individuals at risk of developing MeN. Although we showed that a range of serum and urine markers were not useful as biomarkers for the detection of early disease, pilot proteomic studies in urine identified a number of other urinary candidates, for example, Cys-C, that should be quantified in a larger number of samples. A range of other low molecular proteins were also detected in the urine of individuals with declining kidney function including B-2M, liver-type fatty acid–binding protein (L-FABP), KIM-1 and CD59 as well as degraded forms of collagen) that should also be further explored. More studies should include a broader panel of urinary markers tested and assess which combination can predict better the development of kidney dysfunction. This could be achieved by using low-cost, easy to perform in the field methods such as lateral flow assays (LFA) (584). That would not only help to understand the potential aetiology of the disease but it could help to apply preventative and protective for the individual strategies at the early stages of disease.

It would also be of interest to extend the work on exposure to metals and pesticides. Longitudinal biomonitoring studies collecting serial samples for estimation of metals and pesticides over a harvest season could give better estimation of overall exposure. For example, biological samples could be collected on Mondays (before exposure) and Fridays (after exposure). In this way not only occupational but environmental exposures could be assessed and distinguished. Finally, cohort studies in children following them in time would also give a better understanding of potential early life exposures that could induce kidney injury which would exacerbate with working in the fields.

The aetiology of MeN is potentially multifactorial with more than one exposure acting together in the development of the disease. Increasing the number of study men and women participants (>500) could not only increase the power of the study but also allow the investigation of synergistic effects (e.g. effect of

heavy metals and infections on kidney function when they co-exist) of multiple factors and control for confounders.

A potential genetic factor in MeN has arisen as a hypothesis as a result of appearance of the disease in individuals with a family history of the disease (49). Although this could be through exposure to the same environmental factors, further investigation of the association of genetic variants that could have a role in the development of MeN is needed. For example, variants in genes responsible for the elimination of xenobiotics or pesticides should be further examined (e.g. variants in AS3MT and MT1A genes). Similarly, studies exploring epigenetic changes (e.g. DNA modifications through methylation) and the environment of the individuals (using questionnaires including e.g. diet habits) could give the scientific community a better understanding of the environmental exposures to which the population are exposed.

The limitations of *in vitro* models have been previously discussed. A cell co-culture model could be an important next step to assess cell interactions and provide a better understanding regarding the fibrotic implications of heat, fructose and their combination. Similarly, *in vivo* studies in zebrafish (*Danio rerio)* have various advantages and could be found useful as opposed to experiments in mice. Zebrafish models are easy to maintain, they reproduce a large number of offsprings and share genetic and nephron structural and functional similarities to humans (569). A recent study by Babich et al. (2020), using the experimental model of zebrafish showed that water from endemic areas of Sri-Lanka (containing nephrotoxic compounds and heavy metals) can obstruct kidney development at early stages (570). Similar study designs in zebrafish, exploring the potential effect heat and fructose can have in combinations and alone, would give a better understanding of the role potential environmental and occupational factors have in the development of MeN/CKDu.

One of the leading hypotheses in the development of MeN, is repetitive heat stress and dehydration due to harsh occupational conditions (119). Further well-designed epidemiological cohort studies could give a better understanding as to if and what role heat and dehydration play in the

development of CKDu. Such studies would not only need detailed questionnaires, but also measurements (e.g. SG and colour of the urine) that would access the hydration state of the participants the days of collection. Finally, studies across countries with similar climates would give power as to if heat stress and dehydration could have a leading or just a contributory role in the development of MeN.

6.4 Conclusions

The study participants recruited were healthy and were followed up for a 2-year period with the aim to assess early environmental exposures or biomarkers of injury that could explain the aetiology of MeN. In our study, we did not identify biomarkers that could predict the development of kidney dysfunction but we gathered strong evidence against the metal and pesticides hypotheses. Regardless of whether early diagnostic markers or the cause can be found, it is clear that agricultural workers in these communities have the right to work in a humane environment with sufficient rest, adequate hydration, and access to clean water, as well as appropriate medical care. The working conditions in these areas are harsh and interventional studies that provide shade and water, have been found to benefit individuals by minimising the numbers of AKI episodes, although longer follow-up is needed to establish any protective effect in the development of MeN (571). The research community should continue to search for both early indicators of disease (that can also give a clue for the cause) and for the cause(s) as many questions are still unanswered. Findings will result in strong regulatory changes locally but also globally, to develop protective practices for agricultural workers and especially workers in developing countries who are the most neglected.

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Appendices

Appendix 1

Tables A1.1 and A1.2 summarise the strip-test results of different groups of people between visit 1 and visit 2. Groups tested included gender (male/female), age (below or above 25 years old), residential community (Figure 2.2), quality of well water participants were consuming based on analysed well water samples by Dr Jennifer Le Blond, Imperial College. The hardness of the water was assessed based on the magnesium and calcium concentrations.

