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CHRONIC KIDNEY DISEASE OF UNKNOWN ETIOLOGY IN CENTRAL AMERICA AND SRI LANKA - RENAL MORPHOLOGY AND CLINICAL CHARACTERISTICS

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Chronic Kidney Disease of Unknown Etiology in Central America and Sri Lanka
- Renal Morphology and Clinical Characteristics

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Calle and Sera ♥

ABSTRACT

Background: Chronic kidney disease (CKD) is a global health problem. The most common etiologies of CKD are diabetes mellitus, hypertension, and glomerulonephritis, but over the last several decades a high prevalence of CKD of unknown etiology (CKDu) has been reported from rural communities in some tropical countries, including Central America, and Sri Lanka. The CKDu endemics share some mutual characteristics, they usually affect rural communities and men are more often affected than women. In Central America, the disease often affects male sugarcane workers. In Sri Lanka, male rice farmers in certain areas are at risk of developing CKDu. The cause or causes behind the CKDu endemics have not been elucidated. Furthermore, it has not been determined whether the two endemics are similar diagnostic entities. Kidney biopsy is an important tool to evaluate CKD, but in Central America, biopsy studies from patients with CKDu were lacking at the beginning of our studies.

Aim: The aims were to describe the renal morphology and biochemical characteristics in patients with CKDu in Central America and Sri Lanka in order to find pathogenetic mechanisms, to study the natural history of the disease, and to compare the endemics to determine if they are related diagnostic entities.

Material and Methods: Patients with CKDu were evaluated with kidney biopsy, biochemical tests and a questionnaire. Light- and electron microscopy as well as immunofluorescence evaluation were performed on the biopsies. Follow-up blood and urine samples were collected from subjects in Studies I and II after 1-2.5 years.

Study I: Eight male agricultural workers in El Salvador with an estimated glomerular filtration rate (eGFR) between 27-79 ml/min/1.73m² were included. The kidney biopsies showed a unique renal morphology with extensive glomerulosclerosis, signs of glomerular ischemia, mild to moderate tubular atrophy, and interstitial fibrosis. Vascular structures were generally only mildly changed. In serum, low potassium levels were a common finding.

Study II: Nineteen males from Nicaragua with a history of sugarcane work were included. eGFR was between 33-96 ml/min/1.73m² and low serum sodium, potassium, and magnesium were frequent findings. Sixteen biopsies were representative and the morphology displayed chronic glomerular changes with glomerulosclerosis, glomerular hypertrophy, and signs of glomerular ischemia. Mild to moderate tubulointerstitial changes and mostly mild vascular changes were found. Follow-up samples from 7 participants from Study I and 18 participants from Study II showed a mean eGFR change of -4.4 ± 8.4 ml/min/1.73m² per year.

Study III: Eleven male rice farmers from Sri Lanka with an eGFR between 21-70 ml/min/1.73m² were included. Again, low sodium, potassium, and magnesium were common. The main biopsy findings were chronic glomerular changes (glomerulosclerosis, glomerular hypertrophy) and mild to moderate tubulointerstitial changes. Vascular changes were in most cases mild but in a few cases moderate. The biopsies showed a more mixed morphology compared to Studies I and II. Interstitial inflammation and vascular pathology were more frequent.

Conclusions: Studies I and II show that CKDu in Central America is a unique diagnostic entity with a renal morphology characterized by chronic damage in both glomerular and tubulointerstitial compartments. Although Study III in Sri Lanka revealed a more mixed kidney biopsy morphology, the majority of the subjects had a morphology quite similar to the findings in Central America. Low levels of serum electrolytes were a typical finding in all studies. In summary, CKDu in Sri Lanka and Central America have more similarities than differences in both the morphological and the biochemical characteristics, which supports the theory that the endemics have a common etiology.

LIST OF SCIENTIFIC PAPERS

I.   **WIJKSTRÖM J**, Leiva R, Elinder CG, Leiva S, Trujillo Z, Trujillo L, Söderberg M, Hultenby K, Wernerson A.
Clinical and Pathological Characterization of Mesoamerican Nephropathy: A New Kidney Disease in Central America.
American Journal of Kidney Diseases. 2013; 62(5): 908-918.

II.   **WIJKSTRÖM J**, González-Quiroz M, Hernandez M, Trujillo Z, Hultenby K, Ring A, Söderberg M, Aragon A, Elinder CG, Wernerson A.
Renal Morphology, Clinical Findings, and Progression Rate in Mesoamerican Nephropathy.
American Journal of Kidney Diseases. 2017; 69(5): 626-636.

III.   **WIJKSTRÖM J**, Jayasumana C, Dassanayake R, Priyawardane N, Godakanda N, Siribaddana S, Ring A, Hultenby K, Söderberg M, Elinder CG, Wernerson A.
Morphological and Clinical Findings in Sri Lankan Patients with Chronic Kidney Disease of Unknown Cause (CKDu): Similarities and Differences with Mesoamerican Nephropathy.
Submitted manuscript. 2017.

CONTENTS

LIST OF ABBREVIATIONS

ACR	Albumin-Creatinine Ratio
AKI	Acute Kidney Injury
A1M	α-1-Microglobulin
CI	Confidence Interval
CKD	Chronic Kidney Disease
CKDu	Chronic Kidney Disease of unknown etiology
CRP	C-Reactive Protein
eGFR	Estimated Glomerular Filtration Rate
ESRD	End Stage Renal Disease
GBM	Glomerular Basement Membrane
GFR	Glomerular Filtration Rate
HE	Hematoxylin and Eosin
IFL	Immunofluorescence
NAG	N-Acetyl-Beta-D-Glucosaminidase
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
OR	Odds Ratio
PAS	Periodic Acid-Schiff
PASM	Periodic Acid Silver Methenamine
RAAS	Renin–Angiotensin–Aldosterone System
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
TEM	Transmission Electron Microscopy
WBGT	Wet-Bulb Globe Temperature

1 BACKGROUND

1.1 STRUCTURE AND FUNCTION OF THE KIDNEY

The kidneys are paired organs located retroperitoneal in the abdomen, with an average size of 10-13 cm and weight of 150 g each. The kidney tissue consists of cortex and medulla and is surrounded by a fibrous capsule (Figure 1). Blood enters the kidneys via the renal artery with 20-25% of the cardiac output provided to the kidneys, i.e. renal blood flow is approximately 1-1.25 L/min (1).

The primary function of the kidneys is to produce urine and thereby regulate the volume of fluid in the body, regulate electrolyte and acid/base balance, and dispose of metabolic end products. The functional unit of the kidney is the nephron composed of the glomerulus, Bowman's capsule, and the tubular system ending in the collecting duct (Figure 2a). Around one million nephrons are found in each kidney.
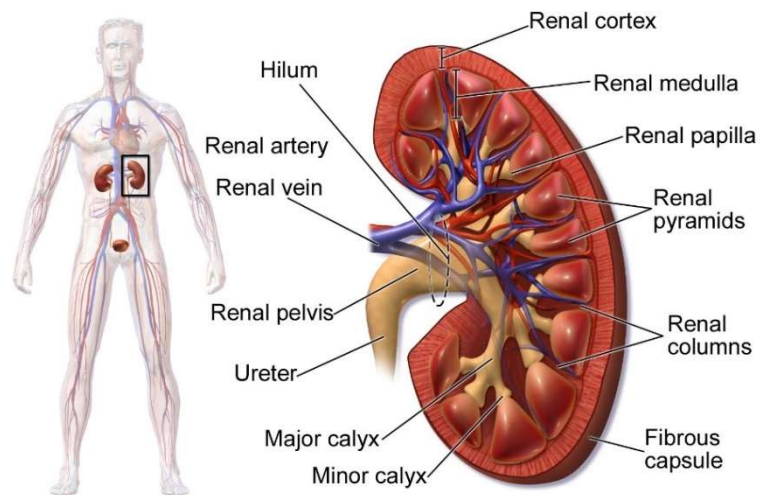
Figure 1: *Anatomy of the kidney. Image by Blausen.com staff (2014)/Wikimedia.*

The glomerulus is a round structure consisting of small capillaries which are lined by endothelial cells on the inner surface and specialized epithelial cells called podocytes on the outer surface (Figure 2b). The capillary loops in the glomerulus are held together by mesangial cells and matrix. Blood enters through the afferent arteriole, and is subsequently filtered through the filtration barrier of the capillaries (endothelium,
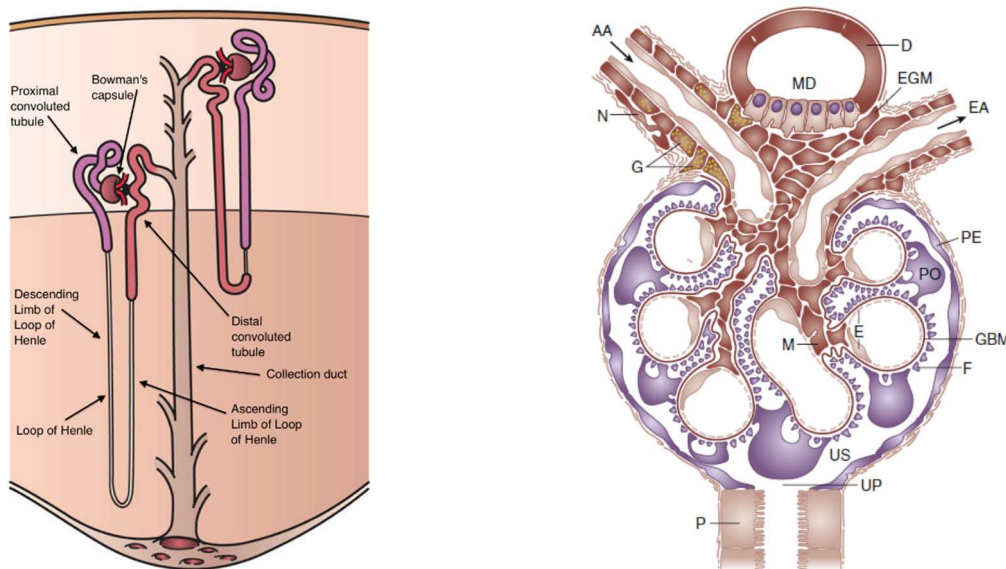
Figure 2a: *Nephron structure. Image by Holly Fisher/Wikipedia commons.* **Figure 2b:** *Cross section of a glomerulus. AA, afferent arteriole; D, distal tubule; E, endothelial cell; EA, efferent arteriole; F, podocyte foot process; G, juxtaglomerular cell; GBM, glomerular basement membrane; M, mesangial cell; MD, macula densa; P, proximal tubule; PE, parietal epithelial cell; PO, podocyte; US, urinary space. Modified from National Kidney Foundation's Primer on Kidney disease 2014 (edt. Gilbert and Weiner).*

glomerular basement membrane and the podocyte slits). An ultrafiltrate (primary urine) is produced and collected by the surrounding Bowman's capsule. The remaining blood exits the glomerulus through the efferent arteriole. Large compounds in the blood such as larger proteins (>60-70 kDa) and blood cells are too big to be filtered and are normally not found in the filtrate (1). Approximately 180 L of filtrate is produced per day. The filtrate is transported and modified through the tubular system to be transformed into the final urine. The vast majority of the filtrate is reabsorbed in the tubules and the final urine output is 1.5-2 L/day. Throughout the tubular structure, various solutes including glucose, electrolytes (sodium, potassium, calcium etc.), amino acids, proteins, vitamins, hydrogen ions, and urea are reabsorbed and secreted (2). The glomerular and tubular structures in kidney tissue can be examined by light microscopy and electron microscopy (Figure 3a and 3b).
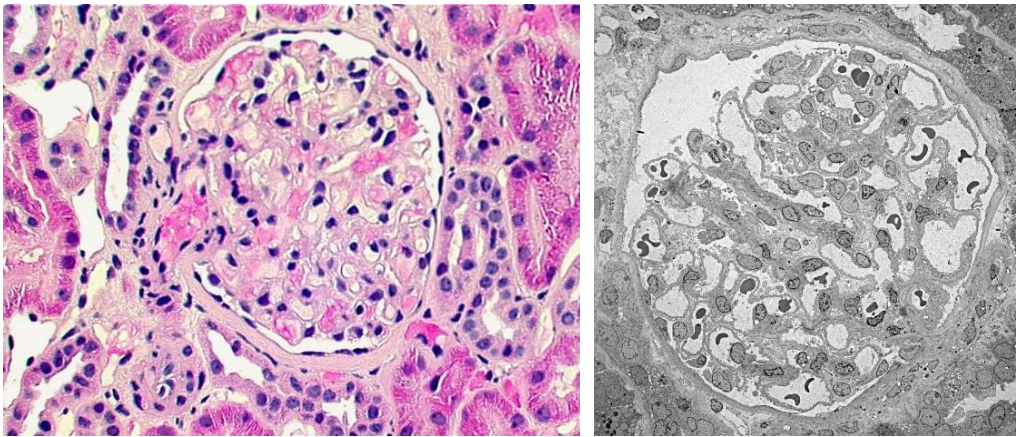
Figure 3a: *Light microscopy image of a normal glomerulus.* **Figure 3b:** *Electron microscopy image of a normal glomerulus.*

Glomerular filtration rate (GFR) is a measurement of the kidney function; specifically it is an estimation of how much blood that is filtered in the glomeruli per minute. GFR is normally ≥ 90 ml/min/1.73m² in healthy individuals. Normal ageing causes a decline in GFR between 0.4-1.2 ml/min/1.73m² per year in adults (3). Many kidney diseases cause a permanent and sometimes progressive decline in GFR. The golden standard of measuring GFR is by analyzing the plasma concentration and excretion (renal clearance) of Inulin, a molecule that is freely filtered through the glomerulus and is not absorbed/excreted in the tubules. However, the method is cumbersome to perform and therefore in practical settings as in hospitals and in most research studies, an estimation of the GFR (eGFR) is made by using an equation including serum creatinine and/or serum Cystatin C.

In addition to producing urine, the kidney has other important functions (1):

- Regulating blood pressure and salt balance by excretion of renin from the juxtaglomerular cells in the glomeruli, thus activating the Renin–Angiotensin–Aldosterone System (RAAS).
- Stimulating the maturation of red blood cells by excretion of erythropoietin from interstitial cells in the cortex.
- Conversion of 25-(OH) vitamin D into the active vitamin D form 1,25 dihydroxy-vitamin D, which is important for the calcium homeostasis.

1.2 CHRONIC KIDNEY DISEASE

Chronic kidney disease (CKD), defined as eGFR <60 ml/min/1.73m², is a serious and increasing health problem worldwide, with a prevalence of around 4.7-8.1% in Europe (4, 5), 4.5-7.7% in the US (6) and 8-16% globally (7, 8). In the "Global burden of diseases" study published in 2010, the age-standardized death rate from CKD actually increased from 1990 to 2010 in contrast to most other diseases (e.g. major cardiovascular disease and most cancer diagnoses) for which death rates fell during this time period (9). CKD affects populations in low-, middle-, and high-income countries, but with somewhat different spectra of causes. Diabetes and hypertension are the two most common CKD causes in most middle- and high-income countries, while glomerulonephritis and unknown causes are more frequent in low-income countries and parts of Asia (8).

CKD may progress to end-stage renal disease (ESRD), a state where the patient needs renal replacement therapy (RRT), i.e. dialysis or kidney transplantation, for survival. RRTs are costly treatments that often are associated with adverse events such as infections, malignancies, increased mortality rates and reduced quality of life (10, 11). However, even if the CKD does not progress to ESRD, the chronic condition of CKD has been identified as a major independent risk factor for morbidity and mortality, especially with regard to cardiovascular disease (12, 13).

In recent years, a remarkably high prevalence of chronic kidney disease of unknown etiology (CKDu) has been reported from several low- and middle-income regions, including Central America, Sri Lanka, India, and Egypt (14).

1.3 CKDu IN CENTRAL AMERICA - MESOAMERICAN NEPHROPATHY

Figure 4: *Map of Central America. Source, Map Data ©2017 Google, INEGI.*

1.3.1 Overview

Mesoamerican nephropathy (MeN) is the name of the endemic form of CKDu affecting mainly male rural agricultural workers living along the pacific (west) coast line of Central America (15) (Figure 4). A high prevalence of CKD in certain areas of Central America has been observed by local physicians and media for several decades but the first scientific report was published in 2002, describing a group of 205 patients with newly diagnosed ESRD. This study concluded that the majority of the patients with ESRD had CKDu, i.e. their disease was not correlated to traditional causes and risk factors for CKD such as hypertension, glomerulonephritis, or diabetes mellitus (16).

Men working with sugarcane cultivation in regions close to the Pacific coast seem to be at greatest risk of developing the disease (17), but women and other agricultural workers and manual laborers in these areas are also affected. Nicaragua and El Salvador are the countries in the region with most reported CKD cases and the highest mortality rate due to CKD (18). In some areas up to 19-40% of the male and 5-14% of the female inhabitants have CKD with eGFR <60 ml/min/1.73m² (19-23). In Central America, where health resources are limited, the diagnosis of CKD can be devastating. Many people with MeN die of ESRD because dialysis treatment facilities are insufficient and there are no functioning kidney transplant programs. In Nicaragua, the sugarcane community La Isla, where the mortality among men due to CKD is very high, is now locally called "La Isla de Viudas", i.e. the Island of Widows (24).

People working in the sugarcane fields work under extreme conditions during the harvest season from mid-November to the end of May. The sugarcane fields are often burned the day before harvest to remove the leafy material of the sugarcane plant. When the sugarcane workers start to harvest there could still be some heat left in the ground. Work is conducted under direct sunlight and the mean daily temperature during harvest season in Central America is 27-30°C, with temperatures during the daytime usually reaching highs of 32-35°C. A sugarcane cutter is usually paid based on performance, and several tons of sugarcane are harvested manually with a machete every day (Figure 5). Working hours are usually from early morning until midday, however, the combination of temperature and workload puts the workers at risk for work-related heat stress already between 9:00 a.m. and 10:00 a.m. (25).

Figure 5: *Sugarcane cutters in El Salvador harvesting sugarcane manually with a machete. Photos courtesy of Tom Laffay for La Isla Network.*

1.3.2 Prevalence studies

Even though there are no completely reliable numbers for the full extent of the epidemic in Central America, local nephrologists and health care workers have reported a steady increase of patients with CKD during the last decades that is overwhelming the health care facilities in the affected regions. According to the World Health Organization (WHO), kidney disease is the third most common cause of death in Nicaragua and the fifth most common health related cause of death in El Salvador (26, 27).

Several local and governmental reports from the 1990s to 2005 have described the rising number of patients with CKD in Central America. In November 2005 a workshop by the Program on Work and Health in Central America (SALTRA), funded by the Swedish International Development Cooperation Agency (SIDA), was held in Leon, Nicaragua, and a summary of available data was

compiled in a report by Cuadra et al (28). The authors concluded that there are areas in El Salvador and Nicaragua with a high prevalence of CKD and that possible risk factors were agricultural work, sugarcane work, pesticide exposure, and alcohol consumption. Data and studies from other Central American countries (Costa Rica, Honduras, Belize, Panama, and Guatemala) were found to be lacking.

Nicaragua and El Salvador are the most affected countries in the region. Several cross-sectional studies and other prevalence studies have been conducted in these two countries, and some studies have been performed in other Central American countries, including Guatemala and Costa Rica. Summaries of the most important studies are presented in the sections below.

1.3.2.1 El Salvador

The first cross-sectional study was published in the Spanish journal Nefrologia in 2005 and was a study of 353 men in El Salvador, of whom 291 were living near the coast and 62 were living in inland. The prevalence of CKD was found to be high, 12.7%, among the men living along the coast. Of the affected individuals, 62% did not have hypertension, diabetes, or any other clear cause for their CKD (29).

In 2011, the first prevalence study of a larger population was published. Seven hundred seventy-five adult individuals from the Baja Lempa region, located along the Pacific coast of El Salvador, were included, and 17% of the men and 4% of the women had reduced kidney function with eGFR <60 ml/min/1.73m². Risk factors associated with CKD were male sex, age, hypertension, and family members with CKD (22).

One year later, Peraza et al (21) published a prevalence study of 664 inhabitants aged 20-60 years living in five different villages, two of which were at low altitude near the coastline with mainly sugarcane cultivation and three of which were located 500 meters above sea level with mainly sugarcane cultivation, coffee cultivation, and urban/service orientation, respectively. They reported that 18% of the men living in the two coastal sugarcane communities had CKD (eGFR <60 ml/min/1.73m²) compared to only 1.8% of the men in the high-altitude sugarcane village and 0% of men in the coffee and service-oriented villages. A significant association was found between CKD and more than 10 years of costal sugarcane work.

A cross-sectional study of 11 communities in three different regions in El Salvador was reported by Orantes et al in 2014 (23). The study was conducted between 2009-2011 and from a door-to-door census they identified 5018 individuals ≥18 years of age of which 2388 individuals were included in the study (23). The prevalence of CKD (CKD defined as eGFR <60 ml/min/1.73m²) was 17% among men and 7% among women, and of these CKD cases 52% did not have diabetes mellitus, hypertension, or proteinuria. Age ≥60 years was associated with CKD in all three regions, and agricultural work was associated with CKD in two regions.

Another descriptive cross-sectional study of 223 individuals living in two Salvadoran rural agricultural communities found that the prevalence of CKD stage 3-5 (eGFR <60 ml/min/1.73m²) was 16%, but contrary to what has been reported from other studies, CKD was more common in women (21% compared to 11% among men). The majority of participants worked in agriculture in the two communities, including 96% and 97% of the men and 57% and 52% of the women. Seventy-seven

percent of all participants reported agrochemical exposure, and 69% reported use of nonsteroidal anti-inflammatory drugs (NSAIDs) (30).

A recent 10-year community registry study reporting the annual incidence of ESRD and CKD mortality was conducted in the Baja Lempa region, one of the MeN "hot-spot" regions (31). The annual incidence rate of ESRD was 1410 cases per million people. Eighty-nine percent of the patients who were diagnosed with ESRD were male, 76% were farmers and 66% did not report hypertension or diabetes mellitus. The annual mortality rate due to CKD was found to be high, 128 per 100 000 inhabitants, compared to other countries in Latin America (32), and the average age at the time of death due to CKD was only 56 years. Furthermore, the study reported that only 35% of the patients with ESRD received RRT, highlighting the lack of health care resources in El Salvador.

Of the patients that do receive RRT in El Salvador, the treatment is often of deficient quality. A nationwide survey reported that only 41% of the patients treated with peritoneal dialysis treatment received optimal treatment (i.e. continuous ambulatory peritoneal dialysis or ambulatory peritoneal dialysis with a soft catheter) and that less than 5% of the patients on hemodialysis received optimal treatments, (i.e. ≥ 3 hemodialysis treatments per week) (33).

1.3.2.2 Nicaragua

In 2010, Torres et al published the first large-scale cross-sectional study from Central America in an international scientific journal (20). They included 1096 participants between 20 and 60 years of age from five villages with different economic profiles in one of the most affected regions, Northwestern Nicaragua. The prevalence of CKD (defined as eGFR <60 ml/min/1.73m²) was highest in the mining/subsistence farming village with 19% of men and 5% of women affected (the definition of subsistence farming is growing crops for personal use). The prevalence was 17% in men and 4% in women in the banana/sugarcane village and 10% in men and 2% in women in the fishing village. Interestingly, CKD prevalence was low in both the service-oriented village (0% in both men and women) and the coffee-cultivation village (7% in men and 0% in women). The coffee-cultivation village was located at a higher altitude, with a colder climate, compared to the other agricultural villages in the study. Decreased renal function in this study was common in agricultural workers working in low-altitude villages, but was also common in craftsmen, constructions workers, and miners.

Sanoff et al studied 997 individuals in the region of Leon and Chinandega, by measuring serum creatinine and blood pressure and by distributing questionnaires. Twelve percent of the participants had reduced kidney function with eGFR <60 ml/min/1.73m². In a logistic regression model agricultural work was significantly associated with higher odds of reduced renal function with an odds ratio (OR) of 2.48 (95% CI 1.59-3.89). Other significant associations with decreased renal function were consumption of local alcohol ("lija") and high water intake (≥5L/day vs. 1L/day) (34).

A high burden of disease among the younger part of the population was reported in a study by O'Donnell et al. That study showed a high prevalence of reduced eGFR (<60 ml/min/1.73m²) among 18-29 year old individuals and among 30-41 year old individuals, 2.6% and 7.4% respectively and this prevalence was significantly higher than data from the US population (0.2% and 0.8% respectively) (35).

The prevalence of CKD in a high-altitude coffee-cultivating village in Nicaragua (1000 meters above sea level) was studied in a cross-sectional study of inhabitants aged 20-60 years. Two hundred ninety-three participants were interviewed, and urine and blood samples from 267 of those inhabitants were collected. No increased prevalence of CKD (eGFR <60 ml/min/1.73m²) was found (0% among men and 1.4% among women). Interestingly, 92% of the participants worked in agriculture and 93% reported working with preparing or applying pesticides (36).

Another study highlighting the high risk for sugarcane workers to develop CKD was reported in 2014 by Raines et al (19). Four hundred twenty-four individuals between 15 and 69 years of age living in a community near the town of Chichigalpa in North Western Nicaragua, a MeN hot spot area, were studied. The prevalence of eGFR <60 ml/min/1.73m² was as high as 42% among men and 10% among females. Hypertension was uncommon, and macroalbuminuria (i.e. >300 mg/dl) was present only in less than 10% of the participants with reduced kidney function. A majority of the men were involved in sugarcane cultivation. Risk factors for lowered eGFR were total days cutting sugarcane, pesticide inhalation, and chewing sugarcane.

Uric acid levels were measured in a cross-sectional study of 266 individuals in 24 CKD-affected families in a MeN hot-spot in Nicaragua (37). Participants with CKD were reported to have 1.2 mg/dl higher serum levels of uric acid compared to CKD patients in a large US cohort (NHANES) when adjusting for age and eGFR. The authors proposed that the higher uric acid levels in MeN patients could contribute to MeN pathogenesis. However, when comparing participants aged ≤ 65 years of age with CKD or when comparing Hispanic participants in the Nicaraguan cohort to the US cohort there was no statistical significant difference in uric acid levels, indicating that the uric acid levels may depend on diet or other factors.

