Validation of urinary nephrin assay as a novel biomarker for determining early glomerular injury

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Copyright declaration

I, Belete Mesfine, affirm that this thesis is my original work towards a Doctor of Philosophy degree. It has not been submitted in any form for the award of another degree at any tertiary education institution. Due acknowledgment has been made in the text to the information derived from the published or unpublished work of others, and a list of references presented. I would be thrilled to hear from any copyright owner who has been incorrectly acknowledged.

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Signature Date

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Abstract

Introduction: Kidney disease is a major public health problem, characterised by gradual decline in kidney function. Damage to the podocytes of the glomeruli can lead to proteinuria and progressive kidney disease. In pregnancy, changes in podocyte function due to hypertension, high blood sugar levels, oxidative stress, pregnancy associated complications and infections can cause glomerular damage. The links between the maternal-fetal environment and increased risk of glomerular injury are increasingly recognised as an opportunity for early intervention. Early intervention for individuals at risk of kidney disease would benefit from novel markers that would enable early detection, prognosis, and monitoring. Laboratory validation of novel markers is a priority in clinical research, in this case, ELISA methods for quantifying urinary nephrin are yet to be validated. Therefore, the aim of this thesis was to validate human nephrin ELISA for both laboratory and clinical diagnostic purposes and investigate the diagnostic performance of urinary nephrin as a marker of early glomerular injury in the cohort of pregnant women and children over the first 24 months.

Methods: The work presented in this thesis was accomplished through three stages. The first stage was a review of the literature and a systematic review and meta-analysis of studies on urinary nephrin as a potential marker for glomerular injury. The second stage was the analytical validation of two commercially available human nephrin ELISAs using a suite of assay validation parameters such as assay precision, limit of detection, assay dynamic range, percent recovery, assay parallelism, and sample stability. The analytical performance of these ELISA kits was compared, common interferences for urinary nephrin measurement were examined and the metrological traceability of the standards of the two ELISA kits was investigated using electrophoresis and size-exclusion chromatography. The third stage was to apply this diagnostic method to a prospective cross-sectional study as part of the Kidney of Mother-Infant and Neonates (KIDMIN) project conducted over 4 years (2019 to August 2023) at Townsville University Hospital, Queensland, Australia for clinical validation of urinary nephrin: creatinine ratio (NCR). The aim was to investigate the use of urinary NCR as a marker for predicting early glomerular injury in pregnant women and, their newborn infants, then to follow these infants for 24 months using serial measurement of urinary nephrin and currently used markers of renal function urinary albumin: creatinine ratio (ACR) and serum creatinine

(SCr) and cystatin C (sCysC). In the third stage, the study established clinically useful reference intervals (RIs) of urinary NCR for term neonates against postnatal age. Finally, longitudinal changes of urinary NCR, ACR, sCysC, and SCr were investigated in these infants and children over the first 24 months of age.

Results: One of the key objectives of this research project was to add to the existing body of knowledge related to urinary nephrin as a marker for early glomerular injury. Existing knowledge demonstrated that increased levels of urinary nephrin could predict early glomerular injury, degree of podocyte damage, and severity of glomerular injury. In particular, these studies demonstrated that nephrinuria positively correlated with albuminuria (**Chapter 2, part A**). A systematic review and meta-analysis (**Chapter 2, part B**) were carried out to determine the diagnostic accuracy of urinary nephrin for predicting glomerular injury. The study found a pooled sensitivity of 86% and a specificity of 73% for the prediction of glomerular injury. The method of choice for the quantification of urinary nephrin was ELISA (sensitivity of 89% and specificity of 72%). No study within the reviewed literature had validated the analytical performance of urinary nephrin ELISA.

The work described in **Chapter 4**, was designed to validate two ELISA kits currently used for determining urinary nephrin. Both assays performed satisfactorily during the validation experiments, therefore, both ELISA kits were considered appropriate for the intended use. The calibration curve was fit to the model for the best description of the data ($r^2 \geq 0.95$). The assay precision was comparable with the guidelines. For both kits, recovery was within the recommended criteria. The assay parallelism shows a similar absorbance vs. concentration-response relationship between the nephrin standard and urinary nephrin. The LifeSpan Bioscience (LS-Bio) ELISA was linear over the measuring range and met the acceptance criteria (CV<20% and recovery: 80-120%). However, the Ethos Biosciences Exocell (Eth-Bio) ELISA did not perform as expected for the linearity and assay dynamic range, particularly around the lowest standard of the kit. No interference from albumin or biotin was detected with the ELISA kit. Sample stability over 3 freeze-thaw cycles was not affected, but nephrin concentration decreased over freeze-thaw cycles by 18% at the fourth and fifth cycles. Interestingly, there was no agreement between the two kits in the measurement of urinary nephrin ($p< 0.05$). There was however a 100-fold difference in concentration of the standards.

These standards were not commutable between the two kits. The standards were also not traceable to an identified primary standard because of antigen specificity for the antibodies provided in the kit. Overall, the LS-Bio ELISA kit was chosen for clinical validation of urinary nephrin due to acceptable analytical performance, ease of use and reliable availability in Australia.

In **Chapter 5** the clinical validation of nephrin was explored utilising a cohort study including a total of 273 pregnant women to investigate the performance of urinary nephrin in this cohort. The participants were grouped based on urinary ACR (Normo, Micro and Macroalbuminuria) to investigate the levels of urinary NCR associated with clinical diagnoses glomerular injury. Urinary NCR correlated with urinary ACR ($r = 0.20$, $p < 0.005$) and increased between the three categories of normo, micro, and macroalbuminuria, $p < 0.05$. Nephrinuria was detected in 64.9% of women with normoalbuminuria, 94.7% of women with microalbuminuria and 100% of women with macroalbuminuria in the entire cohort. The sensitivity of urinary NCR at a cutoff value of 24ng/mg for detecting urinary ACR between women with normo and micro-macro albuminuria was 83% and the specificity was 48% in the entire cohort. There was a significant difference in urinary NCR between women who developed PE compared with women who did not develop PE, $p<0.05$. The sensitivity of urinary NCR to predict glomerular injury of PE was 92.3% and the specificity of 32.4% at a cutoff value of 14ng/mg. Elevated nephrinuria was detected in 92% of women who developed PE.

This thesis also investigates the use of urinary NCR and ACR as primary outcome measures for glomerular integrity and sCysC and SCr as outcome measures for renal function longitudinally in apparently healthy cohort of neonates until 24 months of age. This study included 190 term and 74 preterm neonates at birth. First, the study revealed that urinary NCR is influenced neither by postnatal age nor by gender ($p>0.05$) (**Chapter 6**), then the RIs for urinary NCR were determined in term neonates at birth, 12 months, and 24 months of age. The common upper limit of normal (ULN) of the RIs for males and females was calculated to be 69 ng/mg at birth, 64 ng/mg at 12 months, and 67ng/mg at 24 months of postnatal age. Second, the longitudinal changes of the biomarkers were investigated in both apparently healthy preterm neonates in comparison with term neonates (**Chapter 6**). Indeed, a statistically significant

decline in urinary NCR [13 (39)] to [8.5(20)] ng/mg, p=0.046 and urinary ACR [8.43 (11.7)] to [1.38 (3.1)] mg/mmol, p<0.001 was observed from birth to 24 months of age in preterm neonates. Of concern, preterm neonates had significantly higher urinary NCR [13 (39)] ng/mg at the early neonatal period (birth) compared to term neonates [8.4 (21.4)] ng/mg, p=0.006.

Conclusion: Taken together, the work presented in this thesis provides an important contribution to translating urinary nephrin into clinical practice. Human urinary nephrin ELISA was validated, the performance of urinary NCR was evaluated, and the longitudinal changes of the markers were investigated. The increased level of nephrinuria in women who had normoalbuminuria may indicate a sign of early glomerular injury. However, further study is needed. The RIs of urinary NCR could be utilised for clinical use in neonates and children, and deviation from the ULN could stratify the cohort at risk of early glomerular injury. The increased excretion of urinary NCR in preterm neonates in the early neonatal period could show the vulnerability of preterm kidneys to early glomerular injury. Overall, the finding shows the potential of urinary NCR as a marker of glomerular immaturity and/or early glomerular injury and highlights the need to interpret this marker along with other glomerular integrity and renal function markers, that have the potential to improve early intervention for kidney injury in susceptible cohorts.

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List of abbreviations

ACE	Angiotensin converting enzyme
ACOG	American Congress of Obstetrics and Gynaecology
ACR	Albumin: creatinine ratio
AKD	Acute kidney disease
AKI	Acute kidney injury
ANOVA	Analysis of variance
AUC	Area under the curve
BSA	Bovine serum albumin
CD2AP	CD2 associated protein
CIs	Confidence intervals
CK	Coefficient of kurtosis
CKD	Chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CLSI	Clinical laboratory standards and institute
COM	Comorbidity
CS	Coefficient of skewness
CSA	Chicken serum albumin
CV	Coefficient of variations
DAP	D'Agostino Pearson Test
DM	Diabetes mellitus
DN	Diabetic nephropathy
DOR	Diagnostic odds ratio
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-Linked Immunosorbent Assay
ESKD	End-stage kidney disease
Eth-Bio	Ethos Biosciences
FP	Foot process
GA	Gestational age
GBM	Glomerular basement membrane
GDM	Gestational diabetes mellitus

GFB	Glomerular filtration barrier
ICU	Intensive Care Unit
IDMS	Isotope dilution mass spectrometry
IFCCLM	International Federation of clinical chemistry and laboratory medicine
IGFBP	Insulin-like growth factor binding protein
IQR	Inter quartile range
IUGR	Intrauterine growth restriction
Kd	Kilodalton
KDIGO	Kidney Disease Improving Global Outcome
KIDMIN	Kidney of Mother-Infant and Neonates
KIM-1	Kidney injury molecule 1
L-FABPs	Liver fatty acid binding proteins
LOD	Limit of detection
LOQ	Limit of quantification
LR	Likelihood ratio
LS-Bio	Lifespan Biosciences
MDRD	Modification of Diet in Renal Disease
NATA	National Associations of Testing Authorities, Australia
NCCLS	National Committee of Clinical Laboratory Standards
NCOM	No comorbidity
NCR	Nephrin: creatinine ratio
NGAL	Neutrophil gelatinase associated lipocalin
NHMRC	National Health and Medical Research Council
NICU	Neonatal Intensive Care Unit
NR	Not reported
PBS	Phosphate buffer saline
PE	Preeclampsia
Pr-AKI	Pregnancy-associated AKI
QUADAS	Quality Assessment of Diagnostic Accuracy Studies
RAAS	Renin angiotensin aldosterone system
REDCap	Research Electronic Data Capture

RIs	Reference intervals
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SCr	Serum creatinine
sCysC	Serum cystatin C
Sd	Slit diaphragm
SD	Standard deviation
SEM	Standard error of the mean
SNGFR	Single nephron glomerular filtration rate
SPSS	Statistical package for social sciences
SROC	Summary Receiver Operating Characteristics
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
THHS	Townsville Hospital and Health Service
TIMP1	Tissue inhibitor metalloproteinase 1
TUH	Townsville University Hospital
ULN	Upper limit of normal
ULQ	Upper limit of quantification
UO	Urine output
WB	Western blotting

Foreword Chapter 1

The major focus of this thesis is to validate the human nephrin enzyme linked immunosorbent assay (ELISA) and evaluate the diagnostic performance of urinary nephrin as a potential marker of early glomerular injury. This chapter briefly describes the human kidney and the glomerular filtration barrier's (GFB) structural and functional components. The link between early glomerular injury and the subsequent occurrence of other forms of kidney injury and/or disease is summarised. Then, existing knowledge of traditional and novel biomarkers of glomerular injury is discussed. A brief review of the literature on urinary nephrin as a promising marker for determining and monitoring glomerular injury is explained. Evidence of maternal and pregnancy-related complications and neonatal outcomes, including preterm birth, low birth weight, and other complications that increase the risk of glomerular injury, is presented. Finally, the commonly used diagnostic methods for measuring urinary nephrin are described. However, none of the methods has been validated and urinary nephrin is yet to be translated into clinical practice settings that allow the study of this topic, which will be presented in the experimental chapters.

Chapter 1. General Introduction

1.1. The Human Kidney

The kidney is a vital organ in the human body due to its regulatory function and maintenance of the homeostatic condition of the body (1). Nephrons are structural and functional units of the kidney and comprise renal corpuscles (glomerulus and bowman's capsule), renal tubules, and collecting ducts (2). The glomerulus is the blood-filtering unit of the nephron. It is made up of an intricate capillary network found in the renal corpuscle, which filters the blood of waste products into the urinary space (2, 3).

1.2. The glomerular filtration barrier of the kidney

The kidney contains three distinct physical barriers before the plasma ultrafiltrate can enter the lumen of the Bowman's capsule of the nephron (2). These barriers are the glomerular filtration barrier (GFB) (**Figure 1 A, B, C**). The GFB is formed together with the fenestrated endothelium, the glomerular basement membrane (GBM), and the foot processes of podocytes (2, 4). Between the podocytes is a filtration slit containing a porous filter slit diaphragm (Sd) that regulates the passage of protein-free filtrate (4, 5), and the Sd contains the podocyte-specific protein known as nephrin. Nephrin largely determines the shape, charge, and size selectivity of the ultrafiltrate in the GFB (2, 5, 6). The structural and functional interactions of podocytes and the different layers of the filtration barrier serve as the structural support of the glomerular capillary, regulators of selective permeability of the glomerulus and remodelling of the GBM during injury, and endocytosis of filtered proteins, while restricting the passage of anions and macromolecules (7, 8). Changes in the molecular composition of proteins in the filtration barrier, such as the formation of advanced glycation and rearrangement of the Sd in podocytes, can cause podocyte damage (glomerular injury), resulting in increased proteinuria (9).

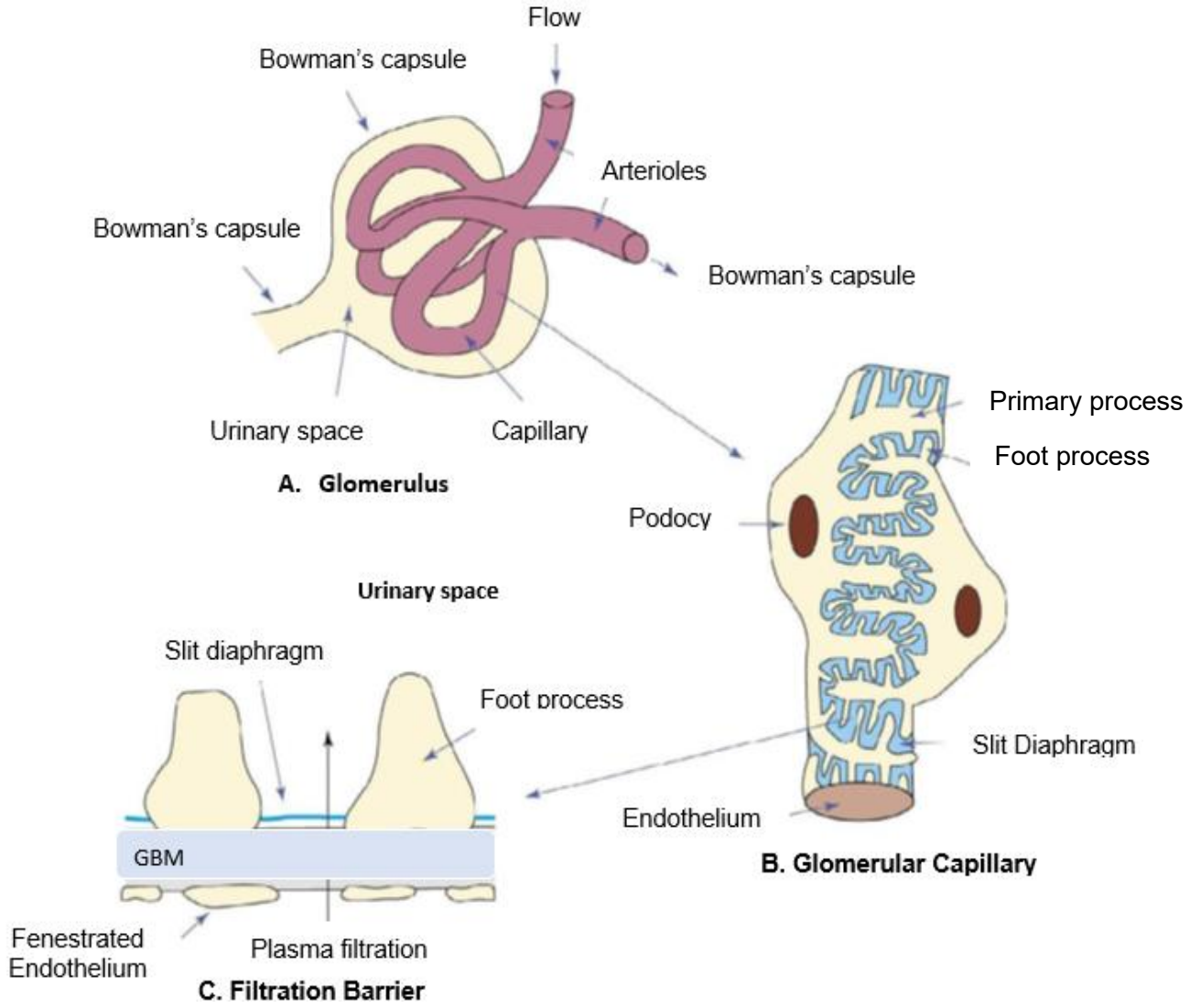


Figure 1.1. The glomerular filtration barrier of the kidney.