Visit 1 (n=350)	Characteristic (n, %)		Leucocytes	Nitrates	Protein	Blood	Ketones	Bilirubin	Glucose
Gender	Male (n=263)	Negative	246 (93.5)	259 (98.5)	224 (85.2)	214 (81.4)	262 (99.6)	140 (53.2)	263 (100.0)
		Trace	5 (1.9)	0 (0.0)	25 (9.5)	30 (11.4)	1 (0.4)	85 (32.3)	0 (0.0)
		Positive	12 (4.6)	4 (1.5)	14 (5.3)	19 (7.2)	0 (0.0)	38 (14.4)	0 (0.0)
	Female (n=87)	Negative	84 (96.6)	87 (100.0)	70 (80.5)	70 (80.5)	87 (100.0)	36 (41.4)	87 (100.0)
		Trace	2 (2.3)	0 (0.0)	13 (14.9)	8 (9.2)	0 (0.0)	34 (39.1)	0 (0.0)
		Positive	1 (1.1)	0 (0.0)	4 (4.6)	9 (10.3)	0 (0.0)	17 (19.5)	0 (0.0)
Age	<25 (n=219)	Negative	206 (94.7)	215 (98.2)	182 (83.1)	178 (81.3)	219 (100.0)	113 (51.6)	219 (100.0)
		Trace	5 (2.3)	0 (0.0)	25 (11.4)	25 (11.4)	0 (0.0)	71 (32.4)	0 (0.0)
		Positive	8 (3.7)	4 (1.8)	12 (5.5)	16 (7.3)	0 (0.0)	35 (16.0)	0 (0.0)
	≥25 (n=131)	Negative	124 (94.7)	131 (100.0)	112 (85.5)	106 (80.9)	130 (99.2)	63 (48.1)	131 (100.0)
		Trace	2 (1.5)	0 (0.0)	13 (9.9)	13 (9.9)	1 (0.8)	48 (36.6)	0 (0.0)
		Positive	5 (3.8)	0 (0.0)	6 (4.6)	12 (9.2)	0 (0.0)	20 (15.3)	0 (0.0)
Community	1 (n=38)	Negative	37 (97.4)	37 (97.4)	33 (86.8)	32 (84.2)	38 (100.0)	21 (55.3)	38 (100.0)
		Trace	0 (0.0)	0 (0.0)	3 (7.9)	2 (5.3)	0 (0.0)	13 (34.2)	0 (0.0)
		Positive	1 (2.6)	1 (2.6)	2 (5.3)	4 (10.5)	0 (0.0)	4 (10.5)	0 (0.0)
	2 (n=45)	Negative	41 (91.1)	44 (97.8)	39 (86.7)	35 (77.8)	45 (100.0)	17 (37.8)	45 (100.0)
		Trace	1 (2.2)	0 (0.0)	5 (11.1)	5 (11.1)	0 (0.0)	17 (37.8)	0 (0.0)
		Positive	0 (0.0)	1 (2.2)	1 (2.2)	5 (11.1)	0 (0.0)	11 (24.4)	0 (0.0)
	3 (n=37)	Negative	36 (97.3)	37 (100.0)	27 (73.0)	31 (83.8)	37 (100.0)	22 (59.5)	37 (100.0)
		Trace	0 (0.0)	0 (0.0)	6 (16.2)	2 (5.4)	0 (0.0)	10 (27.0)	0 (0.0)
		Positive	1 (2.7)	0 (0.0)	4 (10.8)	4 (10.8)	0 (0.0)	5 (13.5)	0 (0.0)
	4 (n=35)	Negative	35 (100.0)	35 (100.0)	33 (94.3)	27 (77.1)	35 (100.0)	20 (57.1)	35 (100.0)
		Trace	0 (0.0)	0 (0.0)	1 (2.9)	4 (11.4)	0 (0.0)	9 (25.7)	0 (0.0)
		Positive	0 (0.0)	0 (0.0)	1 (2.9)	4 (11.4)	0 (0.0)	6 (17.1)	0 (0.0)
	5 (n=26)	Negative	23 (88.5)	25 (96.2)	23 (88.5)	22 (84.6)	26 (100.0)	10 (38.5)	26 (100.0)
		Trace	1 (1.9)	0 (0.0)	2 (7.7)	3 (11.5)	0 (0.0)	12 (46.2)	0 (0.0)
		Positive	2 (3.8)	1 (3.8)	1 (3.8)	1 (3.8)	0 (0.0)	4 (15.4)	0 (0.0)
	6 (n=53)	Negative	50 (94.3)	53 (100.0)	42 (79.2)	43 (81.1)	52 (98.1)	31 (58.5)	52 (98.1)
		Trace	1 (1.9)	0 (0.0)	8 (15.1)	5 (9.4)	1 (1.9)	19 (35.8)	1 (1.9)
		Positive	2 (3.8)	0 (0.0)	3 (5.7)	5 (9.4)	0 (0.0)	3 (5.7)	0 (0.0)
	7 (n=31)	Negative	31 (100.0)	31 (100.0)	26 (83.9)	23 (74.2)	31 (100.0)	16 (51.6)	31 (100.0)
		Trace	0 (0.0)	0 (0.0)	4 (12.9)	6 (19.4)	0 (0.0)	9 (29.0)	0 (0.0)
		Positive	0 (0.0)	0 (0.0)	1 (3.2)	2 (6.5)	0 (0.0)	6 (19.4)	0 (0.0)
	8 (n=40)	Negative	35 (87.5)	40 (100.0)	33 (82.5)	35 (87.5)	40 (100.0)	14 (35.0)	40 (100.0)
		Trace	3 (7.5)	0 (0.0)	6 (15.0)	3 (7.5)	0 (0.0)	19 (47.5)	0 (0.0)

		Positive	2 (5.0)	0 (0.0)	1 (2.5)	2 (5.0)	0 (0.0)	7 (17.5)	0 (0.0)
	9 (n=45)	Negative	42 (93.3)	44 (97.8)	38 (84.4)	36 (80.0)	45 (100.0)	25 (55.6)	45 (100.0)
		Trace	1 (2.2)	0 (0.0)	3 (6.7)	8 (17.8)	0 (0.0)	11 (24.4)	0 (0.0)
		Positive	2 (4.4)	1 (2.2)	4 (8.9)	1 (2.2)	0 (0.0)	9 (20.0)	0 (0.0)
Hardness of well water	Moderately hard (n=97)	Negative	96 (99.0)	97 (100.0)	78 (80.4)	76 (78.4)	97 (100.0)	55 (56.7)	97 (100.0)
		Trace	1 (1.0)	0 (0.0)	13 (13.4)	11 (11.3)	0 (0.0)	26 (26.8)	0 (0.0)
		Positive	0 (0.0)	0 (0.0)	6 (6.2)	10 (10.3)	0 (0.0)	16 (16.5)	0 (0.0)
	Hard (160)	Negative	148 (92.5)	158 (98.8)	137 (85.6)	135 (84.4)	160 (100.0)	77 (48.1)	160 (100.0)
		Trace	5 (3.1)	0 (0.0)	16 (10.0)	14 (8.8)	0 (0.0)	64 (40.0)	0 (0.0)
		Positive	7 (4.4)	2 (1.3)	7 (4.4)	11 (6.9)	0 (0.0)	19 (11.9)	0 (0.0)
	Very hard (n=93)	Negative	86 (92.4)	91 (97.8)	79 (84.9)	73 (78.5)	92 (98.9)	44 (47.3)	93 (100.0)
		Trace	2 (2.2)	0 (0.0)	9 (9.7)	13 (14.0)	1 (1.1)	29 (31.2)	0 (0.0)
		Positive	5 (5.4)	2 (2.2)	5 (5.4)	7 (7.5)	0 (0.0)	20 (21.5)	0 (0.0)
Occupation	Only sugarcane (n=55)	Negative	49 (89.1)	54 (98.2)	46 (83.6)	48 (87.3)	55 (100.0)	24 (43.6)	55 (100.0)
		Trace	4 (7.3)	0 (0.0)	6 (10.9)	5 (9.1)	0 (0.0)	23 (41.8)	0 (0.0)
		Positive	2 (3.6)	1 (1.8)	3 (5.5)	2 (3.6)	0 (0.0)	8 (14.5)	0 (0.0)
	Only agriculture (n=138)	Negative	129 (93.5)	135 (97.8)	116 (84.1)	111 (80.4)	137 (99.3)	76 (55.1)	138 (100.0)
		Trace	2 (1.4)	0 (0.0)	15 (10.8)	15 (10.9)	1 (0.7)	41 (29.7)	0 (0.0)
		Positive	7 (5.1)	3 (2.2)	7 (5.1)	12 (8.7)	0 (0.0)	21 (15.2)	0 (0.0)
	Other occupations (n=157)	Negative	152 (96.8)	157 (100.0)	132 (84.1)	125 (79.6)	157 (100.0)	76 (48.4)	157 (100.0)
		Trace	1 (0.7)	0 (0.0)	17 (10.8)	18 (11.5)	0 (0.0)	55 (35.0)	0 (0.0)
		Positive	4 (2.5)	0 (0.0)	8 (5.1)	14 (8.9)	0 (0.0)	26 (16.6)	0 (0.0)