The only cross-sectional study involving adolescent subjects was reported in 2015 by Ramirez-Rubio (38). They studied 12-18 year-old pupils, with no prior work history, in four different schools from areas with different MeN prevalence in Nicaragua. Questionnaires and urine biomarkers were collected. "Chistata" (see 1.3.4.2) was a common self-reported symptom. Proteinuria and glycosuria were rare (3.5% and 1% respectively) and were similar in all groups, but the tubular injury biomarkers neutrophil gelatinase-associated lipocalin (NGAL) and N-acetyl-beta-D-glucosaminidase (NAG) were higher among adolescents from the area with the highest MeN prevalence, indicating that renal injury might occur before occupational exposure begins.

González-Quiroz and colleagues have started a longitudinal study of 360 young adults (aged 18-30 years) without any known prior diseases living in nine agricultural communities (mainly sugarcane cultivation) in North Western Nicaragua. Baseline findings from this study show that among presumed healthy men a high percentage, 11%, had an eGFR below 90 ml/min/1.73m², whereas in women the prevalence was 1% (39). Future reports from this longitudinal study will add important information on young people living in a MeN hot spot area.

1.3.2.3 *Costa Rica*

The geographical distribution of CKD mortality in different regions of Costa Rica between 1970 and 2012 was reported by Wesseling et al (2015). In the coastal north-west region Guanacaste, where sugarcane cultivation is common, and from where MeN cases have been reported, the standardized CKD mortality rate for men ≥20 years of age increased from 4.4 per 100 000 in the 1970s to 38.5 per 100 000 in 2012 (compared to an increase from 3.6 to 8.4 per 100 000 in the rest of Costa Rica). For

women in Guanacaste, the increase was also apparent, but less pronounced. The report supports that a high CKD mortality rate in Guanacaste, the hottest region of Costa Rica, has been evident since the 1970s and that rates have been increasing ever since.

1.3.2.4 *Guatemala*

In 2015, Laux et al published a study describing enrolment rates of RRT in Guatemala. They reported that dialysis enrolment was higher in south-west Guatemala close to the Pacific coast, where the temperature is higher and sugarcane cultivation is more common, indicating that the MeN endemic is probably also affecting this part of Central America (40). A cross-sectional study by the same team was published one year later, including 242 patients on hemodialysis in southwestern Guatemala, and concluded that 19% of the patients lacked traditional risk factors for CKD, a relatively low percentage compared to what has been reported from El Salvador and Nicaragua. More studies are warranted to determine the extent of MeN in the Guatemalan population, but it is probably not as prevalent as it is in Nicaragua and El Salvador.

1.3.2.5 *Summary*

From these studies, one can conclude that MeN is found in several parts of Central America, and in certain areas the prevalence is very high. Men working in manual labor, especially sugarcane workers, in certain regions located at low altitudes close to the Pacific coastline are mainly affected, but women in these areas also seem to be at some increased risk to develop CKD. One study of adolescents indicated that renal damage might occur already before occupational exposure, but more studies are needed to establish if there is an association with a development of CKD. In some areas, especially in higher altitude environments, inhabitants are not affected by the disease even though they are engaged in similar agricultural plantation work. MeN progresses to ESRD in many cases, and the health care resources in the afflicted areas are not sufficient to treat all affected individuals.

1.3.3 Longitudinal and cross-shift studies

Because sugarcane workers have a high prevalence of MeN and because years of sugarcane work has been reported as a risk factor, a few longitudinal studies and cross-shift studies during workdays have been conducted to assess if there are any biochemical signs of kidney injury related to sugarcane work. However, these types of studies are generally very difficult to perform in Central America, in part because close cooperation needs to be established with the sugarcane companies and because participants might move or change employers during a harvest season. The present security situation in El Salvador, with high rates of crime and violence is also an important factor.

Laws et al (2015 and 2016) (41, 42) investigated 284 sugarcane workers in Nicaragua before and at the end of the six-month long harvest period. Urinary injury markers and serum creatinine were measured, and different task assignments were identified, including cane cutters, seed cutters, seeders, irrigators, agrochemical applicators, drivers and factory workers. All tasks except for driving and factory work were considered to involve medium to high risk for heat stress exposure. A mean eGFR change of -6.9 ml/min/1.73m² was found among field workers compared to non-field workers (drivers and factory workers). Urinary NGAL and IL-18 levels were also elevated compared to non-field workers. The eGFR decrease was significant in seed cutters and irrigators, but it was not significantly decreased in agrochemical applicators compared to factory workers. Albuminuria was found in less than 5% of the workers. This study provide some evidence that the decrease in eGFR might be

associated with field work and heat exposure. However a possible confounding factor might be that eGFR was calculated from serum creatinine levels, which might be influenced by increased muscle mass or changes in diet during the harvest period.

In 2016, Wesseling et al published a study of 29 young sugarcane cutters (age 17-38 years) in Nicaragua, measuring renal biomarkers in blood and urine before and after the workday on the first day of the harvest season, on the sixth day, and after 9 weeks of harvest. At 9 weeks, the pre-shift mean levels of creatinine had increased 20% corresponding to a mean eGFR decreased of 10 ml/min/1.73m². Blood urea nitrogen (BUN) and urinary NGAL also increased significantly during the 9-week period, while serum phosphate and potassium decreased. Significant increases in serum creatinine levels after the workday were reported from all three sampling days (43) indicating renal damage and/or muscle damage.

Another cross-shift study of sugarcane workers, this time in El Salvador, was published in 2015 by Garcia-Trabanino et al and included 189 workers aged 18-49 years from three different plantations. Urine and blood samples were collected before and after a work day (mean 4 hours worktime with a mean temperature of 34-42° C). Fourteen percent of the participants had an eGFR below 60 ml/min/1.73m² pre-shift. During the work day, there was a statistically significant increase in serum creatinine, BUN, and uric acid of about 10% after a work shift as well as increases in urine density, urine creatinine and osmolality. Serum chloride and potassium levels decreased during the work day. The authors concluded that these results were consistent with repeated dehydration due to heavy physical labor in a hot and humid environment (44).

1.3.4   Clinical presentation

Most patients with MeN have no, few, or unspecific symptoms, and they receive the diagnosis based on decreased eGFR based on serum creatinine measurement. According to local physicians, most patients are found in screening programs in affected communities, in screening of sugarcane workers before the harvest season, or when the patients develop symptoms of uremia and seek medical care at the hospital. When we were starting our studies in 2012, no reports on kidney biopsy findings or renal morphology had been published.

1.3.4.1   Blood pressure

Blood pressure is usually normal in patients with MeN and cross-sectional studies in Nicaragua and El Salvador have shown that the prevalence of hypertension is between 18-37% among those participants who have elevated serum creatinine levels (20, 21, 45). Blood pressure levels in patients with MeN seem, from these reports, to be lower than expected in the general CKD population (46).

1.3.4.2   Chistata

Sugarcane workers in Central America often complain about what is locally called "chistata", a group of symptoms including dysuria and lower back pain. In a study by Ramirez-Rubio et al (47) from Nicaragua, 19 physicians and pharmacists working in an area with high prevalence of MeN were interviewed, and they reported that "chistata" symptoms are considered by some professionals to be urinary tract infections and are sometimes treated with antibiotics without a urine culture confirmation. Many of the interviewees also stated that they thought dehydration could be a possible cause of the "chistata" symptoms. A recently published study of sugarcane workers, construction workers and farmers in Nicaragua reported that "chistata" symptoms were common in all three groups

at between 42% and 48% (48). Dysuria has also been reported as a frequent symptom among adolescents in Nicaragua; however, urine dipsticks in the same subjects were mostly normal, i.e. neither leucocyte esterase nor nitrite positivity was observed (38). In a study performed by the Boston University School of Public Health (49), urine cultures were collected from 103 sugarcane workers in western Nicaragua (including 29 workers with dysuria symptoms and 30 workers with positive leukocyte esterase on urine dip stick). All were negative, indicating that "chistata" symptoms probably are not correlated to urinary tract infections, at least not common urinary pathogens. Currently, there is no explanation for what is causing these unspecific urinary tract symptoms.

1.3.4.3 Urine

Proteinuria is often absent or of non-nephrotic range in MeN. In a cross-sectional study in Nicaragua by Torres et al in 2010, 62% of the men and 67% of the women with CKD (eGFR <60 ml/min/1.73m²) displayed no proteinuria, and only 12% and 6% respectively showed proteinuria greater than 300 mg/dl (20). In another cross-sectional study from El Salvador, only 14% of the men and 26% of the women with CKD (eGFR <60 ml/min/1.73m²) were found to have proteinuria and all participants with proteinuria had levels below 300 mg/dl, i.e. of non-nephrotic range (21). In Nicaragua, a slightly higher prevalence of proteinuria has been reported from a cross-sectional study of 424 individuals in a MeN hot spot area. Among those with CKD (eGFR <60 ml/min/1.73m²) 44% had proteinuria ≥30 mg/dl but only 9% had proteinuria ≥300 mg/dl (19).

1.4 CKDu IN SRI LANKA

Figure 6: *Map of Sri Lanka. North Central Province is marked in red. Source, Map Data ©2017 Google.*

1.4.1 Overview

Over the past two decades, a high prevalence of CKDu has been reported from rural areas in Sri Lanka, especially among rice paddy farmers in the North Central Province of Sri Lanka, where the climate is hot and dry (50, 51) (Figure 6). Men seem to be affected more often than women (52, 53). Many different possible etiologies such as pesticides/agrochemicals, heavy metals, fluoride in the drinking water and water hardness have been discussed (50, 54, 55), but as yet no convincing evidence has been presented. Many clinical and biochemical findings seem to be similar to the MeN epidemic in Central America, i.e. patients with mild or moderate kidney failure are often

asymptomatic or complain about back pain or dysuria, urine findings are scarce, proteinuria is usually <1g/day, and ultrasound shows reduced kidney size (56).

One of the first published reports addressing the extent of the CKDu endemic was a retrospective study evaluating clinical records from 492 CKD cases at two nephrology units in which they found that the cause of CKD was unknown, i.e. it was not correlated to diabetes mellitus, hypertension or other known cause, in 54% and 82% of the cases respectively. The patients with CKDu had few symptoms and urine sediment was without active deposits (56).

In general, comprehensive epidemiological data about the CKDu endemic and detailed clinical descriptions of patients with CKDu in Sri Lanka are lacking. Screening for most studies has been based on dip-stick proteinuria, and only participants with proteinuria have been further evaluated with serum creatinine, thus missing patients who only have elevated creatinine levels. Many studies regarding CKDu in Sri Lanka have instead focused on identifying risk factors and possible etiologies.

1.4.2 Prevalence studies

In a publication by Chandrajith et al, preliminary findings from prevalence studies of inhabitants in six different regions were presented. The populations were screened for proteinuria, and the prevalence of CKD was found to range between 2-5% in the different regions. This prevalence is not very high; however, in endemic areas in the North Central Province a high percentage (84%) of the patients with CKD had CKDu compared to a much lower CKDu prevalence in non-endemic regions (54).

A more comprehensive study by Athuraliya et al was published in 2011 and studied 6153 inhabitants in three different regions, the North Central, the Central, and the Southern region. Participants with albuminuria on two out of three occasions were included and further evaluated with serum creatinine, urine sediment, blood pressure, kidney length measurement, etc. The CKD prevalence (defined as proteinuria $\geq$ 1+ on urine dipstick) in the three regions was 5%, 10%, and 2%, respectively, but in the North Central region 87% of the CKD cases were of unknown etiology, while diabetes and hypertension were the main risk factors of CKD in the other two regions. In the North Central province, men were more often affected than women, and in logistic regression models, being a farmer and being >60 years of age were found to be predictors of CKD (53).

Another cross-sectional study of 4957 participants included by screening for dip-stick albuminuria in endemic areas, showed a CKDu prevalence (defined as CKD stage 1-5 without known cause, no diabetes mellitus and blood pressure <160/100) of 17% in women and 13% in men. Among the women, >85% were in CKD stage 1 and 2, while a higher percentage of the men were in more severe CKD stages (55% in stage 1 and 2, and 45% in stage $\geq$3). CKD prevalence increased with older age and a family history of CKD was found in 20% of the CKDu cases (57).

Jayasekara et al (2015) screened 7604 individuals >5 years of age in the North Central Province for dip-stick proteinuria, and individuals with two consecutive positive samples were referred to a renal clinic for CKDu investigation. The authors reported that 6.3% of the screened population had CKDu, but the criteria for CKDu diagnosis were not presented, making an evaluation of the results difficult. They also collected information regarding the water source and reported CKDu to be more prevalent in individuals drinking water from shallow wells compared to natural springs. In the same article, the authors described a cohort of 3996 patients with newly diagnosed CKD from CKDu endemic areas and reported that the male to female ratio was 2.4:1 and that the CKDu prevalence was 70% (58).

In summary, the reported prevalence of CKD in these studies is not very high, but a high percentage of patients with CKD in the North Central Province were reported to have CKDu. However, the prevalence of CKD might be higher than reported because the studies only included participants with proteinuria and thus inhabitants with reduced kidney function without albuminuria were not included. Men and farmers seem to be more often affected by CKDu and the disease becomes more prevalent with older age.

1.4.3 Case-control studies

A few case-control studies have been conducted in Sri Lanka to try to establish possible risk factors and causes behind the endemic.

In one on the first case-control studies, interviewer-administered questionnaires from 183 patients with CKDu in Anuradhapura, in the endemic North Central Province, were collected and compared to 200 controls. Risk factors for CKDu were farming, pesticide use, drinking well water, Ayurvedic medicine use, family history of CKD, and snake bites. However, in logistic regression analysis, the only significant predictors were family history, snake bites, and Ayurvedic treatment (59).

Nanayakkara et al (2014) studied 311 CKD cases and 286 controls in two CKDu endemic areas in the North Central Province. Being a farmer, having a family history of CKD, snake bites, and tobacco chewing were found to be associated with CKDu in a multiple logistic analysis. Another interesting thing in this study is that 43% of the recruited "healthy controls" had to be excluded due to hypertension, elevated HbA1c, proteinuria, or elevated serum creatinine, indicating that non-communicable diseases are frequent in Sri Lanka (60).

1.4.4 Clinical presentation

The patients usually have a rather normal clinical profile, except for increased serum creatinine, although some vague symptoms of backache and dysuria have been reported (56). BMI and blood pressure are in most cases normal (53, 60), but one study has reported that a high percentage of CKDu patients have low BMI (52). In population studies, mean serum sodium, potassium, calcium, phosphate, and uric acid levels have been normal (53), but ultrasound of the kidneys has shown reduced kidney length (53, 56).

In urine, albuminuria is of non-nephrotic range and is usually < 1g/24 h (53), and biomarkers of tubular injury such as α-1-microglobulin (A1M), NAG, NGAL and Kidney Injury Molecule-1 (KIM-1) are often elevated (60-62). Urine dip-stick and sediment are only reported in a few studies, and the majority report normal findings (53), although increased amounts of erythrocytes and leucocytes have been reported in a minority of the patients (63).

1.4.4.1 Kidney morphology

A number of renal biopsy studies or reports of patients with CKDu in Sri Lanka have been published. The morphology is in most studies described as a tubulointerstitial disease with interstitial fibrosis, tubular atrophy, and varying degree of interstitial inflammation, without immunofluorescence (IFL) evidence of immune-complex disease. Chronic glomerular changes such as glomerulosclerosis, glomerular hypertrophy, and mild to moderate vascular damages (intimal thickening, arteriolar hyalinosis) have also been described. Electron microscopy findings have not been reported.

Biochemical and clinical data from biopsied patients are largely lacking. Summaries of the main findings in each biopsy study are presented in the text below, and in all of the studies the majority of the patients had proteinuria or proteinuria was one of the inclusion criteria.

Athuraliya et al (2011) presented a prevalence study of CKD in three rural communities. In one community, Medawachchiya in the northern dry region, 87% of CKD cases did not have a clear cause or risk factor. Of these participants 26 underwent renal biopsy with morphological findings indicating tubulointerstitial disease with varying degrees of interstitial fibrosis. IFL was negative. However, no semi-quantification or details were provided regarding the morphology in this report (53).

A more detailed description of the morphology was provided by Nanayakkara et al in 2012 (64). They evaluated 64 renal biopsies from CKDu patients who underwent renal biopsy at Anuradhapura hospital, one of the CKDu hot spots in Sri Lanka. Patients with diabetes mellitus, malignant hypertension, and other known etiologies were excluded. The mean age of the patients was 45 years, they were in CKD stage 1-4 and 55% (!) had hypertension at the time of the biopsy. The authors described the main morphological findings to be interstitial fibrosis and tubular atrophy, but they also reported a high percentage of global glomerular sclerosis (37%), glomerular enlargement (37%), and collapsing glomeruli (18%) as well as mild to moderate vascular changes. Unfortunately no clinical and biochemical data such as albuminuria or other serum and urine characteristics were reported in the article.

In 2015, Wijetunge et al published results from a retrospective study of 251 renal biopsies (65) collected at the Teaching Hospital Kandy and General Hospital Anuradhapura. Inclusion criteria were residence in a CKDu endemic area for ≥ 5 years, the presence of an interstitial renal disease not secondary to a glomerular or systemic disease, the absence of primary or secondary glomerular disease, and negative IFL. Patients with systemic disease known to cause kidney injury were excluded. The mean age of the patients was 37 years and 77% were men. Proteinuria (dip-stick ≥ 1+) was present in 83% of the cases. The authors presented the morphological findings for CKD stage 1-4. Stage 1 disease is described as mild to moderate interstitial fibrosis, stage 2 as moderate interstitial fibrosis with or without interstitial inflammation, stage 3 as moderate to severe interstitial fibrosis, moderate interstitial inflammation, tubular atrophy, and mild glomerulosclerosis, and stage 4 as, severe interstitial fibrosis, inflammation, tubular atrophy, and glomerulosclerosis. A weakness of the study is that the inclusion criteria "presence of an interstitial renal disease not secondary to a glomerular or systemic disease" probably excluded biopsies with glomerular damage, making it difficult to interpret the results of this study.

An interesting study by Badurdeen et al described renal biopsy findings and clinical data from 59 patients from CKDu endemic regions presenting with acute symptoms (backache, dysuria, fatigue, joint pain, and/or dyspepsia) and elevated creatinine levels (66). The majority of the included patients were male farmers and the mean age was 44 years. C-reactive protein (CRP) was elevated in 49% of the patients, and 49% had leukocyturia. Renal biopsies showed interstitial lymphocyte infiltration in 97% of the biopsies and tubulitis in 81%. Glomerulosclerosis, interstitial fibrosis, and tubular atrophy were also common features. The authors concluded the overall picture to be compatible with acute tubulointerstitial nephritis and proposed that this acute episode is followed by scarring and a pathological process leading to CKDu. A weakness of this study is that vascular renal structures are not described and no morphological pictures are available for the reader to see how the authors have evaluated the biopsies.

1.5 OTHER ENDEMIC NEPHROPATHIES

1.5.1 Aristolochic acid nephropathy / Balkan endemic nephropathy

Balkan endemic nephropathy is one of the most infamous endemic nephropathies. A high incidence of CKD was described in certain villages on the Balkan Peninsula already in the 1950s, and later a close association with upper urinary tract carcinomas became evident (67). The endemic was named Balkan Endemic Nephropathy (BEN). The renal morphology displayed tubulointerstitial atrophy, tubulointerstitial fibrosis and often multifocal urothelial atypia. Typically, glomeruli were spared, although secondary glomerular and vascular lesions could be present (68). For many years the etiology behind the disease was unknown.

In 1993, a report from Belgium was published describing nine women who developed interstitial fibrosis and renal failure after intake of slimming herbs containing aristolochic acid (69). The renal morphology was found to be similar to BEN cases, and the Belgian patients also developed urothelial carcinoma (68). Aristolochic acid had already in the 1970s been proposed as a possible etiology in BEN, because an aristolochic acid-containing plant was found growing in wheat fields in endemic areas and could contaminate the processed flour. However, this hypothesis was forgotten for many years until the report from Belgium appeared.

Further studies have identified aristolactam-DNA adducts and specific TP53 mutations in renal tissue of patients with BEN (70) and in patients with urothelial malignancies associated with the use of herbal medicines containing aristolochic acid in Taiwan (71). Thus, the diseases are regarded to have the same etiology and have thus been given the name aristolochic acid nephropathy.

1.5.2 Heavy metals

Many heavy metals are toxic to humans, and some heavy metals are also known to cause kidney damage. Lead, cadmium, and mercury are the most well-described heavy metals associated with kidney damage, and in most cases the exposure to the metal has been occupational.

Lead poisoning resulting in kidney damage is rare and requires high exposure to lead that would cause symptoms of lead poisoning in other organs. A typical morphological finding is intranuclear lead inclusions in tubular epithelial cells (72), but usually a diagnosis of lead nephropathy is based on a history of lead exposure in combination with reduced kidney function and renal biopsy morphology with non-specific findings, e.g. chronic tubulointerstitial damage and nephrosclerosis (73). A review of cross-sectional studies of occupational lead-exposed individuals by Evans and Elinder (74) concluded that lead nephropathy is a rather infrequent finding and that the presumption that non-toxic lead levels can cause CKD does not yet have supportive evidence.

Cadmium can cause kidney damage characterized by tubular proteinuria, which usually is irreversible (75). Kidney function may deteriorate, but severe CKD is uncommon. Cadmium exposure is in most cases due to smoking, eating contaminated foods, or occupational exposure (e.g. working in the industrial battery industry) (76). Renal biopsy descriptions are scarce, but a case description of the morphology after cadmium poisoning shows severe tubular atrophy, interstitial fibrosis, subcapsular inflammation, and chronic glomerular damage (77).

Mercury-induced kidney damage typically causes nephrotic syndrome and tubular injury where the proteinuria usually vanishes when exposure ends. Typically the morphological findings are

membranous nephropathy, but in some cases minimal change lesions have been reported (78). Exposure to mercury is mainly through diet (fish) and dental amalgam fillings or through occupational exposure, e.g. the chloralkali industry or dentistry. The effects of amalgam fillings on health has been debated, but there is no evidence that mercury from amalgam fillings is nephrotoxic or can cause other significant adverse health effects (73, 79).

1.5.3 CKDu in India / Uddanam nephropathy

A high incidence of CKDu has also been reported from certain regions in India, especially in the rural areas of the Andhra Pradesh region (80). This endemic disease has been named Uddanam nephropathy. Published scientific data is scarce, but local media has reported on the steadily increasing need of dialysis treatment (81). An unpublished cross-sectional study presented in an abstract in 2009 (82) studied 443 individuals in a village in the Srikakulam district in Andhra Pradesh region and reported that 55% of the inhabitants had CKD with eGFR <60 ml/min/1.73m². The study also reported that the high prevalence was not associated with traditional risk factors for CKD like diabetes and hypertension and that only 15% of the affected individuals had proteinuria. Renal biopsies from patients showed normal glomerular structures, lymphocytic peritubulitis, tubular atrophy, interstitial fibrosis, and normal vessels.

The cause of CKDu in India or Uddanam nephropathy is not known, but various etiologies have been suggested such as environmental toxins/pesticides, chemicals in the water and heat exposure (83, 84).

The CKDu endemic in India seems to have some similarities with CKDu endemics in other countries with warm climates, such as MeN in Central America and CKDu in Sri Lanka. All of these CKDu epidemics have in common that they mainly affect poor rural farming communities, and clinically the patients do not have diabetes or hypertension and a majority of patients does not have proteinuria (15, 85). However, the CKDu endemic in India is, as of yet, insufficiently described and further studies are needed to determine the prevalence and clinical characteristics of the disease before a comparison can be made with CKDu endemics in other countries.

2 AIM OF THE STUDIES

The aim of this thesis was to describe and characterize case series of patients with MeN in Central America and CKDu in Sri Lanka with a focus on renal morphology and biochemical characteristics to enhance our understanding of the endemics, including possible pathophysiological processes and etiologies. With this detailed description of case series the aim was also to be able to compare the endemics and elucidate if the diseases are similar diagnostic entities.

The specific objectives for the studies were as follows.

Study I

- To describe the renal morphology in Mesoamerican nephropathy, which has not previously been described.
- To analyze urine and blood samples and clinically relevant data, including work history and medical history in order to provide a detailed description of affected individuals.

Study II

- To describe the renal morphology and biochemical characteristics of Mesoamerican nephropathy in another Central American country using a similar study design as in Study I.
- To study heavy metals in the urine of affected individuals in order to evaluate any toxic exposure to these compounds.
- To collect a detailed assessment of the use of analgesic medicines.
- To study the natural history of Mesoamerican nephropathy by collecting follow-up blood and urine samples from the participants in Studies I and II.

Study III

- To describe the renal morphology and biochemical characteristics in patients with CKDu in Sri Lanka using a similar study design as in Studies I and II.
- To compare the results with Studies I and II in order to elucidate if Mesoamerican nephropathy in Central America and CKDu in Sri Lanka are related diseases.

3   MATERIAL AND METHODS

All studies were conducted in May or June, i.e. outside of harvest season. Participants in all studies were patients who had a CKD diagnosis from a nephrology unit or an occupational health clinic in each country. All participants were recruited by local physicians/nephrologists.

3.1   STUDY SUBJECTS

3.1.1   Study I

Male patients with CKD of unknown cause living and/or working in affected areas in El Salvador were recruited by Dr. Ricardo Leiva and co-workers at the Department of Renal Medicine, Hospital Nacional Rosales, in San Salvador, El Salvador.

Inclusion criteria:

- CKD of unknown cause, i.e. no clinically known kidney disease diagnosis
- 20-60 years of age
- Serum creatinine levels of 120-220 μmol/L (1.36-2.49 mg/dl) or eGFR of 30-60 ml/min/1.73m²

Exclusion criteria:

- Diabetes mellitus, defined as fasting blood glucose >7.0 mmol/L (>126 mg/dl)
- Uncontrolled hypertension, defined as blood pressure >140/90 mm Hg or treatment with more than one antihypertensive drug
- Nephrotic-range proteinuria (24-hour protein excretion >3.5 g).

3.1.2   Study II

Male patients with a history of sugarcane work who had been evaluated due to CKD of unknown cause at the Research Center of Health, Work and Environment at the National Autonomous

University of Nicaragua, León were recruited to the study. Dr. Marvin González-Quiroz was responsible for the recruitment. The enrolment was performed during a 12-month period, and all patients who fulfilled the inclusion criteria were invited to participate. Changes in inclusion/exclusion criteria compared to Study 1 are in bold letters.