Each kidney contains nearly 900,000 to 1,000,000 nephrons, each nephron having a single glomerulus at the tip of the cortex of the kidney. The glomerulus contains a capillary tuft inside the Bowman's capsule (A). Blood in the capillaries of the glomerulus is filtered and filtration occurs through the capillary wall into the urinary space across the capillary wall, which contains an innermost fenestrated endothelium, the GBM, and a layer of podocytes with interdigitated foot processes (B, C). The thin Sd between the foot processes forms the plasma ultrafiltrate. Figure taken from Patrakka et al. 2007 *(10).*

1.3. Glomerular injury

Glomerular injury is damage to the glomerular structure and function that can lead to overall poor kidney function (11). The cause of glomerular injury is multifactorial and is potentially caused by infections, ischemia, hypoxic insults, nephrotoxin, and metabolic disorders (12-16). Glomerular injury is recognised as a major contributor to the increasing burden of kidney disease worldwide (11). It is often the forerunner in the aetiology of acute kidney injury (AKI), chronic kidney disease (CKD), and increased susceptibility to developing end-stage kidney

disease (ESKD) (17, 18). Approximately 90% of all causes of ESKD can be attributed to glomerular disease, which affects glomerular structure and function (19).

Persistent glomerular injury can cause a decrease in renal mass and renal function. The injury increases stress on nephrons, leading to extension of the injury to the remaining nephrons and resulting in glomerular hyperfiltration (20). Glomerular hyperfiltration over time increases the likelihood of progressive kidney injury and severely compromised kidney function (13). Previous investigators studying kidney injury focused on diabetic nephropathy (DN) (21-23) and preeclampsia (PE) (24-26) have shown an increase in the number of podocytes appearing in the urine, suggesting glomerular injury. Glomerular injury might lead to the development of AKI and/or CKD. However, little is known about the role of glomerular injury occurring in neonates, and later in childhood.

1.4. The link between glomerular injury and AKI, CKD, and progression to ESKD

The causes of glomerular injury are multifactorial and result in inflammation or scarring of the glomerulus, sometimes injury to the glomerular membrane is idiopathic. Glomerular injury is the primary outcome of kidney diseases, such as glomerulonephritis and glomerulosclerosis (16, 27, 28), which can progress to severe acute or chronic kidney failure (29). Recent evidence suggests that the major contributor to the increased burden of kidney disease is associated with matrilineal causes during pregnancy (early life) and later in postnatal life (30, 31). Maternal factors, such as smoking, malnutrition, excessive alcohol consumption, and disease during pregnancy, altered the normal renal development of the fetus. These factors result in low birth weight, intrauterine growth restrictions (IUGR), and premature birth, which are significantly associated with low nephron numbers in infants. According to the Brenner-Baker hypothesis (32, 33), a decrease in the number of functional nephrons, causes compensatory hyperfiltration in the remaining nephrons, which can lead to injury to nephrons, eventually resulting in AKI, progression to CKD and ESKD (32-34). If early detection and management are not implemented, CKD can progress to ESKD. Of concern, supporting evidence shows that nearly 70% of children with CKD will progress to ESKD before middle age (35).

In general, glomerular dysfunction is the hallmark of many disease pathologies. These include focal segmental glomerulosclerosis, DN, lupus arthritis, glomerulonephritis, membranous

nephropathy, and minimal change disease that occurs through podocyte effacement and dysfunction of podocytes (36-38). Moderate to severe glomerular injury is characterised by haematuria, proteinuria, or both, and podocyturia because of damage to the GFB (21, 39, 40). However, the aetiologies of all these diseases are linked through initial damage to the podocytes in the glomerulus (5, 21, 41). Hence, the increases in urinary nephrin shown in podocytopathies (21, 22), provide evidence that nephrin could become a novel biomarker heralding adverse changes in glomerular function.

1.5. Diagnostic markers of glomerular injury

Early glomerular injury results in structural changes in the podocytes and is first evident in the Sd. The injury causes a restructuring of the foot processes involving the fusion of filtration slits and apical displacement (21). This results in the detachment of podocytes from the GBM, leading to severe glomerular injury, and allowing the detection of macromolecules, red blood cells, and podocyte proteins in urine (4, 9).

1.5.1. Traditional markers for glomerular injury

Traditional markers for glomerular injury include haematuria, haemoglobinuria, proteinuria, and albuminuria. These markers have been used effectively for diagnosis, prognosis, and monitoring treatment, as markers are a sign of established kidney damage and play a direct role in the progression of glomerular injury. However, the markers are limited in their sensitivity to glomerular injury, often only appearing in urine once membrane damage is severe (42, 43); this is particularly evident in neonates (44, 45).

1.5.2. Novel markers for glomerular injury

Urine podocyte proteins can serve as urinary markers for the diagnosis of glomerular injury, including nephrin, podocalyxin, synaptopodin, podocin, and CD2-associated protein (CD2AP). The markers can be excreted in the urine, and the increased concentrations of podocyte proteins in urine reflect the degree of glomerular injury (40, 46-49).

Several studies have reported novel podocyte proteins as markers for glomerular injury (21, 22, 50). One such study showed podocyte injury in the aetiology of ischemia-reperfusion of kidney injury and post-injury fibrosis (51). The study noted that proteinuria, podocyte damage, and progression to chronic renal fibrosis were observed in mice with AKI, suggesting

that changes in podocyte structure can lead to increased loss of podocytes and podocyte proteins. Likewise, another study using a mouse model observed a decrease in nephrin expression, independent of podocyte loss (52). This shows that nephrin loss was an early event in proteinuria of kidney disease, further highlighting the potential that increased loss of nephrin may play a role in the progression of kidney disease.

Studies have reported a diagnostic value for urinary nephrin and showed a correlation between elevated urinary nephrin levels and kidney injury (53, 54). Similarly, prospective studies show nephrinuria was elevated in patients with normal urinary albumin excretion (23, 55). This suggests that urinary nephrin might emerge as a valuable marker in the diagnosis of early glomerular injury and correlate with albuminuria. This is one of the specific aims of this PhD thesis, to evaluate whether urinary nephrin is a reliable marker for detecting early glomerular injury in pregnant women.

1.6. Nephrinuria and glomerular injury

Nephrin is an integral transmembrane protein primarily expressed in the glomerular podocytes of the kidney (56). Nephrin was discovered in the late 1990s in children with congenital nephrotic syndrome of the Finnish type, due to a mutation of a gene coding for nephrin, NPHS1 (56). The syndrome was first recognised in Finnish populations (57) and later identified elsewhere in the world. Furthermore, Kestilia *et al*. identified the genetic locus of the syndrome in a cohort of 17 Finnish families; the genetic disorder mapped at the long arm of chromosome 19q12-13 (58). Kestilia et al. also showed that patients displaying mutations in the nephrin gene are affected by nephrotic syndrome, resulting in massive proteinuria and podocyturia, and death during the first two years of life (56, 59). Nephrin is demonstrated in glomerular disease not only by its gene mutation but also by a reduction in expression levels in the glomerulus. Studies showed downregulation of nephrin (60), as well as podocytes with low levels of nephrin expression (52), these two studies demonstrated that traces of nephrin were present in urine at the early stage of Proteinuric diseases, prior to all other podocyte proteins detected in urine (52, 60).

Urinary nephrin could be a potential marker for the detection of early pregnancy-related complications. A growing body of evidence has demonstrated that nephrinuria may have a role in the pathogenesis of proteinuria during pregnancy, including disorders such as PE (24,

41). Similarly, Wang et al. and Sun et al. demonstrated that hypertension could cause podocyte injury and detachment from the GBM through increased oxidative stress, resulting in the induction of podocyte protein shedding (25, 61). Therefore, urinary nephrin may be a potential candidate marker for the detection of early glomerular injury of PE.

Several investigations have shown the relationship between prematurity and changes in kidney development (31, 62, 63). In the baboon model, premature birth leads to incomplete nephrogenesis (63), with an increased number of abnormal glomeruli in the outer renal cortex (64). Previous investigations showed that prematurity, low birthweight (30), and IUGR affect normal nephron development and surrogate indices of reduced nephron endowment (65, 66). Importantly, preterm birth (67, 68) and IUGR (69, 70) are indicated as strong independent risk factors for the development of renal disease in later life. In this regard, incomplete organogenesis may lead to nephropathy of prematurity and CKD in childhood and later life (67, 71, 72), and this may come about through direct glomerular injury.

1.7. Diagnostic methods for urinary nephrin

Glomerular injury causes podocyte apoptosis, deficient proliferation, and podocyte detachment from the GBM, which leads to massive proteinuria (21). In patients with glomerular injury, haematuria, proteinuria, and podocyturia can be detected (21). Laboratory diagnosis involves a biopsy to reveal scarring, inflammation, protein deposits, kidney biopsy material, and morphologic changes by using electron microscopy (73). In addition, immunohistochemistry techniques are used to identify and quantify podocyte-specific proteins (49), while genetic analysis is also used in hereditary cases for the detection of specific deoxyribonucleic acid and ribonucleic acid (RNA) sequences (74).

In the past decade, the detection of podocytes and podocyte-specific proteins in urine has become a non-invasive technique of choice for the diagnosis of glomerular injury (50, 75). In secondary nephropathies including DN, PE, and lupus nephritis, urinary podocyte-specific proteins and their messenger RNA have been used for diagnosing and managing the progression of Proteinuric glomerular disease (49, 75). To date, studies have used different methods for detecting and quantifying of urinary nephrin, such as ELISA, Reverse transcription polymerase chain reaction (RT-PCR), and electrophoresis and Western blotting (WB) (76).

Recently, our systematic review and meta-analysis showed that ELISA is the most common and preferred diagnostic method to measure and quantitate urinary nephrin (76).

Several commercially available nephrin ELISA kits are in use, most widely Ethos Biosciences Exocell (Eth-Bio) (Philadelphia, USA). However, other kits are used such as R&D systems (Minneapolis, MN, USA), Huamei (Shenzhen, China), LifeSpan Biosciences (LS-Bio), Inc. (Seattle, USA), EIAab Science Co., Ltd. (Wuhan, China), USCN Life Science Inc. (Wuhan, China), Sunlong Biotech Co., Ltd (Hangzhou, China). Although the kits are used to quantify nephrin, they have different standard formulations, assay dynamic ranges, assay techniques, and assay precisions, and cutoff values are inconsistent. Therefore, nephrin ELISA kits need to be validated to detect and quantify urinary nephrin before this technique is translated into clinical practice, which is one aim of this PhD thesis, to validate nephrin ELISA kits using a suite of assay validation parameters.

1.8. Rationale, hypothesis, and aims of the thesis.

The first 1000 days between a mother's pregnancy and the child's 24-month birthday offer a unique window of opportunity for later development (77). Increasing evidence shows that a significant proportion of kidney diseases arise in utero during fetal renal programming in pregnancy (78). For instance, preterm birth, IUGR, and low birth weight are described as risk factors for glomerular injury (66, 71). Existing evidence from clinical studies in adults showed that nephrinuria is significantly elevated in patients with glomerular injury (21, 50, 68) and might be used as a novel sensitive marker for determining early glomerular injury. Hence, validating methods for determining a marker for glomerular injury is a crucial step in improving kidney health and developing intervention strategies for kidney injury. To our knowledge, there is no validated diagnostic method for urinary nephrin to be used as a tool for determining early glomerular injury (76). A more thorough investigation is needed into the contributions of urinary nephrin in the detection of early glomerular injury in pregnant women, neonates, and infants. The Kidney of Mother Infant and Neonates (KIDMIN) prospective longitudinal cohort study "The Relationship between Maternal Health and Infant Renal Development and Function" that recruits volunteer maternal-infant dyads with no exclusion criteria provides a great opportunity to examine the use of urinary nephrin as a marker for early glomerular injury in pregnancy, infancy and the first 24 months of life.

Therefore, the **hypothesis** of this thesis was that the validation of the nephrin ELISA test can help to explore the clinical utility of the assay as a test for diagnosis of early glomerular injury.

The **objective** of the studies presented in this thesis was to validate and compare two commercially available human nephrin ELISAs (Eth-Bio and LS-Bio), then once the best assay was selected to explore the clinical utility of these assays in pregnancy, infancy, and up to 24 months of life, as a test for identifying early glomerular injury.

This thesis will explore the following **4 specific aims**.

Specific aims of the project

Aim 1: To validate human urinary nephrin ELISAs as a diagnostic marker for early glomerular injury. This aim is addressed in Chapter 4.

Aim 2: To investigate urinary nephrin as a marker for early glomerular injury in unselected pregnant women. This aim is addressed in Chapter 5.

Aim 3: To determine reference intervals of urinary nephrin in neonates against postnatal age. This aim is addressed in Chapter 6.

Aim 4: To investigate the use of urinary nephrin as a marker for early glomerular injury in term and preterm neonates: Longitudinal prospective cohort study. This aim is addressed in Chapter 6.

1.9. Thesis structure

Achieving these aims will help improve the diagnosis of glomerular injury by validating a commercially available urinary nephrin ELISAs. This thesis is presented in the standard doctoral format and provides background information, rationale, hypothesis and aims, literature review, methods, and materials, and three separate experimental chapters, which have a common theme, general discussion, future directions, and conclusions. The figure below shows the structure of the thesis and will be presented at the beginning of each chapter to help guide the reader (**Figure 1.2**).

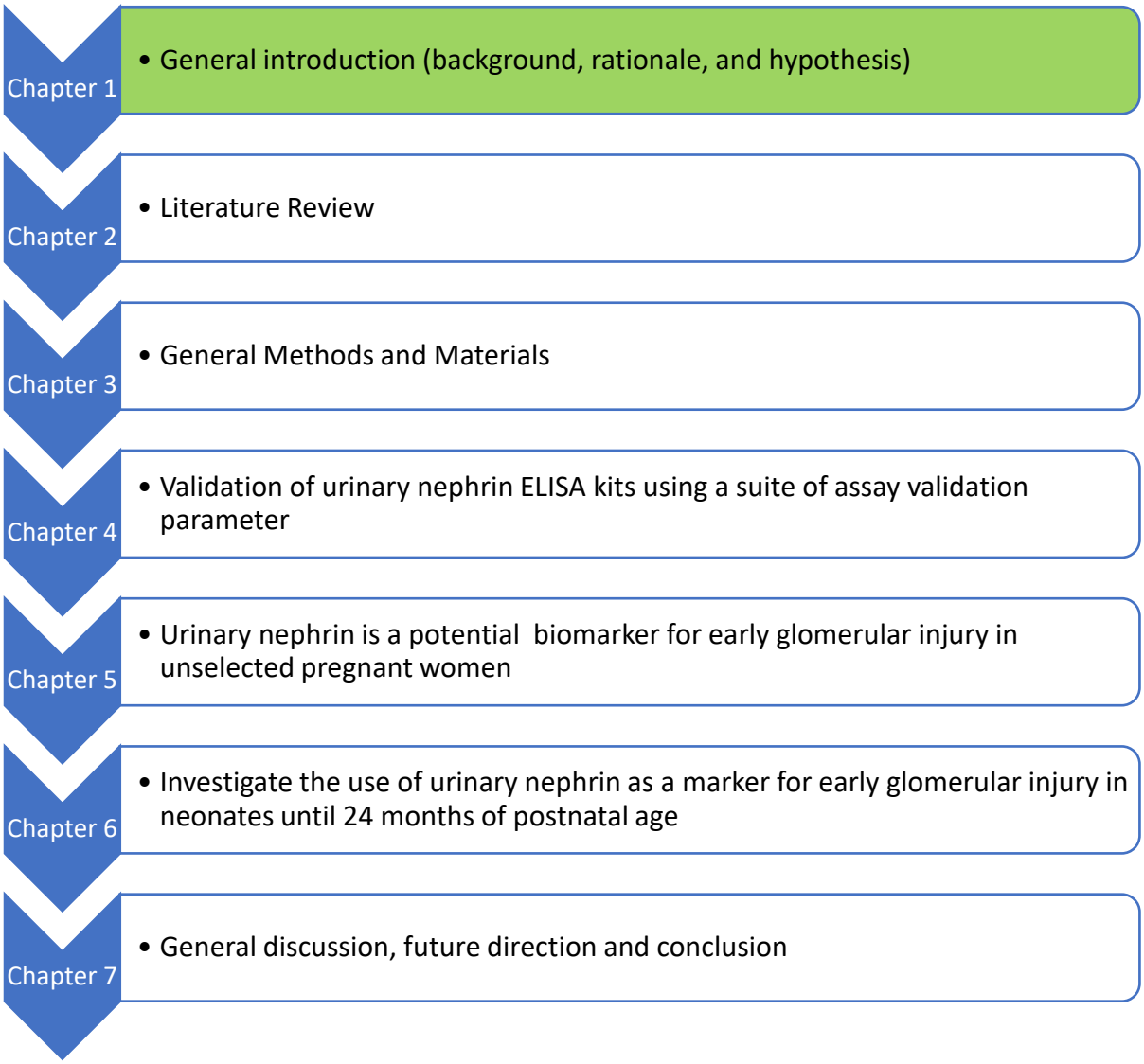


Figure 1.2. Structure of the thesis.

Chapter 2 reviews the literature and is presented in two parts: **Part A** is a narrative review that first describes normal renal development and nephrogenesis in humans, followed by glomerular injury, AKI, and epidemiology of kidney disease. This part of the chapter then discusses maternal and postnatal factors as risks for developing kidney injury during childhood and later in life. Given the importance of early detection of kidney disease, the review highlights the existing literature on urinary nephrin as a marker for the detection of early glomerular injury along with important parameters to be considered in validating and translating the biomarker into clinical practice. **Part B** is a systematic review and meta-analysis published in the Journal of Nephrology https://link.springer.com/article/10.1007/s40620-023-01585-0 (Mesfine et al. 2023) that provides comprehensive evidence of the diagnostic accuracy of urinary nephrin as a potential marker of early glomerular injury.