Table A1.1: Frequencies of different strip-test characteristics among people from different gender, community, consumed water and occupations at visit 1.

Visit 2 (n=291)	Characteristic (n, %)		Leucocytes	Nitrates	Protein	Blood	Ketones	Bilirubin	Glucose
Gender	Male (n=213)	Negative	206 (96.7)	211 (99.1)	175 (82.2)	186 (87.3)	213 (100.0)	100 (46.9)	213 (100.0)
		Trace	7 (3.3)	0 (0.0)	26 (12.2)	20 (9.4)	0 (0.0)	113 (53.1)	0 (0.0)
		Positive	0 (0.0)	2 (0.9)	12 (5.6)	7 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)
	Female (n=78)	Negative	68 (87.2)	74 (94.9)	63 (80.8)	35 (44.9)	78 (100.0)	38 (48.7)	78 (100.0)
		Trace	2 (2.6)	0 (0.0)	11 (14.1)	14 (17.9)	0 (0.0)	23 (29.5)	0 (0.0)
		Positive	8 (10.3)	4 (5.1)	4 (5.1)	29 (37.2)	0 (0.0)	17 (21.8)	0 (0.0)
Age	<25 (n=182)	Negative	172 (94.5)	178 (97.8)	145 (79.6)	145 (79.6)	182 (100.0)	83 (45.9)	182 (100.0)
		Trace	4 (2.2)	0 (0.0)	27 (14.9)	18 (9.9)	0 (0.0)	89 (48.6)	0 (0.0)
		Positive	6 (3.3)	4 (2.2)	10 (5.5)	19 (10.5)	0 (0.0)	10 (5.5)	0 (0.0)
	≥25 (n=109)	Negative	102 (93.6)	107 (98.2)	93 (85.5)	76 (70.0)	109 (100.0)	55 (50.0)	109 (100.0)
		Trace	5 (4.5)	0 (0.0)	10 (9.1)	16 (14.5)	0 (0.0)	47 (43.6)	0 (0.0)
		Positive	2 (1.8)	2 (1.8)	6 (5.5)	17 (15.5)	0 (0.0)	7 (6.4)	0 (0.0)
Community	1 (n=27)	Negative	26 (96.3)	27 (100.0)	21 (77.8)	23 (85.2)	27 (100.0)	12 (44.4)	27 (100.0)
		Trace	1 (3.7)	0 (0.0)	6 (22.2)	3 (11.1)	0 (0.0)	13 (48.1)	0 (0.0)
		Positive	0 (0.0)	0 (0.0)		1 (3.7)	0 (0.0)	2 (7.4)	0 (0.0)
	2 (n=41)	Negative	39 (95.2)	41 (100.0)	35 (85.4)	27 (65.9)	41 (100.0)	17 (41.5)	41 (100.0)
		Trace	1 (2.4)	0 (0.0)	4 (9.8)	8 (19.5)	0 (0.0)	22 (53.7)	0 (0.0)
		Positive	1 (2.4)	0 (0.0)	2 (4.9)	6 (14.6)	0 (0.0)	2 (4.9)	0 (0.0)
	3 (n=29)	Negative	25 (86.2)	28 (96.6)	23 (79.3)	21 (72.4)	29 (100.0)	13 (44.8)	29 (100.0)
		Trace	2 (6.9)	0 (0.0)	4 (13.8)	4 (13.8)	0 (0.0)	14 (48.3)	0 (0.0)
		Positive	2 (6.9)	1 (3.4)	2 (6.9)	4 (13.8)	0 (0.0)	2 (6.9)	0 (0.0)
	4 (n=32)	Negative	31 (96.9)	29 (90.6)	26 (81.2)	26 (81.3)	34 (100.0)	9 (28.1)	34 (100.0)
		Trace	0 (0.0)	0 (0.0)	3 (9.4)	4 (12.5)	0 (0.0)	20 (62.5)	0 (0.0)
		Positive	1 (3.1)	3 (9.4)	3 (9.4)	2 (6.3)	0 (0.0)	3 (9.4)	0 (0.0)
	5 (n=18)	Negative	17 (94.4)	18 (100.0)	10 (55.6)	13 (72.2)	18 (100.0)	6 (33.3)	18 (100.0)
		Trace	0 (0.0)	0 (0.0)	4 (22.2)	1 (5.6)	0 (0.0)	10 (55.6)	0 (0.0)
		Positive	1 (5.6)	0 (0.0)	4 (22.2)	4 (22.2)	0 (0.0)	2 (11.1)	0 (0.0)
	6 (n=50)	Negative	47 (94.0)	50 (100.0)	45 (90.0)	40 (80.0)	50 (100.0)	31 (62.0)	50 (100.0)
		Trace	1 (2.0)	0 (0.0)	4 (8.0)	6 (12.0)	0 (0.0)	19 (38.0)	0 (0.0)
		Positive	2 (4.0)	0 (0.0)	1 (2.0)	4 (8.0)	0 (0.0)	0 (0.0)	0 (0.0)
	7 (n=27)	Negative	26 (96.3)	27 (100.0)	23 (85.2)	20 (74.1)	27 (100.0)	16 (59.3)	27 (100.0)
		Trace	1 (3.7)	0 (0.0)	3 (11.1)	3 (11.1)	0 (0.0)	8 (29.6)	0 (0.0)
		Positive	0 (0.0)	0 (0.0)	1 (3.7)	4 (14.8)	0 (0.0)	3 (11.1)	0 (0.0)
	8 (n=38)	Negative	35 (92.1)	36 (94.7)	29 (76.3)	29 (76.3)	38 (100.0)	19 (50.0)	38 (100.0)
		Trace	2 (5.3)	0 (0.0)	7 (18.4)	2 (5.3)	0 (0.0)	16 (42.1)	0 (0.0)
		Positive	1 (2.6)	2 (5.3)	2 (5.3)	7 (18.4)	0 (0.0)	3 (7.9)	0 (0.0)
	9 (n=29)	Negative	28 (96.6)	29 (100.0)	26 (89.7)	22 (75.9)	29 (100.0)	15 (51.7)	29 (100.0)
		Trace	1 (3.4)	0 (0.0)	2 (6.9)	3 (10.3)	0 (0.0)	14 (48.3)	0 (0.0)
		Positive	0 (0.0)	0 (0.0)	1 (3.4)	4 (13.8)	0 (0.0)	0 (0.0)	0 (0.0)
Hardness of well water	Moderately hard (n=81)	Negative	77 (95.0)	80 (98.8)	68 (84.0)	60 (74.1)	81 (100.0)	37 (45.7)	81 (100.0)