Inclusion criteria:

- CKD of unknown cause
- 20-65 years of age
- Serum creatinine levels **100-220** μmol/L (1.13-2.49 mg/dl) or eGFR **30-80** ml/min/1.73m²

Exclusion criteria:

- Diabetes mellitus, defined as fasting blood glucose >7.0 mmol/L (>126 mg/dl)
- Uncontrolled hypertension, defined as blood pressure >140/90 mm Hg or more than one antihypertensive drug
- 24-hour protein excretion **>3 g**

3.1.3 Study III

Male patients with chronic kidney disease of unknown cause and who were planned for a renal biopsy at Polonnaruwa General Hospital, Sri Lanka, were recruited by Dr. Rajeewa Dassanayake and co-workers at the hospital's renal unit.

Inclusion criteria:

- CKD of unknown cause
- 20-65 years of age
- Serum creatinine 100-220 μmol/L or eGFR 30-80 ml/min/1.73m².

Exclusion criteria:

- Diabetes mellitus, defined as fasting blood glucose >7.0 mmol/L (>126 mg/dl)
- Uncontrolled hypertension, defined as >140/90 mmHg or more than one antihypertensive drug
- Proteinuria >1g/24h

3.2 RENAL BIOPSY PROCEDURE

3.2.1 Overview

Renal biopsy is a clinically established procedure in the investigation of unknown CKD. In most cases, the pathologist's evaluation of the renal tissue can provide a diagnosis and assess prognosis, and the treatment can be altered accordingly. However, complications after renal biopsy may occur. The most common complications are hematuria and bleeding. Hematuria occurs in 3-18% of patients, bleeding severe enough to cause hypotension occurs in 1-2%, and blood transfusions are required in 0.9 - 6% (86, 87) of the patients. Major severe bleeding that requires surgery or arterial embolization is rare and only occurs in 0.1-0.4% of patients (86). Other minor complications include local pain,

drop in hemoglobin level and local soft tissue infection. Late complications, such as the formation of a symptomatic arteriovenous fistula are rare. The risk of mortality is 0 - 0.1% (86-88).

Renal biopsies usually measure 1.2-1.6 × 10-15 mm and are collected from the upper or lower kidney pool. If collected correctly, the biopsy should contain mostly kidney cortex (where the glomeruli are located) and some medulla. Usually two to three renal biopsies from one kidney are required to make a proper morphological evaluation. Risk factors associated with complications are coagulation defects, elevated serum creatinine, elevated blood pressure (>140/90 mmHg), and female gender (86, 89). Some studies have also reported that young age is a risk factor (89, 90). The needle sizes used to collect renal biopsies are normally 14 to 18 Gauge (G). Most studies have not seen any difference in complication rates between the different needle sizes (86), although a recent Swedish study reported more complications with a smaller needle size of 18 G compared to 16 G (91). Furthermore, the larger needle sizes (14 G and 16 G) generally provide better tissue samples (more glomeruli) for morphological evaluation and may require fewer biopsies/passes.

In all three studies, the renal biopsy procedure was conducted according to Swedish and international procedures to minimize the risk of complications. All participants had a clinical check-up before the biopsy to ensure that blood pressure was normal, that the patients did not have a history of coagulation defects, and that the patients had not used any medication affecting blood coagulation (including NSAIDs, aspirin, warfarin, heparin, etc.) during the last 2 weeks. Blood tests from participants were analyzed with complete blood count, platelet count, prothrombin time (PK)/INR, and APTT before the biopsy to ensure that no measurable coagulation defect was present. Ultrasound of the kidneys was performed before the biopsy, and if solitary kidney, small hyperechoic kidneys, or other major abnormalities were found no biopsy was taken.

The renal biopsies were collected by experienced local nephrologists using an ultrasound-guided percutaneous technique with a spring-loaded biopsy needle (14 G in Study I and 16 G in Study II and III) (Figure 7). After the biopsy procedure, the patients remained in a lying position for a minimum of 6 hours, blood pressure was checked regularly and the urine was controlled for macroscopic hematuria. The patients stayed at the hospital for observation for 24 hours after the biopsy. After discharge, all patients were followed up with a visit at the nephrology outpatient clinic to receive information about the test results including biopsy findings.

Figure 7: Dr Luis Trujillo and Dr Ricardo Leiva preparing for a renal biopsy procedure at Hospital Rosales, San Salvador, El Salvador (Study I). The patient is awake and lying on his stomach. Ultrasound was performed before and during biopsy procedure. Photo courtesy of Annika Wernerson.

3.2.2 Tissue preparation

In Study I, the renal biopsies were divided into two parts immediately after the biopsy procedure. One part was placed in phosphate-buffered 4% formaldehyde and stored at ambient temperature and the other part was snap frozen in liquid nitrogen and then stored in a freezer at -18°C.

In Study II, the renal biopsies were divided into three parts immediately after the biopsy procedure. One part was placed in phosphate-buffered 4% formaldehyde at ambient room temperature, and one part was snap frozen in liquid nitrogen and stored at -20°C. The third part for transmission electron microscopy (TEM) was put in a 0.1 M phosphate buffer with 2.5% glutaraldehyde and 0.5% paraformaldehyde.

In Study III, the renal biopsies were divided into three parts immediately after the biopsy procedure. One part was placed in phosphate-buffered 4% formaldehyde, the second part was placed in Zeus fixative solution, and the third part for TEM was placed in 0.1 M phosphate buffer with 2% glutaraldehyde and 1% paraformaldehyde. All samples were stored at room temperature.

Within one week after renal biopsy collection the specimens were transported to Karolinska University Hospital in ambient temperature and on dry ice respectively.

3.2.3 Light microscopy

Light microscopy is the most important part in evaluating the morphological findings in kidney tissue from patients with acute and chronic kidney diseases. The tissue is sectioned and stained according to different protocols to facilitate the evaluation of specific structures in the renal tissue. In many cases it is possible to find a specific diagnosis after light microscopy evaluation by an experienced pathologist.

In the studies for this thesis, the formaldehyde-fixed tissues were further embedded in paraffin, sectioned into 1-2 µm sections, and stained according to standard protocols with hematoxylin and eosin (HE), Ladewig, periodic acid Schiff (PAS), and periodic acid silver methenamine (PASM). Polarized light was used to detect any birefringent crystals in the paraffin-embedded tissue (Studies I, II, and III) and in frozen sections (Study II, in the nine patients from whom frozen material was available). De Galantha´s staining (92) was performed on paraffin-embedded material in Study II to detect any urate crystals.

Light microscopic evaluation of the renal biopsies was performed by Annika Wernerson and Magnus Söderberg, two experienced senior consultants at the Department of Clinical Pathology and Cytology, Karolinska University Hospital. The initial evaluation was independently performed and blinded to study number and biochemical data. After initial evaluation the two pathologists discussed their findings to reach consensus (differences between the pathologists' evaluations were found in about every tenth semi-quantifications, but these only changed the results by one step in a four-graded scale). The histological findings were semi-quantified according to the scheme in Table 1. Kidney specimens with > 10 glomeruli were considered representative, specimens with 7-9 glomeruli as marginally representative and specimens with <7 glomeruli as not representative.

Table 1: *Morphological parameters evaluated with light microscopy in Studies I, II, and III.*

	Scale	Comment
Glomerular pathology		
Total number of glomeruli	Counts	
Globally sclerosed glomeruli	Counts	
Segmentally sclerosed glomeruli	Counts	
Endocapillary cell proliferation	0-1	0=no, 1=yes
Matrix increase	0-3	0=no, 1=mild, 2=moderate, 3=severe
Cell proliferation	0-3	0=no, 1=mild, 2=moderate, 3=severe
Glomerular size	0-3	0=no, 1=mild, 2=moderate, 3=severe
Wrinkling of GBM/ Thickening of Bowman's capsule	0-1	0=no, 1=yes
Tubulointerstitial pathology		
Tubular atrophy	0-3	0=no 1=mild, 6%-25% 2=moderate, 26%-50% 3=severe, >50% of the cortical area
Tubulitis	0-3	0=no, 1=mild, 2=moderate, 3=severe
Granulocytes in tubuli	0-1	0=no, 1=yes
Interstitial fibrosis	0-3	0=no 1=mild, 6%-25% 2=moderate, 26%-50% 3=severe, >50% of the cortical area
Interstitial inflammation	0-3	0=no 1=mild, 6%-25% 2=moderate, 26%-50% 3=severe, >50% of the cortical area
Vascular pathology		
Intimal thickening	0-3	0=no, 1=mild, 2=moderate, 3=severe
Smooth muscle hyperplasia	0-3	0=no, 1=mild, 2=moderate, 3=severe
Arteriolar hyalinosis	0-3	0=no, 1=mild, 2=moderate, 3=severe

3.2.4 Immunofluorescence (IFL)

IFL is an immunohistochemistry technique used to localize a specific protein in the tissue section. The technique uses antibodies that have been conjugated to fluorescent dyes and when the antibody binds to an antigen epitope (the protein of interest) in the tissue section, the location and intensity of the fluorescence are evaluated using a fluorescence microscope.

In this study, the specimens for IFL (the snap-frozen tissues from Studies I and II and Zeus solution tissues from Study II that had been rinsed and snap frozen) were sectioned into 5 μm cryosections and were incubated with FITC-conjugated antibodies against immunoglobulins (IgA, IgG, and IgM), complement (C1q, C3), light chains (kappa and lambda), and fibrinogen (antibody manufacturer Dako, Glostrup, Denmark). Evaluations were performed by renal pathologist Annika Wernerson using a fluorescence microscope (Microphot-FXA, Nikon, Tokyo, Japan).

3.2.5 Transmission electron microscopy (TEM)

An electron microscope has a much higher resolution than a light microscope and allows tissue samples to be studied on a cellular and subcellular level. Instead of using ordinary light (photons), the electron microscope uses a beam of accelerated electrons, and because the electrons have a shorter wavelength a more detailed image can be produced. The electron microscopy image is in gray scale and reflects the density of different structures in the tissue sample. TEM is the most commonly used electron microscopy technique when evaluating tissue samples including renal biopsy samples.

TEM is utilized in the routine diagnostic evaluation of renal specimens, in particular to study the glomerular compartment of the nephron. The pathology of subcellular structures can be evaluated, e.g. the thickness of the glomerular basement membrane (GBM), podocyte foot-process effacement, immune complex deposits, etc.

In the studies for this thesis, the renal biopsy specimens for TEM were embedded in epoxy resin and cut into 60 nm sections (in Study I, tissues stored in formaldehyde were fixed in a 0.1 M phosphate buffer with 2.5% glutaraldehyde and 0.5% paraformaldehyde before being embedded in epoxy resin). The sections were then evaluated by Kjell Hultenby (head of the Electron Microscopy Department) and Annika Wernerson using an electron microscope (Tecnai 12, FEI, Eindhoven, The Netherlands). GBM thickness was measured in five areas in five randomly selected capillaries per glomerulus, and the mean thickness was calculated. In the same capillaries, podocyte foot-process effacement was semi-quantified by calculating the numbers of slits per μm GBM (Figure 8). Effacement was defined as widespread if ≥80% of the measured areas had <1.0 slit/μm GBM, or segmental if 21% to 79% of evaluated areas had <1.0 slit/μm GBM.
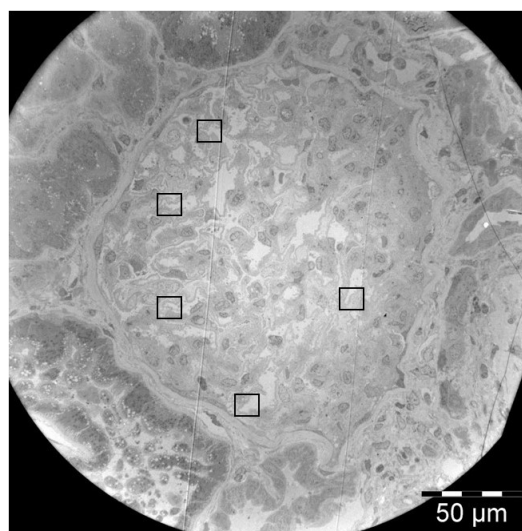

Figure 8: *Transmission electron microscopy image in low magnification showing a glomerulus. For assessment of GBM thickness and degree of foot-process effacement five randomly capillaries (squares) were studied.*

3.3 BLOOD AND URINE SAMPLES

Within two weeks before the renal biopsy procedure, whole blood analysis was performed on participants to measure electrolytes, complete blood cell count (including platelets), and relevant coagulation tests (PK/INR, APTT, bleeding time, etc.). In Studies II and III urine samples were collected before the biopsy for microscopic examination of urine sediment, urine dip stick, urine culture, and 24 hour urine collection (Study II only). In Study I the same samples were collected 2-3 months after the biopsy. All of these tests were performed by local laboratories (Hospital Nacional Rosales laboratory (Study I), the laboratory of the faculty of medicine at National Autonomous University of Nicaragua at León (Study II), and General Hospital Polonnaruwa (Study III)).

New blood samples and urine samples were collected on the morning before the biopsy procedure. All participants were fasting, and no intravenous fluid had been given before the sample collection. Blood samples were centrifuged, and the supernatant was pipetted into transporting vials. Samples were stored in freezers (-18°C, -20°C and -40°C respectively) and then transported within one week on dry ice to Karolinska University Hospital where the samples were stored at -70°C pending analysis.

Table 2 presents the plasma/serum and urine constituents analyzed at Karolinska University Hospital Laboratory for the different studies. Urine heavy metals were analyzed in Studies II and III by ALS Scandinavia AB Laboratory, Luleå, Sweden. eGFR was calculated using the CKD-EPI equations for creatinine, $eGFR_{Cr}$ (93); Cystatin C, $eGFR_{CystC}$ (94); and Creatinine + Cystatin C, $eGFR_{Cr+CystC}$ (94).

Table 2: *Plasma/serum and urine constituents analyzed in the study.*

Plasma/Serum	Study I	Study II	Study III
Creatinine	x	x	x
Cystatin C	x	x	x
Urea nitrogen	x	x	x
CRP	x	x	x
Sodium	x	x	x
Potassium	x	x	x
Magnesium	x	x	x
Calcium	x	x	x
Uric acid	x	x	x
Albumin	x	x	x
Phosphate	x	x	x
ALAT*	x	x	x
ANA screening	x	x	x
ANCA screening	x	x	x
Anti-GBM-ab	x	x	x
Complement	x	x	x
β2-microglobulin	x		x
Renin	x	x	x
Aldosterone	x	x	x
Osmolality		x	
HIV	x	x	x
Hepatitis B & C	x	x	x
Leptospira			x
Hatanvirus			x

Urine	Study I	Study II	Study III
Albumin	x	x	x
Creatinine	x	x	x
Uric Acid	x	x	x
Osmolality			x
NAG	x	x	
A1M	x	x	x
NGAL			x
Sodium	x	x	x
Potassium	x	x	x
Magnesium			x
Arsenic		x	x
Cadmium		x	x
Mercury		x	x
Lead		x	x
Uranium			x
Vanadium			x

3.3.1 Blood and urine samples follow-up in Study II

In Study II, follow-up blood and urine samples were collected from patients in Study I (El Salvador) and Study II (Nicaragua). From El Salvador, 7 of the 8 original patients were included in the follow-up and in Nicaragua 18 of the 19 original participants were included, thus 2 of the 27 participants were lost to follow up.

In El Salvador, the samples were collected 19 to 26 months after the renal biopsy. Current employment and whether the patient was dead or in dialysis were also reported. Four of the seven samples were transported and analyzed at Karolinska University hospital, and three samples were analyzed at Hospital Rosales. Creatinine, sodium, and potassium were measured in serum/plasma, and albumin, creatinine and NAG were measured in urine. Change in eGFR was calculated.

In Nicaragua, the samples were collected 13 to 17 months after the renal biopsy. Samples were analyzed at Karolinska University Hospital for creatinine, cystatin C, sodium, potassium, and uric acid in plasma and for albumin, creatinine, and A1M in urine. Change in eGFR was calculated.

3.4 QUESTIONNAIRES

In all three studies, patients were interviewed by local physicians/researchers in their native tongue, and a questionnaire was filled out by the interviewer. The base of the questionnaire was the same in all studies and included questions about employment (current and previous), current use of medications (including antihypertensive medicines, NSAIDs and paracetamol/acetaminophen), any use of antibiotics in the last 6 months, use of herbal medicines, daily liquid intake, and smoking habits. In Study II, the part of the questionnaire regarding analgesic use was more detailed to allow a better

assessment of lifetime exposure. In Study III, snake bites, family history of CKD, and pesticide exposure were added to the questionnaire.

3.5 STATISTICS

Since all three studies were relatively small case series, usually only the mean and standard deviation and range were reported. However, in Study II, where follow-up samples from both Study I and II were combined (n=25), simple linear regression was used to predict change in eGFR per year, based on blood and urine results at baseline. In the same study, unpaired t-test was used to compare the mean change in $eGFR_{Cr}$ per year between groups with different morphology.

3.6 ETHICAL CONSIDERATIONS

It is standard procedure to collect renal biopsies in the clinical evaluation of chronic kidney disease in most middle and high-income countries. Nevertheless, the procedure carries a risk of serious complications. One of the most feared complications is post-biopsy bleeding that might require blood transfusions, or in severe and rare cases, may require surgery or interventional radiology. In all three studies, our first priority was to ensure that the biopsy procedure was conducted safely and according to international standards, as described in the section "Renal biopsy procedure", to minimize the risk of complications and to ensure that the biopsies were conducted in facilities where blood transfusions could be given and surgeons, if needed, could intervene in case of uncontrolled bleeding. In Studies I and II, our local collaborators had experience in performing renal biopsies; however, clinically motivated biopsies were rarely carried out due to lack of renal pathologists. In Sri Lanka, on the other hand, renal biopsies are more often collected in the clinical evaluation of patients with CKD, and all of our included patients in Study III were already planned for a clinically motivated renal biopsy by the local physician/nephrologist. Another ethical consideration was to ensure that the participating patients received the results from the morphological and biochemical analyses performed in Sweden. This was achieved by sending all of the results to the nephrologist in charge in each country who then presented the results to the patient and, if needed, altered the treatment accordingly.

3.6.1 Ethics committee approvals

Ethics approvals for the studies were received from the Ethics Committee at Hospital Nacional Rosales, San Salvador, El Salvador (ACTA N°:03-2012 and ACTA EXP. N°: 11/2016)); the Ethics Committee at the National Autonomous University of Nicaragua at León (ACTA No 83, 2013; ACTA No 31); the Ethics Review Committee at the Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka (ERC/2016/06); and from the Ethics Committee in Stockholm, Sweden (Dnr 2012/441-31/3, 2013/1225-32, 2015/849-32 and 2016/747-32). All participants gave informed consent.

4 RESULTS

4.1 STUDY I (EL SALVADOR)

Eight male agricultural/plantation workers with a mean age of 44 (range 22-57) years were included in the study. All participants had normal blood pressure of 100-130/60-80 mmHg and their mean BMI was 26 (range 20-35) kg/m².

4.1.1 Questionnaire

The number of years working on plantations varied from 8 to 44 (mean 32) years and sugarcane, corn, beans and sorghum were the most common crops cultivated at the plantations. Only two patients reported any chronic disease (one hypertension and one hyperuricemia), but three patients were on antihypertensive medication. NSAID use was frequent, and two patients reported daily use of NSAIDs and five reported weekly use. Estimated daily liquid intake was 2.5-5 liters, and water was the main liquid consumed.

4.1.2 Biochemical characteristics

The mean plasma creatinine was 172 ± 50 μmol/L (1.94 ± 0.57 mg/dl) and the mean eGFR$_{Cr}$ was 46 ± 20 (range 27-74) ml/min/1.73m² in the study group, with two patients in CKD stage 2, four in CKD stage 3, and two patients in CKD stage 4. Electrolyte disturbances were common, and low levels of plasma potassium and/or sodium were found in six of the eight patients. Low magnesium levels were found in four patients, and uric acid was elevated (>7.0 mg/dl) in six of the eight patients. Most other biochemical parameters measured in blood showed normal values. Urine dip-stick and sediment showed trace, 1+, and 2+ albumin, respectively, in three patients, and in the other patients no pathology was found (i.e. no casts, hematuria, glucosuria etc.). Albuminuria was rare, only two patients had increased albumin-creatinine ratio (ACR), and both were of non-nephrotic range. Markers of tubular damage NAG and/or A1M were elevated in all but one patient.

4.1.3 Renal morphology

Renal biopsies of representative quality were collected from all patients. Surprisingly, all eight biopsies had a very similar morphology, which did not resemble any other known kidney disease, i.e. the results indicate that MeN is a unique diagnostic entity with a specific morphology. The main findings are presented in the following sections.

4.1.3.1 Light microscopy and immunofluorescence

Extensive glomerulosclerosis, ranging from 29% to 78%, of the included glomeruli was observed in the specimens (Figure 9A). In all patients, glomerular hypertrophy and signs of glomerular ischemia with wrinkling of the glomerular capillaries and/or thickening of Bowman's capsule were seen (Figure 9B, 9D). Segmentally sclerosed glomeruli were observed in two patients.

The tubulointerstitial compartments showed chronic damage with mild to moderate tubular atrophy, interstitial fibrosis, and interstitial inflammation (Figure 9A, 9C), although no specimens displayed severe damage. Mild tubulitis was found in one patient, but no neutrophil granulocytes were observed in the tubular lumina. In one patient, a few eosinophils were seen in peritubular capillaries.
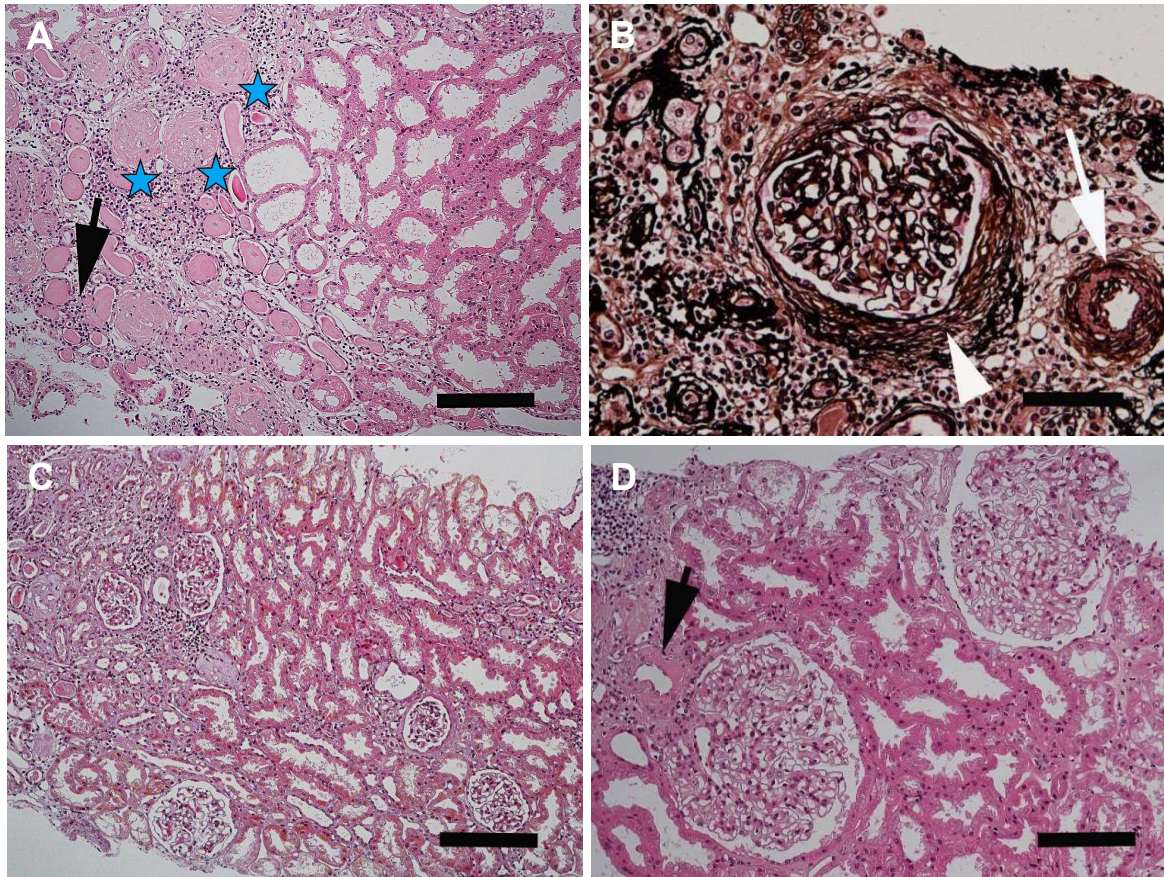
Figure 9: *Light microscopy images from Study I. Glomerulosclerosis (stars in A) and glomerular hypertrophy (D) were seen in all biopsies. Signs of glomerular ischemia with thickening of Bowman's capsule (white arrow head in B) were common. Tubular atrophy (black arrow in A), interstitial inflammation (A and C) and interstitial fibrosis (C) were of mild to moderate degree. Arteries were usually normal or had mild changes such as smooth muscle hyperplasia (white arrow in B). Arterioles were usually normal, but a few had mild hyalinosis (black arrow in D). A: HE staining from patient 6, bar=200 μm. B: PASM staining from patient 2, bar=50 μm. C: Ladewig staining from patient 3, bar=200 μm. D: HE staining from patient 2, bar=200 μm.*

Arteries and arterioles in the specimens were normal or had mild pathology (Figure 9B, 9D). In arteries, mild intimal thickening was found in only two specimens, but mild smooth muscle hyperplasia was found in all but one of the biopsies. Arterioles were mostly normal, although three specimens showed mild hyalinosis. IFL on the frozen material did not show any signs of immune-complex disease, i.e. was negative for immunoglobulins, complement, light chains and fibrinogen.