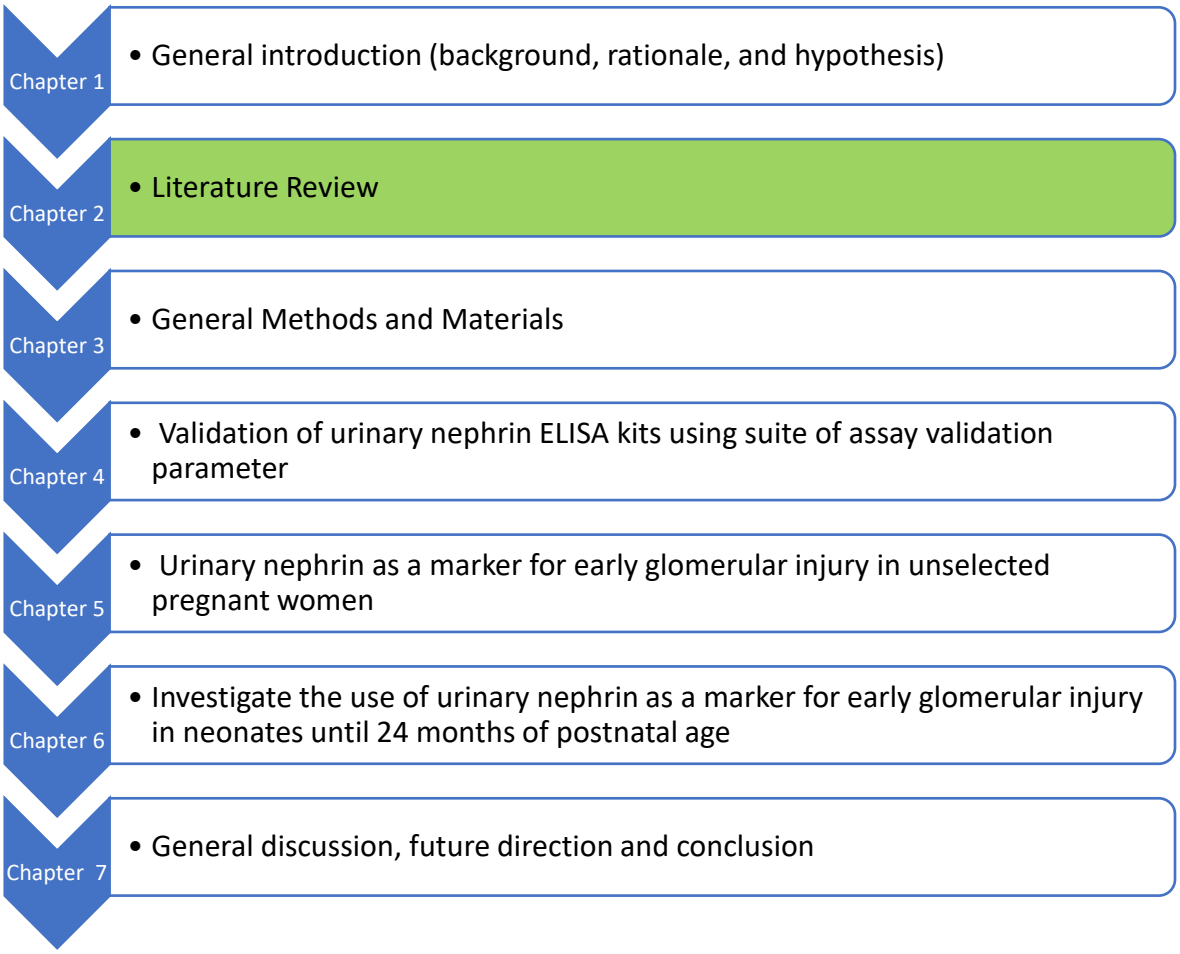
Chapter 3 describes the methods and materials of the overall study, and **Chapter 4** aim 1 of the thesis**,** investigates the validation of urinary nephrin ELISA. Initially, validating commercially available urinary nephrin ELISAs was important to determine the most appropriate and efficient method to measure urinary nephrin concentration for the detection of early glomerular injury. In this chapter, the analytical performance of urinary nephrin ELISA was evaluated using a suite of assay validation parameters. These include establishing a standard calibration curve and confirming the accuracy and precision of the kits, the level of interference of albumin and biotin for measuring urinary nephrin, and then correlate and compare the two kits based on their analytical performance to recommend a method of choice for clinical validation. Finally, this chapter investigates the accuracy and metrological traceability of the standard of both ELISA kits using electrophoresis and size exclusion chromatography. The results provided in this chapter were used as the basis for the next methods used throughout the subsequent chapters presented in the thesis.

Chapter 5 aim 2 of the thesis, investigates urinary nephrin: creatinine ratio (NCR) as a marker of early glomerular injury in unselected pregnant women. This chapter hypothesises that urinary NCR could be a marker for early glomerular injury in unselected pregnant women and that nephrinuria precedes albuminuria. The chapter explores the association of urinary NCR with the development of pregnancy-associated complications known to involve the kidney and examines the diagnostic sensitivity and specificity of urinary NCR to predict glomerular injury and the diagnosis of PE during pregnancy. **Chapter 6** aim 3 of the thesis determines reference intervals (RIs) for urinary NCR in neonates against postnatal age. This chapter hypothesises that urinary NCR has no dynamic physiological variations and would not require postnatal age and gender specific RIs. RIs were determined using nonparametric percentile and robust methods and compared with the parametric method for the coherent interpretation of test results and clinical decision-making. Following this, the longitudinal prospective cohort study **Chapter 6** aim 4 of the thesis investigates longitudinal changes of urinary NCR, albumin: creatinine ratio (ACR), and renal function markers serum creatinine (SCr), serum Cystatin C (sCysC), and estimated glomerular filtration rate (eGFR) from birth, 12 and 24 months of postnatal age. This chapter hypothesises that increased urinary NCR (above the ULN of the RIs) recognises neonates with early glomerular injury and/or immaturity across the term and preterm neonates in the first 24 months of postnatal age. Lastly, **Chapter 7**

discusses the findings of this project and their implications for future research. **Appendices** presents a copy of published article and article under review from this thesis.

1.10. Chapter Summary

This chapter describes the background information related to the thesis, rationale, hypothesis, and aims of the project. The thesis structure is stated, and each experimental chapter comprises an abstract, introduction, results, discussion, conclusion, and recommendation. The experimental chapters may encompass similar experimental methods and concepts in two different cohorts. The last chapter is the synthesis, where findings from all chapters are brought together in discussion and avenues for future research direction are highlighted, and a list of references is provided at the end of the thesis. The next chapter (**Chapter 2)** will provide a detailed review of literature relevant to the thesis.



Foreword Chapter 2

This chapter reviews the existing literature related to the focus of the thesis. It has two parts (Part A and B). Part A first describes normal renal development and nephrogenesis in humans, followed by an explanation of glomerular injury, AKI, and epidemiology of kidney disease, and then goes on to discuss maternal and postnatal factors as a risk of developing renal dysfunction. Given the importance of early detection of kidney disease, the review highlights the existing literature on markers of kidney injury and function, particularly urinary nephrin as a biomarker for the detection of early glomerular injury along with important parameters to be considered for validating and translating the biomarker into clinical practice. Part B comprises a systematic review and meta-analysis of urinary nephrin as a potential marker of early glomerular injury. This section aimed to determine the pooled diagnostic sensitivity, specificity, and other estimates of diagnostic accuracy of urinary nephrin in predicting glomerular injury and evaluate the discriminatory power of urinary nephrin for determining glomerular injury in patients with secondary nephropathies.

Chapter 2. Literature Review

2.1. Introduction

Kidney disease is a major public health problem that affects an estimated 750 million people worldwide; from various causes of kidney disease (26, 79). About 10% of the world's population is affected by CKD, which is the fastest growing cause of death (80). In a Lancet review, Cockwell and Fisher reported an increase in the global burden of CKD from 9.1% in 1990 to 29.3% in 2017 (81). Moreover, the authors found that age-standardised incidence of ESKD treated by renal replacement therapy with dialysis increased by 43.1%, and kidney transplantation increased by 34.4%.

The nephron is the functional and structural unit of the kidney. It consists of the glomerulus, Bowman's capsule, and renal tubules located within the cortex and medulla of the kidney. For this reason, kidney disease can be described in different clinical situations as glomerular injury, tubular injury, AKI, CKD, and ESKD. Of these, glomerular injury is often a forerunner in the aetiology of AKI (12), CKD, and increased susceptibility to developing ESKD (82). Furthermore, AKI is also a significant driver of CKD, and it affects more than 13 million people worldwide (83). Like AKI and CKD, glomerular injury at an early age not only leads to significant morbidity and mortality but also results in renal dysfunction later in life (67, 71, 83).

The problem of kidney disease varies considerably throughout the world (83, 84)**.** While the magnitude and effect of the disease are well defined in industrialised countries, growing evidence implies that developing nations have a comparable kidney disease burden (84, 85) (**Figure 2.1**).

The risk factors for kidney disease are multifactorial, including environmental, genetic, sociodemographic, and clinical factors. The disease is well described to have an association with identified factors in most peoples across the world. This phenomenon is well recognised in developed countries where people with low socioeconomic status and ethnic differences, with a high burden of the disease, for example, African Americans, Indigenous Australians, and Indo-Asians in the United Kingdom, are disproportionally affected by progressive kidney disease (86-89).

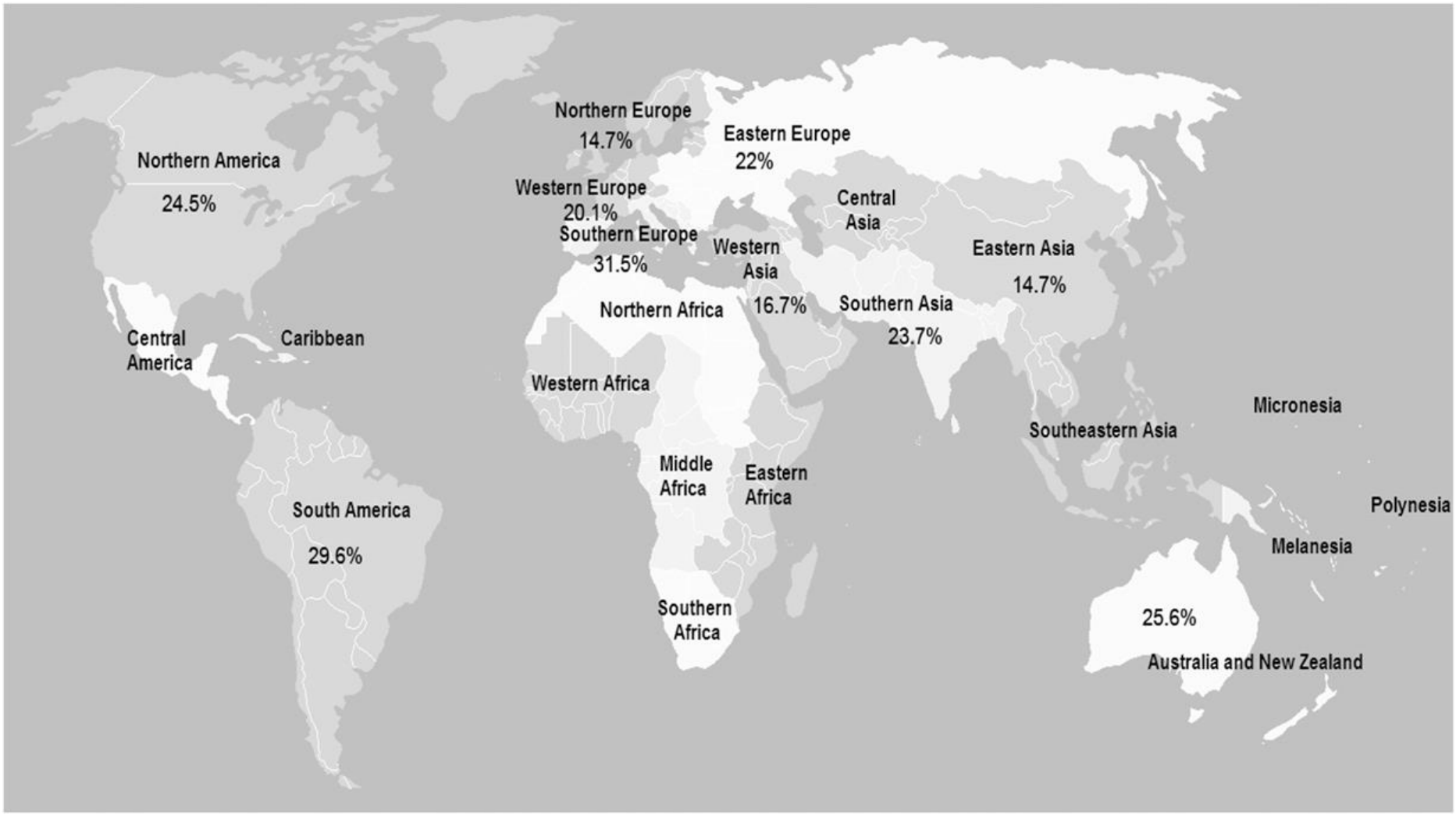


Figure 2.1. Pooled prevalence of AKI by world geographic regions using a Kidney Disease: Improvement Global Outcome (KDIGO) equivalent SCr based AKI definition, (Susantitaphong et al. 2013) (84).

2.2. Kidney Development and Function

Altered kidney development and/or function leading to kidney injury has been shown to cause different adverse complications early in childhood and later in life (21, 90). To understand the main risk factors, and diagnose using a novel biomarker, it is crucial to first clearly understand normal human kidney development and function. In mammalian renal development, complete nephrogenesis and glomerular formations are the hallmarks of the normal functioning of the kidney. To date, studies speculate that incomplete nephrogenesis (prematurity) (68) and glomerular dysfunction are the hallmarks of many glomerular diseases (91) that cause glomerular injuries. Indeed, podocytopathies (discussed in section **2.9**) have demonstrated that nephrin is a significant element for maintaining glomerular structure and function (5, 92). Hence, understanding normal human kidney development and function is crucial to validate a biomarker like nephrin as a novel marker for early glomerular injury and translation into clinical practice.

2.2.1. Normal Human Kidney Function

Humans have a pair of kidneys ranging from roughly 900,000 to 1000,000 nephrons per kidney (93). The nephron is a functional and structural unit of the kidney. It comprises the Malpighian body (glomerulus and Bowman's capsule) within the cortex of the kidney, convoluted tubules within the cortex and medulla of the kidney, and the collecting ducts which are found within the pyramids of the kidney's medulla. The nephron regulates the filtration of blood, selective reabsorption of substances, maintains fluid and electrolyte balance, acid-base equilibrium, excreting metabolic waste products, and regulates blood pressure. The nephron is connected to the collecting duct network from which the filtrate passes to leave the kidney and moves to the bladder (94) (**Figure 2.2**).

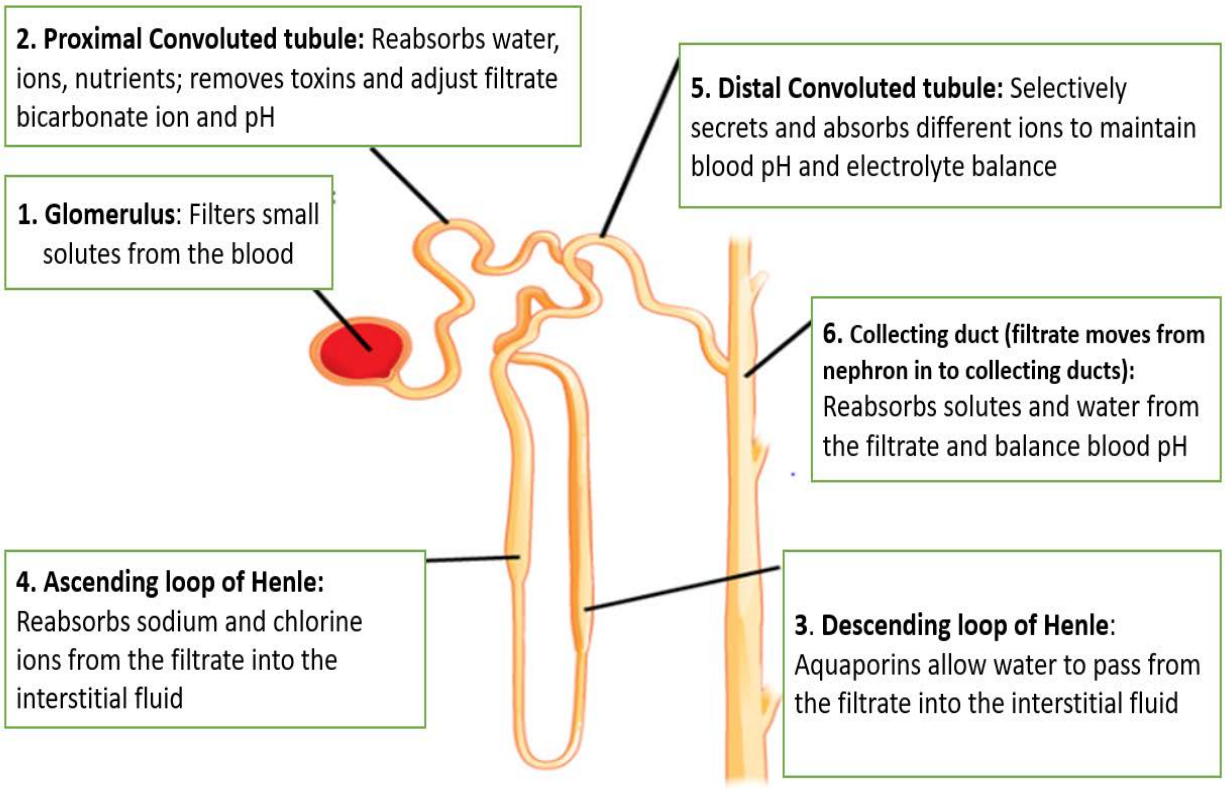


Figure 2.2. Parts of the nephron involved in filtering waste and keeping homeostatic balance.

(Adapted from: https://courses.lumenlearning.com/wm-biology2/chapter/kidney-function-and-physiology/).

2.2.2. Branching morphogenesis in the human kidney

Branching morphogenesis is a central feature of the development of several organs, such as the kidneys, lungs, and mammary glands (95). Branching normally commences with the formation of a bud-like organ anlage which then continues to grow and divide, typically by bifurcation (95), and morphogenesis of the ureteric tree mainly determines the final structure of the kidney (95, 96). During renal development, the ureteric bud eventually gives rise to the collecting duct system such as the ureter, renal pelvis, renal calyces, and collecting duct tubules, and the metanephric mesenchyme differentiates into the glomeruli, and tubule segments of the nephrons and the interstitium (97). As the process of branching proceeds in the kidney, a ureteric bud tip (ampullae) is formed in response to coordinated interactions between cells at the tips of the branching epithelium and specialised surrounding mesenchymal cells; this causes the tips to elongate into the developing mesenchyme (95, 96). These stages of branching morphogenesis and nephrogenesis in the developing kidney are illustrated in **Figure 2.3**.