		Trace	2 (2.5)	0 (0.0)	7 (8.6)	11 (13.6)	0 (0.0)	37 (45.7)	0 (0.0)
		Positive	2 (2.5)	1 (1.2)	6 (7.4)	10 (12.3)	0 (0.0)	7 (8.6)	0 (0.0)
	Hard (137)	Negative	128 (93.4)	132 (96.4)	107 (78.1)	110 (80.3)	137 (100.0)	69 (50.4)	137 (100.0)
		Trace	5 (3.6)	0 (0.0)	23 (16.8)	12 (8.8)	0 (0.0)	61 (44.5)	0 (0.0)
		Positive	4 (2.9)	5 (3.6)	7 (5.1)	15 (10.9)	0 (0.0)	7 (5.1)	0 (0.0)
	Very hard (n=73)	Negative	69 (94.6)	73 (100.0)	63 (86.3)	51 (69.8)	73 (100.0)	32 (43.8)	73 (100.0)
		Trace	2 (2.7)	0 (0.0)	7 (9.6)	11 (15.1)	0 (0.0)	38 (52.1)	0 (0.0)
		Positive	2 (2.7)	0 (0.0)	3 (4.1)	11 (15.1)	0 (0.0)	3 (4.1)	0 (0.0)
Occupation	Only sugarcane (n=113)	Negative	110 (97.3)	113 (100.0)	89 (78.8)	99 (87.6)	113 (100.0)	55 (48.7)	113 (100.0)
		Trace	2 (1.8)	0 (0.0)	20 (17.7)	8 (7.1)	0 (0.0)	57 (50.4)	0 (0.0)
		Positive	1 (0.9)	0 (0.0)	4 (3.5)	6 (5.3)	0 (0.0)	1 (0.9)	0 (0.0)
	Only agriculture (n=60)	Negative	56 (93.3)	60 (100.0)	50 (83.2)	46 (76.7)	60 (100.0)	23 (38.4)	60 (100.0)
		Trace	3 (5.0)	0 (0.0)	5 (8.4)	13 (21.7)	0 (0.0)	35 (58.3)	0 (0.0)
		Positive	1 (1.7)	0 (0.0)	5 (8.4)	1 (1.6)	0 (0.0)	2 (3.3)	0 (0.0)
	Banana (n=18)	Negative	16 (88.9)	18 (100.0)	16 (88.9)	16 (88.8)	18 (100.0)	10 (55.6)	18 (100.0)
		Trace	2 (11.1)	0 (0.0)	2 (11.1)	1 (5.6)	0 (0.0)	8 (44.4)	0 (0.0)
		Positive	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)
	Other occupations (n=100)	Negative	92 (92.0)	94 (94.0)	83 (83.0)	60 (60.0)	100 (100.0)	50 (50.0)	100 (100.0)
		Trace	2 (2.0)	0 (0.0)	10 (10.0)	12 (12.0)	0 (0.0)	36 (36.0)	0 (0.0)
		Positive	6 (6.0)	6 (6.0)	7 (7.0)	28 (28.0)	0 (0.0)	14 (14.0)	0 (0.0)

Table A1.2: Frequencies of different strip-test characteristics among people from different gender, community, consumed water and occupations at visit 2.

A number of urine samples with a positive protein strip-test and negative for albumin after bromocresol green quantification, were run on a 20% SDS-Polyacrylamide gel followed by Silver staining. The sections of the gel containing lower molecular weight proteins (<25 kDa) were excised and sent to the Advanced Mass Spectrometry Facility, School of Biosciences, University of Birmingham. Table A1.3 and A1.4 show the results of the analysis for both samples with the following abbreviations:

Description: UniprotKB protein description. Provides the name of the protein exclusive of the identifier that appears in the Accession column.
Score: The protein score, is the sum of the scores of the individual peptides. SEQUEST search algorithm was used, for which the score is the sum of all peptide Xcorr values above the specified score threshold. The Proteome Discoverer application uses only the highest scored peptide. When it performs a search using dynamic modifications, one spectrum might have multiple matches because of permutations of the modification site.
Coverage: The percent coverage calculated by dividing the number of amino acids in all found peptides by the total number of amino acids in the entire protein sequence (the higher the better).
Proteins: The number of identified proteins in the protein group of a master protein. Proteins are grouped based on sequence homology and/or isoforms.
Unique peptides: The number of peptide sequences unique to a protein group.
Peptides: The number of distinct peptide sequences in the protein group.
PSMs: The total number of identified peptide sequences (peptide spectrum matches) for the protein, including those redundantly identified.
AAs, MW [kDa], calc. pI: The calculated parameters of the protein based on the amino acid sequence in the FASTA database used to generate the report. The Proteome Discoverer application calculates the molecular weight without considering post-translational modifications.