4.1.3.2 Electron microscopy (EM)

In the glomeruli, podocyte foot-process effacement was found in three patients. Vacuoles and fat droplets were frequently found in the podocyte cytoplasm (Figure 10). The GBM, endothelial cells, and mesangium were normal in most patients. In one patient, small amounts of electron-dense deposits were found in the mesangium (subsequent IFL on pronase-treated paraffin-embedded material showed small amounts of IgG-deposits in the mesangium (IgA and IgM negative), suggesting that the immune complexes could represent a remnant of a previous episode of glomerulonephritis in this patient, e.g. postinfectious glomerulonephritis). In the tubuli the evaluation was difficult due to fixation artifacts, but no lead inclusions could be seen in the tubular cells.
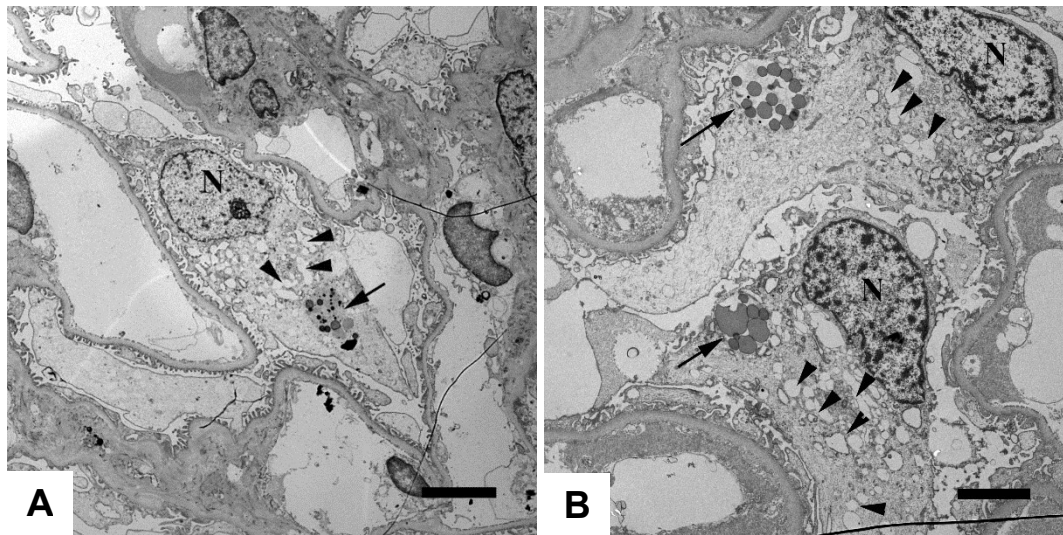
Figure 10: *Transmission electron microscopy images from patient 1 (A) and patient 7 (B) showing vacuoles (arrow heads in A and B) and lipid droplets (arrows in A and B) in the podocyte cytoplasm. N=nucleus. Bars=5 μm.*

4.2 STUDY II (NICARAGUA)

Nineteen male sugarcane workers in Nicaragua with a mean age of 33 (range 24-54) years were included in the biopsy study. All participants had normal blood pressure of 105-133/56-88 mmHg and their mean BMI was 25 (range 19-37) kg/m². In the follow-up study, 25 patients were included (18 from the Nicaragua biopsy study and 7 from the El Salvador biopsy study (Study I)).

4.2.1 Questionnaire – Biopsy study

The 19 participants had been working with sugarcane cultivation for a mean of 12 (range 2.5-38) years. None of the participants were on hypertensive medicines. Among the participants, 32% reported previous regular NSAID use (defined as weekly use for ≥ 2 years), and four patients reported current use of NSAIDs. Herbal remedy use was common (37%). Mean water intake on work days and non-work days was 9 liters and 5.3 liters respectively. Seven subjects reported that they had fainted during work (mean 3 times, range 1-6 times).

4.2.2 Biochemical characteristics – Biopsy study

The mean plasma creatinine concentration was 146 ± 34 µmol/L (1.66 ± 0.38 mg/dl) and the mean eGFR$_{Cr}$ was 57 (range 33-96) ml/min/1.73m². Electrolyte disorders were common, and low potassium was found in 21% of the participants, low sodium in 47%, and low magnesium in 37%. Hyperuricemia was found in 58% of the participants, and 47% had 24 h urine volume > 3 liters, i.e. polyuria. Urinary potassium levels indicated renal potassium losses in the patients with hypokalemia. Urinary heavy metals were all below toxic levels.

4.2.3 Renal morphology – Biopsy study

All 19 participants underwent renal biopsy with 13 biopsy specimens being representative, 3 marginally representative, and 3 being not representative. The latter were excluded from the group analysis. The morphological findings were homogenous and were very similar to the morphological findings in Study I. The main findings were as follows.

4.2.3.1 Light microscopy and immunofluorescence

All specimens showed glomerulosclerosis of varying degree (7-70%) (Figure 11) and glomerular hypertrophy. Segmental scleroses were found in two patients, and signs of glomerular ischemia were found in all but one patient (Figure 11A). Tubular atrophy and interstitial inflammation were generally mild (Figure 11B), and interstitial fibrosis was in most cases mild to moderate. None of the participants had severe tubulointerstitial changes. An unspecific finding of a few neutrophil granulocytes in the tubuli were observed in two patients and a few interstitial eosinophils were observed in two other patients. Arteries were in most cases normal or mildly damaged with mild intimal thickening and/or mild to moderate smooth muscle hyperplasia (Figure 11B). Arterial hyalinosis was found in 31% of the patients. In all but four patients, material for IFL was available. In the available specimens, there were no signs of immune complex disease.
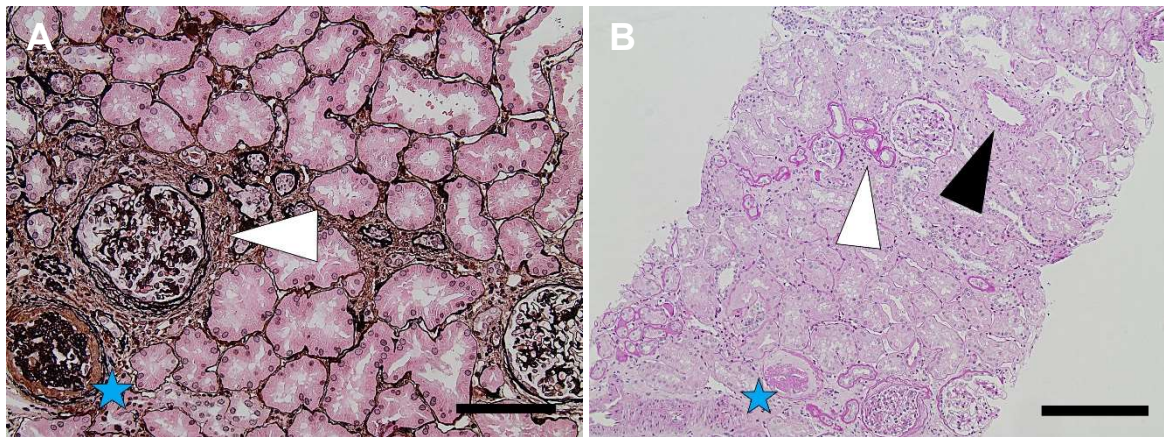

Figure 11: *Light microscopy findings from Study II. Glomerulosclerosis (stars in A and B) of varying degree was found in all participants. Thickening of Bowman's capsule (white arrow head in A) was a frequent finding. Tubulointerstitial changes were generally mild to moderate, with mostly mild tubular atrophy (white arrow head in B). Arteries were normal (black arrow head in B) or mildly changed. A: PASM staining, patient 13, bar=100 μm. B: PAS staining, patient 9, bar=200 μm.*

4.2.3.2 Electron microscopy

Podocyte foot-process effacement was observed in 56% of the specimens (Figure 12A) and the GBM

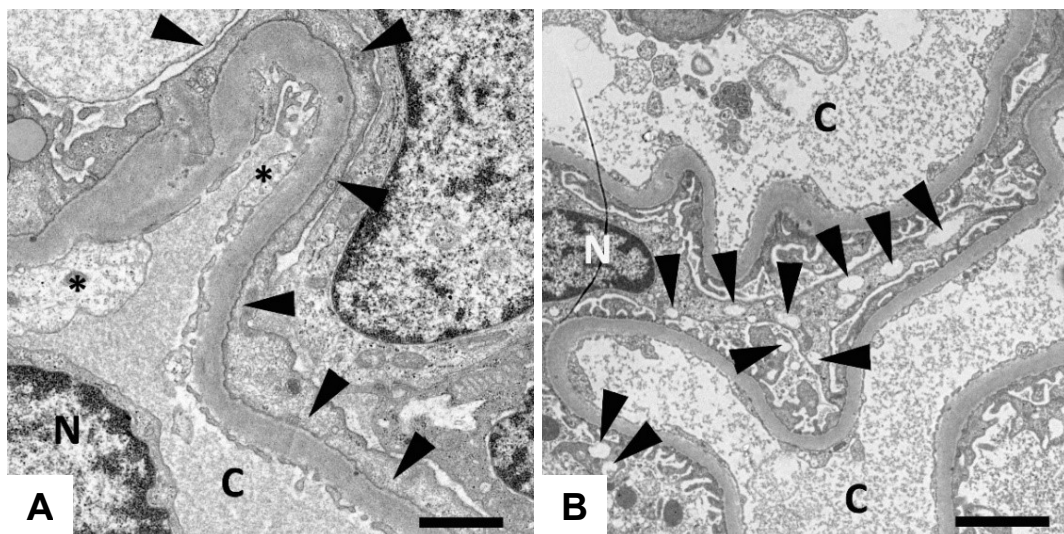**Figure 12:** *Transmission electron microscopy findings in Study II. Podocyte foot-process effacement (black arrow heads in A, patient 17) and podocyte cytoplasm inclusion such as vacuoles (arrow heads in B, patient 10) or lipofuscin-like bodies were found in the majority of the patients. Endothelial cells were in some cases focally swollen (* in A). C=capillary space. N=nucleus. Bars: A=1μm, B=5μm.*

was thickened in a few cases. The endothelium was swollen in 38% of the specimens. Vacuoles and lipofuscin-like bodies in the podocyte cytoplasm were a common finding (Figure 12B). Tubular cells showed signs of atrophy but no other evident pathology. No immune-complexes were found in any of the specimens.

4.2.4 Follow-up study

The mean $eGFR_{Cr}$ was 45 ± 25 (range 9-76) ml/min/1.73m² in the El Salvador patient group (n=7) and 52 ± 20 (range 20-87) ml/min/1.73m² in the Nicaragua patient group (n=18) at follow-up, which corresponds to a mean change in $eGFR_{Cr}$ of -4.4 ± 8.4 (range -27.7-10.2) per year when both studies were combined. Electrolyte disturbances were still common, and low sodium levels were found in 11 of the 25 patients (44%) and low potassium levels were found in 6 of the 25 patients (24%). Elevated urinary ACR of non-nephrotic range was found in four patients, three of them had elevated ACR at the time of the biopsy and at follow-up the levels had increased. In one patient the elevated ACR was new and measured only 5.1 mg/mmol.

When studying possible biochemical or morphological predictors of a decrease in $eGFR_{Cr}$, low serum sodium or severe glomerular enlargement in the biopsy at baseline was significantly correlated with a larger decrease in eGFR, however, due to the limited sample size, these associations might not be valid and need to be confirmed in larger cohorts.

4.3 STUDY III (SRI LANKA)

Eleven male patients with a mean age of 48 ± 11 (range 27-61) years with a history of rice paddy farming were included in the study. Mean BMI was 20 (range 16-28) kg/m², and mean blood pressure was 122/78 mmHg.

4.3.1 Questionnaire

Three of the participants had a family history of CKD. The patients reported that they had been farmers between 7 and 40 years, and all reported exposure to multiple pesticides and fertilizers. Mean liquid intake was 4.3 liters per day and water was the main liquid intake. Few patients used analgesics, but the majority of participants reported prior use of traditional herbal medicines (Ayurvedic medicines). Three participants used antihypertensive medicines and two participants were on potassium-chloride supplements.

4.3.2 Biochemical characteristics

Mean serum creatinine was 184 ± 50 μmol/L (2.08 ± 0.56 mg/dl), and the mean $eGFR_{Cr}$ was 40 ± 14 (range 21-70) ml/min/1.73m². Electrolyte disturbances in serum were common, and low sodium was found in one, low potassium in two, and low magnesium in four patients. Uric acid levels were elevated in six patients. Serological tests for Hantavirus were positive for Hantaan virus IgG in eight patients (IgM negative), indicating previous infection. Only three patients had elevated urine ACR (all three <500mg/g). In the two patients with hypokalemia, urine samples indicated renal potassium losses and in 10 patients the urine test indicated renal wasting of magnesium. Urinary heavy metals were all below toxic levels.

4.3.3 Renal morphology

All 11 participants underwent renal biopsy and the biopsies contained 7-29 glomeruli. The morphological findings were more heterogeneous compared to the morphological findings in Studies I and II. A summary of the main findings is reported in the text below.

4.3.3.1 *Light microscopy and immunofluorescence*

Global glomerulosclerosis of varying degree (8-75% of included glomeruli) (Figure 13A, 13B, 13D) and glomerular hypertrophy were found in all patients (Figure 13A). Signs of glomerular ischemia (thickening of Bowman's capsule and/or wrinkling of capillaries) were observed in seven patients; in three of these cases the signs of ischemia may be explained by a concurrent moderate thickening of the arterial intima. Bowman's capsule showed focal cystic dilatation in three patients (Figure 13B). Tubular atrophy was mostly mild, and interstitial fibrosis was mostly mild to moderate (Figure 13C, 13D). Interstitial inflammation of varied greatly, from no inflammation to severe (Figure 13A, 13B, 13D). In three patients, tubulitis was observed, and in two patients clusters of neutrophil granulocytes were observed in the tubular lumina, indicating acute pyelonephritis. In most cases, arteries were
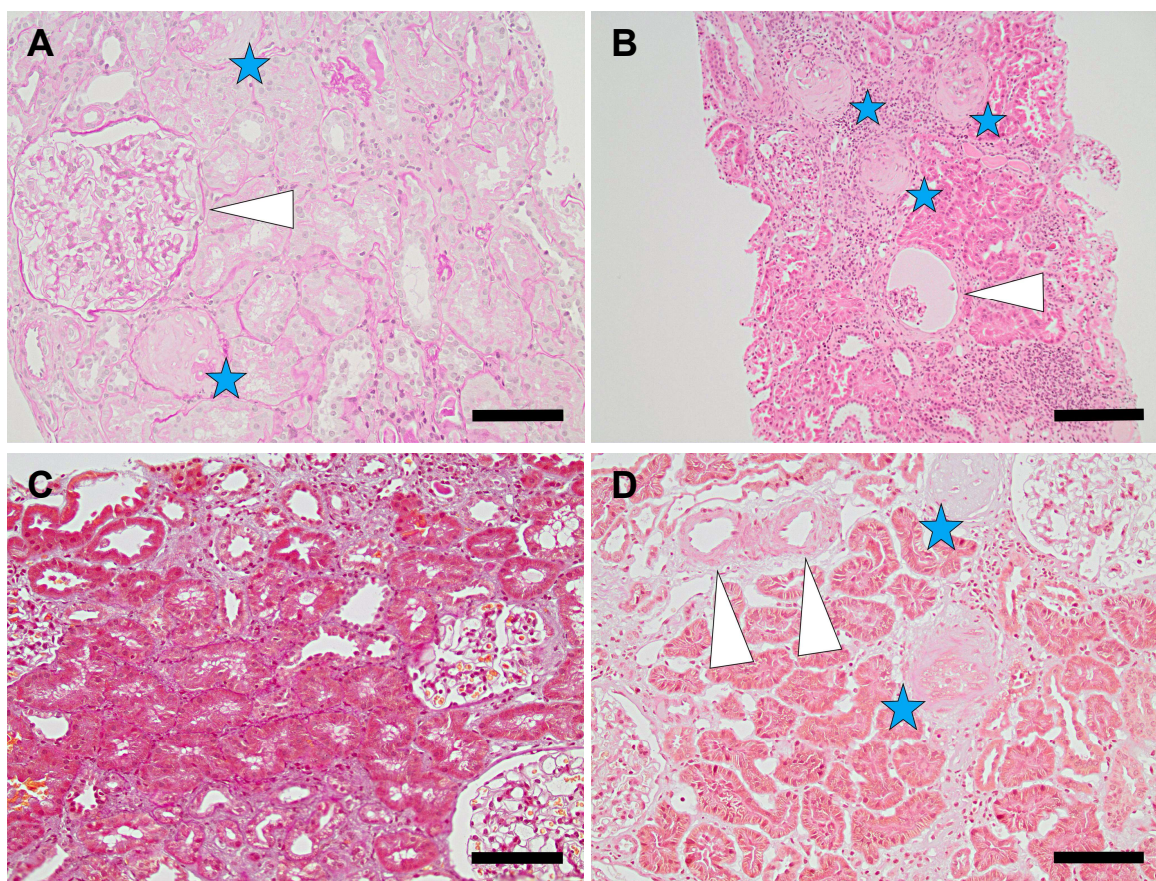

Figure 13: *Light microscopy findings in Study III. Glomerular hypertrophy (arrow in A) and global glomerulosclerosis (stars in A, B and D) were observed in all patients. Cystic dilation of Bowman's capsule was found in some patients (arrow in B). Tubular atrophy and interstitial fibrosis (C, D) were mostly mild to moderate, but interstitial inflammation was of greatly varying degree from no inflammation (A) to mild (D), moderate (B), and severe inflammation. Arteries were in most cases normal or mildly changed (arrows in D). A: PAS staining from patient 2, bar= 100 μm. B: HE staining from patient 7, bar= 200 μm. C: Ladewig staining from patient 10, bar= 100 μm. D: Ladewig staining from patient 4, bar= 100 μm.*

normal or only mildly changed, but in three cases the intimal thickening was moderate. Mild to moderate arteriolar hyalinosis was observed in most patients. IFL were all negative, thus not showing any signs of immune-complex disease. The individual biopsy results showed that six patients as main

findings showed a combination of glomerulosclerosis, glomerular enlargement, and mild to moderate tubulointerstitial damage, i.e. findings similar to MeN as described in Studies I and II. In two patients the morphology diagnosis was chronic tubulointerstitial nephritis, in two other patients the findings suggested acute and chronic pyelonephritis and in the last patient the morphology indicated nephrosclerosis.

4.3.3.2 Electron microscopy

Segmental podocytic foot-process effacement was seen in two patients (Figure 14A) and most patients showed an increased number of vacuoles or lipofuscin-like bodies in the podocyte cytoplasm (Figure 14B). No electron-dense immune complexes were observed.
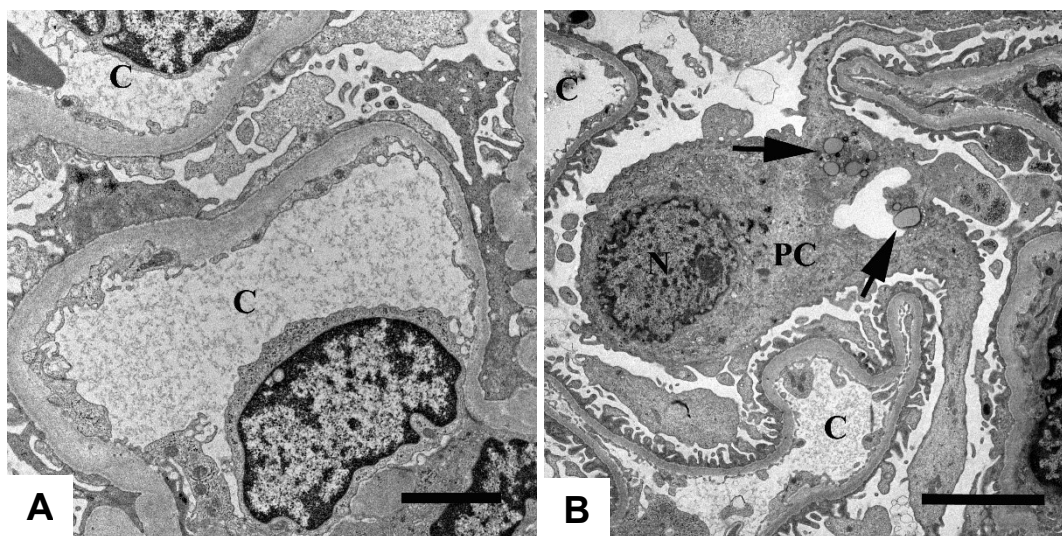

Figure 14: *Transmission electron microscopy images from Study III. Podocytic foot-process effacement was seen in two patients (A, patient 4). Nine patients had increased numbers of vacuoles and/or lipofuscin-like bodies (arrows in B, patient 6) in the podocyte cytoplasm. C=capillary space. N=nucleus. PC=podocyte. Bars: A=2µm, B=5µm.*

4.4   STRENGTH AND LIMITATIONS OF THE STUDIES

The main strength of the studies is the detailed description of groups of patients with CKDu including detailed kidney morphology in combination with thorough clinical examinations, and biochemical data from urine and blood samples. Another important strength of the studies is the similarities in study design, which enable a comparison of CKDu in the different regions. In all three studies the renal biopsies were handled in a similar manner and were stained according to the same protocols in the same renal biopsy laboratory, thus ensuring that the preparation of the biopsy slides for pathological evaluation were consistently of high quality. Evaluations of the renal specimens was conducted by two senior renal pathologists according to a standardized protocol, partly based on the Banff criteria used for renal allograft pathology (95, 96). To ensure reliability and consistency, most of the biochemical tests (urine and blood samples) were analyzed at accredited laboratories such as the Karolinska University Hospital Laboratory and the ALS Scandinavia Laboratory.

A limitation in all of the studies is the small number of participants. However, the studies were intentionally designed to study a small number of patients, because the renal biopsy procedure is an invasive procedure with a non-negligible risk of complications. A severe complication would probably have added more to the disease burden in patients from these poverty-stricken communities.

Another limitation is that there was no control group with regards to the biochemical and exposure data from the questionnaires. The ideal study would have had a control group with CKD of other known causes (e.g. diabetes, hypertension or glomerulonephritis) matched for age, sex and eGFR, and healthy controls would also have been interesting to include. However, since the sample size was small, conclusions regarding differences may be difficult to interpret. Another limitation of the studies is the selection of patients, which was not conducted in a randomized way in the population. The patient selection in Study I was performed by local nephrologists by asking patients attending a kidney out-patient clinic matching the inclusion and exclusion criteria if they wanted to participate in the study. This selection process may have caused a selection bias. In Study II, patient recruitment was more structured and all patients attending an occupational and environmental medicine out-patient clinic during a 12 month-period and who fit the inclusion/exclusion criteria were invited to the study. A total of 50 patients were invited, of whom 19 patients agreed to participate (20 patients did not respond). By only inviting patients from an occupational and environmental clinic, the included participants would, however, not reflect a cross-section of the whole CKDu population. In Study III, patients already planned for clinical renal biopsy and who were eligible according to the inclusion criteria were asked to participate. However, no record was kept on how many potential participants were invited, which increases the risk of selection bias.

All studies were conducted outside of harvest season and this could be a limitation, because occupational exposure has been suggested to be of importance in the development of the disease. However, to perform kidney biopsies in a safe manner hospitalization is required and the patients have to refrain from hard physical activity for 1-2 weeks post biopsy. Thus, obtaining biopsies during harvest season was not possible, since the participants would lose their income or even employment during this period.

5   COMPARISON AND DISCUSSION OF STUDIES I-III

Patient characteristics and clinical data were quite similar between the studies (Table 3). Noteworthy differences were that a higher percentage of patients in Study I used NSAIDs regularly. Patients in Study II were generally younger, did not use any hypertensive medicines, used less herbal medicines, and reported a lower exposure to agrochemicals than in the other studies. Furthermore, patients in Study III were older, had a lower BMI (probably due to lower muscle mass), and did not use NSAIDs. In summary, Study II was the youngest and "healthiest" study group suggesting that any age-related changes in the kidney tissue, such as changes secondary to general arteriosclerosis are probably less frequent in this group. Study III had the oldest participants, and the majority had been smokers.

Table 3: *Patient characteristics in Study I (El Salvador), Study II (Nicaragua), and Study III (Sri Lanka). Results are presented as the mean ± SD (range) or cases (%).*

	Study I, n=8	Study II, n=19	Study III, n=11
Age (years)	44 (22-57)	33 (24-54)	48 (27-61)
Height (m)	1.68 (1.55-1.78)	1.68 (1.52-1.90)	1.66 (1.57-1.75)
Weight (kg)	73 (55-100)	71 (50-100)	56.6 (46.9-77)
BMI (kg/m²)	26 (20-35)	25 (19-37)	20 (16-28)
Systolic BP (mmHg)	112 (100-130)	121 (105-133)	122 (97-160)
Diastolic BP (mmHg)	71 (60-80)	75 (56-88)	78 (58-90)
Kidney length (mm)	-	98 (87-109)	87 (78-98)
Years working in agriculture	32 (8-44)	15 (3-40)	24 (3-40)
Agrochemical exposure	8 (100%)	7 (37%)	11 (100%)
Current liquid intake (L/day)	3.9 (2.5-5)	5.3 (1.0-10)	4.3 (3.0-6.0)
Liquid intake a work day (L)	-	9.0 (3.4-15.1)	-
Hypertensive medicines	3 (38%)	0 (0%)	3 (27%)
Regular NSAID use	5 (63%)	6 (32%)	0 (0%)
Regular paracetamol use	5 (63%)	10 (53%)	2 (18%)
Ever used herbal medicines	5 (63%)	1 (5%)	7 (64%)
Ever smoked	4 (50%)	6 (32%)	8 (73%)
Pack years	5.8 (0-40)	0.9 (0-10.5)	1.6 (0-6.2)

5.1 BLOOD CHARACTERISTICS

The results of the blood tests were rather similar in all three studies (Table 4), especially with regards to serum electrolyte changes. eGFR, was somewhat similar in Studies I and III, but was significantly higher in Study II.