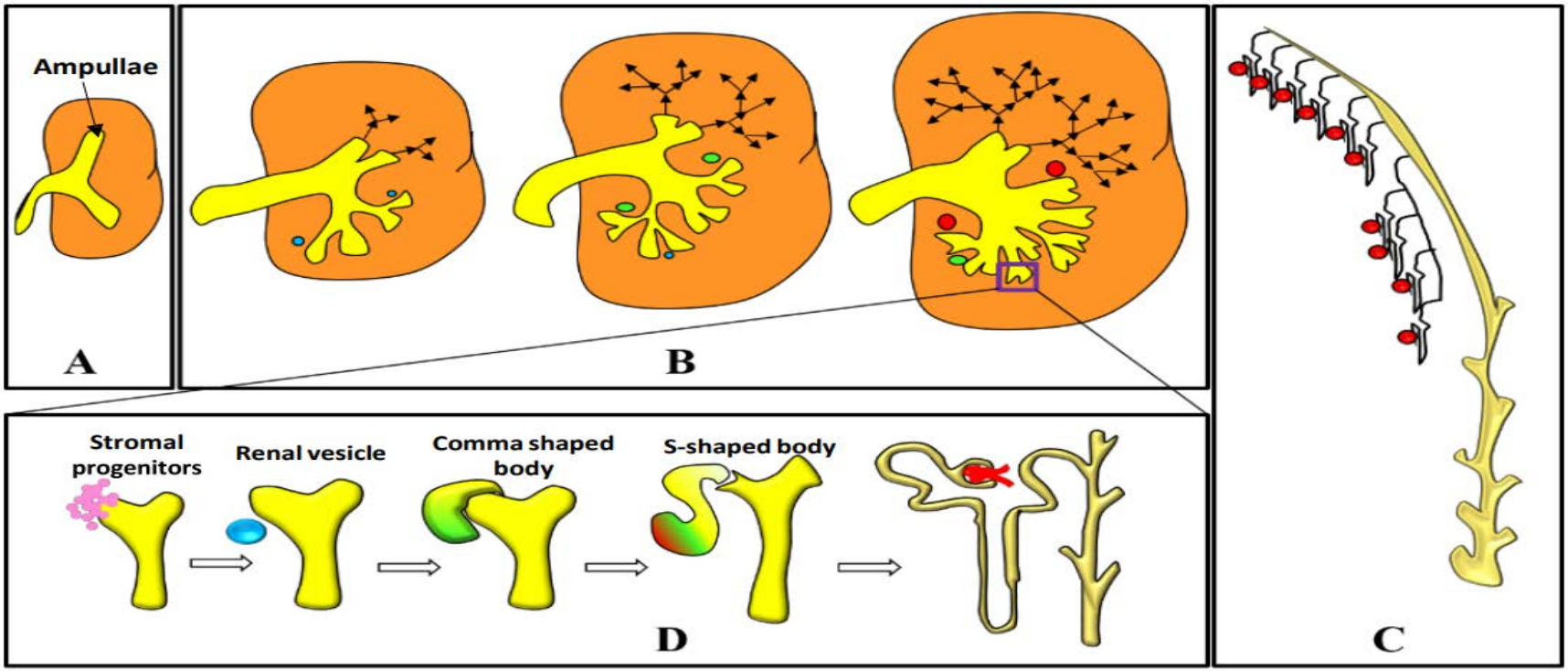


Figure 2.3. Stages of branching morphogenesis and nephrogenesis in the developing human kidney.

Initially, the ureteric bud ampullae divides into 2 lateral branches (A). After that, the branches continue to bifurcate up to 3-4 generations of ureteric budding (B). Concurrently, nephrons are induced at the ampulla, where only half of the branch tip encourages nephron formation (B). These newly formed nephrons remain either connected to a single collecting duct, or to the connecting tubules of another nephron, forming in an 'arcade' manner (C). Signals from the ampullae induce a mesenchymal to epithelial differentiation, forming the renal vesicles, which produce the comma-shaped and S-shaped bodies, and finally differentiate and form the tubular structures, Bowman's capsule, and glomerular capillaries (D), (the figure taken from Vojisavljevic. 2016) (96).

2.2.3. Nephrogenesis

Nephrogenesis during normal human renal development begins in the first trimester of pregnancy and is complete from 32-36 completed weeks of gestation (98). There are 3 developmental forms of the human kidney described as pronephros, mesonephros, and metanephros. The early developmental phases are pronephros and mesonephros (99) and the mature phase is the metanephros which develops into the functional kidney and becomes the permanent kidney (100) (**Figure 2.4**).

The early developmental phases are a vestigial structure seen in early life and formed approximately at the 3rd week of gestation in the cervical regions of the segmented nephrogenic cord, which comprises simple tubules. The pronephros then regresses at approximately 4th week of gestation, and the mesonephros form and become responsible for renal function during early fetal development (101). The mesonephros is able to produce small volumes of urine from the 6th to 10th weeks of gestation (102). During this time, an important structure of the developing excretory system is formed, known as the Wolffian duct (101).

At approximately 20-27 days gestation, the metanephros begins to develop forming the permanent (mature) kidney in humans (102). The caudal extension of the mesonephric duct, referred to as the ureteric bud, occupies the surrounding metanephric mesenchyme during the 5th week of gestation and will continue to form the permanent kidney (102). The ureteric bud undergoes branching morphogenesis, which involves each branch bifurcating with each division. Only one-half of the newly divided branches continue to further divide, whilst the other branch tip induces nephron formation. This forms an arcade structure of the kidney and ultimately nephron endowment (99, 103).

Signals between the ureteric bud and metanephric mesenchyme cause the initiation of nephron formation, with the differentiation of metanephric-mesenchyme to renal epithelia (94). The ureteric tree forms the pelvis, ureters, and collecting ducts. Nephrogenesis proceeds as the renal vesicle (the most embryonic and recognised as a precursor of nephron) and continues to develop into a comma-shaped body, and then differentiates into an S-shaped body, and eventually forms the glomerulus, proximal tubule, the loop of Henle,

and distal convoluted tubules (101). The kidney becomes functional within the 6th to 10th week of gestation and fetal urine passed into the amniotic cavity mixes with amniotic fluid (102).

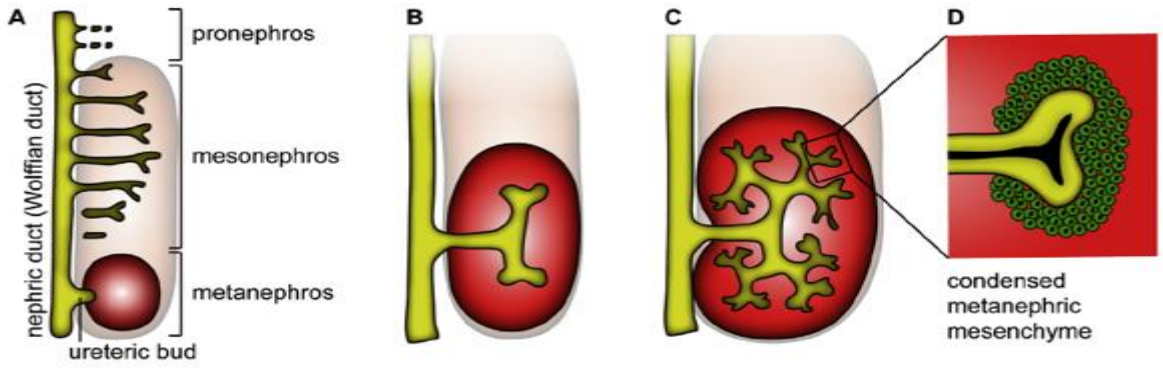


Figure 2.4. The stages in the development of the human kidney

The stages of development and formation of the pronephros and mesonephros, and the induction of the ureteric bud and metanephric mesenchyme, and the formation of functional nephrons during the development of the human kidney are described. (A) In mammals, the kidney develops from the metanephric mesenchyme upon invasion of the ureteric bud out of the nephric duct. (B and C) The ureteric bud branches within the growing metanephric mesenchyme. (D) The mesenchyme condenses around the ureteric bud tips forming the six 2-positive cap mesenchyme (Schell et al. 2014)(100).

2.2.4. Glomerular formation (glomerulogenesis)

The glomerular formation, represented in **Figure 2.5** (adapted from Seely et al. 2017), encompasses the development of the glomerulus and Bowman's capsule, which arises from the lower limb of the S-shaped body of the kidney developmental phase and the process occurs concurrently with nephrogenesis. The S-shaped body has 3 sections, the proximal, medial, and distal. The proximal cells in the S-shaped body differentiate to form the parietal epithelium of the glomerulus, coating the Bowman's capsule and the visceral epithelium of the glomerulus called the podocytes. Glomeruli development begins when the podocyte precursor lining the S-shaped body forms vascular endothelial growth factor 2, hence attracting endothelial cells and forming a vascular tuft (104), this then forms the afferent and efferent arterioles of the glomerulus.

Podocytes are cells found in the Bowman's capsule which develop from epithelial cells of the S-shaped body and wrap around the capillaries of the glomerulus. They are a functional unit for the GFB, composed of 3 components, that is, the fenestrated endothelial cells of the glomerular capillary loop, the GBM, and the Sd, which is designed by connecting

adjacent foot process of podocyte cells (92, 105).

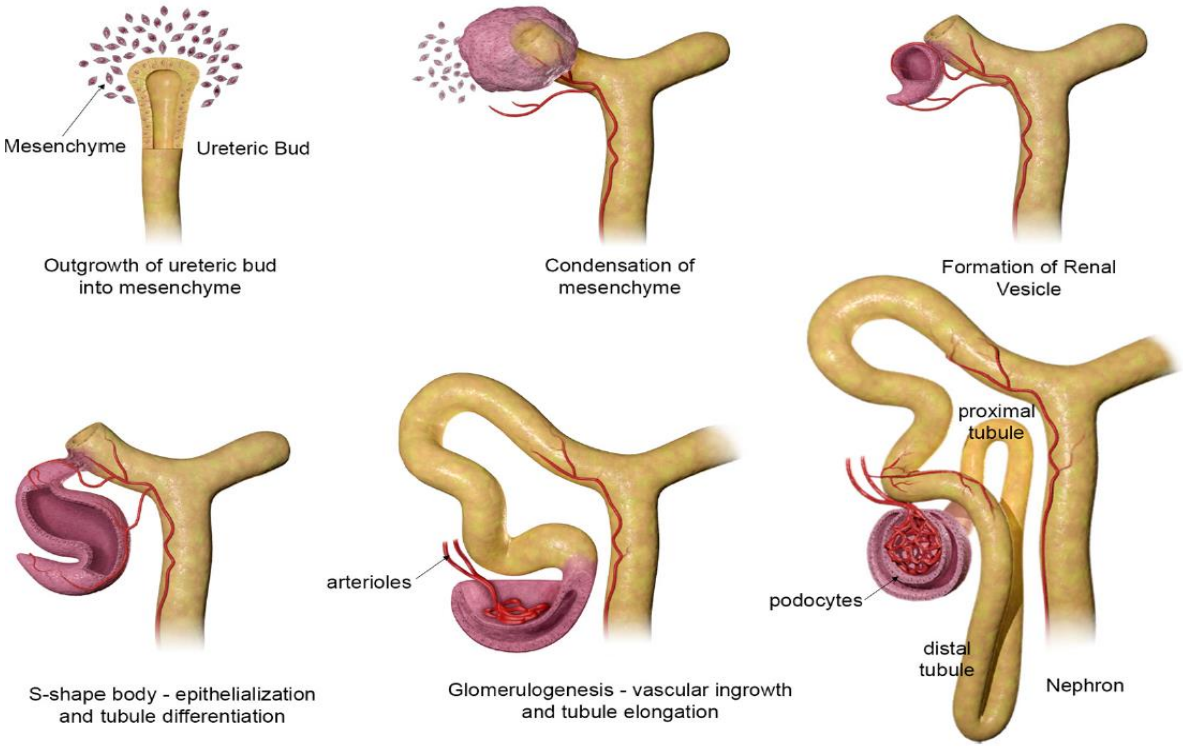


Figure 2.5. Configurational changes during nephrogenesis and glomerulogenesis

Nephrogenesis and glomerulogenesis show configurational changes as nephrons develop (Seely et al. 2017) (106). Podocytes conjoin with mesangial cells to maintain the structure and function of the glomerulus. Podocytes consist of three segments: a large cell body, major extending processes, and foot processes (107), where the podocyte foot processes fasten themselves to glomerular capillaries at the basement membrane creating an intracellular junction that forms Sd and maintains normal kidney function (8). Alterations in podocyte structure and function can lead to glomerular dysfunction and play a major role in the pathogenesis of proteinuria (8, 108). Note that the structural and functional protein nephrin is specifically located on the Sd of podocytes of the glomerulus.

To date, scientific researchers' attention has been drawn to the significance of Sd in the pathogenesis of proteinuria (21, 37, 46). Early structural changes in podocyte injury from various causes including glomerular disease occur in the Sd (46); this initiates restructuring of the foot process, and filtration slits and leads to apical displacement. This will result in detachment of the podocyte from the GBM leading to the insufficiency of the GFB and glomerular injury, eventually leading to the progression of kidney injury, and compromising normal renal function.

2.3. Glomerular injury and its pathophysiology

Glomerular injury is defined as damage to the glomerular structure and function that can lead to overall poor kidney function (11). Persistent glomerular injury can cause a decline in the renal mass, the functioning of the nephrons, and increased stress on other nephrons. Eventually, this can lead to compensatory hyperfiltration of remaining nephrons and consequently, an extension of the injury to the remaining nephrons (20); which increases the likelihood of reduced nephron endowment, increased susceptibility towards progressive kidney injury, and potentially compromised kidney function (13).

The kidney response to damage from diseases or exposure to toxins and hypoxic insults can be projected based on 3 hypotheses such as the "intact nephron hypothesis'', the theory of ''hyperfiltration'', and the ''complex deposition'' theory.

2.3.1. Intact nephron hypothesis

In 1960, Bricker et al. developed the "intact nephron hypothesis"(109). The assumption aims to emphasise the different observations regarding the glomerular response following injury and restated the fact that the nephron is a structural and functional unit of the kidney. Bricker et al. in mouse models and clinical cases of kidney dysfunction in humans proposed that injury to one part of the nephron and a reduce in the number of entire functioning nephrons result in a compensatory growth and hyperfunction of the remaining nephrons.

Most importantly, support for this assumption was provided by micropuncture studies by Carl Gottschalk and colleagues in the 1970s (110-112). The investigators in the rat model of chronic glomerulonephritis showed that GFR for a single nephron varied from 1/3rd to 3 times normal. Injury to one of the components causes injury to the entire nephron via defects in the peritubular capillary network, changes in the composition of urinary fluid passing down to the tubule, a decrease in oxygen supply, and consequent metabolic abnormalities. The consequence of nephron damage increases the single nephron glomerular filtration rate (SNGFR) to balance for the performance of greater fractions of the total renal excretion, but it has a negative long-term impact. Furthermore, the increase in SNGFR is revealed by the rate of loss of nephron, the GFR, the capacity of the renal system to remove metabolic waste products, and the sustainability of single nephron hyperfunction. In the long term, the consequences of glomerular damage result in lower protein resorption, reduced synthesis of

renal hormones, failure of glomerular filtration, and leads to progressive damage to the kidney (109, 113, 114).

2.3.2. The hyperfiltration theory

In 1982, Brenner et al. suggested the “hyperfiltration” theory to describe the progressive nature of glomerular disease following injury and aging (115). It is well understood from studies of kidney disease in hypertensive patients that progressive glomerular injury leading to albuminuria is a result of chronic hypertension. The mechanism has been observed in experimental rodent models subjected to subtotal renal ablation and in those with induced DM, developed progressive glomerulosclerosis triggered by hyperfiltration across the glomerulus, because of increased hydrostatic pressure in the glomerulus. Brenner et al. proposed that the increased hydraulic pressure of the glomerular capillary due to deviations in arterial blood pressure and/or variations in efferent and afferent arteriolar resistances are the causes of glomerular damage due to augmented hydrostatic pressure in the glomerulus. According to the hypothesis, glomerular damage reduces the number of ‘’intact’’ nephrons, this causes the remaining nephrons to undergo functional and morphological hypertrophy, with increased SNGFR. The hypothesis also stated that a low nephron number at birth puts individuals at risk for renal damage later in postnatal life. In the absence of intervention strategies, GFR in patients reduces gradually in a parallel further increase in urine albumin, which can eventually lead to ESKD (116, 117).

2.3.3. The complex deposition theory

The kidney is a key organ for removal of metabolic waste products, including macromolecules, from the circulation. The kidney response to depositions of macromolecules is the third mechanism of glomerular injury (113). This theory mainly describes the large molecules in these soluble immune complexes (118). These complexes are distinct from the ordinary immunological mechanism due to interactions of an excess of either antigens and/or antibodies. These immune complexes are filtered out by the kidney and deposited in different sites depending on chemical and physical properties. Some deposits are in the GBM, while others are observed in the mesangium. The effects of antigen-antibody complex deposition, complement activation (119), and leukocyte recruitment result in inflammatory reactions that lead to glomerular injury. Other molecules may activate mesangial cells or provoke the secretion of excess proteins, leading to permanent sclerosis of glomeruli (118).