Description	Score	Coverage	Proteins	Unique Peptides	Peptides	PSMs	AAs	MW (kDa)	Calc. pI
Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	49.29	23.60	1	11	13	16	644	66.0	8.12
Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	39.94	25.51	16	12	15	15	584	58.8	5.21
Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	35.77	13.32	1	8	8	13	623	62.0	5.24
Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2 - [ALBU_HUMAN]	34.64	19.05	1	11	11	13	609	69.3	6.28
Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	29.87	15.34	4	6	10	10	639	65.4	8.00
Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	29.06	23.04	18	4	9	10	473	51.2	5.05
Keratin, type II cytoskeletal 6C OS=Homo sapiens GN=KRT6C PE=1 SV=3 - [K2C6C_HUMAN]	28.18	21.99	7	7	10	10	564	60.0	8.00
Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	21.42	18.01	18	2	8	8	472	51.5	5.16
Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	19.66	14.07	2	5	7	7	590	62.3	7.74
CD59 glycoprotein OS=Homo sapiens GN=CD59 PE=1 SV=1 - [CD59_HUMAN]	17.11	25.00	1	3	3	5	128	14.2	6.48
Beta-2-microglobulin OS=Homo sapiens GN=B2M PE=1 SV=1 - [B2MG_HUMAN]	16.38	42.86	1	5	5	7	119	13.7	6.52
Protein AMBP OS=Homo sapiens GN=AMBP PE=1 SV=1 - [AMBP_HUMAN]	13.16	18.75	1	5	5	7	352	39.0	6.25
Immunoglobulin superfamily member 8 OS=Homo sapiens GN=IGSF8 PE=1 SV=1 - [IGSF8_HUMAN]	12.25	8.97	1	4	4	4	613	65.0	8.00
Non-secretory ribonuclease OS=Homo sapiens GN=RNASE2 PE=1 SV=2 - [RNAS2_HUMAN]	10.65	19.88	1	3	3	3	161	18.3	8.73
Keratin, type I cytoskeletal 17 OS=Homo sapiens GN=KRT17 PE=1 SV=2 - [K1C17_HUMAN]	10.42	8.80	12	1	4	4	432	48.1	5.02
Hemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=2 - [HBB_HUMAN]	9.41	23.81	5	3	3	3	147	16.0	7.28
Cystatin-M OS=Homo sapiens GN=CST6 PE=1 SV=1 - [CYTM_HUMAN]	8.98	14.09	1	1	1	2	149	16.5	8.09
Hemoglobin subunit alpha OS=Homo sapiens GN=HBA1 PE=1 SV=2 - [HBA_HUMAN]	8.98	16.90	1	2	2	3	142	15.2	8.68
Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1 - [CYTA_HUMAN]	8.17	39.80	1	3	3	3	98	11.0	5.50
Lithostathine-1-alpha OS=Homo sapiens GN=REG1A PE=1 SV=3 - [REG1A_HUMAN]	7.81	20.48	2	3	3	3	166	18.7	5.94
Procollagen C-endopeptidase enhancer 1 OS=Homo sapiens GN=PCOLCE PE=1 SV=2 - [PCOC1_HUMAN]	7.52	7.80	1	3	3	3	449	47.9	7.43
Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1 - [IGHG1_HUMAN]	6.22	7.88	4	2	2	3	330	36.1	8.19
Secreted and transmembrane protein 1 OS=Homo sapiens GN=SECTM1 PE=1 SV=2 - [SCTM1_HUMAN]	6.21	7.66	1	1	1	2	248	27.0	7.43

Agrin OS=Homo sapiens GN=AGRN PE=1 SV=5 - [AGRIN_HUMAN]	6.04	1.26	1	2	2	2	2067	217.1	6.39
Collagen alpha-3(VI) chain OS=Homo sapiens GN=COL6A3 PE=1 SV=5 - [CO6A3_HUMAN]	5.74	0.79	1	2	2	2	3177	343.5	6.68
Plasminogen OS=Homo sapiens GN=PLG PE=1 SV=2 - [PLMN_HUMAN]	5.46	3.46	1	2	2	2	810	90.5	7.24
Fibrillin-1 OS=Homo sapiens GN=FBN1 PE=1 SV=3 - [FBN1_HUMAN]	5.26	0.70	1	2	2	2	2871	312.0	4.93
Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1 - [IGKC_HUMAN]	4.89	18.87	1	1	1	1	106	11.6	5.87
Lipocalin-1 OS=Homo sapiens GN=LCN1 PE=1 SV=1 - [LCN1_HUMAN]	4.86	12.50	2	2	2	2	176	19.2	5.58
SH3 domain-binding glutamic acid-rich-like protein 3 OS=Homo sapiens GN=SH3BGRL3 PE=1 SV=1 - [SH3L3_HUMAN]	4.17	16.13	1	1	1	1	93	10.4	4.93
Peptidoglycan recognition protein 1 OS=Homo sapiens GN=PGLYRP1 PE=1 SV=1 - [PGRP1_HUMAN]	4.10	8.16	1	1	1	1	196	21.7	8.59
Ig kappa chain V-III region SIE OS=Homo sapiens PE=1 SV=1 - [KV302_HUMAN]	3.81	16.51	4	1	1	1	109	11.8	8.48
Protein shisa-5 OS=Homo sapiens GN=SHISA5 PE=1 SV=1 - [SHSA5_HUMAN]	3.62	2.92	1	1	1	2	240	25.6	6.68
Lysosomal protective protein OS=Homo sapiens GN=CTSA PE=1 SV=2 - [PPGB_HUMAN]	3.54	2.71	1	1	1	1	480	54.4	6.61
Resistin OS=Homo sapiens GN=RETN PE=1 SV=1 - [RETN_HUMAN]	3.49	13.89	1	1	1	1	108	11.4	6.86
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Homo sapiens GN=HSPG2 PE=1 SV=4 - [PGBM_HUMAN]	3.37	0.32	1	1	1	1	4391	468.5	6.51
Ly-6/neurotoxin-like protein 1 OS=Homo sapiens GN=LYNX1 PE=1 SV=3 - [LYNX1_HUMAN]	3.29	9.92	1	1	1	1	131	14.0	6.71
Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3 - [G3P_HUMAN]	3.20	4.18	1	1	1	1	335	36.0	8.46
Inactive caspase-12 OS=Homo sapiens GN=CASP12 PE=2 SV=2 - [CASPC_HUMAN]	3.16	6.16	1	1	1	1	341	38.8	6.02
Uromodulin OS=Homo sapiens GN=UMOD PE=1 SV=1 - [UROM_HUMAN]	3.10	2.50	1	1	1	1	640	69.7	5.24
Superoxide dismutase [Cu-Zn] OS=Homo sapiens GN=SOD1 PE=1 SV=2 - [SODC_HUMAN]	3.09	9.09	1	1	1	1	154	15.9	6.13
Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1 PE=1 SV=2 - [ZA2G_HUMAN]	2.99	3.36	1	1	1	1	298	34.2	6.05
Mannan-binding lectin serine protease 2 OS=Homo sapiens GN=MASP2 PE=1 SV=4 - [MASP2_HUMAN]	2.99	2.33	1	1	1	1	686	75.7	5.63
Leucine-rich alpha-2-glycoprotein OS=Homo sapiens GN=LRG1 PE=1 SV=2 - [A2GL_HUMAN]	2.89	2.88	1	1	1	1	347	38.2	6.95
Ig kappa chain V-II region TEW OS=Homo sapiens PE=1 SV=1 - [KV204_HUMAN]	2.86	11.50	4	1	1	1	113	12.3	6.00
Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1 - [HPT_HUMAN]	2.85	3.20	1	1	1	1	406	45.2	6.58