Table 4: *Blood test results in Study I (El Salvador), Study II (Nicaragua), and Study III (Sri Lanka). Results are presented as the mean (range) or cases (%).*

Serum/plasma			
	Study I, n=8	Study II, n=19	Study III, n=11
Creatinine (µmol/L)	172 (100-241)	146 (92-222)	184 (120-267)
eGFR$_{Cr}$ (ml/min/1.73m²)	46 (27-79)	57 (33-96)	40 (21-70)
eGFR$_{CystC}$ (ml/min/1.73m²)	40 (23-60)	57 (38-109)	38 (20-66)
eGFR$_{Cr+CystC}$ (ml/min/1.73m²)	42 (24-67)	56 (36-96)	38 (20-66)
Sodium (mmol/L)	136 (122-141)	137 (133-139)	140 (134-147)
Low Sodium (<137 mmol/L)	2 (25%)	9 (47%)	1 (9%)
Potassium (mmol/L)	3.3 (2.4-4.7)	3.8 (2.2-4.9)	4.3 (2.2-6.3)
Low Potassium (<3.5 mmol/L)	6 (75%)	4 (21%)	2 (18%)
Magnesium (mmol/L)	0.74 (0.44-1.01)	0.70 (0.51-0.82)	0.70 (0.43-0.88)
Low Magnesium (<0.7 mmol/L)	4 (50%)	7 (37%)	4 (36%)
Uric acid (µmol/L)	457 (375-572)	443 (264-715)	420 (201-583)
High Uric acid (> 416 µmol/L)	6 (75%)	11 (58%)	6 (55%)
Renin (ng/L or mIU/L)	94 (29-352) ng/L	68 (18-615) mIU/L	28 (3-90) mIU/L
High Renin (>33 ng/L or >40mIU/L)	7 (88%)	8 (42%)	2 (18%)
Aldosterone (pmol/L)	926 (211-2520)	265 (86-1080)	364 (94-953)
High Aldosterone (>650 pmol/L)	4 (50%)	1 (5%)	2 (18%)

Low serum levels of sodium, potassium and magnesium were a frequent finding in patients in all three studies. Generally, in CKD patients, both hypo and hypernatremia may occur, in most cases due to other comorbidities or medications (97). Serum potassium and magnesium levels usually increases gradually when GFR is declining and both hyperkalemia and hypermagnesemia are frequent findings in advanced stages of CKD (98, 99). Moranne et al, reported that already in CKD stage 3, i.e. GFR 30-59 ml/min/1.73m², hyperkalemia can be found in approximately 4-14% of patients, and with further deteriorating kidney function, the prevalence of hyperkalemia gradually increase to >40% in patients with GFR <20 ml/min/1.73m² (100) and, in fact, other studies have reported an even higher prevalence (98). Thus, the low levels of serum potassium (Figure 15) and magnesium found in our studies differ from what is normally seen in patients with CKD.
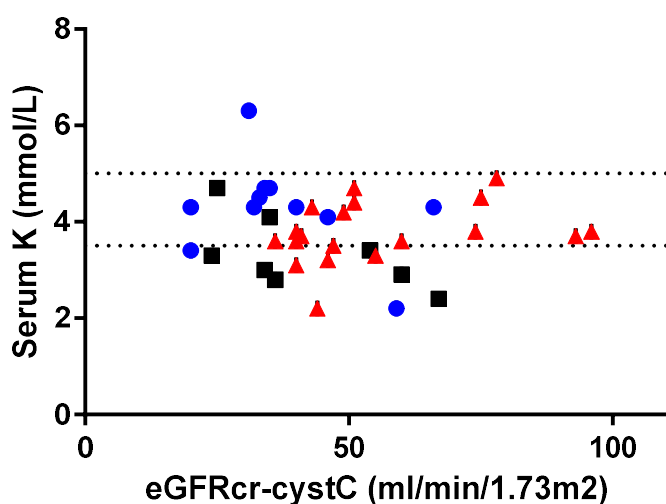

Figure 15: *The association between serum potassium and eGFR$_{Cr+CystC}$ in Study I (squares), Study II (red triangles), and Study III (blue dots). Dotted lines indicates the normal reference value for serum potassium, 3.5-5.0 mmol/L.*

Our findings regarding the serum electrolytes are consistent with other reports from patients with MeN in Central America. Herrera et al (45) reported from a cohort of 46 participants in El Salvador with CKDu who also underwent renal biopsy (101) that 47.8% had hyponatremia, 30.4% hypokalemia, and 19.6% had hypomagnesemia. In cross-sectional studies similar electrolyte disturbances have been reported. For example Kupferman et al reported that among 242 individuals from a MeN hot spot area 10% of the CKD patients had hyponatremia, 9% had hypokalemia and 25% had hypomagnesemia (37). The similarities in electrolyte disturbances seen in Study III from Sri Lanka support the hypothesis that CKDu in Central America and Sri Lanka are related diseases with similar pathophysiology.

Elevated serum levels of uric acid were seen in 55-75% of the participants in the three studies and are probably secondary to the reduced GFR because hyperuricemia is a prevalent finding in patients with CKD. The prevalence of hyperuricemia has been reported from a US cohort to be approximately 30% in patients with GFR 60 ml/min/1.73m² and to increase to almost 80% in patients with GFR 30 ml/min/1.73m² (102). The prevalence of elevated uric acid is somewhat higher in our studies compared to the US data, but our sample size is small and might not be comparable to a population study from another country. Furthermore, differences in diet and genetics may influence uric acid levels (103).

5.2 URINE CHARACTERISTICS

Results from the urine samples were also quite similar in Study I, Study II, and Study III (Table 5). Only a minority of the participants in each study had elevated ACR levels, and in all cases the albuminuria was of non-nephrotic range. Even though an exclusion criterion in the studies was proteinuria above 3.5, 3.0, and 1.0 g per 24 h respectively in the studies, the majority of the patients did not have albuminuria at all, and the individuals with increased ACR all had values ≤91 mmol/L (i.e. approximately <1 g/24 h). The low or absent albuminuria in MeN reported in epidemiological studies (19-21), is in agreement with our findings. In previous Sri Lankan studies of CKDu, proteinuria/albuminuria has also been described as low-range (53). However, it should be noted that most epidemiological studies used dip-stick albuminuria as a screening method. In Study III, we used the exclusion criterion of proteinuria >1 g /24 h to be able to study a group of patients comparable to those of Studies I and II from Central America. Indeed, the results show that in Study III the majority of the included patients with CKDu also did not have any albuminuria, and the four patients who did have albuminuria all had levels ≤47.2 mg/mmol (i.e. approximately <0.5 g/24 h). Similar results have been reported from another biopsy study of patients with MeN in El Salvador in which only 1 of 46 participants displayed proteinuria >1 g/24 hours (45). In our studies, most participants had increased levels of urinary biomarkers for tubular injury, A1M and NAG (104, 105). Other reports from both Central America and Sri Lanka have also described a high prevalence of tubular injury markers in urine (45, 61, 62).

Table 5: *Urine spot sample test results in Study I (El Salvador), Study II (Nicaragua), and Study III (Sri Lanka). Results are presented as the mean (range) or cases (%).*

Urine	Study I, n=8	Study II, n=19	Study III, n=11
ACR (mg/mmol)	14.5 (0.1-89.1)	7.2 (0.1-91.4)	7.2 (0.1-47.2)
High ACR (>3.4 mg/mmol)	2 (25%)	2 (11%)	4 (36%)
A1M/creatinine (mg/mmol)	6.3 (0.5-13.4)	3.1 (0.3-15.9)	5.2 (0.8-14.5)
High A1M/creatinine (>0.7 mg/mmol)	7 (88%)	15 (79%)	10 (91 %)
NAG (nkat/mmol)	13 (4-19)	8 (1-21)	-
High NAG (>8 nkat/mmol)	6 (75%)	8 (42%)	-
Uric Acid (mmol/L)	1.2 (<0.3-2.4)	1.6 (0.5-3.9)	1.3 (0.5-2.6)
Sodium (mmol/L)	64 (23-90)	119 (21-254)	104 (36-299)
FENa (%)	1.3 (0.5-2.6)	1.6 (0.9-3.0)	1.8 (0.5-3.3)
Potassium (mmol/L)	40 (15-76)	33 (4-61)[b]	27 (5-50)
Potassium/creatinine	5.5 (3.2-9.7)	4.3 (2.7-8.9)	3.3 (1.1-3.7)
FEK (%)	30 (15-71)	17 (8-32)	15 (8-25)
Magnesium (mmol/L)	-	-	2.1 (1.1-3.7)
FEMg	-	-	11 (4-17)

As in Central America, the findings in urine dip-stick and sediment in most CKDu cases from Sri Lanka were bland. However, in Study III one patient had a few granular casts and one patient had slightly increased red blood cell count in urine. Urine cultures were negative in all but one patient in our three studies, and other studies of MeN patients have also reported negative urine cultures (45). In Sri Lanka, there is to our knowledge as of yet no published report on the findings in urine sediment or urine cultures in patients with CKDu.

Mean urine uric acid levels were relatively similar in all three studies and were within normal range. Urine uric acid crystals were found in one patient (with serum uric acid 650 mmol/L) in Study II.

Interestingly, signs of renal electrolyte wasting (sodium, potassium and magnesium) were found in the urine of a number of patients from all three studies (Table 5), and this wasting was probably responsible for or contributed to the low electrolyte levels seen in serum. Urinary sodium excretion is normally 40-220 mmol/24h, but the secretion of sodium is difficult to interpret because it is highly variable depending on e.g. dietary salt intake, serum glucose etc. Our results are also limited to spot urine samples. Nevertheless, out of the 12 patients with low serum sodium in our studies, and who were non-symptomatic and presumably euvolemic, 10 had urine sodium excretion > 30 mmol/L, which could indicate renal salt wasting (106).

The normal range for urinary potassium secretion is 25-125 mmol/24h, but in hypokalemic patients, the secretion normally declines to 5-25 mmol/24h. In hypokalemic patients, the potassium-creatinine ratio in a spot urine sample should be <1.5, and the fractional excretion of potassium (FEK) should be <10%, and higher ratios indicate renal potassium losses (107, 108). In the three studies, hypokalemia was found in 12 patients, and these patients had a mean urinary potassium concentration of 38 (range 12-76) mmol/L, and all 12 had a potassium-creatinine ratio >1.5 and FEK >10% indicating renal potassium wasting.

Urinary magnesium was measured in Study III and interestingly the fractional excretion of magnesium, FEMg, was high in all patients (i.e. >4%) indicating renal magnesium wasting (109, 110). However, it has been shown previously that the FEMg may increase in moderate CKD as a way to compensate for the decreases in GFR and to keep plasma magnesium levels within the normal range (99). Four of the 11 patients in Study III had hypomagnesemia indicating that at least in these patients the excretion of magnesium was excessive.

In Study II, 24 h urine was collected and showed that almost half of the participants had polyuria, i.e. 24 h urine volume >3 L. Similar result with polyuria and electrolyte losses in urine have also been reported by Herrera et al in El Salvador (45). The combination of polyuria and urinary electrolyte levels further indicates renal electrolyte wasting, probably due to tubulointerstitial damage, and this might explain the low levels of electrolytes found in plasma. An aspect that needs to be considered regarding the polyuria is that patients diagnosed with CKD in MeN-endemic areas are aware of the hypothesis regarding dehydration and probably have been instructed by physicians and/or the media to drink more water, thus the high urine output might be due to increased water intake.

In Sri Lanka, published data about urine electrolytes is scarce. Jayatilake et al studied metals and heavy metals in urine and reported normal levels of sodium, magnesium, and potassium (57); however the serum concentrations of the ions were not reported.

Serum osmolality was measured in Study II and was normal in 16 of the 19 patients. The three remaining patients had values between 304 and 316 mosmol/kg. Urine osmolality was measured only in Study III, and the only two values that were not within the reference range were those of the two patients with morphological signs of pyelonephritis at 126 and 139 mosmol/kg, respectively. However, both serum and urine osmolality samples had been frozen and stored, which might give false high values, especially in serum (111), thus limiting the interpretation of these results.

5.3   KIDNEY MORPHOLOGY

The clinical characteristics of the patients with low-grade or absence of proteinuria and few other significant symptoms or laboratory findings led many clinicians and researchers to believe that MeN was a tubulointerstitial nephritis. However, before 2012 no information regarding the renal morphology of MeN had been reported or published in the scientific literature. In fact, in both El Salvador and Nicaragua, only a few clinical renal biopsies had been performed before 2012, to some extent due to financial limitations, but mainly due to a lack of pathologists specialized in renal pathology.

The aim of Studies I and II was to investigate and report the renal morphology in patients with MeN in detail, together with a clinical and biochemical description of the patients. Our research group conducted the first study of renal biopsies together with clinical and biochemical data from eight patients with suspected MeN in El Salvador in 2012 (Study I), and the results were published in 2013. We found a unique renal morphology in patients with MeN, characterized by chronic damages to both glomerular and tubulointerstitial compartments with extensive glomerulosclerosis, glomerular hypertrophy, and mild to moderate tubulointerstitial changes with mostly normal vessels (112).

In 2014, another study of biopsies from patients with MeN in El Salvador was published by López-Marín et al and described the morphological findings from 46 patients with CKD stage 2-3 (i.e. GFR 30-90 ml/min/1.73m²). The authors concluded the findings to be a chronic tubulointerstitial nephropathy with secondary glomerular damage; however, they also reported substantial chronic glomerular changes. Furthermore, they also reported that 37% of the patients had ≤ 5% interstitial fibrosis, 87% had <25 % tubular atrophy, and 89% had <25 % interstitial inflammation, i.e. the results were very similar to our findings in Studies I and II (Table 6), even though the authors' conclusions with regard to the morphology (chronic tubulointerstitial nephropathy with secondary glomerular damage) differs from ours.

It is well known that interstitial changes can cause glomerular changes and vice versa. The two pathologists in our studies have made the assessment that the tubulointerstitial changes seen in our studies are likely to be too mild to alone cause the rather extensive glomerular changes. They have made this assessment from their experience as pathologists evaluating other tubulointerstitial renal diagnoses; however a more systematic comparison with other tubulointerstitial diseases has, as of yet, not been made. From our studies, we cannot conclude whether the primary lesion is tubulointerstitial or glomerular, but the morphology does not correspond to a classic chronic tubulointerstitial nephritis, where glomeruli usually are unremarkable until advanced stages of the disease (113). To some, the discussion of whether the renal morphology in MeN (and CKDu in Sri Lanka) is a tubulointerstitial disease or not, might seem irrelevant. Nevertheless, since the etiology of the disease is still not fully known, it is important to be objective and to report all morphological findings as they may be of importance in the pathophysiological mechanisms behind the disease.

In Sri Lanka, one of the first reports of renal biopsy findings was published in 2011 (53), and since then a number of studies have been published. They describe a tubulointerstitial disease with interstitial fibrosis and tubular atrophy as the main finding, but in addition, they also report glomerular changes, such as global and segmental sclerosis, ischemia induced glomerular changes, etc. The main difference between studies conducted in Central America and Sri Lanka is that patients in the Sri Lankan biopsy studies have been selected by screening for dip-stick albuminuria, thus deselecting

individuals with CKDu without albuminuria. Therefore, Study III was designed to include Sri Lankan patients with CKDu with low-range or without albuminuria.

5.3.1 Comparison of Studies I, II and III

In Table 6, a summary of the morphological findings in Studies I, II and III is presented. When comparing Studies I and II (Table 6) there are some interesting similarities and differences. The main differences between Studies I and II are that the percentage of glomerulosclerosis was lower and the tubulointerstitial changes were less pronounced in Study II. These findings suggest that MeN at earlier stages (better kidney function) has a similar morphology but with less chronic changes. Interestingly, glomerular hypertrophy was more pronounced in Study II even though subjects in Study II had better renal function, indicating that glomerular enlargement might be an early pathophysiological or compensatory mechanism in MeN. Similar findings, with a higher percentage of glomerular hypertrophy in patients with better renal function, have also been reported by López-Marín et al in their biopsy study in El Salvador (101). Glomerular enlargement might be compensatory due to the loss of functioning nephrons or low nephron count due to other causes (114), but can also be a result of chronic ischemia (115). The glomerular enlargement might also increase the risk for the development of segmental sclerosis, which has been observed in our studies (116).

Table 6: *Renal biopsy results from Study I (El Salvador), Study II (Nicaragua), and Study III (Sri Lanka). Results are presented as the mean ± SD (range) or cases (%).*

	Study I, n=8	Study II, n=16	Study III, n=11
Mean number of glomeruli	20 ± 6 (10-30)	17 ± 7 (7-28)	17 ± 6 (7-29)
Light microscopy			
Glomerular changes	n=8	n=16	n=11
Globally sclerosed glomeruli (%)	52 ± 17 (29-78)	38 ± 21 (7-70)	43 ± 20 (8-75)
% globally sclerosed glomeruli			
<25%	0 (0%)	4 (25%)	2 (18%)
25-50%	5 (63%)	7 (44%)	4 (36%)
>50%	3 (38%)	5 (31%)	5 (45%)
Segmental scleroses	2 (25%)	2 (13%)	0 (0%)
Glomerular hypertrophy			
0	0 (0%)	0 (0%)	0 (0%)
1	3 (38%)	0 (0%)	6 (55%)
2	3 (38%)	11 (69%)	4 (36%)
3	2 (25%)	5 (31%)	1 (9%)
Wrinkled GBM / Periglom. fibrosis			
Yes	8 (100%)	15 (94%)	7 (64%)
No	0 (0%)	1 (5%)	4 (36%)
Tubulointerstitial changes	n=8	n=16	n=11
Tubular atrophy			
No, 0-5%	0 (0%)	1 (6%)	0 (0%)
Mild, 6-25%	4 (50%)	13 (81%)	10 (91%)
Moderate, 26-50%	4 (50%)	2 (13%)	1 (9%)
Severe, >50%	0 (0%)	0 (0%)	0 (0%)
Interstitial fibrosis			
No, 0-5%	0 (0%)	1 (6%)	0 (0%)
Mild, 6-25%	3 (38%)	8 (50%)	6 (55%)
Moderate, 26-50%	4 (50%)	7 (44%)	4 (36%)

Severe, >50%	1 (13%)	0 (0%)	1 (9%)
Interstitial inflammation			
No, 0-5%	1 (13%)	2 (13%)	2 (18%)
Mild, 6-25%	4 (50%)	12 (75%)	5 (45%)
Moderate, 26-50%	3 (38%)	2 (13%)	2 (18%)
Severe, >50%	0 (0%)	0 (0%)	2 (18%)
Vascular changes			
Intimal thickening	n=8	n=15	n=10
0	6 (75%)	11 (73%)	5 (50%)
1	2 (25%)	3 (20%)	2 (20%)
2	0 (0%)	1 (7%)	3 (30%)
3	0 (0%)	0 (0%)	0 (0%)
Smooth muscle hyperplasia	n=8	n=15	n=10
0	1 (13%)	5 (33%)	6 (60%)
1	5 (63%)	6 (40%)	4 (40%)
2	2 (25%)	4 (27%)	0 (0%)
3	0 (0%)	0 (0%)	0 (0%)
Arteriolar hyalinosis	n=8	n=16	n=11
0	5 (63%)	11 (69%)	1 (9%)
1	3 (38%)	3 (19%)	7 (64%)
2	0 (0%)	2 (13%)	3 (27%)
3	0 (0%)	0 (0%)	0 (0%)
Electron microscopy	n=8	n=16	n=11
GBM thickness (nm)	419 ± 93 (340-625)	441 ± 63 (333-566)	307 ± 39 (259-349)
Podocyte foot processes (slits/μm GBM)	1.3 ± 0.3 (0.9-1.7)	1.0 ± 0.5 (0.1-1.7)	1.5 ± 0.2 (1.2-1.8)
Podocyte foot process effacement			
No (normal)	5 (63%)	7 (44%)	11 (100%)
Segmental effacement	3 (38%)	4 (25%)	2 (18%)
Widespread effacement	0 (0%)	5 (31%)	0 (0%)
Endothelial cells			
Normal	8 (100%)	10 (62%)	11 (100%)
Swollen	0 (0%)	6 (38%)	0 (0%)
Podocyte cytoplasm inclusions			
Yes	6 (75%)	12 (75%)	9 (82%)
No	2 (25%)	4 (25%)	2 (18%)

In Study III, the glomerular changes were not as pronounced as in Studies I and II, especially with regards to glomerular enlargement and signs of glomerular ischemia (wrinkled GBM and/or thickening of Bowman´s capsule). Tubular atrophy and interstitial fibrosis in Study III were relatively similar to the results in Studies I and II, but the interstitial inflammation was more diverse and some patients displayed severe inflammation, which was not observed in Studies I and II. Vascular changes were also more common in Study III, which might be due to the older age of the participants.

When comparing Study III (Sri Lanka CKDu) with Studies I and II (MeN/CKDu in Central America), the findings at the group level are quite similar. However, when studying the individual biopsies, the biopsies from Study III display a much more mixed morphology compared to the studies from Central America where the findings have been more homogenous. Six of the eleven participants in Study III had a MeN-like morphology, i.e. findings very similar to the findings in Studies I and II, supporting the hypothesis that the two endemics are related diseases. However the five remaining biopsies

showed chronic tubulointerstitial nephritis, pyelonephritis, and nephrosclerosis. One could speculate that these biopsies represent different stages or a variation of the disease, or one could hypothesize that the CKDu endemic in Sri Lanka could have several different etiologies. Nanayakkara et al reported that in some biopsies from CKDu patients they found massive interstitial inflammation that they suggested to be the primary lesion in CKDu (64). We also found severe interstitial inflammation in two patients in the Sri Lankan study, but these specimens also displayed chronic tubulointerstitial changes indicating a more chronic state. A repeated inflammatory state might explain these findings. The results in Study III with some of the individual biopsy results indicating pyelonephritis, despite negative urine cultures, and chronic tubulointerstitial nephritis, call for more research focusing on infectious and inflammatory diseases.

In summary, the majority of the CKDu patients in our study in Sri Lanka (Study III) had a similar renal morphology as the CKDu patients we studied in Central America (Studies I and II), supporting a common diagnostic entity and thus also a common etiology. However, glomerular changes were less pronounced in Sri Lanka and the Sri Lankan patients had more mixed morphology and more frequently displayed interstitial inflammation and vascular pathology. Additional and larger biopsy studies are needed to elucidate these differences.

6 DISCUSSION REGARDING POSSIBLE ETIOLOGIES

Many different hypotheses regarding possible etiologies and contributing factors in the development of MeN in Central America and CKDu in Sri Lanka have been proposed, but as of yet no definite etiology or pathophysiological mechanism has been scientifically proven in any of the regions. Proposed etiologies include pesticides, heavy metals, infections, drinking locally brewed alcohol, genetic factors, and nephrotoxic drugs. Still, the leading hypothesis regarding etiology is occupational heat exposure causing repeated volume and salt depletion resulting in kidney damage that eventually develops into CKD (85, 117). A summary of the evidence for these hypotheses and their relation to our findings will be presented in this chapter.

6.1 DEHYDRATION / HEAT STRESS / SALT DEPLETION

Complications due to heat exposure such as heat stroke, heat collapse, heat rashes and heat fatigue are known problems among people working in warm environments. To measure heat exposure during work outside wet-bulb globe temperature (WBGT) is usually used, because this measurement takes in to account temperature, humidity, wind speed, as well as solar radiation. The Occupational Safety and Health Administration (OSHA) in the US has guidelines for how much rest a worker needs per hour during hard physical work in hot climates, to avoid being at risk for heat exposure related illnesses. The heat exposure threshold limit values in these guidelines are 25°C for continuous work, but at 26°C there is a need for 15 min of rest each hour, at 28°C the worker needs 30 min of rest each hour, and at 30°C there is a need for 45 min of rest each hour (118).

Several studies have reported an increase in hospital admissions in renal diagnoses during high temperatures and heat waves, e.g. in Australia (119) and Rhode Island (120). In Taiwan, it was reported that the relative risks of hospital admissions due to a renal diagnosis are 1.45 (95% CI 1.27–1.64) at 30°C compared to 25°C (the temperature with the lowest incidence of hospital admissions). A study from Brisbane, Australia, reported an increased number of renal disease emergency hospital

admission among children during heat waves, and interestingly, tubulointerstitial nephritis was one of the most frequent diagnoses according to this study (121). Chronic interstitial nephritis has also been described as a consequence of heatstroke in an old publication studying mine workers in South Africa (122). Another study indicating an association between heat exposure and kidney disease was published in 2012, and that study reported a significant association between self-reported occupational heat stress and renal disease diagnosis among 37816 male workers in Thailand (OR = 1.48, 95% CI 1.01–2.16) (123).

Repeated subclinical or undiagnosed acute kidney injury (AKI) due to heavy work in a hot climate is one of the suggested main mechanisms in the pathophysiology of MeN in Central America. Several different causes may be involved, including dehydration/volume depletion, rhabdomyolysis, RAAS activation, salt depletion due to sweating, but also exposure to nephrotoxins. In the last decades there has been growing body of evidence indicating that episodes of AKI per se are risk factors for the development of CKD (124, 125). A recent study from the UK reported that patients with recovery of renal function after one AKI episode actually have a subsequent increased risk for decreasing kidney function over a ten-year period (126).

In Study II, the extreme conditions during sugarcane harvest are evident in the amount of liquids consumed during a work day (Results, Table 3), when daily liquid almost doubles and almost half of the participants reported a daily intake of > 10 liters. Salt losses due to increased sweating in combination with a large intake of water might play a role in the development of the electrolyte disturbances seen in the patients. However, in all three studies, samples were collected outside of harvest season indicating a more chronic condition.

Occupational studies of sugarcane workers in Central America also reveal their difficult working conditions. In a study of sugarcane workers in Costa Rica, WBGT and workload were assessed during workdays. The results showed that after 9:15 a.m. the workers' heat exposure was of such extent that according to OSHA guide lines the workers should only work 15 minutes per hour to avoid heat-related health risks (25). The frequency of heat-related symptoms among 106 sugarcane harvesters in Costa Rica compared to non-harvesters was evaluated by Crowe et al. They reported that heat and dehydration symptoms were significantly more frequent among harvesters and that with increasing heat exposure symptoms became more prevalent (127). Another study by Garcia-Trabanino et al, measured the heat index during harvest in three different sugarcane plantations in El Salvador and found that a heat index of 103°F (39.4°C), considered "high risk" according to OSHA was reached already at 7:00-7:30 a.m. and that a heat index of 115°F (46°C), considered "very high to extreme", was reached between 7:30 and 10:00 a.m. (44).

Among sugarcane workers in Central America, cross-shift studies have indeed demonstrated that many sugarcane workers have an increase in serum creatinine levels during a workday (44) and during/after the harvest season (41-43) indicating an occupation-associated decrease in eGFR /kidney function.

Interestingly, two studies of agricultural workers conducted in warm regions outside of known CKDu areas have reported that a high percentage of workers have a serum creatinine increase during a work shift equivalent to AKI. Santos et al reported from 28 sugarcane workers in Brazil that 19% of the workers had a serum creatinine increase during a work shift consistent with AKI (128). Moyce et al (2017) studied 283 agricultural workers in California during the summer and found that 12% developed changes between pre and post-shift serum creatinine consistent with AKI. In the latter

study, heat strain was assessed in the participants by measuring core body temperature and pulse rate, and in logistic regression models heat strain was associated with a higher risk of AKI, while changes in body mass indicating volume depletion did not show any statistically significant association (129).