In general, glomerular injury is a common element in the pathogenesis of both AKI and CKD and progression to ESKD. The cause of glomerular injury is multifactorial and can come about due to either disease, hypoxia, toxins, inflammation, or hyperglycaemia (12-14). If the glomerular function and structure cannot be restored following inflammatory and proliferative episodes, the glomerulus will become injured. Noticeably, such injured glomerulus led to decline in nephron number and function, that may be further injured by the altered glomerulus. Therefore, the diagnosis of early glomerular damage is important to predict and prevent ongoing renal damage. Importantly, delay in early diagnosis and management of the injury can cause the progression of the injury to AKI, acute kidney disease (AKD), CKD, and ESKD (**Figure 2.6**).

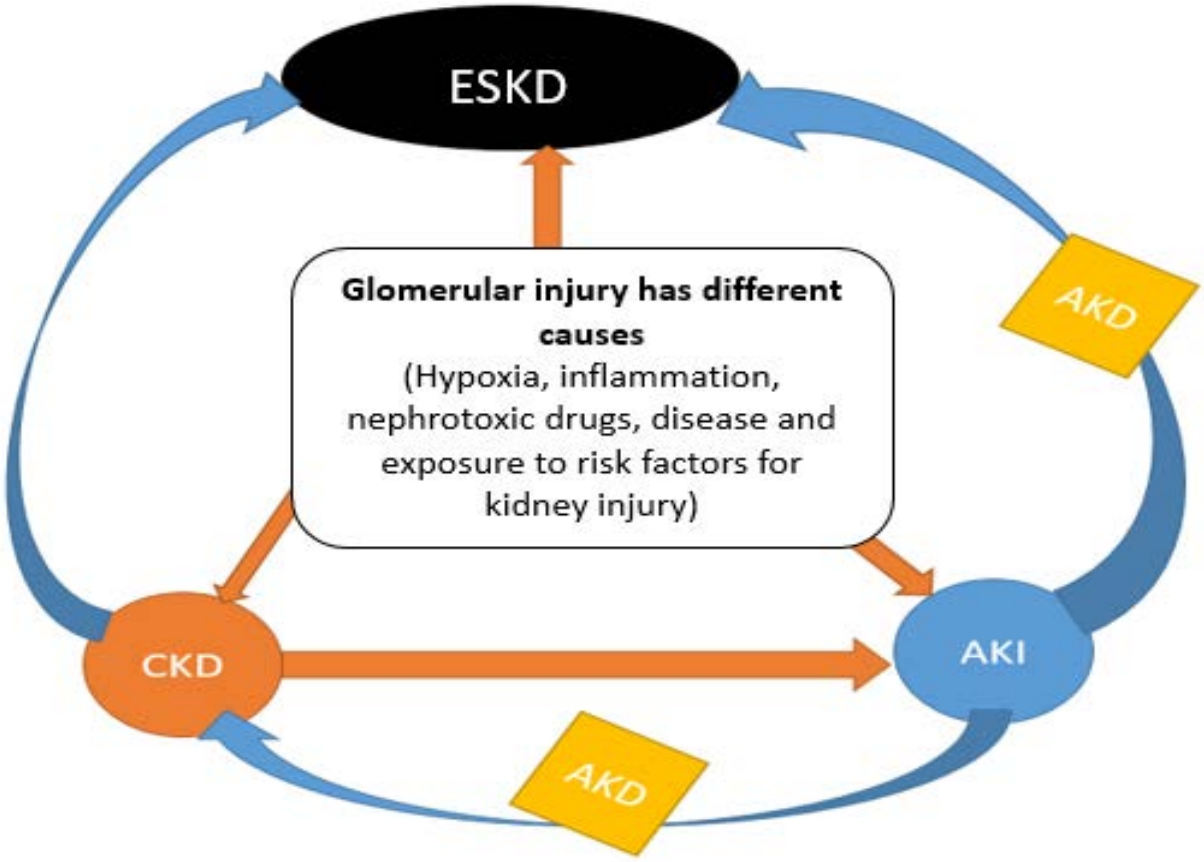


Figure 2.6. The link between glomerular injury, AKI, AKD, CKD, and ESKD.

2.4. Acute Kidney Injury

AKI in the past, defined as “acute renal failure”, is a clinical syndrome independently and strongly linked to increased morbidity, longer hospital stays, and increased healthcare costs in hospitalised patients (120-123). It is characterised by varying clinical manifestations, from

patients with a rapid increase in absolute SCr to patients with a total failure of renal function (124, 125).

AKI also arises from multiple causes and leads to compromised fluid and electrolyte balance, acid-base balance, and removal of waste products (uraemia), and can induce generalised immunological responses that result in functional defects of distant organs (126, 127) (**Figure 2.7**). The functional and structural defects of the kidney and other organs can be determined by blood, tissue, and urinalysis and imaging studies (128-130). Diagnostically, AKI is related to either the absolute increase in SCr by ≥ 0.3mg/dl (≥26.5µmol/L) over 48 hours, and/or an increase in SCr to ≥1.5 times the baseline within the prior 7 days, and/or a significant decrease in urine output (UO) (<0.5ml/kg/hour) for 6 hours (124).

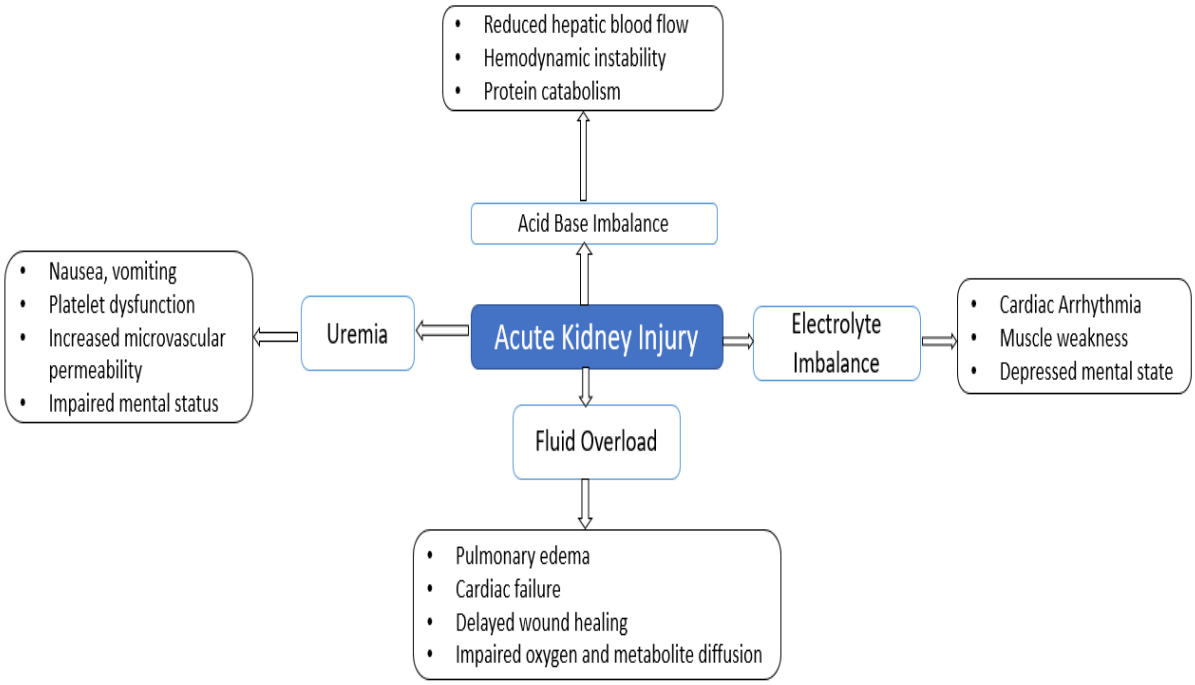


Figure 2.7. Short-term effects of AKI that occur due to acute deterioration in renal function. (*Adapted from Doyle et al. 2016*)(127).

2.4.1. Aetiology and pathogenesis of acute kidney injury

The aetiologies of kidney injury are multifactorial. They are classified based on pathophysiological mechanisms into 3 major categories as prerenal injury, intrinsic renal injury, and postrenal causes of injury.

Prerenal injury occurs because of defects in the function of the kidneys without actual injury to the renal parenchyma (12). It is related to reduced renal blood flow or hypo-perfusion pressure because of intravascular volume contraction or decreased cardiac output (131). It also results from increased capillary permeability or decreased oncotic pressure from hypoalbuminemia (131).

Postrenal kidney injury can occur anywhere in the urinary tubules (12) and is the less common cause of kidney injury in neonates (132). It occurs because of intrinsic obstruction of urinary flow like fungal balls; extrinsic compression, including tumours, which increases renal tubular pressure and decreases GFR. Further, it may lead to declined renal blood flow and an impaired inflammatory process that reduces the amount of blood entering the kidney for filtration and leads to diminished GFR (133).

Intrinsic renal injury is a consequence of damage to the renal parenchyma of the kidney (12). It is the predominant cause of kidney injury in neonates next to pre-renal causes, accounting for 11% of all causes of kidney injury (133). Its aetiologies are difficult to evaluate because of a range of injuries that can occur to the kidney, such as to the renal tubules, glomerulus, interstitium, or intra-renal blood vessels.

Kidney damage occurs from glomerular injury secondary to acute inflammation of the glomerulus (glomerulonephritis) (134). Injury to capillaries of the glomerulus can occur due to damage in intra-renal vessels that cause a decline in renal perfusion pressure and decreases GFR (135). Kidney damage also arises from an allergic reaction to different medications or infections which results in inflammation of the glomeruli (12).

Structural injuries in the kidneys are the distinctive characteristic of intrinsic kidney injury, and the injury is associated with glomerular disease (134, 136). Glomerular disease, including glomerulonephritis, is a cause of kidney injury and 90% of all causes of ESKD are attributed to glomerular disease (19). Glomerular injury as a result of glomerular disease was also found in rapidly progressive glomerulonephritis in renal biopsies (134, 135). A renal biopsy is recognised as the best standard test for the diagnosis of glomerular disease. The clinical presentations of glomerular disease are nephrotic syndrome and glomerulonephritis which can be worsened by AKI (136). The traditional markers for glomerular injury are massive

proteinuria, albuminuria, haematuria, and red blood cell casts present in the urine; these can also be involved in the development of progressive kidney injury (135, 136).

2.5. Epidemiology of Kidney Injury and Kidney Disease

AKI is a significant public health problem, which is related to substantial morbidity and mortality (137). However, the lack of a comprehensively accepted definition of kidney disease has affected the described incidence and clinical course of AKI (137); and varying figure representations depending on the definition used, study population, and geographic area studied.

A review article on the global epidemiology of AKI reported that the worldwide prevalence of AKI is estimated to be from 1% to 66% (138), and in a large population-based cohort study, 8-17% of hospital admissions have been reported (139). A longitudinal electronic health records study predicted 55.8% AKI in all inpatient episodes, of which 90.2% required dialysis with a lead time of 48 hours (140).

Of concern, AKI remains to be a major contributor to increased mortality in the hospital setting. A meta-analysis conducted in the United States of America (USA), aiming to assess the worldwide incidence of AKI in a large cohort of patients conducted in hospital settings, showed the pooled morbidity for AKI to be 21.6% in adults and 33.7% in children (84). A pooled mortality rate of 23.9% in adults and 13.8% in children was reported, using the KDIGO AKI definitions (84). Other studies in USA and China reported the incidence of AKI ranges from 2.4 to 22.7% with a fourfold likelihood of death in hospitalised patients (3, 91, 141); a death rate of 63.2% in AKI and an overall in-hospital mortality rate of 60.8% in critically ill patients was reported (141).

In Australia, the Australian Institute of Health and Welfare (AIHW 2015) reported the number of hospital admissions increased from 42 in 2000 to 71 in 2012, with 19-21 dying per 100,000 population over the 12 years (142). This report shows that 16% of deaths in the Indigenous Australians population were due to CKD, which is 3.5 times higher than that for the non-Indigenous population (142). Further, using the National Aboriginal and Torres Strait Islander Health Measures Survey, the report revealed that the risk of developing CKD is 2 times higher in the Indigenous than in the non-Indigenous population. This highlights the unequal

distribution of CKD across the Australian population (143) (**Figure 2.8**). The AIHW also reports that a clear disparity in hospitalisation is related to age, sex, geographical location (remote residences), and low socioeconomic status; this may contribute to the difference in the disease's burden between Indigenous and non-Indigenous status (142).

Depending on geographical location, Indigenous Australians have a higher incidence of ESKD, with a female preponderance, when compared to non-Indigenous Australians (144). Of concern, the median age of Indigenous Australians patients of Kimberley region beginning renal replacement therapy is reported to be markedly lower in Indigenous Australians (47.6 years old) compared to non-Indigenous Australians (63.5 years old) (145). Likewise, another study looking at the eGFR amongst Indigenous Australians adults showed a rapid loss of kidney function in Indigenous populations across over 20 sites in urban, regional, and remote Australia (146). These concerning findings were further supported in a retrospective population-based study on Indigenous peoples living in the Kimberley regions of Western Australia, reporting a higher incidence of kidney injury (28%) in the younger age group. Considering that the National Australian database shows that the incidence is higher in the older aged population (147), these findings emphasise the urgent need for improved detection and monitoring of kidney injury in Australian Indigenous populations; it highlights as well that kidney disease is not isolated to the older Australian population.

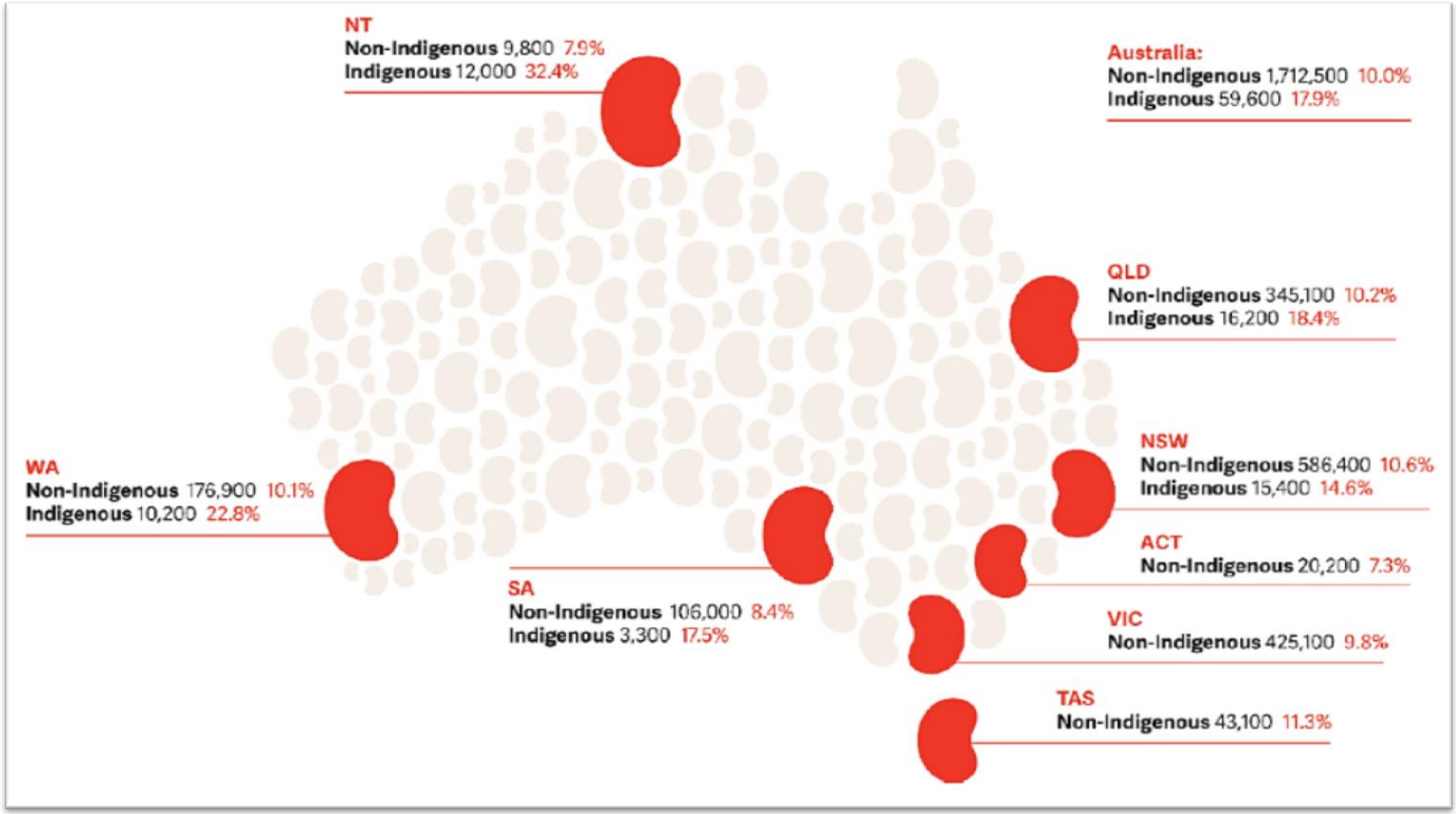


Figure 2.8. People living with signs of CKD: Kidney Health Australia State of the Nation Report 2016 *(143).*

The incidence of Kidney injury and the disease continues to increase in children (148, 149) and this increased risk begins with maternal health during pregnancy. Evidence for this comes from studies (**discussed in section 2.6**), among them the worrying observations of increased incidence of kidney disease in pregnancy, caused by PE (150) as well as an atypical haemolytic syndrome (151). Furthermore, kidney damage is linked with poor pregnancy outcomes even after recovery from AKI (152).

The Assessment of Worldwide Acute Kidney Injury, Renal Angina, and Epidemiology multinational prospective study covering 32 tertiary care paediatric intensive care units worldwide (153), reported a 27% incidence of AKI among 4683 seriously ill children. The investigators reported that patients with a SCr rise of ≥2x the baseline had a mortality rate of 11%. Moreover, the Assessment of Worldwide Acute Kidney Injury Epidemiology in Neonates study on 2022 neonates from 24 neonatal intensive care units, also showed a 29.9% incidence rate of AKI; the mortality rate of infants with AKI is 10% (122). The investigators concluded that AKI was a risk factor for mortality, demonstrating that infants and children die of AKI.