Neutrophil defensin 1 OS=Homo sapiens GN=DEFA1 PE=1 SV=1 - [DEF1_HUMAN]	2.80	9.57	2	1	1	1	94	10.2	6.99
Receptor-type tyrosine-protein phosphatase S OS=Homo sapiens GN=PTPRS PE=1 SV=3 - [PTPRS_HUMAN]	2.79	0.67	1	1	1	1	1948	216.9	6.46
Cystatin-C OS=Homo sapiens GN=CST3 PE=1 SV=1 - [CYTC_HUMAN]	2.79	7.53	1	1	1	1	146	15.8	8.75
Filamin-C OS=Homo sapiens GN=FLNC PE=1 SV=3 - [FLNC_HUMAN]	2.77	0.59	1	1	1	1	2725	290.8	5.97
Cadherin-13 OS=Homo sapiens GN=CDH13 PE=1 SV=1 - [CAD13_HUMAN]	2.71	1.54	1	1	1	1	713	78.2	4.98
Lipocalin-15 OS=Homo sapiens GN=LCN15 PE=1 SV=1 - [LCN15_HUMAN]	2.70	5.43	1	1	1	1	184	20.4	4.94
Glutaredoxin-1 OS=Homo sapiens GN=GLRX PE=1 SV=2 - [GLRX1_HUMAN]	2.70	10.38	1	1	1	1	106	11.8	8.09
Vitelline membrane outer layer protein 1 homolog OS=Homo sapiens GN=VMO1 PE=1 SV=1 - [VMO1_HUMAN]	2.65	8.91	1	1	1	1	202	21.5	5.07
Trefoil factor 2 OS=Homo sapiens GN=TFF2 PE=1 SV=2 - [TFF2_HUMAN]	2.62	11.63	1	1	1	1	129	14.3	5.81
Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3 - [DESP_HUMAN]	2.55	0.35	1	1	1	1	2871	331.6	6.81
Fibronectin OS=Homo sapiens GN=FN1 PE=1 SV=4 - [FINC_HUMAN]	2.54	0.63	1	1	1	1	2386	262.5	5.71
Lactotransferrin OS=Homo sapiens GN=LTF PE=1 SV=6 - [TRFL_HUMAN]	2.52	1.41	1	1	1	1	710	78.1	8.12
Ribonuclease UK114 OS=Homo sapiens GN=HRSP12 PE=1 SV=1 - [UK114_HUMAN]	2.49	11.68	1	1	1	1	137	14.5	8.68
Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1 - [LYSC_HUMAN]	2.38	12.16	1	2	2	2	148	16.5	9.16
Titin OS=Homo sapiens GN=TTN PE=1 SV=4 - [TITIN_HUMAN]	2.32	0.03	1	1	1	1	34350	3813.7	6.35
Ig kappa chain V-IV region Len OS=Homo sapiens PE=1 SV=2 - [KV402_HUMAN]	2.30	7.89	4	1	1	1	114	12.6	7.93
Alpha-1B-glycoprotein OS=Homo sapiens GN=A1BG PE=1 SV=4 - [A1BG_HUMAN]	2.25	2.42	1	1	1	1	495	54.2	5.86
Macrophage migration inhibitory factor OS=Homo sapiens GN=MIF PE=1 SV=4 - [MIF_HUMAN]	2.25	7.83	1	1	1	1	115	12.5	7.88
Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2 - [H4_HUMAN]	2.24	9.71	1	1	1	1	103	11.4	11.36
Thioredoxin OS=Homo sapiens GN=TXN PE=1 SV=3 - [THIO_HUMAN]	2.23	12.38	1	1	1	1	105	11.7	4.92
Kininogen-1 OS=Homo sapiens GN=KNG1 PE=1 SV=2 - [KNG1_HUMAN]	2.20	1.40	1	1	1	1	644	71.9	6.81
Guanylin OS=Homo sapiens GN=GUCA2A PE=1 SV=2 - [GUC2A_HUMAN]	2.13	8.70	1	1	1	1	115	12.4	4.59
Ganglioside GM2 activator OS=Homo sapiens GN=GM2A PE=1 SV=4 - [SAP3_HUMAN]	1.97	10.88	1	2	2	2	193	20.8	5.31
Nuclear transport factor 2 OS=Homo sapiens GN=NUTF2 PE=1 SV=1 - [NTF2_HUMAN]	1.92	5.51	1	1	1	1	127	14.5	5.38

Filamin-A OS=Homo sapiens GN=FLNA PE=1 SV=4 - [FLNA_HUMAN]	1.88	0.45	1	1	1	1	2647	280.6	6.06
Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1 SV=1 - [S10A8_HUMAN]	1.86	7.53	1	1	1	1	93	10.8	7.03
Putative helicase MOV-10 OS=Homo sapiens GN=MOV10 PE=1 SV=2 - [MOV10_HUMAN]	1.70	1.69	1	1	1	1	1003	113.6	8.82
Multimerin-2 OS=Homo sapiens GN=MMRN2 PE=1 SV=2 - [MMRN2_HUMAN]	1.65	0.95	1	1	1	1	949	104.3	5.86
Ig kappa chain V-III region VG (Fragment) OS=Homo sapiens PE=1 SV=1 - [KV309_HUMAN]	0.00	7.83	1	1	1	1	115	12.6	4.96
Complement C4-A OS=Homo sapiens GN=C4A PE=1 SV=2 - [CO4A_HUMAN]	0.00	0.86	2	1	1	1	1744	192.7	7.08
Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 PE=1 SV=5 - [PRDX2_HUMAN]	0.00	5.56	2	1	1	1	198	21.9	5.97
Coiled-coil domain-containing protein 87 OS=Homo sapiens GN=CCDC87 PE=1 SV=2 - [CCD87_HUMAN]	0.00	0.82	1	1	1	1	849	96.3	8.59
TBC1 domain family member 2B OS=Homo sapiens GN=TBC1D2B PE=1 SV=2 - [TBD2B_HUMAN]	0.00	0.73	1	1	1	1	963	109.8	6.16

Table A1.3: The proteomic analysis results from sample 1 with positive result for protein strip-test but negative for albumin.