If AKI can develop in a significant number of workers, as shown in these studies, after only one work shift, it is likely that repeated kidney injury will occur during harvest season, further increasing the risk of developing CKD.

An intervention program based on OSHA's Water.Rest.Shade. initiative (130) was carried out during a five-month harvest period in a sugarcane plantation in El Salvador. The intervention started half-way through harvest season and included approximately 60 workers who were provided with water in individual backpacks, scheduled rest periods, mobile shaded rest areas, and lighter and more ergonomically designed machetes. Anthropometric data, blood and urine samples, and questionnaires were collected before and after the intervention along with daily temperatures (WBGT) and data regarding productivity. The first report from this intervention was published in 2016 by Bodin et al (131) and the authors found that workers drank 25% more water during the intervention and that heat stress-related symptoms decreased. Actually, productivity increased during the intervention, indicating that better working conditions can maintain or even increase productivity on sugarcane plantations. A recent publication presents the biochemical results from the intervention group compared to a non-intervention group (132). A cross-shift decrease in eGFR of -10.5 ml/min/1.73m² was found in both intervention and non-intervention groups. Both groups also had a decrease in eGFR over the harvest, although somewhat lower in the intervention group, -3.4 ml/min/1.73m² compared to -5.3 ml/min/1.73m² in the non-intervention group, suggesting that intervention may reduce the eGFR decline. Most likely a longer intervention time than 2-3 months is needed to study the long-term effects of the intervention on renal function and the development of chronic kidney injury.

6.1.1 Uric acid / fructokinase / dehydration mechanism hypothesis

Roncal-Jimenez and co-workers argue that MeN might be a uric acid disorder (133). Since uric acid can develop during heat stress due to muscle cell damage, and because workers exposed to occupational heat stress with the risk of volume depletion probably develop increased urinary concentration, the authors hypothesized that sugarcane workers might be subjected to repeated uricosuria with the formation of urate crystals causing tubular damage and hyperuricemia causing glomerular hypertension. The authors refer to a pilot study of sugarcane workers in which uric acid crystals were common findings in the urine samples. The data from the original study of sugarcane workers by Garcia-Trabanino et al 2015 (44) showed that serum uric acid levels were elevated (>7.0 mg/dl) in 26% of sugarcane workers before a work shift and in 43% after the work shift compared to serum creatinine which was increased in 20% of the workers pre-shift and in 25% of the workers post-shift. Interestingly, urine samples from the same workers demonstrated the presence of urate crystals in 16% of the pre-shift samples and in 22% in the post-shift samples. Another study of sugarcane workers by Wesseling et al showed that uric acid in serum did not change significantly during the workday, even though the urine uric acid increased significantly during the workday (43). Kupferman reported in 2016 that serum uric acid levels where higher in MeN patients compared to CKD participants in a large US cohort (NHANES); however, the two cohorts were very different in age composition, and when comparing men ≤65 years of age there was no statistical difference in uric acid levels (37). Whether hyperuricemia per se might cause or worsen kidney damage has been heavily debated (134).

A possible mechanism behind dehydration-induced kidney damage involving fructose metabolism and uric acid has been proposed in an animal study conducted by Jimenez and colleagues. In that study, both fructokinase-knockout mice and wild-type mice were exposed to repetitive dehydration by placing the mice in a warm chamber. Some mice had access to water during the entire day, and some mice only received water at night after suffering dehydration during the day. Renal injury markers (serum creatinine, urinary NGAL and renal tissue findings of tubule-interstitial damage) were increased in the wild-type mice that received water only at night, while the fructokinase-deficient mice with delayed rehydration did not show any renal damage. The authors suggested the pathological mechanism to be injury to the tubuli caused by end products in fructose metabolism (uric acid, inflammatory mediators, and oxidants) (135).

In Studies I, II, and III the uric acid levels were above the reference value of 416 µmol/L (7.0 mg/dl) in 75%, 58%, and 55% of the participants, respectively. None of the participants had values over 715 µmol/L. In one patient from Study II (Nicaragua), uric acid crystals were found in the urine, but none of the other patients included in Studies I, II, and III had urine crystals reported. The urine uric acid levels were measured in all three studies and were within the normal range. When evaluating the renal biopsies, there were no signs of chronic urate crystal depositions, such as microtophi formation or granulomas, surrounding giant cells, or scars indicating prior granulomas. Furthermore, no feathery or needle shaped crystals were found, nor any empty spaces indicating dissolved crystals (136, 137). Thus the results from our three studies do not support the uric acid hypothesis because, we did not find any typical acute or chronic uric acid deposits in the kidney tissue. Uric acid levels in serum were typical of what is to be expected in patients with CKD. Nevertheless, it should be noted that our samples were all collected outside of harvest season, i.e. not during periods of occupational heat stress when uric acid crystals in urine has been reported to be found in sugarcane workers (133), which limits the interpretation of our samples in relation to the hypothesis.

6.2 PESTICIDES

Although exposure to various pesticides has been reported to cause damage to a number of different organ systems, AKI from pesticides is mainly seen in connection to severe systemic toxicity (138).

In both the Central American and Sri Lankan CKDu endemics, pesticides have been proposed as possible contributing factors. Several studies including cross-sectional, case-control and case studies have examined pesticide exposure mainly by self-reported exposure in questionnaires and with varying assessment of exposure (often yes or no). To my knowledge, the only longitudinal study (with 6 months follow-up) of pesticide exposure is that published by Laws et al (as described below).

Most studies from Central America have not found an association between pesticides and CKD. Gracia et al, studied 297 men living in coastal areas of El Salvador and found that 13% of the participants had CKD (29). The authors reported that being a farmer, pesticide exposure, and alcohol consumption were common characteristics in participants both with and without CKD, thus not supporting an association between CKD and pesticides (29). A cross-sectional study from Nicaragua by Sanoff et al included 124 CKD cases and 873 participants without CKD and showed that self-reported pesticide exposure was significantly associated with CKD in a univariate analysis, but not in a multivariable analysis (34). In Nicaragua, Laux et al (36) measured renal function among 267 inhabitants aged 20-60 years in a coffee growing village located 1000 meters above sea level. The

prevalence of reduced eGFR (<60 ml/min/1.73m²) was <1% in this farming community, although 98% of the male participants reported exposure to pesticides (either preparing or applying pesticides), suggesting that high pesticide exposure is not correlated to decreased kidney function. Laws et al followed a cohort of 284 sugarcane workers during harvest season and found that pesticide applicators did not have a significant decrease in eGFR during harvest period, while sugarcane workers with other tasks (seed cutters and irrigators) did have a significant decrease in eGFR. Furthermore, the urinary biomarkers NGAL and IL-8 did not increase during harvest among pesticide applicators (p=0.9), but they rose significantly in cane cutters, seed cutters and irrigators forking in the same sugarcane fields (41, 42).

In contrast to these studies, there are also some reports from Central America of a significant association with pesticides. For example, Raines et al (2014) reported from a cross-sectional study of 424 individuals in Nicaragua, that non-deliberate pesticide inhalation was a risk factor for CKD (age and sex-adjusted OR 3.14, 95% CI 1.12-8.78); however ,other indicators of pesticide exposure such as "lifetime days mixing pesticides", "lifetime days applying pesticides", and "lifetime days working in fields with pesticide use" were not different between CKD cases and non-CKD participants (19). Garcia-Trabanino et al found that among 189 sugarcane cutters in El Salvador, carbamate pesticide use was more frequent among sugarcane workers with CKD compared to workers without CKD (74% vs 29%). Furthermore, in a logistic regression model "any use of pesticides ever" was not associated with CKD, but if replaced with "use of carbamate pesticides ever" in the model a significant association was found (44).

In Sri Lanka, pesticides as a possible cause for the CKDu endemic has been heavily debated, and a number of studies of varying quality have been performed. In a case-control study by Wanigasuriya et al (2007), including 138 CKDu cases and 200 controls, the initial analysis indicated that the use of pesticides was a risk factor for CKDu, but in multivariate logistic regression analysis no association was found (59). Athuralia et al, studied 246 patients with proteinuric CKD in three different regions, including one CKDu endemic region and two non-endemic regions. In a multiple logistic regression model adjusted for hypertension and diabetes mellitus, agrochemical exposure was only found to be a significant predictor of CKD in one of the non-endemic regions (53).

Jayasumana et al have argued for the hypothesis that a combination of the pesticide glyphosate, hard water and nephrotoxic metals might be the cause for the CKDu endemic in Sri Lanka. The authors' arguments are mostly based on the geographical distribution of water hardness in ground water and the prevalence of CKDu, and they hypothesize that a glyphosate and metal complex causes the kidney damage (139). A case-control study by the same author was published in 2015, comparing risk factors among 125 CKDu cases and 180 controls using questionnaires. In addition, drinking water was analyzed for metals, hardness, and glyphosate. Using a multivariable analysis, the study showed that risk factors for CKDu were drinking water from abandoned wells (OR 2.52, 95% CI 1.12-5.70), a history of drinking water from abandoned wells (OR 5.43, 95% CI 2.88-10.26), and using the pesticide glyphosate (OR 5.12, 95% CI 2.33-11.26). Analysis of water from abandoned wells showed higher levels of glyphosate and more hardness compared to other water sources, but glyphosate levels were far below the no-observed-adverse-effect levels used in animal models (140). Even though this study to some extent supports Jayasumana´s hypothesis, there is, as of yet, no clear evidence for the prevalence or nephrotoxicity of this glyphosate-metal complex.

In Sri Lanka, Jayatilake et al (57) found residues of seven different pesticides, of the eleven measured in urine samples from CKDu patients in Sri Lanka. However, because the authors did not report the levels of pesticide residues in controls these findings are almost impossible to interpret.

A detailed review of 21 published articles (11 from Central America, 5 from Sri Lanka, and 6 from other countries) related to pesticide exposure and CKDu endemics concluded that there is very scarce evidence for an association between CKDu and pesticide exposure (141). Thirteen studies showed a positive association and eight studies showed no association between CKDu and pesticide exposure. However, most articles were considered to be of low or medium explanatory scientific value due to study design, unspecific and/or unquantified pesticide exposure, confounding factors and selection bias. Even though the authors of the review concluded that the evidence is insufficient, they called for further studies with better design and assessment of specific pesticide exposure and interactions with other risk factors such as heat stress before fully discarding the possible involvement of pesticides in CKDu development.

A recent publication by Lebov et al (2016) studied the association between work related exposure to 39 pesticides and ESRD among a cohort of 55580 male pesticide applicators in Iowa and North Carolina in the US. Three hundred twenty individuals in the cohort who were diagnosed with ESRD during a 14-18 year follow-up answered self-administered questionnaires regarding pesticide usage. A large number of statistical associations were examined, but only a few showed statistical significance. Positive dose-related associations were reported for six pesticides: alachlor, atrazine, metolachlor, paraquat, and pendimethalin and the permethrin. Hospital stay due to pesticide use (HR 3.05, 95% CI 1.67-5.58) and multiple medical visits after pesticide use (HR 2.13, 95% CI 1.17-3.89) were also found to be associated with ESRD (142).

In summary, the evidence for pesticides contributing to the CKDu endemics in Central America and Sri Lanka is weak, and even though, an association has been observed in some studies, the alleged compounds differ between the studies, which challenges a joint etiology. One can also argue that if pesticides were to cause permanent kidney damage, other organ systems would probably also be affected, but this is not a common feature in MeN or CKDu in Sri Lanka. However, as Wesseling and co-authors have stated, the available studies are not of satisfying quality and better studies, preferably with better life-time pesticide exposure assessments and, if possible, longitudinal studies, are warranted.

6.3 HEAVY METALS

The measurement of urine heavy metals is often performed to evaluate the extent of heavy metal exposure. However, urine samples might not reflect the lifetime exposure of inorganic compounds even though some compounds, including urinary cadmium, have been proposed to be correlated with the accumulation of cadmium in kidney tissue (143). Nevertheless, urinary metals are frequently analyzed to assess heavy metal levels in exposed individuals, and these give a relatively accurate reflection of the current exposure. When evaluating urinary heavy metal levels it is important to know that in patients with impaired kidney function, urinary creatinine-adjusted values may be misleading due to a lower clearance of creatinine.

Urine heavy metals were all of non-toxic levels in Studies II and III, thus not supporting the hypothesis that heavy metal exposure is involved in the pathogenesis of CKDu in Central America or

Sri Lanka, although it must be noted that the study samples were small. Urine heavy metals have also been measured in other studies from both regions. In Sri Lanka, the debate regarding heavy metals has been more intense, and many researchers in the area are studying if there is an association to CKDu, but so far these studies have provided varying and contradictory results (50, 144).

In one study from Sri Lanka by Jayasumana et al, 19 different heavy metals and the pesticide glyphosate were analyzed in urine samples from 10 patients with CKDu from one endemic region, 10 controls from the same endemic region and 10 controls from non-endemic areas. The study found that median urinary levels (µg/L) of 18 of the 19 heavy metals and glyphosate were highest in the endemic control-group. When adjusted for urine creatinine (µg/g), 15 of the 19 heavy metals and glyphosate were higher in the CKDu patients. In the control group from the non-endemic areas, the urinary heavy metal levels and glyphosate levels were generally lower (145). Compared to our heavy metal analysis in Study II, the levels reported in the study by Jayasumana are substantially higher, both in cases and controls, including cadmium, lead and vanadium, indicating that the high levels might be a locally occurring problem. Another study from Sri Lanka, evaluating urinary heavy metals among CKDu cases (n=311) and controls (n=286), reported that all of the 18 measured heavy metal levels (µg/L) were higher in controls than in CKDu cases. When adjusting for urine creatinine, Al, As, Co, Cu, Mn, and Zn (µg/g creatinine) were significantly higher in cases than controls; however, cadmium levels were significantly higher in controls (60).

Arsenic:

Arsenic is found in both a highly toxic inorganic form and a non-toxic organic form. The organic form is common in fish and seafood and thus diets high in these foods increases the total amount of arsenic in urine, without influencing the toxicity. Even though inorganic arsenic might cause severe systemic toxicity, kidney damage has rarely been reported (73).

In Sri Lanka, arsenic levels were measured in urine and hair samples in 125 CKDu cases and 180 controls by Jayasumana et al. They reported that 68% of the CKDu patients had total urine arsenic levels of >21 µg/g creatinine, compared to 28% in controls, and they suggested that arsenic might be of importance in the pathogenesis of CKDu (146). However, the differences could be due to different diets in the two groups. Another study of urine arsenic in CKDu patients (n=495) and controls, showed no significant difference in arsenic levels (57). In Studies II (Nicaragua) and III (Sri Lanka), the mean urinary arsenic levels were 10 µg/L (12 µg/g creatinine) and 37 µg/L (42 µg/g creatinine), respectively, which is moderately higher than in the US population (8.3 µg/g creatinine)(147), but slightly lower compared to levels in a Korean cohort (41 µg/g creatinine)(148). Furthermore, all samples in Studies I and II and the values reported by Jayasumana et al (146) were within what is considered a normal range, i.e. <100µg/L (149). Furthermore, in a study by Nanayakkara et al specific arsenic levels were measured in CKDu patients and controls, showing that there was no significant difference between groups (60). Taken together, arsenic is not likely to be an important factor in CKDu in Central America or Sri Lanka.

Cadmium:

Cadmium can cause kidney damage characterized by tubular proteinuria and may develop or contribute to decreasing kidney function (75). In Sri Lanka, one of the first hypotheses regarding CKDu etiology was cadmium exposure. Bandara et al reported high levels cadmium in water, fish and plants in endemic areas as well as high urinary cadmium levels (7-11µg/g creatinine) from both CKD

patients and controls without CKD (150). However, these values were later proven to be incorrect when two other studies showed that urine cadmium levels were actually about ten times lower in CKDu patients (the mean urinary cadmium levels were 0.79 and 0.32-0.67 μg/g creatinine respectively) (54, 61), which corresponds well to the cadmium levels found in Study III (mean 0.49 μg/g creatinine). These levels are non-toxic (143), thus not supporting a role of cadmium in the development of CKDu in Sri Lanka. Cadmium levels have also been rejected as a possible causative factor in Central America because measurements from sugarcane workers in endemic regions were generally equivalent to the levels found in the US population (49). In our study from Nicaragua (Study II), we could confirm this finding, since our observed urinary cadmium levels were low (0.1 μg/g creatinine).

Lead:

Low levels of lead have been reported from MeN patients in Central America and without association with renal function (35). In Studies I and II, low, or even unmeasurable levels, of lead were found in urine samples. Contradictory results, i.e. both high and low/normal urinary levels have been reported from Sri Lanka (60, 145). The typical morphological finding of intranuclear lead inclusion bodies (72) was not observed in our three studies, nor have they been reported in renal biopsies from other MeN or CKDu cases (53, 101).

Mercury:

Mercury-induced kidney damage typically causes nephrotic syndrome with membranous nephropathy as the classic morphological lesion (78). The clinical picture of CKDu in Central America and Sri Lanka, with no or low-range proteinuria suggests that the risk of mercury-induced kidney damage is low. Furthermore, urinary mercury levels in Studies I and II were low or even below detection levels.

In summary, there is very scarce evidence for heavy metals as a contributing factor in CKDu, and the available studies measuring urinary levels report contradictory results. In addition, the morphological picture described in our studies and described in other biopsy studies does not correspond to the typical findings described in heavy meta- induced nephropathy.

6.4   NONSTEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDS)

NSAIDs are available over the counter in many rural supermarkets in Central America, and their use has been described as high in areas with MeN, especially in El Salvador. Local physicians and pharmacists have reported that inhabitants involved in agriculture frequently use NSAIDs due to muscle ache or "chistata" (47). In a cross-sectional study of 976 men and 1412 women in an area in El Salvador with a high prevalence of CKD, 84% of the included participants reported use of NSAIDs (23). Two other studies in El Salvador reported that >70% of both CKD cases and controls and 41% of CKD patients used NSAIDs respectively (19, 45). In our studies in Central America, we could observe this pattern of NSAID use in some of our patients. In Study I, five of the eight participants reported weekly use of NSAIDs even though they were known CKD patients with regular visits to a renal clinic (112), and in Study II six of the 19 patients (32%) reported that they had used NSAIDs on a weekly basis for ≥2 years (151). However, the majority of the participants did not report NSAID use. Quite contrary to our findings in Central America, in Study III in Sri Lanka, none of the participants reported any NSAID usage (152).

NSAIDs can cause kidney injury through drug-induced/allergic acute tubulointerstitial nephritis, which is characterized by tubulitis, interstitial edema, and interstitial inflammation containing eosinophils. In our three studies, none of the renal biopsy specimens displayed such a morphology. Nevertheless, NSAIDs can also affect the kidney by inhibiting the production of prostaglandins, which are important substances to increase renal blood flow by dilatation of afferent renal arterioles. The prostaglandins' abilities to increase or maintain renal blood flow is especially important under conditions of decreased renal perfusion, such as hypotension and decreased renal arterial blood flow (153). It is well known that NSAIDs can cause or contribute to AKI under conditions of hypotension. Intake of NSAIDs in combination with decreased renal blood flow (e.g. dehydration/volume depletion secondary to work related heat stress) might contribute to the signs of glomerular ischemia seen in our patients from Central America (Studies I and II). However, since not all participants in our studies reported use of NSAIDs, this seems very unlikely to be the main cause behind the disease. Nevertheless patients diagnosed with MeN or CKDu should avoid NSAIDs and sugarcane workers, and other individuals with heavy physical occupations in hot environments, should probably also avoid NSAID use, at least before and during heat stress exposure.

6.5 INFECTIONS

Infectious etiologies have been proposed in CKDu both in Central America and Sri Lanka (154, 155), but no specific pathogen has yet been found to have a role in the pathogenesis. Urine cultures from sugarcane workers both with, and without, dysuria or "Chistata" symptoms have been negative in several studies (49, 156). In our three studies, urine cultures were negative in all but one patient (Study I, E.coli), indicating that urine infections due to bacteria are rare. Leptospirosis has been suggested to be a possible pathogen involved in the development of CKDu, although it has not yet been proven to cause CKD in humans (15). Because CKDu cases are often asymptomatic, while leptospirosis infection causes rather severe symptoms (flu-like symptoms, jaundice, hemorrhage, renal failure, and myocarditis), a causative connection seems unlikely, but needs to be further investigated.

Hantavirus is a common pathogen in rural areas in Sri Lanka and a recent publication reported that >50% of patients with CKDu were found to be seropositive for Hantavirus (14% in controls) (157). In Study III we performed serological tests for both leptospirosis and Hantavirus because interstitial inflammation was more severe in renal biopsies in some cases. The tests were actually positive for Hantaan virus (IgG positive, IgM negative) in eight of eleven patients, indicating a previous infection. Since our patients did not report any hospitalizations or treatments for hantavirus-associated hemorrhagic fever with renal syndrome (acute tubulointerstitial nephritis) or cardiopulmonary syndrome, a subclinical or mild infection could have been the case in our patients. As of yet, there are no reports that a mild or subclinical Hantavirus infection could cause kidney damage, but a few reports indicate a higher prevalence of Hantavirus antibodies in renal failure cohorts (158, 159). Data regarding Hantavirus prevalence in Central America is scarce (160), but to date there is no evidence for Hantavirus involvement in CKDu in Central America. Investigation of serum prevalence of Hantavirus antibodies in MeN would be interesting. Even if Hantavirus itself might not be the primary etiology behind CKDu, one could speculate whether patients with a previous Hantavirus or other potential infection might be more vulnerable and susceptible to other types of kidney damage, including CKDu.

6.6 GENETIC FACTORS

Although a family history of CKD has been shown to be a risk factor in both Central America and Sri Lanka (22, 59), this increased risk might be explained by the fact that families tend to live in the same region and have the same profession as their parents. In a study from Sri Lanka, 10 patients with a familial clustering of CKDu were found in a cohort of 106 patients. When studying pedigrees, no evidence was found for autosomal dominant or recessive inheritance, nor for X-linked inheritance (61). However, genetic susceptibility may be of importance since both Brazil and Cuba, do not have increasing rates of CKDu even though they produce sugarcane with the same technique and have a similar climate.

One study supporting genetic susceptibility was published by Nanayakkara et al. They performed a genome-wide association study on whole blood DNA from 311 CKDu cases and 286 controls from CKDu endemic areas in Sri Lanka. They found a single-nucleotide polymorphism (SNP), rs6066043 close to the SLC13A3 gene that was significantly associated with CKDu, and the population attributable fraction/risk was 50% with an odds ratio of 2.13. The SLC13A3 gene encodes "high-affinity sodium dicarboxylate transporter 3", a protein found in the basolateral membranes of renal proximal tubules, but also in the brain, liver, and placenta. Although these novel findings are interesting, further studies are needed to establish if there is indeed a link between SNP rs6066043 and CKDu.

To our knowledge, no genetic studies have been performed regarding MeN/CKDu in Central America.

7 SUMMARY AND CONCLUDING REMARKS

The three studies included in this thesis have provided detailed information regarding renal morphology in the CKDu endemics in Central America and Sri Lanka. Study I was the first study to show that Mesoamerican nephropathy has a unique morphology and thus constitutes a new diagnostic entity. Study II could confirm the morphological findings in patients from a different region in Central America, and the results from Study III indicate that the disease might affect other regions. Furthermore, the clinical and biochemical characteristics reported in the studies have provided important information that can be used in the clinical evaluation process as well as for ongoing and future research regarding the etiology and pathophysiology behind the disease.

The morphological, biochemical, and clinical data from our three studies do not resemble any previously described endemic nephropathy such as Balkan nephropathy/aristolochic acid nephropathy or heavy metal-induced nephropathy. Aristolochic acid nephropathy is also correlated to an increased risk of urothelial cancer (68), something that has not been observed in CKDu regions. Even though the use of herbal remedies is relatively common in Central America and Sri Lanka, aristolochic acid is not a common ingredient in Central American remedies. In Sri Lanka, aristolochia species in traditional Ayurvedic treatments are rarely used, and if used the doses are negligible (161).

In Study I, the findings of low sodium and potassium were quite surprising, and Study I was actually the first report on this phenomenon. Several subsequent studies have been able to confirm that these changes are present in other MeN cohorts. We have speculated whether the low levels of sodium and/or potassium could have a role in the pathogenesis, because changes in these substances might

stimulate hormonal systems, e.g. RAAS, vasopressin, etc. The morphological findings in Studies I and II, and to some extent also Study III, with glomerular ischemia and a mix of chronic glomerular and tubulointerstitial changes, might support the hypothesis of recurrent volume depletion, causing direct glomerular and tubulointerstitial damage as well as secondary damage through RAAS activation and constriction of glomerular arterioles. However, all three studies were of descriptive design and cannot, as of yet, provide strong evidence for this hypothesis.

Low serum magnesium was the most common electrolyte disturbance in our three studies and might be partly responsible for the low potassium levels, since concomitant hypomagnesemia is known to aggravate hypokalemia (162).

One of the most interesting observations in our studies was the urinary findings in the participants with low serum levels of sodium, potassium, and magnesium, which indicates that renal wasting of these compounds is likely to be a main reason behind the low serum levels. An explanation for the renal losses could be chronic tubulointerstitial damage. In support of this hypothesis, Wesseling et al (43) found that serum potassium was significantly lower in sugarcane workers after 9 weeks of harvest compared to potassium at the start of the harvest, even though serum creatinine increased during this period. In the same cohort, the urinary tubular injury marker NGAL also increased significantly, supporting the hypothesis that the tubular injury might be responsible for the drop in serum potassium level. One could also speculate whether the high output of electrolytes could have a role in the pathophysiology of CKDu, probably will it make patients more susceptible to volume- and further salt depletion.

Hypokalemic nephropathy is a diagnosis mostly found in old scientific literature and is described as a chronic interstitial nephritis seen in patients with chronic hypokalemia (163). Findings in renal biopsies are swelling and vacuolization of the proximal tubular epithelium, tubular dilatation and atrophy, but with normal glomerular structures (164). Even though hypokalemic nephropathy in humans nowadays rarely is described in the scientific literature, several animal studies, where the dietary intake of potassium can be limited, have described hypokalemic nephropathy and suggest that chronic hypokalemia induces renal vasoconstriction, renal ischemia, and progressive interstitial fibrosis (165, 166). The morphological pattern in our studies does not resemble the findings reported from human hypokalemic nephropathy, because our patients had pronounced glomerular damage. However, one might speculate whether the chronic serum depletions of potassium seen in a subset of our patients could have a role in the pathophysiology and deterioration of kidney function.