Timely diagnosis of kidney injury/disease enables supportive management of kidney health, such as maintenance of fluid and electrolyte balance, controlling blood pressure, and prevention of exposure to nephrotoxin (124) and other modifiable risk factors are valuable to avoid further negative outcomes.

Undiagnosed and unmanaged AKI leads to higher healthcare costs in terms of chronic dialysis treatment (154), hospital mortality, and risk of new or progressive CKD and ESKD (154-156). The link between AKI, CKD, and progression to CKD is increasingly recognised (**Figure 2.9**). In patients with CKD, the diseased kidney has reduced renal reserve (157) and reduced capacity to handle stress such as low blood pressure (50, 157) and exposure to nephrotoxic insults (50, 158).

The exact pathophysiological mechanism for the relationship between AKI and CKD is not well understood, many studies suggest that pre-existing CKD is a factor in the occurrence of AKI (156, 159). On the other hand, a consensus report of the acute dialysis quality initiative workgroup has found that AKI can lead to kidney injury, thus CKD (158). The report also presents a timeline for the progression of AKI to CKD (**Figure 2.9**). Thus, AKI is described as a

rapid failure in kidney function happening within 7 days or less, whereas CKD is described as the tenacity of kidney disease for a period of greater than 3 months. The report also describes AKD as acute or subacute injury and /or loss of kidney function for 7 days to 3 months after initiation of AKI (**Figure 2.9**).

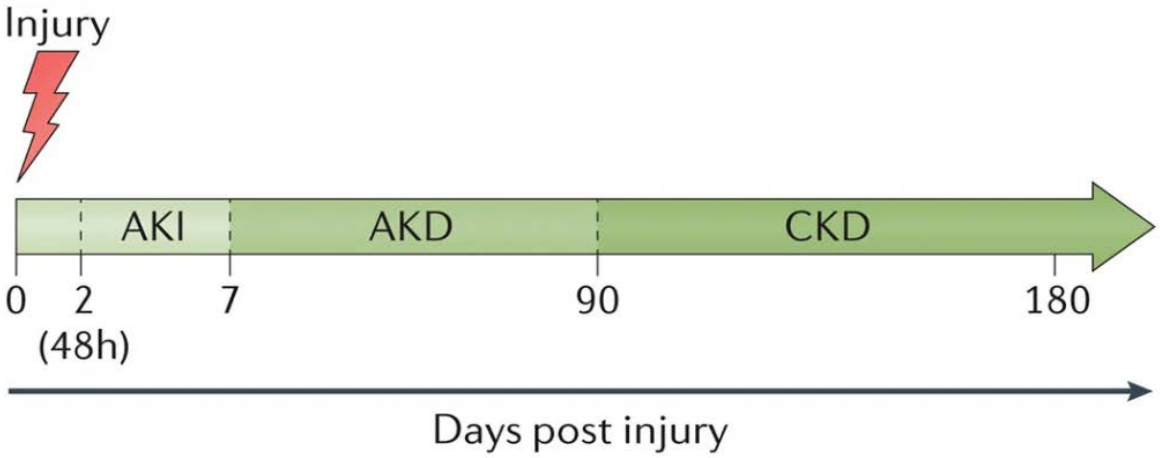


Figure 2.9. The continuum of AKI, AKD, and CKD.

The AKI, AKD, and CKD can form a continuum through which initial kidney injury can lead to insistent renal injury, finally leading to CKD (Chawla, et al., 2017, acute dialysis quality initiative 16) (158). The above studies provide evidence that first, AKI is the main risk factor and directly associated with the progression of CKD, and second, that pre-existing CKD is a factor for the occurrence of AKI.

2.6. Risk factors for kidney injury

Kidney injury is the fastest-growing public health problem worldwide. Recent evidence has shown that maternal factors during pregnancy, genetic predisposition, and postnatal environmental factors play a key role in increasing the risk of developing kidney injury in neonates and the child in later life.

2.6.1. Maternal risk factors for kidney injury

The major causes of kidney injury related to maternal risk are multifactorial such as sepsis, ischemia-reperfusion injury, and toxic insults (126). However, the injury involves inflammation, and tubular and vascular damage which may be acquired from healthcare facilities due to exposure to infectious and nephrotoxic agents (126). In most cases, kidney injury is diagnosed in hospitalised patients and patients receiving contrast agents such as radiocontrast (160), which may impact neonatal outcomes.

Studies have showed that maternal renal dysfunction during pregnancy can affect fetal outcomes (79, 152). Growing evidence shows that maternal factors can contribute to the prevalence of kidney injury in neonates and children (148, 161-163). A Canadian investigation has revealed that approximately one in ten thousand pregnancies required dialysis (164), and that this was associated with maternal morbidity, poor perinatal outcome, and postnatal life of the children.

Animal and human studies have shown maternal nutrition during pregnancy, maternal diabetes mellitus, smoking, obesity, and alcohol consumption all result in pregnancy-related problems including premature birth, low birth weight, IUGR, which is linked with reduced nephron endowment and are a predisposing factor for kidney injury (165-167).

2.6.2. Maternal nutrition during pregnancy

A systematic review revealed that maternal nutrition during pregnancy influences fetal kidney structure and function (168). This review indicated that deficiency in maternal folate, vitamin A, and deficiency in total energy during pregnancy is linked with unfavourable effects on fetal kidney structure and function, estimated by kidney volume, eGFR, proteinuria, and mean creatinine clearance in the offspring. Likewise, a review article, investigating the impact of birth weight, malnutrition, and associated kidney outcomes showed that maternal, fetal, and childhood nutrition was a vital contributing factor to the normal development of fetal kidneys (169). The review showed that maternal diet deficiency in protein, total calories, and/or iron, all reduced nephron number in the infant; furthermore, low birth weight, prematurity, and small size for gestational age correlated with reduced nephron number (169). These are risk factors for neonatal kidney injury (170) and kidney disease in later life (171). In addition, maternal protein and common micronutrient deficiencies in iron, iodine, vitamin ,A and zinc are risk factors for intrauterine growth restriction/ low birth weight (172).

2.6.3. Maternal diabetes during Pregnancy

Several studies have revealed that gestational exposure to maternal diabetes or obesity undesirably impacts renal programming in offspring (168) and are major factor related to an increased risk of kidney disease in later life (169). In a human study, investigating gestational diabetes mellitus (GDM) and renal consequences in term neonates (173), the authors evaluated renal volume, urinary biomarkers for renal function, and tubular impairment in 30-

40-day-old neonates of GDM mothers who needed insulin therapy during pregnancy, and compared patients compliant and non-compliant to the management of GDM. The urinary biomarker levels, renal volume, and cortical volume were considerably different in non-compliant GDM mother neonates compared to the compliant GDM mother neonates. The authors highlighted that GDM impairs both renal development and tubular integrity. The investigators concluded that strict glycaemic control and compliance with the GDM management program may prevent this negative effect.

Indeed, a recent study in women with GDM, showed that treated GDM supports fetal kidney growth, showed by normal fetal kidney size and volume measured by obstetric ultrasound at 32-34 weeks of pregnancy (174, 175). Furthermore, studies have shown that renal function reserve was decreased in adult children whose mothers had type 1 diabetes mellitus (T1DM), compared with those who had a diabetic father, suggesting that maternal diabetes was a factor in the reduction in nephron number (176, 177).

In an experimental mouse model, maternal impaired glucose tolerance during pregnancy resulted in reduced nephron number and was linked with glomerular hypertrophy in offspring (167). Similar experimental models support these results, showing, maternal hyperglycaemia was associated with significantly reduced nephron number in pups (178). The consequence of reduced nephron number could lead to declined renal function and result in an increased SNGFR and hyperfiltration, which are associated risk factors for kidney disease (179).

2.6.4. Maternal smoking

Smoking during pregnancy is accountable for both maternal and fetal health disorders, including adverse pregnancy outcomes and overall health in both the mother and offspring. The studies that aimed to evaluate prenatal exposure to smoking across pregnancy in relationship with preterm birth showed that maternal smoking and exposure to household smoking are linked with preterm birth (165, 180), low birth weight, and childhood proteinuria (79). Another study in a cohort of Indigenous Australians women noted that smoking was linked with preterm birth, low birth weight, and reduced renal volume (181). Renal volume can be a marker for nephron number (182), so the lower renal volume can suggest a reduced nephron number, which is a risk factor for neonatal AKI and kidney disease in the later life of the child.

2.6.5. Alcohol consumption during pregnancy

The connotation of heavy alcohol consumption during pregnancy and adverse birth outcomes has been covered in previous literature. For instance, experimental studies on a rat model revealed that exposure to moderate alcohol drinking during pregnancy and prenatal environment has a significant impact on foetal renal development (183, 184). Further in experimental mice models, the effects of alcohol exposure during pregnancy on nephron endowment and renal function of the offspring were investigated (113, 115). Kidneys were collected at embryonic day 20 or postnatal day 30 (183), and 1 month of age (185), and the nephron number was determined. Both studies reported that ethanol exposure impairs kidney development, leading to permanent nephron deficit, probably as a consequence of repressed ureteric branching morphogenesis, consequently leading to reduce renal function. In human studies, a systematic review highlights that heavy alcohol consumption during pregnancy increases the risk of low birth weight, small for gestational age, and preterm birth (186). In addition, an Australian cohort study, comprising 1626 offspring, also showed that maternal alcohol consumption during pregnancy is linked with kidney disease in the later life of the offspring at 30 years (166).

2.6.6. Effect of postnatal environment on renal function

Nephrogenesis in humans is ongoing until 36 weeks of gestation (187). In premature neonates, nephrogenesis is still not completed at the time of birth and continues after birth but glomerular abnormalities are observed (187), this phenomenon has linked preterm birth to decreased total kidney volume and decline in renal function (182, 188). In an experimental mouse model, Ding et al. reported that both prenatal and postnatal maternal environments (maternal blood pressure, healthy weight, normal renal function) have a considerable effect on increasing nephron endowment and reducing the severity of polycystic kidney disease (184).

A study on preterm and term neonates showed that the tubular and glomerular function of the neonates is affected by gestational age at birth and postnatal age (78). The authors showed that pathological proteinuria and high level of renal tubule damage associated with marker neutrophil gelatinase-associated lipocalin (NGAL), was reported in preterm neonates

(189) during the first month of life and renal tubular damage was showed by raised levels of NGAL, however, this marker still needs further investigation in preterm neonates.

In another study, Sutherland et al. observed renal dysfunction in preterm-born Australian Indigenous infants (90). The investigators showed that renal dysfunction was evident in the first month of life in Indigenous preterm infants, where preterm Indigenous babies showed higher rates of renal injury compared to non-Indigenous infants; this was evident in the extremely preterm cohorts. Further, Kandasamy et al. also found that the total kidney volume (surrogate nephron number marker) of Indigenous preterm neonates was significantly lower compared to non-Indigenous neonates (182). The authors also investigated the renal function of neonates. They found that extremely preterm neonates and low birth weight infants achieve similar eGFR as term-born neonates, probably through single nephron hyperfiltration which occurs very early in postnatal life (190). However, it has been shown that extremely preterm and low birth weight neonates show evidence of glomerular injury (190). Therefore, these studies indicate that premature neonates, low birth weight neonates, and neonates of Indigenous ethnicity have a negative effect on renal development and that in the early postnatal period, the renal function might reflect the immaturity of the podocytes of the glomerulus, particularly in extremely preterm neonates.

2.6.7. Postnatal factors that lead to kidney injury

Different postnatal factors can lead to adverse effects on renal function including nephrotoxic medications during neonatal cares, ventilation, and hemodynamic changes (191) (**Figure 2.10**). The factors cause adverse effects on kidney metabolism and oxygenation and activation of inflammatory cytokines (192), as well as increased demand for filtration due to the administration of fluid. It has been shown that critically ill neonates, who are frequently given medications, may increase vulnerability to toxic renal injury and risk of AKI, and experience long term kidney dysfunction (79); a common example is Indomethacin (known to be nephrotoxic) used in preterm infants for treating patent ductus arteriosus (193). Moreover, the International Society of Nephrology zero by the year two thousand twenty-five (0 by 2025), which aims to reduce deaths from AKI to zero by 2025, found that in a cohort of 354 children, dehydration, hypotension, infection and exposure to nephrotoxic agents as common causes of AKI in infants and children (194).

Nutrition during the postnatal period also plays an important role in the renal development of the infant. Ece et al. recruited 74 children with malnutrition and compared them with healthy controls (195). The authors found that malnutrition (children who were severely wasted and had minimal subcutaneous tissue without oedema defined as malnourished) during infancy is significantly associated with reduced renal volume and body weight. In addition, protein intake in infancy is positively linked to a lesser ACR and a higher eGFR (196). Breastfeeding may also play an important role in renal development. Miliku et al. compared infants that had exclusive breastfeeding for 4 months to nonexclusive breastfeeding in the first 4 months (196), and the results showed that nonexclusive breastfeeding for 4 months was significantly linked with smaller combined kidney volumes and lower eGFR. The authors concluded that breastfeeding is related to subclinical changes in kidney outcomes.

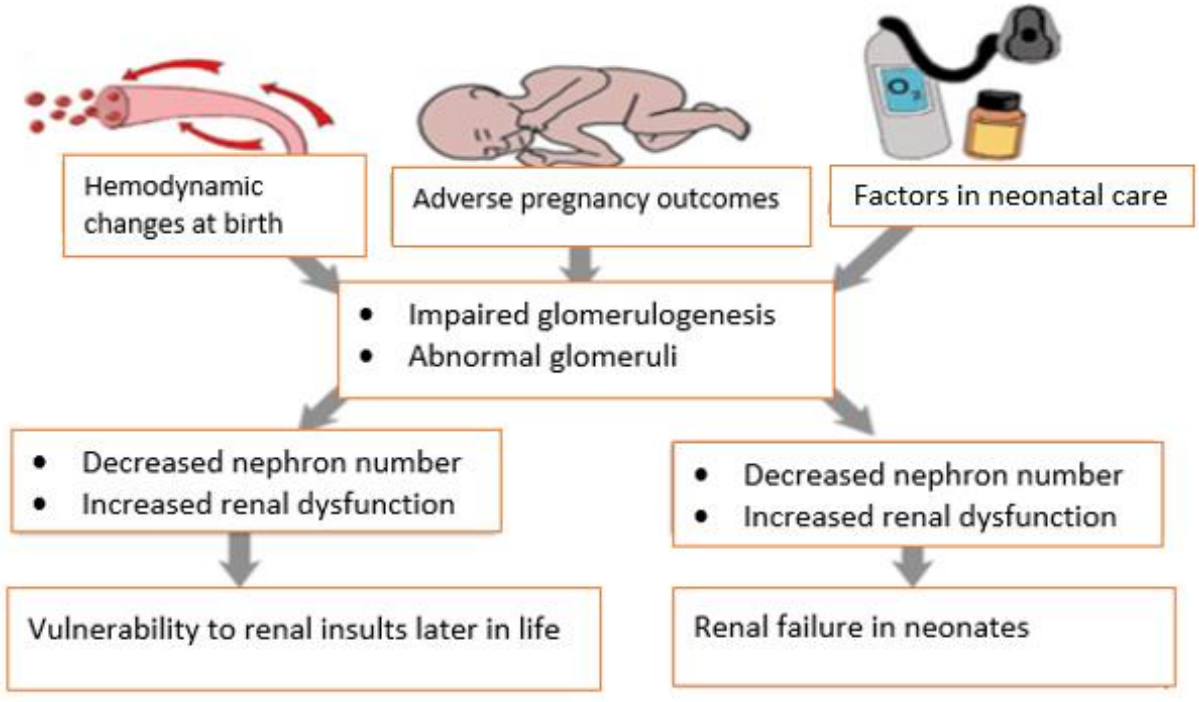


Figure 2.10. Factors that may contribute to the formation of abnormal glomeruli in kidneys in infants and the health consequences of reduced nephron endowment at birth.

2.6.8. Genetic predisposition to kidney injury

The risk of developing kidney injury and triggers for disease progression has been investigated by gene association studies such as investigations on inflammatory pathways, cell metabolism and maintenance of hemodynamic status.

Systematic reviews by Fernandez et al. and Vilander et al. investigated the genetic influence on acute renal damage (197, 198). These studies showed changes in Angiotensin-converting enzyme (ACE), Tumour Necrosis Factor-alpha, Interleukin-6, Interleukin-10, Nicotinamide Dinucleotide Phosphate Oxidase, and Apo lipoprotein-E gene polymorphism were all associated with the risk and progression of AKI.

There is also existing evidence that genetic differences in the population correlate with a predisposition to the disease. A study in Australia showed the renin-angiotensin-aldosterone system (RAAS) single nucleotide polymorphisms were differentially expressed in an Indigenous Australians desert community compared with non-Indigenous Australians (199). It was proposed that polymorphism in RAAS in the Indigenous population may influence genes that regulate renal growth and development. It should be noted that the activity of the RAAS, which is a perilous period in the course of renal development, is affected by single nucleotide polymorphisms in genes rs5186 of angiotensin II type 1 receptor, which controls the synthesis of various RAAS proteins (200).

The prevalence of the insertion/deletion (I/D) polymorphism of the ACE gene (rs4646994) is also different in Indigenous communities compared with the non-Indigenous population (201). In addition, a study in the Southeast Asian population also showed that ACE-D (ACE-D) alleles are accompanied by an increased risk of AKI but that the Interleukin-6-572C allele reduces the risk of kidney injury (202). The investigators noted that the effect of IL-6 is inconsistent with the findings of previous studies in Caucasian populations. The authors' hypothesis for this inconsistency is the multifaceted interaction between the gene, environment, and population-specific modifying factors. Overall, the pattern of expression and activities of intra-renal RAAS, which is essential for normal kidney development, may be affected by differences in the prevalence of RAAS gene polymorphisms among the population, via different levels of RAAS proteins, such as angiotensinogen, or ACE.

2.7. Functional markers of the kidney

Diagnostic biomarkers of kidney injury are important for the early diagnosis of patients. Understanding the role of the biomarker in early screening and prognosis helps the scientific community to translate the marker into clinical practice.