Description	Score	Coverage	Proteins	Unique Peptides	Peptides	PSMs	AAs	MW (kDa)	calc. pI
Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	34.98	21.58	7	10	11	12	584	58.8	5.21
Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2 - [ALBU_HUMAN]	26.37	15.11	1	8	8	9	609	69.3	6.28
Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	23.17	12.58	1	5	7	7	644	66.0	8.12
Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	18.45	10.17	5	4	6	6	639	65.4	8.00
Beta-2-microglobulin OS=Homo sapiens GN=B2M PE=1 SV=1 - [B2MG_HUMAN]	13.02	37.82	1	3	3	5	119	13.7	6.52
Cystatin-M OS=Homo sapiens GN=CST6 PE=1 SV=1 - [CYTM_HUMAN]	11.04	20.81	1	2	2	3	149	16.5	8.09
Transthyretin OS=Homo sapiens GN=TTR PE=1 SV=1 - [TTHY_HUMAN]	7.73	9.52	1	2	2	2	147	15.9	5.76
Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1 - [LYSC_HUMAN]	7.22	8.11	1	1	1	2	148	16.5	9.16
CD59 glycoprotein OS=Homo sapiens GN=CD59 PE=1 SV=1 - [CD59_HUMAN]	6.57	18.75	1	2	2	2	128	14.2	6.48
Protein AMBP OS=Homo sapiens GN=AMBP PE=1 SV=1 - [AMBP_HUMAN]	5.42	7.39	1	2	2	2	352	39.0	6.25
Hemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=2 - [HBB_HUMAN]	5.31	15.65	5	2	2	2	147	16.0	7.28
Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	4.84	3.81	6	1	2	2	472	51.5	5.16
Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	4.42	2.25	1	1	1	1	623	62.0	5.24
Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1 - [IGKC_HUMAN]	4.20	18.87	1	1	1	4	106	11.6	5.87
Cystatin-B OS=Homo sapiens GN=CSTB PE=1 SV=2 - [CYTB_HUMAN]	3.43	12.24	1	1	1	1	98	11.1	7.56
Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1 - [HPT_HUMAN]	3.39	3.20	1	1	1	1	406	45.2	6.58
Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1 SV=1 - [S10A8_HUMAN]	3.38	11.83	1	1	1	1	93	10.8	7.03
Thioredoxin OS=Homo sapiens GN=TXN PE=1 SV=3 - [THIO_HUMAN]	3.21	12.38	1	1	1	1	105	11.7	4.92
Fibronectin OS=Homo sapiens GN=FN1 PE=1 SV=4 - [FINC_HUMAN]	3.20	0.63	1	1	1	1	2386	262.5	5.71
Glutaredoxin-1 OS=Homo sapiens GN=GLRX PE=1 SV=2 - [GLRX1_HUMAN]	3.16	10.38	1	1	1	1	106	11.8	8.09
Guanylate cyclase activator 2B OS=Homo sapiens GN=GUCA2B PE=1 SV=1 - [GUC2B_HUMAN]	3.10	11.61	1	1	1	1	112	12.1	6.48
SH3 domain-binding glutamic acid-rich-like protein OS=Homo sapiens GN=SH3BGRL PE=1 SV=1 - [SH3L1_HUMAN]	2.92	8.77	1	1	1	1	114	12.8	5.25
Profilin-1 OS=Homo sapiens GN=PFN1 PE=1 SV=2 - [PROF1_HUMAN]	2.91	10.00	1	1	1	1	140	15.0	8.27

Cystatin-C OS=Homo sapiens GN=CST3 PE=1 SV=1 - [CYTC_HUMAN]	2.91	7.53	1	1	1	1	146	15.8	8.75
Plasma kallikrein OS=Homo sapiens GN=KLKB1 PE=1 SV=1 - [KLKB1_HUMAN]	2.83	4.55	1	1	1	1	638	71.3	8.22
Antileukoproteinase OS=Homo sapiens GN=SLPI PE=1 SV=2 - [SLPI_HUMAN]	2.80	9.09	1	1	1	1	132	14.3	8.75
Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1 - [CYTA_HUMAN]	2.80	12.24	1	1	1	1	98	11.0	5.50
Collagen alpha-3(VI) chain OS=Homo sapiens GN=COL6A3 PE=1 SV=5 - [CO6A3_HUMAN]	2.62	0.38	1	1	1	1	3177	343.5	6.68
Desmocollin-2 OS=Homo sapiens GN=DSC2 PE=1 SV=1 - [DSC2_HUMAN]	2.52	1.55	1	1	1	1	901	99.9	5.34
Lithostathine-1-alpha OS=Homo sapiens GN=REG1A PE=1 SV=3 - [REG1A_HUMAN]	2.47	6.02	1	1	1	1	166	18.7	5.94
Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2 - [H4_HUMAN]	2.43	9.71	1	1	1	1	103	11.4	11.36
Fatty acid-binding protein, liver OS=Homo sapiens GN=FABP1 PE=1 SV=1 - [FABPL_HUMAN]	2.25	8.66	1	1	1	1	127	14.2	7.18
Ig lambda-2 chain C regions OS=Homo sapiens GN=IGLC2 PE=1 SV=1 - [LAC2_HUMAN]	2.12	9.43	2	1	1	1	106	11.3	7.24
Ig kappa chain V-I region Lay OS=Homo sapiens PE=1 SV=1 - [KV113_HUMAN]	1.97	8.33	4	1	1	1	108	11.8	7.96
Ig kappa chain V-III region VG (Fragment) OS=Homo sapiens PE=1 SV=1 - [KV309_HUMAN]	1.95	7.83	1	1	1	1	115	12.6	4.96
Protein shisa-5 OS=Homo sapiens	1.94	2.92	1	1	1	1	240	25.6	6.68

Table A1.4: The proteomic analysis results from sample 2 with positive result for protein strip-test but negative for albumin.

Appendix 2

Tables A2.1 and A2.2 summarise the results of metal analysis on different groups of people between visit 1 and visit 2.