The natural history of the disease has not been sufficiently described in Sri Lanka or in Central America. Study II was the first longitudinal report of the natural history of the disease and showed that, on average, eGFR deteriorated by 4.4 ml/min/1.73m² per year, but in some cases eGFR was stable or even increased. Even though our follow-up study was small, these findings suggest that not all affected patients have a rapid disease progression of the disease. Data from our study also indicated that patients with low serum sodium and severe glomerular hypertrophy might have faster deterioration of kidney function. Further studies in larger cohorts are needed to study the natural history of the disease, which is of great importance for identify risk factors for deteriorating kidney function as well as for suggesting which clinical findings indicate a worse prognosis so that patients at higher risk can be identified and more closely monitored.

In my opinion, the available studies from the CKDu endemic in Sri Lanka have, as of yet, not sufficiently described the characteristics of the endemic. Well-designed epidemiological studies using

screening for increased serum creatinine in combination with urine dip-stick would give a better perspective of the true prevalence of CKDu in Sri Lanka. Clinical case descriptions of larger cohorts, including detailed blood and urine testing, are needed to give a more comprehensive picture of the typical CKDu patient in Sri Lanka. Data from our study in Sri Lanka suggest that electrolytes both in serum and urine should be included in such studies, in combination with a broader testing panel of blood and urine characteristics, similar to what we have used in our studies. More comprehensive epidemiological and clinical studies would surely give important insights regarding etiology and would also provide more evidence for whether the endemics in Sri Lanka and Central America truly have a common etiology or not.

Even though it is not established that any nephrotoxic compound in the water has any role in the pathophysiology of CKDu in Sri Lanka and Central America, providing clean water to individuals living and working in a warm climate is of course important. If there are any potential nephrotoxic substances in the water, these will then be reduced, which will be overall beneficial for individuals with CKD.

Interestingly, two recent separate reports, one from a sugarcane mill based hospital in Nicaragua (167) and the other from Sri Lanka (66), describes findings of "acute" CKDu, defined as an acute illness, where the patient present at the hospital with diffuse inflammatory symptoms (e.g. back pain, feverish fatigue, nausea, etc.) and elevated creatinine levels. Clinically the patients often have increased inflammatory parameters in the both blood and urine. In the study from Sri Lanka, renal biopsies were collected and indicated acute interstitial nephritis. From Nicaragua there are no published biopsy findings, but preliminary findings from 11 patients showed interstitial nephritis (abstract) (168). The patients in our studies did not report any acute inflammatory illnesses in conjunction with increases in serum creatinine. However, some of the sugarcane workers in Study II have reported that they had been treated for work-related heat symptoms/exhaustion at the sugar mill hospital, but we did not have access to their medical records (personal communication, Marvin González-Quiroz). An interesting hypothesis is whether heat stress or heat stroke can cause episodes of systemic inflammation that also affects the kidneys. One might also suspect an unknown infectious pathogen, and thus further studies are warranted in this field in both regions.

From our three studies we can conclude that there are many similarities between the two CKDu endemics in Central America and Sri Lanka, but also some differences. The main difference in morphology is the more heterogeneous picture in Sri Lanka, even though more than half of the patients had a morphology very similar to the CKDu findings in Central America. One cannot completely rule out different etiologies of CKDu in Central America and Sri Lanka; however, they probably have a similar pathophysiological mechanism since both the clinical picture, the biochemical findings, and the renal morphologies have so many resemblances.

8 FUTURE PERSPECTIVES

Even though the knowledge about CKDu in Central America and Sri Lanka has increased significantly during the last ten years, there are still numerous of unresolved questions about the etiology, pathogenesis, and natural history of the disease(s).

One of the most interesting findings in our studies is the low serum electrolytes levels of sodium, potassium and magnesium and the signs of renal wasting of these compounds in the urine. Further studies of the urinary biochemical profile in CKDu patients are warranted and, in my opinion also more in-depth studies of the tubular system including tubular ion-channels involved in the transport of these electrolytes. Moreover, the role of electrolyte depletion in patients with MeN/CKDu and the importance in the pathogenesis should be further studied. One can hypothesize that low levels of sodium, potassium, and/or magnesium with, or without concurrent volume depletion, may affect different hormonal systems such as the RAAS or vasopressin/antidiuretic hormone (ADH). Activation of these pathways may be responsible for some of the chronic damages seen in the kidneys. In future studies, it would be interesting to measure different RAAS components and ADH in an occupational setting with heat exposure.

Our studies support a common etiology of the two endemics, but larger studies examining both kidney biopsies and blood and urine characteristics are needed to confirm this. As discussed previously, the epidemiological and clinical profile of the CKDu endemic in Sri Lanka need to be better described, especially the prevalence of CKD by measurement of serum creatinine and eGFR in epidemiological studies. A better description would give more information regarding resemblances with the CKDu endemic in Central America and also provide more knowledge about risk factors for CKDu in Sri Lanka. Another way to compare the two endemics would be to analyze the transcriptome, i.e. RNA profiles, in the kidney tissue. Matching or similar profiles would further support a common etiology. Transcriptome analysis provides information on which genes that are up or down regulated in the tissue cells and may provide valuable information regarding the pathogenesis and etiology of the disease.

Another important step is to evaluate possible endemics of CKDu in other regions. One interesting region where a high prevalence of CKD has been reported is India, and especially rural areas in the Andhra Pradesh region. Published studies from this region are largely lacking and therefore well-designed epidemiological and clinical studies from this region would be of great interest. Since our studies support that the two CKDu endemics in Central America and Sri Lanka are similar diagnostic entities, studies of other CKDu endemics around the world would most certainly provide important information regarding etiologies and risk factors. If the cause behind CKDu is a specific exposure, similar exposure is likely to be found in all endemic areas. Thus, if the disease is found in professions involving physically strenuous work in more tropical or sub-tropical regions this would support the occupational heat stress hypothesis. In this context the current and future contributing effect of global warming should also be further addressed.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakgrund: Kronisk njursvikt, d.v.s. permanent nedsatt njurfunktion, är ett globalt hälsoproblem. De vanligaste bakomliggande orsakerna till kronisk njursvikt är diabetes, högt blodtryck och kronisk njurinflammation. Under de senaste årtiondena har förekomsten av kronisk njursvikt av okänd orsak (CKDu) ökat kraftigt på landsbygden i vissa tropiska områden, bland annat i Centralamerika och på Sri Lanka. Gemensamt för de utbrott av CKDu som rapporterats är att de oftare drabbar män än kvinnor och att CKDu i huvudsak ses hos landsbygdsbefolkning. I Centralamerika drabbas främst män som skördar sockerrör medan det i Sri Lanka främst är manliga risodlare som insjuknar. Den underliggande orsaken eller orsakerna till CKDu har ännu inte klarlagts, men i Centralamerika har man kunnat fastställa att hårt fysiskt arbete i varmt klimat med risk för upprepad intorkning är en riskfaktor. Det är fortfarande oklart om det rör sig om samma sjukdomstillstånd eller om det är olika sjukdomar i Centralamerika respektive Sri Lanka. Vävnadsprov från njurarna, så kallade njurbiopsier, är ett viktigt redskap för att undersöka orsakerna till kronisk njursvikt, vilket genomförs rutinmässigt vid utredning av kronisk njursvikt i Sverige och andra länder med utvecklad sjukvård. I Centralamerika hade inga studier av njurbiopsier från patienter med CKDu genomförts när detta projekt startade.

Mål: Målet med studierna i denna avhandling var att beskriva vilka förändringar som kan ses i njurbiopsier samt i blod och urinprover hos patienter med CKDu i Centralamerika och Sri Lanka. Detta är av avgörande betydelse för att kunna förstå orsaken till och i förlängningen kunna förebygga sjukdom. Vi ville också studera sjukdomsförloppet samt jämföra CKDu i dessa länder för att kunna klarlägga om det är samma sjukdom.

Material och metoder: Studierna planerades och genomfördes i samarbete med lokala läkare och forskare. Tillstånd från etiska kommittéer inhämtades både i Sverige och i samarbetsländerna och alla patienter samtyckte till att delta i studierna. Njurbiopsier, blod och urinprover insamlades från patienter med njursvikt av okänd orsak. Deltagarna fick även fylla i ett frågeformulär om sjukdomshistoria, medicinering, livsstilsfaktorer mm. Ljus- och elektronmikroskopi användes för att utvärdera biopsierna. Uppföljningsprover insamlades från patienterna i Studie I och II efter 1-2,5 år.

Studie I: Åtta manliga jordbruksarbetare i El Salvador med mild till måttligt nedsatt njurfunktion (uppskattad glomerulär filtrationshastighet (eGFR) mellan 27-79 ml/min/1,73m2) och utan känd orsak till sin nedsatta njurfunktion, d.v.s. CKDu, inkluderades i studien. Njurbiopsierna visade en unik bild med omfattande ärromvandling av njurens kärlnystan (glomeruloskleros), tecken på försämrat blodflöde i njurens kärlnystan (glomerulär ischemi) samt milda till måttliga kroniska skador på njurens rörsystem och bindvävsomvandling av njurvävnaden (tubulär atrofi och interstitiell fibros). Blodkärlen i njurvävnaden var oftast normala eller uppvisade lättare förändringar. Blodproverna uppvisade låga nivåer av saltet kalium hos flertalet av patienterna.

Studie II: Nitton män från Nicaragua med misstänkt CKDu som arbetat på sockerplantager inkluderades i studien. Njurfunktionen var mild till måttligt nedsatt (eGRF mellan 33-96 ml/min/1,732) och låga halter av natrium, kalium och magnesium i blodet var vanliga fynd. Sexton njurbiopsier var av tillräckligt god kvalitet för att ingå i studien. De visade kroniska förändringar på njurens kärlnystan med ärromvandling (glomeruloskleros), förstorade kärlnystan (glomerulär hypertrofi) och tecken på försämrat blodflöde (glomerulär ischemi). Milda till måttliga kroniska skador på njurens rörsystem och bindvävsomvandling av njurvävnaden (tubulointerstitiella förändringar) samt milda förändringar i njurens blodkärl observerades. Uppföljningsprover från 7

studiedeltagare från Studie I och 18 studiedeltagare från Studie II visade att den genomsnittliga försämringen av njurfunktionen som mäts med eGRF var 4.4 ± 8.4 ml/min/1,73m² per år.

Studie III: Elva risbönder från Sri Lanka med mild till uttalat nedsatt njurfunktion (eGRF mellan 21-70 ml/min/1,73m²) inkluderades i studien. Låga halter av natrium, kalium och magnesium i blod var vanliga observationer. De huvudsakliga fynden från biopsierna var kroniska förändringar på njurens kärlnystan såsom ärromvandling (glomeruloskleros) förstorade kärlnystan (glomerulär hypertrofi) samt milda till måttliga skador på njurens rörsystem och bindvävsomvandling av njurvävnaden (tubulär atrofi och interstitiell fibros). Härtill sågs varierande grad av inflammation (interstitiell inflammation). Njurens blodkärl hade i de flesta fall endast milda förändringar men var i vissa fall måttliga. Njurbiopsierna hos olika patienter uppvisade större variation jämfört med Studie I och II, och interstitiell inflammation och kärlförändringar var mer vanligt förekommande.

Slutsatser: Studie I och II visade att kronisk njursvikt av okänd orsak (CKDu) i Centralamerika är ett unikt sjukdomstillstånd med typiska fynd i njurvävnaden som karaktäriseras av kroniska skador på både glomerulära och tubulointerstitiella delar. Fynden ger visst stöd för hypotesen att arbetsrelaterad värmeexponering med risk för upprepad intorkning är den bakomliggande orsaken, men fortsatt forskning är nödvändig för att bevisa detta. Vid jämförelse mellan CKDu i Centralamerika (Studie I och II) och Sri Lanka (Studie III) noteras det att biopsifynden i den senare studien hade en mer varierad bild, men trots detta uppvisade majoriteten av studiedeltagarna i Sri Lanka liknande fynd som i Centralamerika. Låga halter av natrium, kalium och magnesium i blod var vanliga observationer i alla tre studierna. Sammantaget tyder resultaten från de tre studierna på att CKDu i Sri Lanka och Centralamerika uppvisar fler likheter än olikheter, både vad gäller fynd i njurvävnaden samt i blod och urinprover, vilket stödjer hypotesen att de har en gemensam orsak.

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11 REFERENCES

1.	Gilbert SJ, Weiner DE, Gipson DS, Perazella MA, Tonelli M, National Kidney Foundation. National Kidney Foundation's primer on kidney diseases. Sixth edition ed. xvii, 646 pages p.
2.	Floege Jr, Johnson RJ, Feehally J. Comprehensive clinical nephrology. 4th ed. Philadelphia, PA: Saunders/Elsevier; 2010. xxii, 1286 p. p.
3.	Chapter 2: Definition, identification, and prediction of CKD progression. Kidney Int Suppl (2011). 2013;3(1):63-72.
4.	Zhang QL, Rothenbacher D. Prevalence of chronic kidney disease in population-based studies: systematic review. *BMC public health.* 2008;8:117.
5.	Bruck K, Stel VS, Gambaro G, Hallan S, Volzke H, Arnlov J, et al. CKD Prevalence Varies across the European General Population. *JASN.* 2015.
6.	Coresh J, Selvin E, Stevens LA, Manzi J, Kusek JW, Eggers P, et al. Prevalence of chronic kidney disease in the United States. *JAMA.* 2007;298(17):2038-47.
7.	Hill NR, Fatoba ST, Oke JL, Hirst JA, O'Callaghan CA, Lasserson DS, et al. Global Prevalence of Chronic Kidney Disease - A Systematic Review and Meta-Analysis. *PloS one.* 2016;11(7):18.
8.	Jha V, Garcia-Garcia G, Iseki K, Li Z, Naicker S, Plattner B, et al. Chronic kidney disease: global dimension and perspectives. *Lancet.* 2013;382(9888):260-72.
9.	Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet.* 2012;380(9859):2095-128.
10.	Liem YS, Bosch JL, Arends LR, Heijenbrok-Kal MH, Hunink MGM. Quality of life assessed with the medical outcomes study short form 36-item health survey of patients on renal replacement therapy: A systematic review and meta-analysis. *Value Health.* 2007;10(5):390-7.
11.	Vogelzang JL, van Stralen KJ, Noordzij M, Diez JA, Carrero JJ, Couchoud C, et al. Mortality from infections and malignancies in patients treated with renal replacement therapy: data from the ERA-EDTA registry. *Nephrol Dial Transplant.* 2015;30(6):1028-37.
12.	Go AS, Chertow GM, Fan DJ, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med.* 2004;351(13):1296-305.
13.	Stenvinkel P. Chronic kidney disease: a public health priority and harbinger of premature cardiovascular disease. *J Intern Med.* 2010;268(5):456-67.
14.	Weaver VM, Fadrowski JJ, Jaar BG. Global dimensions of chronic kidney disease of unknown etiology (CKDu): a modern era environmental and/or occupational nephropathy? *BMC Nephrol.* 2015;16:145.
15.	Wesseling C, Crowe J, Hogstedt C, Jakobsson K, Lucas R, Wegman D, et al. Mesoamerican Nephropathy: Report from the First International Research Workshop on MeN. Heredia, C.R: SALTRA / IRET-UNA; 2013 [Available from: http://www.regionalnephropathy.org/wp-content/uploads/2013/04/Technical-Report-for-Website-Final.pdf.
16.	Trabanino RG, Aguilar R, Silva CR, Mercado MO, Merino RL. [End-stage renal disease among patients in a referral hospital in El Salvador]. Revista panamericana de salud publica = Pan American journal of public health. 2002;12(3):202-6.
17.	Correa-Rotter R, Wesseling C, Johnson RJ. CKD of unknown origin in Central America: the case for a Mesoamerican nephropathy. *Am J Kidney Dis.* 2014;63(3):506-20.
18.	Ordunez P, Saenz C, Martinez R, Chapman E, Reveiz L, Becerra F. The epidemic of chronic kidney disease in Central America. *Lancet Glob Health.* 2014;2(8):e440-1.
19.	Raines N, Gonzalez M, Wyatt C, Kurzrok M, Pool C, Lemma T, et al. Risk factors for reduced glomerular filtration rate in a Nicaraguan community affected by Mesoamerican nephropathy. *MEDICC Rev.* 2014;16(2):16-22.
20.	Torres C, Aragon A, Gonzalez M, Lopez I, Jakobsson K, Elinder CG, et al. Decreased kidney function of unknown cause in Nicaragua: a community-based survey. American journal of kidney diseases : the official journal of the National Kidney Foundation. 2010;55(3):485-96.
21.	Peraza S, Wesseling C, Aragon A, Leiva R, Garcia-Trabanino RA, Torres C, et al. Decreased kidney function among agricultural workers in El Salvador. *Am J Kidney Dis.* 2012;59(4):531-40.
22.	Orantes CM, Herrera R, Almaguer M, Brizuela EG, Hernandez CE, Bayarre H, et al. Chronic Kidney Disease and Associated Risk Factors in the Bajo Lempa Region of El Salvador: Nefrolempa Study, 2009. *MEDICC Rev.* 2011;13(4):14-22.
23.	Orantes CM, Herrera R, Almaguer M, Brizuela EG, Nunez L, Alvarado NP, et al. Epidemiology of chronic kidney disease in adults of Salvadoran agricultural communities. *MEDICC Rev.* 2014;16(2):23-30.
24.	Krinsky LM, Levine WJ. An island of widows: the human face of Mesoamerican endemic nephropathy. *Kidney Int.* 2014;86(2):221-3.
25.	Crowe J, Wesseling C, Solano BR, Umana MP, Ramirez AR, Kjellstrom T, et al. Heat exposure in sugarcane harvesters in Costa Rica. *Am J Ind Med.* 2013;56(10):1157-64.

26.   WHO. El Salvador: WHO statistical profile 2012 2012 [Available from: http://www.who.int/gho/countries/slv.pdf?ua=1.
27.   WHO. Nicaragua: WHO statistical profile 2012 2012 [Available from: http://www.who.int/gho/countries/nic.pdf?ua=1.
28.   Cuadra SN JK, Hogstedt C,Wesseling C. Chronic kidney disease: Assessment of current knowledge and feasibility for regional research collaboration in Central America. Heredia, Costa Rica: SALTRA, IRET-UNA, 2006. 76 p. ISSN:1659-2670. http://www.iret.una.ac.cr/Publicaciones/01-0008.pdf.
29.   Gracia-Trabanino R, Dominguez J, Jansa JA, Oliver A. Proteinuria and chronic renal failure in the coast of El Salvador. *Nefrologia*. 2005;25(1):31-8.
30.   Vela XF, Henriquez DO, Zelaya SM, Granados DV, Hernandez MX, Orantes CM. Chronic kidney disease and associated risk factors in two Salvadoran farming communities, 2012. *MEDICC Rev.* 2014;16(2):55-60.
31.   Garcia-Trabanino R, Hernandez C, Rosa A, Dominguez Alonso J, en nombre del Fondo Social de Emergencia para la Salud del canton Tierra Blanca ddUES. Incidence, mortality, and prevalence of end-stage chronic renal disease in the Bajo Lempa region of El Salvador: A ten-year community registry. *Nefrologia.* 2016;36(5):517-22.
32.   (PAHO) PAHO. Renal Failure and Chronic Kidney Disease (CKD) mortality visualization 2007 [Available from: http://www.paho.org/hq/index.php?option=com_content&view=article&id=9402%3A2014-renal-failure-chronic-kidney-disease-ckd-mortality-visualization&catid=2390%3Aregional-health-observatory-themes&Itemid=2391&lang=en.
33.   Garcia-Trabanino R, Trujillo Z, Colorado AV, Magana Mercado S, Henriquez CA, En nombre de la Asociacion de Nefrologia e Hipertension Arterial de El S. Prevalence of patients receiving renal replacement therapy in El Salvador in 2014. *Nefrologia*. 2016;36(6):631-6.
34.   Sanoff SL, Callejas L, Alonso CD, Hu Y, Colindres RE, Chin H, et al. Positive association of renal insufficiency with agriculture employment and unregulated alcohol consumption in Nicaragua. *Ren Fail.* 2010;32(7):766-77.
35.   O'Donnell JK, Tobey M, Weiner DE, Stevens LA, Johnson S, Stringham P, et al. Prevalence of and risk factors for chronic kidney disease in rural Nicaragua. *Nephrol Dial Transplant.* 2011;26(9):2798-805.
36.   Laux TS, Bert PJ, Barreto Ruiz GM, Gonzalez M, Unruh M, Aragon A, et al. Nicaragua revisited: evidence of lower prevalence of chronic kidney disease in a high-altitude, coffee-growing village. *J Nephrol.* 2012;25(4):533-40.
37.   Kupferman J, Amador JJ, Lynch KE, Laws RL, Lopez-Pilarte D, Ramirez-Rubio O, et al. Characterization of Mesoamerican Nephropathy in a Kidney Failure Hotspot in Nicaragua. *Am J Kidney Dis.* 2016;68(5):716-25.
38.   Ramirez-Rubio O, Amador JJ, Kaufman JS, Weiner DE, Parikh CR, Khan U, et al. Urine biomarkers of kidney injury among adolescents in Nicaragua, a region affected by an epidemic of chronic kidney disease of unknown aetiology. *Nephrol Dial Transplant.* 2016;31(3):424-32.
39.   Gonzalez-Quiroz M, Camacho A, Faber D, Aragon A, Wesseling C, Glaser J, et al. Rationale, description and baseline findings of a community-based prospective cohort study of kidney function amongst the young rural population of Northwest Nicaragua. *BMC Nephrol.* 2017;18(1):16.
40.   Laux TS, Barnoya J, Guerrero DR, Rothstein M. Dialysis enrollment patterns in Guatemala: evidence of the chronic kidney disease of non-traditional causes epidemic in Mesoamerica. *BMC Nephrol.* 2015;16:54.
41.   Laws RL, Brooks DR, Amador JJ, Weiner DE, Kaufman JS, Ramirez-Rubio O, et al. Changes in kidney function among Nicaraguan sugarcane workers. *Int J Occup Env Heal.* 2015:2049396714Y0000000102.
42.   Laws RL, Brooks DR, Amador JJ, Weiner DE, Kaufman JS, Ramirez-Rubio O, et al. Biomarkers of Kidney Injury Among Nicaraguan Sugarcane Workers. Am J Kidney Dis. 2016;67(2):209-17.
43.   Wesseling C, Aragon A, Gonzalez M, Weiss I, Glaser J, Bobadilla NA, et al. Kidney function in sugarcane cutters in Nicaragua - A longitudinal study of workers at risk of Mesoamerican nephropathy. *Environ Res.* 2016;147:125-32.
44.   Garcia-Trabanino R, Jarquin E, Wesseling C, Johnson RJ, Gonzalez-Quiroz M, Weiss I, et al. Heat stress, dehydration, and kidney function in sugarcane cutters in El Salvador--A cross-shift study of workers at risk of Mesoamerican nephropathy. *Environ Res.* 2015;142:746-55.
45.   Herrera R, Orantes CM, Almaguer M, Alfonso P, Bayarre HD, Leiva IM, et al. Clinical characteristics of chronic kidney disease of nontraditional causes in Salvadoran farming communities. *MEDICC Rev.* 2014;16(2):39-48.
46.   Rao MV, Qiu Y, Wang C, Bakris G. Hypertension and CKD: Kidney Early Evaluation Program (KEEP) and National Health and Nutrition Examination Survey (NHANES), 1999-2004. *Am J Kidney Dis.* 2008;51(4 Suppl 2):S30-7.
47.   Ramirez-Rubio O, Brooks DR, Amador JJ, Kaufman JS, Weiner DE, Scammell MK. Chronic kidney disease in Nicaragua: a qualitative analysis of semi-structured interviews with physicians and pharmacists. *BMC public health.* 2013;13:350.

48.   Wesseling C, Aragon A, Gonzalez M, Weiss I, Glaser J, Rivard CJ, et al. Heat stress, hydration and uric acid: a cross-sectional study in workers of three occupations in a hotspot of Mesoamerican nephropathy in Nicaragua. *BMJ Open.* 2016;6(12):11.

49.   McClean M, Amador J, Laws R, Kaufman J, Weiner D, Sanchez-Rodriguez J, et al. Biological Sampling Report: Investigating biomarkers of kidney injury and chronic kidney disease among workers in Western Nicaragua http://wwwcao-ombudsmanorg/cases/document-links/links-82aspx [Internet]. 2012 April 26, 2012. Available from: http://www.cao-ombudsman.org/cases/document-links/links-82.aspx.

50.   Wanigasuriya K. Update on uncertain etiology of chronic kidney disease in Sri Lanka's north-central dry zone. *MEDICC Rev.* 2014;16(2):61-5.

51.   Jayasekara JM, Dissanayake DM, Adhikari SB, Bandara P. Geographical distribution of chronic kidney disease of unknown origin in North Central Region of Sri Lanka. *Ceylon Med J.* 2013;58(1):6-10.

52.   Senevirathna L, Abeysekera T, Nanayakkara S, Chandrajith R, Ratnatunga N, Harada KH, et al. Risk factors associated with disease progression and mortality in chronic kidney disease of uncertain etiology: a cohort study in Medawachchiya, Sri Lanka. *Environ Health Prev Med.* 2012;17(3):191-8.

53.   Athuraliya NT, Abeysekera TD, Amerasinghe PH, Kumarasiri R, Bandara P, Karunaratne U, et al. Uncertain etiologies of proteinuric-chronic kidney disease in rural Sri Lanka. *Kidney Int.* 2011;80(11):1212-21.

54.   Chandrajith R, Nanayakkara S, Itai K, Aturaliya TN, Dissanayake CB, Abeysekera T, et al. Chronic kidney diseases of uncertain etiology (CKDue) in Sri Lanka: geographic distribution and environmental implications. *Environ Geochem Health.* 2011;33(3):267-78.

55.   Jayasumana C, Orantes C, Herrera R, Almaguer M, Lopez L, Silva LC, et al. Chronic interstitial nephritis in agricultural communities: a worldwide epidemic with social, occupational and environmental determinants. *Nephrol Dial Transplant.* 2017;32(2):234-41.