2.7.1. Glomerular filtration rate (GFR)

The GFR evaluates the amount of blood passing through the glomeruli into the Bowman's capsule each minute. There are different formulas to estimate GFR in adults and children by considering age, sex, creatinine concentration, ethnicity, height, and weight. GFR can be estimated by intravenous administration of exogenous constituents that are easily filtered through the glomerulus. For example, the Inulin clearance test is measured as a gold standard for eGFR. However, it is not routinely performed in ICU because of practical reasons, such as being invasive and expensive for routine clinical use (203). Instead, the eGFR is derived from creatinine clearance computed from 24-hour urine collection. Importantly, the GFR is commonly estimated from SCr using different formulas as illustrated in (**Table 2.1)**. More recently, a new Big equation has been established for small kidneys to estimate neonatal GFR (204) but remains to be validated.

AKI studies suggest using creatinine clearance /GFR equations to estimate baseline creatinine for patients lacking the baseline creatinine. The currently available classification of AKI guidelines (Risk, injury, failure and loss, and ESKD and KDIGO) used the baseline SCr to classify and stage AKI. If the baseline creatinine concentration is unknown and if the patient has no history of CKD, it can be back-calculated using the MDRD equation (205), by considering the baseline GFR of 75ml/min/1.73m^2. The back-calculation of creatinine has limitations. Pickering et al. (206) reported that the back-calculation misclassifies AKI in ICU patients, and another multicentre cohort study on ICU patients reported overestimation of AKI using Risk, injury and failure and loss and ESKD (207, 208). The investigators advised using the measured baseline creatinine rather than the estimated value in trial or epidemiologic studies of AKI. In 2009 Levey et al. (209) proposed the CKD-EPI equation, which is more precise than the MDRD, to calculate the GFR with less bias and better accuracy. The CKD-EPI equation estimates GFR based on creatinine and sCysC and other variables for the adult and paediatric population (**Table 2.1**).

Table 2 1: Equation for determination of estimated glomerular filtration rate.

Equation	Variables	Formulae
2009 CKD-EPI creatinine equation for adult [209]	Age, sex, race, SCr	eGFR = 141 * min (SCr/κ,1) $^{\alpha}$ * max (SCr/κ, 1)$^{-1.209}$ * 0.993 ^{Age} * 1.018 [if female] * 1.159 [if black]
Schwartz Creatinine based equation for paediatric [210]	Height, SCr, BUN	eGFR =41.3 (height in meter/SCr) eGFR 1B= 40.7 (height/SCr)$^{0.64}$ x (30/BUN)$^{0.202}$
2012 CKD-EPI cystatin C equation [209]	sCysC	eGFR=133 min (sCysC/0.8, 1)$^{-0.499}$ x max (sCysC/0.8, 1)$^{-1.328}$ x 0.996^{Age} [0.932 if female]
CKD-EPI sCysC based equation for paediatric [209]	sCysC	eGFR= 70.69 x (sCysC)$^{-0.931}$
2012 CKD-EPI creatinine–cystatin C equation [209]	SCr, sCysC, age, sex, race	eGFR= 135 min (SCr/k, 1) $^{\alpha}$ *x* max (SCr/k, 1)$^{-0.601}$ x min (sCysC/0.8, 1)$^{-0.375}$ x max (sCysC/ 0.8, 1)$^{-0.711}$ x 0.995^{Age} [0.969 if female] [1.08 if black]
Cockcroft-Gault [211]	Age, weight, sex, SCr	CrCl (male) = ([140-age] × weight in kg)/ (SCr × 72) * (0.85 if female)
Modification of Diet in Renal Disease (MDRD) [205]	Age, weight, sex, BUN, SCr	eGFR (ml/min/1.73 m^2) = 186 x SCr (mg/dl)$^{-1.154}$ x age$^{-0.203}$ x 0.742 (if woman)
Zappitelli et al. 2006 [212]	sCysC	eGFR (ml/min per 1.73 m^2) = 75.94/ [sCysC$^{1.17}$])

BUN: blood urea nitrogen in mg/dl, CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration, eGFR= Estimated Glomerular Filtration Rate, SCr: Serum creatinine (mg/dl), sCysC: Serum Cystatin C (mg/L), κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males for CKD-EPI creatinine equation, min indicates the minimum of SCr/κ or 1, and max indicates the maximum of SCr/κ or 1, α is -0.248 for females and -0.207 for males for CKD-EP SCr and sCysC equation, CrCl: Creatinine clearance, BUN: Blood urea nitrogen.

2.7.2. Serum creatinine and urine output: a conventional marker of kidney function

The classifications for AKI (Risk, Injury, Failure, Loss and ESKD, Acute Kidney Injury Network, KDIGO) are based on SCr and UO. However, these tests are recognised as screening tests for GFR, and both are likely to have a substantial bias when used as indicators of kidney function (209, 213, 214).

Using SCr level as a measure of kidney injury has several limitations. SCr is not sensitive to changes unless a significant reduction in GFR is observed (215). The concentration of SCr starts to rise only after 50% of the kidney function has already declined (215). SCr is a marker of excretory function only and are a non-specific marker of kidney injury, leading to delay in diagnosis and treatment opportunities (216). SCr levels also vary with age, gender, ethnicity, muscle mass, nutritional status, non-steroidal anti-inflammatory drug use, and fluid status (216). In addition, SCr is affected by volume depletion and other causes that decrease renal perfusion pressure without underlined kidney injury. Laboratory test methods for determining SCr concentration can be inconsistent due to several conditions including haemolysis, acetone, usage of some antibiotics, and increased bilirubin in the sample, and can be further affected by certain diagnostic techniques such as Jaffe's kinetic method (217).

The UO is a good clinical indicator of kidney injury, (218) but it is not renal specific. Low UO can result from obstructive uropathies in the neonates born from a mother who has DM, maternal drug use and other maternal factors (such as smoking, and alcohol drinking) and may also be acquired in the postnatal period because of hypoxic and toxic insults (79). Likewise, lower UO (less than 0.2mL/kg/hr) of longer duration (6-12 hours) is associated with AKI (219). This study also showed that low UO, even in the absence of AKI but a high concentration of SCr, is independently associated with mortality. Therefore, there is a need for further interpretation within the clinical context because patients who fulfill the AKI definition using UO and SCr do not have AKI, on the contrary patients with clear evidence of AKI do not meet SCr and UO criteria for AKI (216).

Detecting AKI in neonates remains to be a complex challenge and the current use of SCr and UO is not a reliable marker for GFR in this cohort due to low urine production in the first few days of life and compensatory hyperfiltration of nephrons producing normal GFR estimates

(220, 221). It is important to note, the correlation of GFR and UO is not linear in most clinical situations in neonates and adult populations; in fact, UO may persist until kidney function ceases, this means the UO may be normal during an investigation, but the patient has other evidence of AKI (216, 222). A good example showing the discrepancy between GFR and UO is highlighted in very obese patients, where direct utilisation of UO calculated per weight (ml/kg/h) will cause the GFR to appear significantly reduced (influenced by body weight), leading to overestimation of AKI in this cohort (154).

In summary, these conventional markers exhibit variations with the course of AKI as surrogates of GFR while having limitations; The limitations of these parameters and the overwhelming clinical implications of AKI lead to a search for a new non-invasive biomarker for the early detection of AKI (**Figure 2.11**).

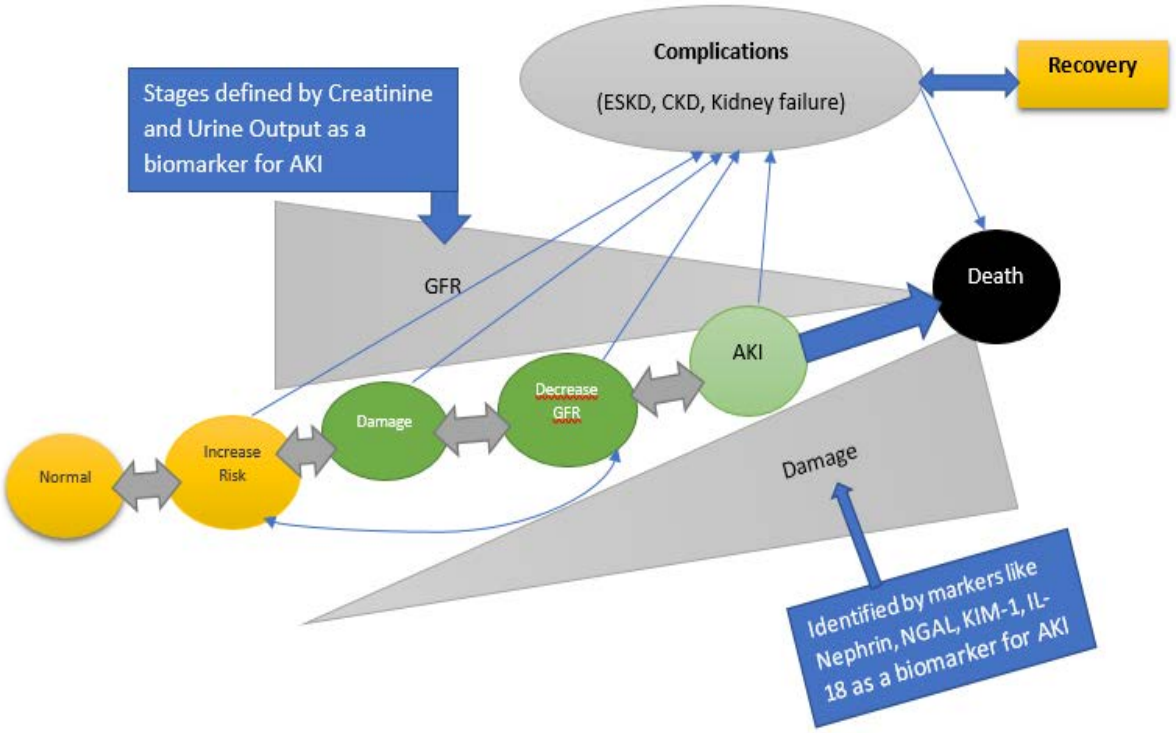


Figure 2.11. Theoretical model for traditional and injury associated markers in the evaluation of kidney injury.

*The arrows between circles signify risk factors related to the beginning and progression of the disease that can be affected or identified by interventions (223). "**Complications"** designates to all problems of AKI, including efforts at prevention and treatment, and impediments in other organ systems, and the triangle for GFR (base to the tail of the triangle shows decreasing sensitivity to detect interventions and complications of AKI) and damage shows the sensitivity of biomarkers in detecting the severity of AKI from tail to the base of the triangle.*

2.7.3. Cystatin C as a functional marker of kidney

Cystatin C (CysC) is a low molecular weight cysteine protease inhibitor formed by all nucleated cells at a persistent rate (224). It is encoded by the CST3 gene and circulates in plasma not bound to proteins (224). Cys C is freely filtered through the glomerulus and fully reabsorbed at proximal convoluted tubules of the kidney, and it is not secreted by the renal tubules; this makes, CysC based estimation of GFR a reliable marker for predicting AKI (224), and it has all features of an ideal marker of kidney function (225).

A systematic review assessed the accurateness of sCysC in the prediction of AKI in 1948 children aged < 18 years old (1302 non-AKI children and 645 AKI cases) by assessing 24 studies related to the prognostic performance characteristics of sCysC in the prediction of AKI. The investigators showed sCysC has a good prognostic value for predicting AKI within the first 24 hours of admissions (226). Similar findings were seen in infants where Kandasamy et al. recruited a cohort of 80 (31 terms and 49 premature) infants at term postmenstrual age and found that SCr had a significant correlation with body weight which was not evident for sCysC (221). The authors in this study recommended that sCysC-based GFR measurement is a more reliable indicator for diagnosis of AKI in smaller neonates than SCr. Likewise, in critically ill children (n =62 with ages ranging from 1 month to 18 years of age) admitted to the paediatric ICU, sCysC was more sensitive to AKI compared to SCr on admission at 12 hours (227).

More importantly, El-sadek et al. showed that sCysC was found to be a timely predictor of AKI in 60 critically ill neonates in NICU, showing great promise as an early detector of AKI (228). The sCysC level increased 48 hours before SCr in the critically ill neonates who developed AKI. Encouragingly, in a cohort of 63 identified patients, sCysC also proved to be a good predictor of renal recovery from AKI, compared to SCr (229), which decreases before creatinine in most hospitalised patients with AKI. Together, these studies have provided evidence that sCysC is a timelier predictor of AKI and possible renal recovery from AKI compared to the commonly used SCr marker of AKI in infants and children. However, it is important to note, that there need to be further studies in critically ill neonates regarding sCysC and other more reliable markers of AKI as well as their association with the long-term outcomes in these cohorts.

2.8. Damage-associated biomarkers of kidney injury

A novel biomarker is a distinctive marker that can be empirically measured and assessed as a sign of normal physiological and disease processes (230). It is a fundamental tool in preclinical and clinical research and helps to predict, diagnose and understand the development of the disease and the outcome of treatment (231).

Several potential damages associated biomarkers for kidney injury and its adverse outcomes have been studied to date, such as NGAL, Kidney Injury Molecule 1 (KIM-1), Insulin-Like Growth Factor Binding Protein 7, and Tissue Inhibitor Metalloproteinase 2 (IGFBP-7.TIMP-2), IL-18 (232-236), but none of these have so far been recognised to be superior, in terms of sensitivity or specificity to others for detection of early glomerular injury or prediction of AKI.

The marker, NGAL is elevated in plasma during septic conditions irrespective of the kidney injury status (132, 225). Likewise, the clinical use of IGBP.TIMP-2 has been also assessed for the risk of AKI (237), but the investigators suggested a need for further clarification before using these biomarkers for diagnosis, and prognosis of AKI in different clinical situations. Further, Nadkarni et al. (238) studied routine urinalysis findings and urinary kidney injury biomarkers. The investigators found a significant association of leukocyte esterase with NGAL, and haematuria and proteinuria with NGAL, IL-18, Liver Fatty Acid Binding Protein (L-FABP), and KIM-1, without the underlined clinical kidney injury. The authors showed that significant interference existed in routine urinalysis and kidney injury biomarkers and the biomarkers were not specific for a site of injury.

2.8.1. Traditional markers for glomerular injury

Traditional markers of glomerular injury include haemoglobinuria, haematuria, proteinuria and albuminuria (43, 239). These markers have been used effectively for diagnosis, prognosis, and for monitoring treatment since the markers are a sign of established kidney damage and have a direct role in the progression of glomerular injury. Proteinuria and albuminuria are also indicators of glomerular injury and other pregnancy-related complications including PE during pregnancy (240). However, these markers have limitations in the timing and sensitivity for prediction, diagnosis, and prognosis of glomerular injury (42, 43). For example, studies have shown that the timing in detecting injury using urinary albumin concentration as a marker does not show early prediction of glomerular injury (45, 241). A study by Hana et al. indicated

that there was no significant difference in the concentration of urinary albumin detected between AKI and control groups (45). Instead, they found that urinary albumin concentrations were the same in infants without kidney injury, infants who recently developed kidney injury (day 1 of diagnosis), and infants that were recently diagnosed with AKI. Urinary albumin also showed low sensitivity in predicting the early detection of AKI in neonates. Hence, urinary albumin may not be used as an early marker for the detection and monitoring of AKI regardless of glomerular injury.

Existing evidence showed that albuminuria described as urinary ACR is a marker for glomerular injury in neonates as well as in the adult population. For example, the KDIGO guideline 2012 categorised ACR as microalbuminuria 3-30mg/mmol and macroalbuminuria ≥30mg/mmol as a marker of glomerular damage and management of CKD (242). A population-based cross-sectional study of the general Australian population used the above categories to assess albuminuria concordance in children and parents in six and eight regional cities and home visits across Australia (243), the authors hypothesised that ACR predicts the risk of kidney disease at a later life of the children.

The Australian guideline for albuminuria categorises urinary ACR in mothers with 3.5-35mg/mmol microalbuminuria and >35mg/mmol macroalbuminuria (244). Likewise, a cohort study among Aboriginals people in central Australia showed that a baseline urinary ACR level of ≥3.5 mg/mmol was associated with a 10x increased risk of ESKD (146), the authors conclude that a single measure of increased spot urinary ACR was a strong predictor of renal failure. Recent studies also described the upper limit of normal ACR for preterm neonates and term neonates in the first 3 days of age (≥34mg/mmol) (245) for the detection and diagnosis of glomerular injury in daily clinical practice. Therefore, ACR can be used for diagnosis, prognosis, and monitoring treatment for glomerular injury by considering the limitations in terms of timing and sensitivity to detect early glomerular injury.

2.8.2. Potential novel markers for glomerular injury

Potential novel biomarkers that may be more specific for early detection and monitoring of glomerular injury are urine podocyte proteins, including nephrin, podocalyxin, synaptopodin, podocin and CD2AP. These markers are excreted in the urine during glomerular injury, and a high concentration of podocytes in urine suggests glomerular injury and damage to the

integrity of the GFB (46). The podocyte proteins have a structural component and functional role for podocytes. For example, podocalyxin is the main podocyte surface antigen that prevents podocyte foot processes from collapsing; nephrin is a structural protein that scaffolds the podocyte Sd and is connected to the actin cytoskeleton through podocin and CD2AP to maintain functional integrity and allows tight junctions between podocyte foot processes (47).

Damage to the glomerular membrane or downregulation of podocyte protein expression shows structural and functional alterations such as loss of stress fibres, the uncharacteristic formation of filopodia, and compromised cell migration (48), and may allow macromolecules and cells to leak into the urine, unlike in normal glomerular function. For example, Hara et al. showed elevated levels of podocalyxin in DN patients with normoalbuminuria (40) and Jim et al. reported downregulation of synaptopodin, podocin, and nephrin in kidney biopsies of DN compared with controls (49).

Recently, a promising and emerging new novel biomarker for early glomerular injury and prediction of AKI has been nephrin because of its biological plausibility, a functional, and structural component of the glomerulus (50, 246). Therefore, understanding the source and aetiology of each biomarker is important for interpreting the value of the biomarker for glomerular injury. Some examples of biomarkers for glomerular injury and AKI are described in **Table 2.2**.