Visit 1 (n=350)	Element (units)		Al (µmol/mol)	Total As (µmol/mol)	Cd (µmol/mol)	Cr (µmol/mol)	Co (µmol/mol)	Cu (µmol/mol)	Pb (µmol/mol)	Mn (µmol/mol)	Hg (µmol/mol)	Se (µmol/mol)	Si (mmol/mol)	Sr (µmol/mol)
Age	<25 (n=219)	Mean	480.6	150.2	1.9	1.5	1.2	27.5	0.5	5.1	0.11	38.6	220.0	356.4
	≥25 (n=131)	Mean	634.4	157.9	2.4	1.3	1.2	19.5	0.4	5.6	0.15	40.9	215.7	327.7
Community	1 (n=38)	Mean	680.1	171.2	15.9	1.3	1.4	24.0	0.58	5.9	0.11	142.9	279.0	369.1
	2 (n=45)	Mean	781.5	110.1	0.29	1.5	1.0	17.2	0.25	4.1	0.12	29.9	221.4	283.5
	3 (n=37)	Mean	468.3	135.1	0.34	1.2	1.2	19.1	0.22	4.9	0.03	33.3	211.4	271.6
	4 (n=35)	Mean	515.6	128.5	0.49	1.0	1.3	21.5	0.68	4.3	0.13	28.8	191.8	457.9
	5 (n=26)	Mean	408.4	153.5	0.36	0.98	1.7	81.0	1.7	6.2	0.10	25.6	234.9	652.6
	6 (n=53)	Mean	605.3	174.8	0.48	2.0	1.3	20.2	0.3	6.6	0.12	23.0	267.8	378.6
	7 (n=31)	Mean	502.0	145.0	0.43	1.0	1.2	19.8	0.35	4.7	0.23	21.5	74.7	114.6
	8 (n=40)	Mean	298.2	174.0	0.37	1.0	0.90	16.7	0.40	3.8	0.17	26.2	170.7	285.1
	9 (n=45)	Mean	483.9	175.7	0.45	2.0	1.2	22.0	0.34	6.6	0.15	26.7	262.1	354.1
Hardness of well water	Moderately hard (n=97)	Mean	496.5	140.7	0.57	1.1	1.2	20.6	0.34	4.8	0.12	28.6	195.0	318.2
	Hard (160)	Mean	512.8	167.7	4.0	1.4	1.3	29.7	0.66	5.6	0.13	52.4	221.6	380.2
	Very hard (n=93)	Mean	625.2	140.9	0.3	1.7	1.1	19.7	0.32	5.3	0.14	28.5	237.2	315.1
Occupation	Only sugarcane (n=55)	Mean	389.1	148.0	3.5	1.24	1.1	19.3	0.36	4.1	0.14	48.2	223.0	343.6
	Only agriculture (n=138)	Mean	584.0	148.4	1.5	1.2	1.1	18.9	0.40	4.7	0.11	35.2	232.0	363.3
	Other occupations (n=157)	Mean	550.1	159.0	2.1	1.6	1.4	31.4	0.60	6.2	0.14	40.2	204.9	331.0

Table A2.1: The mean concentration of each metal and metalloids found in the urine in people of different age group, community, consumption of water and occupation at visit 1.

Visit 2 (n=291)	Element (units)		Al (µmol/mol)	Total As (µmol/mol)	Cd (µmol/mol)	Cr (µmol/mol)	Co (µmol/mol)	Cu (µmol/mol)	Pb (µmol/mol)	Mn (µmol/mol)	Hg (µmol/mol)	Se (µmol/mol)	Si (mmol/mol)	Sr (µmol/mol)
Age	<25 (n=182)	Mean	365.7	116.8	0.38	3.7	2.1	15.1	0.43	5.2	0.11	24.6	183.5	217.7
	≥25 (n=109)	Mean	418.63	136.5	0.45	5.9	2.6	16.8	0.41	6.0	0.13	29.5	197.9	207.7
Community	1 (n=27)	Mean	653.0	158.7	0.41	1.3	1.01	14.6	0.28	5.7	0.14	21.8	247.9	260.5
	2 (n=41)	Mean	334.7	105.0	0.24	0.72	0.85	10.9	0.21	4.5	0.11	43.6	206.8	193.2
	3 (n=29)	Mean	379.3	108.6	0.29	0.92	0.91	14.8	0.39	4.4	0.00	25.8	193.7	169.8
	4 (n=32)	Mean	444.1	80.5	0.19	0.47	1.05	12.4	0.42	2.1	0.13	22.7	202.2	202.5
	5 (n=18)	Mean	290.0	85.6	0.21	0.42	0.88	12.8	0.46	3.1	0.12	20.3	178.1	230.3
	6 (n=50)	Mean	350.3	145.8	0.76	3.2	7.66	19.1	0.79	8.4	0.13	25.3	160.8	219.9
	7 (n=27)	Mean	436.6	123.6	0.42	5.0	1.71	15.0	0.41	6.9	0.22	20.3	162.2	237.1
	8 (n=38)	Mean	324.8	158.1	0.52	23.0	1.04	23.7	0.40	7.2	0.14	28.6	183.3	217.6
	9 (n=29)	Mean	301.9	126.0	0.30	0.96	2.34	14.3	0.28	4.2	0.08	19.6	176.0	209.9
Hardness of well water	Moderately hard (n=81)	Mean	355.9	105.7	0.32	2.3	1.76	16.0	0.42	4.5	0.11	23.4	173.8	208.3
	Hard (137)	Mean	439.0	141.9	0.53	7.8	3.10	17.4	0.52	6.6	0.13	24.1	195.8	224.6
	Very hard (n=73)	Mean	318.1	111.5	0.27	0.81	1.47	12.3	0.25	4.4	0.10	34.1	192.5	200.0
Occupation	Only sugarcane (n=113)	Mean	314.6	107.4	0.38	2.8	2.34	14.0	0.42	3.9	0.13	22.1	179.8	215.1
	Only agriculture (n=60)	Mean	389.4	129.2	0.36	5.0	1.6	16.0	0.35	4.7	0.10	29.5	202.5	206.6
	Banana (n=18)	Mean	360.7	176.3	0.51	20.9	1.03	15.7	0.43	7.2	0.13	26.8	215.6	217.0
	Other occupations (n=100)	Mean	467.9	130.8	0.44	3.2	2.9	17.5	0.46	7.4	0.12	29.3	186.2	216.4

Table A2.2: The mean concentration of each metal and metalloids found in the urine in people of different age group, community, consumption of water and occupation at visit 2.