56.   Athuraliya TN, Abeysekera DT, Amerasinghe PH, Kumarasiri PV, Dissanayake V. Prevalence of chronic kidney disease in two tertiary care hospitals: high proportion of cases with uncertain aetiology. *Ceylon Med J.* 2009;54(1):23-5.

57.   Jayatilake N, Mendis S, Maheepala P, Mehta FR, Team CKNRP. Chronic kidney disease of uncertain aetiology: prevalence and causative factors in a developing country. *BMC Nephrol.* 2013;14:180.

58.   Jayasekara KB, Dissanayake DM, Sivakanesan R, Ranasinghe A, Karunarathna RH, Priyantha Kumara GW. Epidemiology of chronic kidney disease, with special emphasis on chronic kidney disease of uncertain etiology, in the north central region of Sri Lanka. *J Epidemiol.* 2015;25(4):275-80.

59.   Wanigasuriya KP, Peiris-John RJ, Wickremasinghe R, Hittarage A. Chronic renal failure in North Central Province of Sri Lanka: an environmentally induced disease. *Trans R Soc Trop Med Hyg.* 2007;101(10):1013-7.

60.   Nanayakkara S, Stmld S, Abeysekera T, Chandrajith R, Ratnatunga N, Edl G, et al. An Integrative Study of the Genetic, Social and Environmental Determinants of Chronic Kidney Disease Characterized by Tubulointerstitial Damages in the North Central Region of Sri Lanka. *J Occup Health.* 2014;56(1):28-38.

61.   Nanayakkara S, Senevirathna ST, Karunaratne U, Chandrajith R, Harada KH, Hitomi T, et al. Evidence of tubular damage in the very early stage of chronic kidney disease of uncertain etiology in the North Central Province of Sri Lanka: a cross-sectional study. *Environ Health Prev Med.* 2012;17(2):109-17.

62.   De Silva P, Abdul KSM, Eakanayake E, Jayasinghe SS, Jayasumana C, Asanthi HB, et al. Urinary Biomarkers KIM-1 and NGAL for Detection of Chronic Kidney Disease of Uncertain Etiology (CKDu) among Agricultural Communities in Sri Lanka. *PLoS Negl Trop Dis.* 2016;10(9):17.

63.   Selvarajah M, Weeratunga P, Sivayoganthan S, Rathnatunga N, Rajapakse S. Clinicopathological correlates of chronic kidney disease of unknown etiology in Sri Lanka. *Indian J Nephrol.* 2016;26(5):357-63.

64.   Nanayakkara S, Komiya T, Ratnatunga N, Senevirathna ST, Harada KH, Hitomi T, et al. Tubulointerstitial damage as the major pathological lesion in endemic chronic kidney disease among farmers in North Central Province of Sri Lanka. *Environ Health Prev Med.* 2012;17(3):213-21.

65.   Wijetunge S, Ratnatunga NV, Abeysekera TD, Wazil AW, Selvarajah M. Endemic chronic kidney disease of unknown etiology in Sri Lanka: Correlation of pathology with clinical stages. *Indian J Nephrol.* 2015;25(5):274-80.

66.   Badurdeen Z, Nanayakkara N, Ratnatunga NV, Wazil AW, Abeysekera TD, Rajakrishna PN, et al. Chronic kidney disease of uncertain etiology in Sri Lanka is a possible sequel of interstitial nephritis! *Clin Nephrol.* 2016;86 (2016)(13):106-9.

67.   Grollman AP. Aristolochic acid nephropathy: Harbinger of a global iatrogenic disease. *Environ Mol Mutagen.* 2013;54(1):1-7.

68.   Gokmen MR, Cosyns JP, Arlt VM, Stiborova M, Phillips DH, Schmeiser HH, et al. The Epidemiology, Diagnosis, and Management of Aristolochic Acid Nephropathy A Narrative Review. *Ann Intern Med.* 2013;158(6):469-U132.

69.	Vanherweghem JL, Depierreux M, Tielemans C, Abramowicz D, Dratwa M, Jadoul M, et al. Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs. *Lancet.* 1993;341(8842):387-91.

70.	Jelakovic B, Karanovic S, Vukovic-Lela I, Miller F, Edwards KL, Nikolic J, et al. Aristolactam-DNA adducts are a biomarker of environmental exposure to aristolochic acid. *Kidney Int.* 2012;81(6):559-67.

71.	Chen CH, Dickman KG, Moriya M, Zavadil J, Sidorenko VS, Edwards KL, et al. Aristolochic acid-associated urothelial cancer in Taiwan. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(21):8241-6.

72.	Goyer RA. Lead toxicity: a problem in environmental pathology. *Am J Pathol.* 1971;64(1):167-82.

73.	Barregard L, Elinder C-G. Renal Effects from Exposure to Metals In: Nordberg GF, Fowler BA, Nordberg M, editors. Handbook on the Toxicology of Metals I General Considerations. 4th edition ed. Amsterdam: Academic Press/Elsevier; 2014 p. 333-50.

74.	Evans M, Elinder CG. Chronic renal failure from lead: myth or evidence-based fact? *Kidney Int.* 2011;79(3):272-9.

75.	Hellstrom L, Elinder CG, Dahlberg B, Lundberg M, Jarup L, Persson B, et al. Cadmium exposure and end-stage renal disease. *Am J Kidney Dis.* 2001;38(5):1001-8.

76.	Elinder CG. Epidemiology and toxicity of cadmium: UpToDate; [Available from: http://www.uptodate.com/contents/epidemiology-and-toxicity-of-cadmium.

77.	Bonnell JA, Ross JH, King E. Renal lesions in experimental cadmium poisoning. *Br J Ind Med.* 1960;17:69-80.

78.	Li SJ, Zhang SH, Chen HP, Zeng CH, Zheng CX, Li LS, et al. Mercury-induced membranous nephropathy: clinical and pathological features. *CJASN.* 2010;5(3):439-44.

79.	Gillian Beauchamp M, Shana Kusin M, Carl-Gustaf Elinder M, PhD. Mercury toxicity: UpToDate; [Available from: https://www.uptodate.com/contents/mercury-toxicity.

80.	Rajapurkar MM, John GT, Kirpalani AL, Abraham G, Agarwal SK, Almeida AF, et al. What do we know about chronic kidney disease in India: first report of the Indian CKD registry. 2012;13:8.

81.	Ganguli A. Uddanam Nephropathy/Regional Nephropathy in India: Preliminary Findings and a Plea for Further Research. *Am J Kidney Dis.* 2016;68(3):344-8.

82.	Machiraju RY, K. Gowrishankar, S. Edwards, KL. et al. . Epidemiology of Udhanam Endemic Nephropathy. [Abstract]. *J Am Soc Nephrol* 2009;20:643A.

83.	Siddarth M, Datta SK, Mustafa MD, Ahmed RS, Banerjee BD, Kalra OP, et al. Increased level of organochlorine pesticides in chronic kidney disease patients of unknown etiology: Role of GSTM1/GSTT1 polymorphism. *Chemosphere.* 2014;96:174-9.

84.	Reddy DV, Gunasekar A. Chronic kidney disease in two coastal districts of Andhra Pradesh, India: role of drinking water. *Environ Geochem Health.* 2013;35(4):439-54.

85.	Wegman D CJ, Hogstedt C, Jakobsson K, Wesseling C. Mesoamerican nephropathy: report from the second international research workshop on MeN. Heredia, Costa Rica.: SALTRA / IRET- UNA.; 2016 [1 ed.:[Available from: http://repositorio.una.ac.cr/bitstream/handle/11056/13142/MeN%202015%20Scientific%20Report%20high%20resolution_corregida.pdf?sequence=3&isAllowed=y.

86.	Whittier WL. Complications of the percutaneous kidney biopsy. *Adv Chronic Kidney Dis.* 2012;19(3):179-87.

87.	Corapi KM, Chen JL, Balk EM, Gordon CE. Bleeding complications of native kidney biopsy: a systematic review and meta-analysis. *Am J Kidney Dis.* 2012;60(1):62-73.

88.	Tondel C, Vikse BE, Bostad L, Svarstad E. Safety and complications of percutaneous kidney biopsies in 715 children and 8573 adults in Norway 1988-2010. *CJASN.* 2012;7(10):1591-7.

89.	Manno C, Strippoli GF, Arnesano L, Bonifati C, Campobasso N, Gesualdo L, et al. Predictors of bleeding complications in percutaneous ultrasound-guided renal biopsy. *Kidney Int.* 2004;66(4):1570-7.

90.	Peters B, Andersson Y, Stegmayr B, Molne J, Jensen G, Dahlberg P, et al. A study of clinical complications and risk factors in 1,001 native and transplant kidney biopsies in Sweden. *Acta Radiol.* 2014;55(7):890-6.

91.	Peters B, Molne J, Hadimeri H, Hadimeri U, Stegmayr B. Sixteen Gauge biopsy needles are better and safer than 18 Gauge in native and transplant kidney biopsies. Acta Radiol. 2017;58(2):240-8.

92.	Armed Forces Institute of Pathology (U.S.), Luna LG. Manual of histologic staining methods of the Armed Forces Institute of Pathology. Edited by Lee G. Luna. 3d ed. New York,: Blakiston Division; 1968. xii, 258 p. p.

93.	Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, 3rd, Feldman HI, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med.* 2009;150(9):604-12.

94.	Inker LA, Schmid CH, Tighiouart H, Eckfeldt JH, Feldman HI, Greene T, et al. Estimating glomerular filtration rate from serum creatinine and cystatin C. *N Engl J Med.* 2012;367(1):20-9.

95.	Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, et al. The Banff 97 working classification of renal allograft pathology. *Kidney Int.* 1999;55(2):713-23.

96.   Solez K, Colvin RB, Racusen LC, Haas M, Sis B, Mengel M, et al. Banff 07 classification of renal allograft pathology: updates and future directions. *Am J Transplant.* 2008;8(4):753-60.
97.   Kovesdy CP. Significance of hypo- and hypernatremia in chronic kidney disease. *Nephrol Dial Transplant.* 2012;27(3):891-8.
98.   Einhorn LM, Zhan M, Hsu V, Walker LD, Moen MF, Seliger SL, et al. The Frequency of Hyperkalemia and Its Significance in Chronic Kidney Disease. *Arch Intern Med.* 2009;169(12):1156-62.
99.   Cunningham J, Rodriguez M, Messa P. Magnesium in chronic kidney disease Stages 3 and 4 and in dialysis patients. *Clin Kidney J.* 2012;5(Suppl 1):i39-i51.
100.   Moranne O, Froissart M, Rossert J, Gauci C, Boffa JJ, Haymann JP, et al. Timing of onset of CKD-related metabolic complications. *JASN.* 2009;20(1):164-71.
101.   Lopez-Marin L, Chavez Y, Garcia XA, Flores WM, Garcia YM, Herrera R, et al. Histopathology of chronic kidney disease of unknown etiology in Salvadoran agricultural communities. *MEDICC Rev.* 2014;16(2):49-54.
102.   Juraschek SP, Kovell LC, Miller ER, 3rd, Gelber AC. Association of kidney disease with prevalent gout in the United States in 1988-1994 and 2007-2010. *Semin Arthritis Rheum.* 2013;42(6):551-61.
103.   Choi HK, Zhu Y, Mount DB. Genetics of gout. *Curr Opin Rheumatol.* 2010;22(2):144-51.
104.   Yu H, Yanagisawa Y, Forbes MA, Cooper EH, Crockson RA, Maclennan ICM. ALPHA-1-MICROGLOBULIN - AN INDICATOR PROTEIN FOR RENAL TUBULAR FUNCTION. *J Clin Pathol.* 1983;36(3):253-9.
105.   Bazzi C, Petrini C, Rizza V, Arrigo G, Napodano P, Paparella M, et al. Urinary N-acetyl-beta-glucosaminidase excretion is a marker of tubular cell dysfunction and a predictor of outcome in primary glomerulonephritis. *Nephrol Dial Transplant.* 2002;17(11):1890-6.
106.   Spasovski G, Vanholder R, Allolio B, Annane D, Ball S, Bichet D, et al. Clinical practice guideline on diagnosis and treatment of hyponatraemia. *Eur J Endocrinol.* 2014;170(3):G1-G47.
107.   UpToDate. Evaluation of the adult patient with hypokalemia [Available from: http://www.uptodate.com/contents/evaluation-of-the-adult-patient-with-hypokalemia.
108.   Elisaf M, Siamopoulos KC. FRACTIONAL EXCRETION OF POTASSIUM IN NORMAL SUBJECTS AND IN PATIENTS WITH HYPOKALEMIA. *Postgrad Med J.* 1995;71(834):211-2.
109.   Elisaf M, Panteli K, Theodorou J, Siamopoulos KC. Fractional excretion of magnesium in normal subjects and in patients with hypomagnesemia. *Magnes Res.* 1997;10(4):315-20.
110.   Agus ZS. Hypomagnesemia. *JASN.* 1999;10(7):1616-22.
111.   Sureda-Vives M, Morell-Garcia D, Rubio-Alaejos A, Valina L, Robles J, Bauca JM. Stability of serum, plasma and urine osmolality in different storage conditions: Relevance of temperature and centrifugation. *Clin Biochem. 2017.*
112.   Wijkstrom J, Leiva R, Elinder CG, Leiva S, Trujillo Z, Trujillo L, et al. Clinical and pathological characterization of Mesoamerican nephropathy: a new kidney disease in Central America. *Am J Kidney Dis.* 2013;62(5):908-18.
113.   Fogo AB, Lusco MA, Najafian B, Alpers CE. AJKD Atlas of Renal Pathology: Chronic Interstitial Nephritis. *Am J Kidney Dis.* 2017;70(1):e1-e2.
114.   Fogo A, Ichikawa I. Evidence for a pathogenic linkage between glomerular hypertrophy and sclerosis. *Am J Kidney Dis.* 1991;17(6):666-9.
115.   Heyman SN, Khamaisi M, Rosen S, Rosenberger C. Renal parenchymal hypoxia, hypoxia response and the progression of chronic kidney disease. *Am J Nephrol.* 2008;28(6):998-1006.
116.   Fogo A, Hawkins EP, Berry PL, Glick AD, Chiang ML, MacDonell RC, Jr., et al. Glomerular hypertrophy in minimal change disease predicts subsequent progression to focal glomerular sclerosis. *Kidney Int.* 1990;38(1):115-23.
117.   Roncal-Jimenez CA, Garcia-Trabanino R, Wesseling C, Johnson RJ. Mesoamerican Nephropathy or Global Warming Nephropathy? *Blood Purif.* 2016;41(1-3):135-8.
118.   (OSHA) TOSaHA. Heat stress [Available from: https://www.osha.gov/dts/osta/otm/otm_iii/otm_iii_4.html#1.
119.   Hansen AL, Bi P, Ryan P, Nitschke M, Pisaniello D, Tucker G. The effect of heat waves on hospital admissions for renal disease in a temperate city of Australia. *Int J Epidemiol.* 2008;37(6):1359-65.
120.   Kingsley SL, Eliot MN, Gold J, Vanderslice RR, Wellenius GA. Current and Projected Heat-Related Morbidity and Mortality in Rhode Island. *Environ Health Perspect.* 2016;124(4):460-7.
121.   Wang XY, Barnett A, Guo YM, Yu WW, Shen XM, Tong SL. Increased risk of emergency hospital admissions for children with renal diseases during heatwaves in Brisbane, Australia. *World J Pediatr.* 2014;10(4):330-5.
122.   Kew MC, Abrahams C, Seftel HC. Chronic interstitial nephritis as a consequence of heatstroke. *Q J Med.* 1970;39(154):189-99.
123.   Tawatsupa B, Lim LLY, Kjellstrom T, Seubsman SA, Sleigh A, Thai Cohort Study T. Association Between Occupational Heat Stress and Kidney Disease Among 37 816 Workers in the Thai Cohort Study (TCS). *J Epidemiol.* 2012;22(3):251-60.

124.   Coca SG, Singanamala S, Parikh CR. Chronic kidney disease after acute kidney injury: a systematic review and meta-analysis. *Kidney Int.* 2012;81(5):442-8.
125.   Chawla LS, Kimmel PL. Acute kidney injury and chronic kidney disease: an integrated clinical syndrome. *Kidney Int.* 2012;82(5):516-24.
126.   Sawhney S, Marks A, Fluck N, Levin A, McLernon D, Prescott G, et al. Post-discharge kidney function is associated with subsequent ten-year renal progression risk among survivors of acute kidney injury. *Kidney Int.* 2017.
127.   Crowe J, Nilsson M, Kjellstrom T, Wesseling C. Heat-Related symptoms in sugarcane harvesters. *Am J Ind Med.* 2015;58(5):541-8.
128.   Paula Santos U, Zanetta DM, Terra-Filho M, Burdmann EA. Burnt sugarcane harvesting is associated with acute renal dysfunction. *Kidney Int.* 2015;87(4):792-9.
129.   Moyce S, Mitchell D, Armitage T, Tancredi D, Joseph J, Schenker M. Heat strain, volume depletion and kidney function in California agricultural workers. *Occup Environ Med.* 2017;74(6):402-9.
130.   (OSHA) OSaHA. Water.Rest.Shade. [Available from: https://www.osha.gov/heat/index.html.
131.   Bodin T, Garcia-Trabanino R, Weiss I, Jarquin E, Glaser J, Jakobsson K, et al. Intervention to reduce heat stress and improve efficiency among sugarcane workers in El Salvador: Phase 1. *Occup Environ Med.* 2016;73(6):409-16.
132.   Wegman DH, Apelqvist J, Bottai M, Ekstrom U, Garcia-Trabanino R, Glaser J, et al. Intervention to diminish dehydration and kidney damage among sugarcane workers. *Scand J Work Environ Health.* 2017.
133.   Roncal-Jimenez C, Garcia-Trabanino R, Barregard L, Lanaspa MA, Wesseling C, Harra T, et al. Heat Stress Nephropathy From Exercise-Induced Uric Acid Crystalluria: A Perspective on Mesoamerican Nephropathy. *Am J Kidney Dis.* 2016;67(1):20-30.
134.   Johnson RJ, Nakagawa T, Jalal D, Sanchez-Lozada LG, Kang DH, Ritz E. Uric acid and chronic kidney disease: which is chasing which? *Nephrol Dial Transplant.* 2013;28(9):2221-8.
135.   Jimenez CAR, Ishimoto T, Lanaspa MA, Rivard CJ, Nakagawa T, Ejaz AA, et al. Fructokinase activity mediates dehydration-induced renal injury. *Kidney Int.* 2014;86(2):294-302.
136.   Colvin RB, Chang A. Diagnostic Pathology: Kidney Diseases: AMIRSYS,; 2015.
137.   Gonick HC, Rubini ME, Gleason IO, Sommers SC. The Renal Lesion in Gout. *Ann Intern Med.* 1965;62:667-74.
138.   D'Haese PC, Elsevier MM, Yaqoob M, De Broe M. Organic solvents, silicon-containing compounds and pesticides. In: De Broe ME, Porter GA, editors. Clinical Nephrotoxins Renal Injury from Drugs and Chemicals. Third Edition ed. New York, NY Springer; 2008. p. 827-41.
139.   Jayasumana C, Gunatilake S, Senanayake P. Glyphosate, hard water and nephrotoxic metals: are they the culprits behind the epidemic of chronic kidney disease of unknown etiology in Sri Lanka? *Int J Environ Res Public Health.* 2014;11(2):2125-47.
140.   International Program on Chemical Safety., Inter-Organization Programme for the Sound Management of Chemicals. The WHO recommended classification of pesticides by hazard and guidelines to classification. Geneva: World Health Organization. p. volumes.
141.   Valcke M, Levasseur ME, Soares da Silva A, Wesseling C. Pesticide exposures and chronic kidney disease of unknown etiology: an epidemiologic review. *Environ Health.* 2017;16(1):49.
142.   Lebov JF, Engel LS, Richardson D, Hogan SL, Hoppin JA, Sandler DP. Pesticide use and risk of end-stage renal disease among licensed pesticide applicators in the Agricultural Health Study. *Occup Environ Med.* 2016;73(1):3-12.
143.   Roels HA, Hoet P, Lison D. Usefulness of biomarkers of exposure to inorganic mercury, lead, or cadmium in controlling occupational and environmental risks of nephrotoxicity. *Ren Fail.* 1999;21(3-4):251-62.
144.   Wimalawansa SJ. The role of ions, heavy metals, fluoride, and agrochemicals: critical evaluation of potential aetiological factors of chronic kidney disease of multifactorial origin (CKDmfo/CKDu) and recommendations for its eradication. *Environ Geochem Health.* 2016;38(3):639-78.
145.   Jayasumana C, Gunatilake S, Siribaddana S. Simultaneous exposure to multiple heavy metals and glyphosate may contribute to Sri Lankan agricultural nephropathy. *BMC Nephrol.* 2015;16:103.
146.   M.A.C.S.Jayasumana PAP, M.D. Amarasinghe, K.M.R.C.Wijewardane, K.S.Dahanayake, S.I.Fonseka, K.D.L.M.P.Rajakaruna, A.M.P.Mahamithawa, U.D.Samarasinghe, V.K.Senanayake. Possible link of Chronic arsenic toxicity with Chronic Kidney Disease of unknown etiology in Sri Lanka. *Journal of Natural Sciences Research*. 2013;3(1):64-73.
147.   Caldwell KL, Jones RL, Verdon CP, Jarrett JM, Caudill SP, Osterloh JD. Levels of urinary total and speciated arsenic in the US population: National Health and Nutrition Examination Survey 2003-2004. *J Expo Sci Environ Epidemiol.* 2009;19(1):59-68.
148.   Lee JW, Lee CK, Moon CS, Choi IJ, Lee KJ, Yi SM, et al. Korea National Survey for Environmental Pollutants in the Human Body 2008: Heavy metals in the blood or urine of the Korean population. *Int J Hyg Environ Health.* 2012;215(4):449-57.

149.		Registry AfTSaD. ToxGuideTM for Arsenic 2007 [Available from: http://www.atsdr.cdc.gov/toxguides/toxguide-2.pdf.
150.		Bandara JM, Senevirathna DM, Dasanayake DM, Herath V, Bandara JM, Abeysekara T, et al. Chronic renal failure among farm families in cascade irrigation systems in Sri Lanka associated with elevated dietary cadmium levels in rice and freshwater fish (Tilapia). *Environ Geochem Health.* 2008;30(5):465-78.
151.		Wijkstrom J, Gonzalez-Quiroz M, Hernandez M, Trujillo Z, Hultenby K, Ring A, et al. Renal Morphology, Clinical Findings, and Progression Rate in Mesoamerican Nephropathy. *Am J Kidney Dis.* 2017;69(5):626-36.
152.		Wijkstrom J, Jayasumana C, Dassanayake R, Priyawardane N, Godakanda N, Siribaddana S, et al. Morphological and clinical findings in Sri Lankan patients with chronic kidney disease of unknown cause (CKDu): similarities and differences with Mesoamerican Nephropathy. *Submitted manuscript.* 2017.
153.		Hao CM, Breyer MD. Physiological regulation of prostaglandins in the kidney. *Annu Rev Physiol.* 70. Palo Alto: Annual Reviews; 2008. p. 357-77.
154.		Murray KO, Fischer RS, Chavarria D, Duttmann C, Garcia MN, Gorchakov R, et al. Mesoamerican nephropathy: a neglected tropical disease with an infectious etiology? *Microbes Infect.* 2015;17(10):671-5.
155.		Gamage CD, Sarathkumara YD. Chronic kidney disease of uncertain etiology in Sri Lanka: Are leptospirosis and Hantaviral infection likely causes? *Med Hypotheses.* 2016;91:16-9.
156.		Wijkstrom J, Leiva R, Elinder CG, Leiva S, Trujillo Z, Trujillo L, et al. Clinical and Pathological Characterization of Mesoamerican Nephropathy: A New Kidney Disease in Central America. *Am J Kidney Dis.* 2013;62(5):908-18.
157.		Gamage CD, Yoshimatsu K, Sarathkumara YD, Kulendiran T, Nanayakkara N, Arikawa J. Serological evidence of hantavirus infection in Girandurukotte, an area endemic for chronic kidney disease of unknown aetiology (CKDu) in Sri Lanka. *Int J Infect Dis.* 2017;57:77-8.
158.		George J, Patnaik M, Bakshi E, Levy Y, Ben-David A, Ahmed A, et al. Hantavirus seropositivity in Israeli patients with renal failure. *Viral Immunol.* 1998;11(2):103-8.
159.		Peter JB, Patnaik M, Gott P, Weins B, Souw PT. Antibodies to different strains of hantavirus in end-stage renal disease in USA and Japan. *Lancet.* 1994;343(8890):181.
160.		Hotez PJ, Woc-Colburn L, Bottazzi ME. Neglected tropical diseases in Central America and Panama: Review of their prevalence, populations at risk and impact on regional development. *Int J Parasit.* 2014;44(9):597-603.
161.		Wijesinghe W PS, Hettiarchchi P, Wijerathne B, Siribaddana S. Regulation of herbal medicine use based on speculation? A case from Sri Lanka. *J Tradit Complement Med.* 2016.
162.		Huang CL, Kuo E. Mechanism of hypokalemia in magnesium deficiency. *JASN.* 2007;18(10):2649-52.
163.		Bock KD, Cremer W, Werner U. Chronic hypokalemic nephropathy: a clinical study. *Klin Wochenschr.* 1978;56 Suppl 1:91-6.
164.		Relman AS, Schwartz WB. The nephropathy of potassium depletion; a clinical and pathological entity. *N Engl J Med.* 1956;255(5):195-203.
165.		Reungjui S, Roncal CA, Sato W, Glushakova OY, Croker BP, Suga S, et al. Hypokalemic nephropathy is associated with impaired angiogenesis. *JASN.* 2008;19(1):125-34.
166.		Suga SI, Phillips MI, Ray PE, Raleigh JA, Vio CP, Kim YG, et al. Hypokalemia induces renal injury and alterations in vasoactive mediators that favor salt sensitivity. *Am J Physiol Renal Physiol.* 2001;281(4):F620-9.
167.		Fischer RSB, Mandayam S, Chavarria D, Vangala C, Garcia MN, Garcia LL, et al. Clinical Evidence of Acute Mesoamerican Nephropathy. *Am J Trop Med Hyg.* 2017.
168.		Fischer RS. HISTOPATHOLOGIC CHARACTERIZATION OF ACUTE MESOAMERICAN NEPHROPATHY (MeN): INSIGHTS INTO THE MECHANISM OF DISEASE (Abstract). 2017.