Table 2 2: Potential aetiology and source underlying increases and biomarker rationale for glomerular injury and/or AKI

Biomarker	Potential aetiology	Source of biomarker	Biomarker rationale
Plasma NGAL	Ischemia	Proximal and distal tubular leukocytes, the loop of Henle, and collecting ducts	Inflammatory marker
Urinary NGAL	Non-specific	Proximal and distal tubular leukocytes, epithelial cells, the loop of Henle, and collecting ducts	Inflammatory marker
Urinary KIM-1	Non-specific	Proximal tubular cells	Cell injury marker
Urinary IL-18	Inflammation	Proximal tubular cells, Monocytes, dendritic cells, macrophages	Inflammatory marker
Urinary TIMP-2	Non-specific	Tubular epithelial cells	Cell cycle marker
Urinary IGFBP-7	Non-specific	Endothelial, vascular, epithelial cells, and others	Cell cycle marker
Urinary L-FABPs	Oxidative stress and hypoxemia	Hepatocytes, proximal tubular cells	Cell injury marker
sCysC	Non-specific	All nucleated cells	Glomerular filtration marker
Angiotensinogen	Renal tubular injury	Kidney vasoconstrictor	Renin-angiotensin system activation marker
Urinary nephrin	Glomerular injury	Glomerulus	Glomerular injury marker
Urinary protein	Glomerular injury	Blood leaks through the filtration barrier	Glomerular filtration marker
Urinary albumin	Glomerular injury	Blood leaks through the filtration barrier	Glomerular filtration marker

2.9. Nephrin as a novel biomarker for determining early glomerular injury.

Studies have reported novel podocyte proteins, including nephrin, as potentially superior markers of glomerular injury (91, 247). Likewise, human studies have reported a diagnostic value of nephrin and have shown a correlation with kidney injury (24, 68). A novel highly specific and sensitive non-invasive biomarker to recognise early glomerular injury which fulfills the ideal characteristics of the biomarker is needed. Although albuminuria is used as a marker of glomerular injury and reflects abnormal permeability of the glomerulus to protein (215), there is now evidence that nephrinuria occurs early in glomerular injury, preceding albuminuria (21, 246, 248), and shows a positive correlation of nephrinuria with the severity of kidney injury (21). Hence, urinary nephrin analysis has the potential to become a novel marker of early glomerular injury.

2.9.1. Definition and function of nephrin

Nephrin is a 180 kilodalton (Kd) integral transmembrane protein, which has a major role in the proper development and functioning of the GFB (56). The GFB comprises negatively charged glycocalyx which covers the laminal surface of endothelium and covers the opening of fenestrae of endothelial cells; negatively charged fenestrated endothelium (allows passage of water and small molecules); GBM and the foot process (FP) of podocytes. Podocytes, cells in the inner wall of Bowman's capsule, are covering finger-like projections round the capillaries of the glomerulus and culminate in a network of cooperating FPs that subsidise the GFB (5, 213). The Sd, a dedicated podocyte-podocyte junction found between interdigitating FPs, basically regulates the size-selectivity of the filtration barrier (5, 6, 213), molecules approximately <40 nanometres in diameter can pass through the filtration barrier into the urinary space**.** The structural and functional component of the filtration barrier, nephrin is a situated at the Sd of glomerular podocytes, between the FPs and GBM. Nephrin is primarily expressed in glomerular podocytes (56). It is also expressed in non-renal tissues such as the human pancreas (249), and human lymphoid tissues (250).

2.9.2. Discovery and molecular structure of nephrin

Nephrin was discovered in the late 1990s by Kestilia et al. (56), in children with congenital nephrotic syndrome of the Finnish type, due to a mutation of a gene coding for nephrin (NPHS1). The syndrome was first recognised in the Finnish Population (57) and later identified elsewhere in the world. Kestilia et al. in 1994 went on further to identify the genetic locus of the syndrome in a cohort of 17 Finnish families and found that the genetic disorder was mapped at the 19q12-13 chromosome (58).

Patients with nephrin gene mutations are affected by nephrotic syndrome resulting in massive proteinuria, loss of Sd and FPs (56, 59), and death during the first two years of life. Hence, the role of nephrin is supported by not only its gene mutation being associated with nephrotic syndrome but also with decreased nephrin concentration and /or reduced expression in the glomerulus in glomerular disease. Importantly, studies found that downregulation of nephrin or alteration in its function has been reported at the early stage of all types of proteinuria disease before any change in other podocyte proteins is detected (52, 251). Besides recognising the gene mutations of nephrin, Kestilia et al. sequenced 150Kd critical regions of NPHS1 and predicted the molecular structure, as a transmembrane protein of immunoglobulin superfamily of cell adhesion molecules (56). Further investigation showed that nephrin has an extracellular domain with an amino (N)-terminal signal peptide, followed by 8 immunoglobulin-like modules and one fibronectin type III-like module, a single transmembrane domain, and an intracellular carboxylic (C)-terminal domain (**Figure 2.12**). The extracellular portion of nephrin is glycosylated and has binding sites for heparan sulphate. Kestilia et al. also demonstrated that nephrin is likely to be an adhesion receptor and signalling protein. The cytosolic domain has tyrosine residues, which are phosphorylated during the binding of nephrin with its ligand (56).

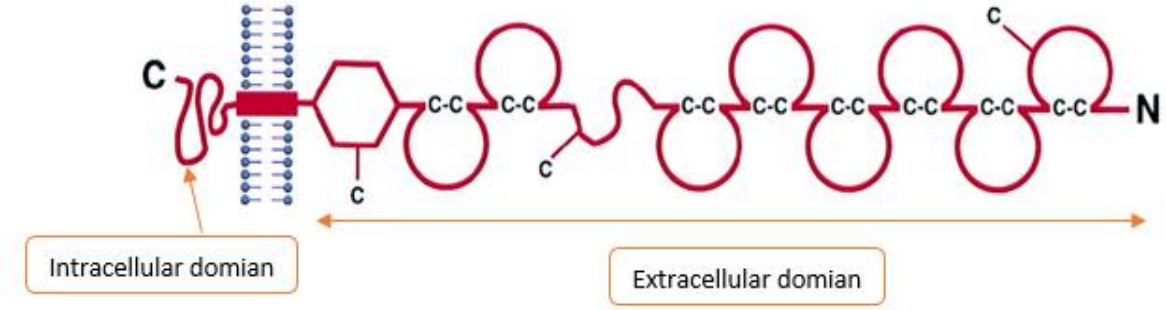


Figure 2.12. Schematic presentation of the domain structure of nephrin

Disulfide bridges (C-C) connect incomplete circles to show the immunoglobulin repeats. The locations of free cysteine residues are indicated by a (—C). The red box shows the transmembrane domain. N: extracellular amino terminal; C: Intracellular Carboxyl Terminal (Routsalainen et al. (59)). The free cysteine residues on the extracellular domain of nephrin are important for disulphide bond formation with adjacent molecules, with itself and nephrin family proteins, to provide stability and to keep the integrity of the Sd (252, 253).

The Sd/slit membrane is a molecular sieve made up of nephrin molecules. The repetitive nature and precise designing of extracellular nephrin-nephrin regions form pores that allow for discriminating filtration of the blood. Nephrin's cytoplasmic region recruits a range of signalling molecules that control the cytoskeletal organisation and foot process shape as well as barrier turnover, each of which contributes to slit membrane maintenance post-injury (5). The model of nephrin association to form the isoporous filter of the podocytes Sd is illustrated in **Figure 2.13**.

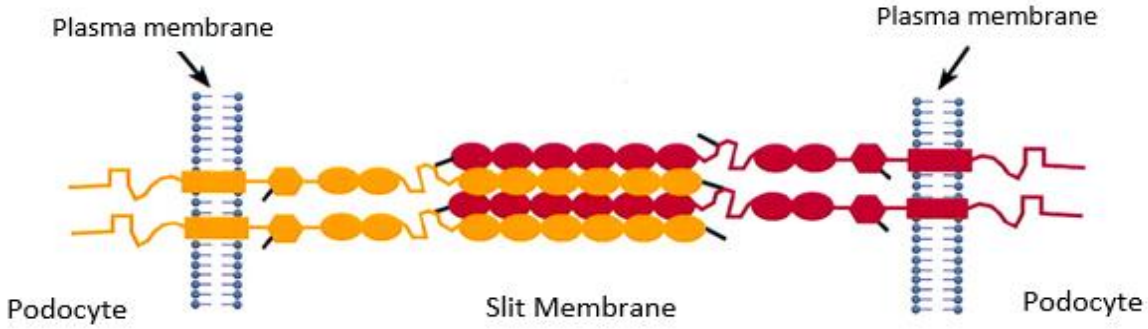


Figure 2.13. Theoretical model of nephrin association to form the isoporous filter of the podocytes slit diaphragm.

Probable mode of the interdigitating relationship of four nephrin molecules in the slit between two-foot processes. Nephrin molecules from opposite foot processes are shown in different colours. In this model, it is supposed that immunoglobulin repeats 1–6 of a nephrin molecule of a one-foot process subordinate in an interdigitating fashion with repeats 1–6 in a neighbouring molecule from the opposite foot process. Cysteine residues are illustrated by black lines and two potential disulfide bridges crosslinking four nephrin molecules in the centre of the slit are demonstrated. The remaining single free cysteine present in the fibronectin domain may react with another nephrin molecule or some other yet unknown molecule, which may connect with the plasma membrane or cytoskeleton (59).

2.9.3. Nephrinuria and glomerular disorder

Nephrinuria is the presence of nephrin in urine and it shows damage to the GFB (21). In glomerular filtration, podocytes prevent plasma proteins from entering the urinary ultrafiltrate. The structural and functional interactions of podocytes are important to understand, as the distinct layers of the filtration barrier serve as the structural support of the glomerular capillary. These characteristics allow (1) regulation of selective permeability of the glomerulus (molecule size and charge-dependent), (2) enable the filtration of cationic molecules, electrolytes, and small solutes, but restrict the passage of anionic and macromolecules; and (3) contribute to the remodelling of the GBM during injury and endocytosis of filtered proteins (7, 8). Adverse changes in molecular structures and arrangement in the podocytes of the Sd of the glomerulus causes podocyte injuries. The injury contributes to the advance of glomerular diseases due to the insufficiency of the GFB, resulting in massive proteinuria (9); the conditions that cause podocyte injury are called podocytopathies.

2.9.4. The relationship between nephrinuria and glomerular injury.

It is briefly described in animal and human models that nephrinuria may be an early marker for glomerular injury. Nephrinuria has also an association with the severity of the glomerular injury.

2.9.4.1. Preclinical studies-the relationship between nephrinuria and glomerular injury.

It is well established in animal models that nephrinuria is a reliable indicator of glomerular injury and monitoring of the progression of injury. In a recent study in an experimental model of mice, Chen et al. assessed the role of podocyte impairment in the causes of ischemia-reperfusion of AKI and fibrosis after injury (50). The investigators revealed that proteinuria, podocyte injury, and advancement into chronic renal fibrosis were perceived in mice with AKI. In a similar experimental model, Verma et al. explored the role of nephrin in podocyte recovery after injury (52). The investigators produced an inducible model of nephrin deletion using tamoxifen induction in healthy adult mice. They observed a 75% decrease in nephrin expression by 14 days independent of podocyte loss. The authors proposed nephrin as an initial event in proteinuria kidney disease and suggested it may also play a role in kidney

disease progression. In addition, other experimental studies have also shown that nephrinuria is associated with glomerular injury (37, 91, 239, 247) **(Table 2.3).**

Table 2 3: Pre-clinical studies showing the relation between nephrinuria and glomerular injury.

Authors	Animal	Disease Model	Nephrin assay	Findings
Nakatsue et al. (2005) [37]	Wistar Rats	Membranous nephropathy	Western blotting	• Nephrin is detected in urine in the early stages of nephritis
Alter et al. (2012)[239]	Wistar rats	Streptozotocin-induced diabetes mellitus	ELISA (USCN Life Sci. Inc. Burlington, NC, USA)	• Nephrinuria occurs in urine preceding albuminuria
Chang et al. (2012)[91]	Akita Mice	T1DM and diabetic nephropathy	Eth-Bio ELISA (Philadelphia, USA)	• Nephrinuria is noticeable early in kidney disease process. • Nephrinuria has a positive correlation with albuminuria
O'Brien et al. (2013) [247]	KK-A^{y} Mice	Type 2 diabetes mellitus (T2DM)	Eth-Bio ELISA (Philadelphia, USA)	• Nephrinuria increased in glomerular injury. • Nephrin correlates with albuminuria
Verma et al. (2018)[52]	Mice	Tamoxifen induced Proteinuric kidney disease	Histochemistry	• Reduced nephrin expression correlates with heavy proteinuria. • Reduced nephrin expression occurs preceding podocyte loss

2.9.4.2. Clinical studies showing the relation between nephrinuria and glomerular injury.

Over the past decade, human studies have made nephrin a biomarker of interest in glomerular injury because it is one of the structural and functional components of the glomerulus which appears in urine at the early stage of the glomerular injury (**Table 2.4**).

Glomerular injury can originate as early as the time of birth and it is well known that prematurity is a risk factor for glomerular injury (79). Kandasamy et al. assessed the influence of prematurity on kidney development and function (68). The investigators included 53 premature neonates born at < 28 weeks of gestation, with birth weight between the 10^{th} and 90^{th} centile (appropriate for gestational age), and 31 term neonates. The median gestational age of premature neonates was 26.4 weeks. They undertook assessments at 28, 32, and 37 weeks postmenstrual age. The authors reported a statistically substantial decline in urinary NCR and ACR from 32 to 37 weeks of postmenstrual age. Importantly, urinary NCR was higher in preterm neonates in early postnatal life, which suggests a sign of glomerular injury and/or glomerular immaturity because of being born early.

In more established glomerular disease in children, Wang et al. investigated urinary podocytes and nephrin as markers of glomerular disease (54). In this study, 65 children with nephrotic syndrome were recruited. Investigators found 53.8% urinary podocyte and 50.8% nephrin positivity rate in urine sediment of children with glomerular disease using ELISA, Western blotting, and indirect immunofluorescence techniques to measure podocyte and nephrin concentrations. In podocyte and nephrin-positive children, ACR and SCr were significantly elevated compared to their negative counterparts. The results showed that urinary nephrin expression was positively related to podocyte and urinary ACR concentration. The investigators concluded that urinary podocytes and nephrin can be used as non-invasive early markers for children with glomerular disease.

In the adult population, similar findings were reported, for example, in adult patients with T2DM, an increased level of nephrin was evident compared to albumin in the presence of nephropathy. Lioudaki et al. recruited 71 T2DM patients with normoalbuminuria and 39 non-diabetic controls, to determine mRNA abundance of podocyte-specific markers (synaptopodin, podocin, and nephrin) in urine sediment using PCR (23). The investigators

showed nephrin and/ or podocin were detected in a higher proportion of T2DM patients with normoalbuminuria compared to controls, which may indicate early podocyte injury. Similarly, Petrica et al. recruited the same cohort of patients (38 T2DM with normoalbuminuria, 32 with microalbuminuria, and 21 healthy controls) to evaluate proximal tubule dysfunction with podocyte biomarker excretion using ELISA (Elabscience Biotech Co. Ltd, Wuhan, Hubei Province, China) methods (55). The investigators indicated the association of proximal tubule dysfunction with podocyte injury biomarkers, even in the normoalbuminuria stage. The study further highlighted a correlation between nephrinuria and albuminuria. Therefore, urinary nephrin could be a valuable marker in the early diagnosis of kidney injury preceding albuminuria.

Similarly, in pregnant women who experience PE, there is some evidence that nephrin precedes protein in the urine. A growing body of evidence has demonstrated that nephrinuria may have a role in the pathogenesis of proteinuria during pregnancy, including disorders such as PE (24, 41), this suggests that nephrin could be a good marker of glomerular injury. In the past, the gold standard test for diagnosis of proteinuria or glomerular injury during pregnancy was renal biopsy to identify PE from other glomerular diseases. However, the test is invasive and not performed frequently (130). Instead, high blood pressure was used as a clinical marker for PE, but for women who have pre-existing hypertensive disease, the diagnosis was clinically challenging. For these reasons, nephrin may have the potential for the prediction and diagnosis of PE.

Comparably, a systematic review by Kerley et al. and Kandasamy et al. aimed at the role of nephrin in glomerular injury during PE, showed that urinary nephrin suggested a better marker for PE (24, 41). The investigators decided nephrinuria was a good indicator of glomerular injury. In addition, Yang et al. demonstrate the predictive ability of nephrin for PE, in relation to sensitivity (67-73%) and specificity (89%) (149). Furthermore, Sun et al. also indicated that hypertension (also experienced by women with PE) may cause podocyte injury and detachment from the GBM, resulting in podocyturia (50). This was also supported by Wang et al. in a study that reported that hypertension associated with increased oxidative stress plays a significant role in inducing podocyte protein shedding in PE (254). The authors recommend that nephrinuria detected early in the development of hypertensive renal injury.

Moreover, the predictive ability of 3 urinary biomarkers (podocyturia, nephrinuria, and albuminuria) was compared on 91 pregnant women who developed PE (255). The sensitivity and specificity of podocyturia were 70% and 43%, albuminuria was 36% and 96%, and nephrinuria was 57% and 58%, highlighting that at the late stage of PE, the nephrinuria may be gradually decreased because of podocyte loss and detachment from GBM. The ACR had high specificity (96%) which shows severe damage in the GFB. However, individually the three markers had low positive predictive values (14-63%), which signifies less sensitive markers for PE, but a good negative predictive value (89-91%) which indicates these markers may be used as early predictors for PE. Together, the authors reported that they believe the predictive ability of the three markers are minimal for clinical use.

In general, there is sufficient evidence to suggest that urinary nephrin may be used as a marker for detecting early glomerular injury as it precedes albuminuria. However, the measurement of urinary nephrin requires further validation for the purpose of introducing the test from pre-clinical to clinical practice to identify, monitor and prognosticate kidney injury.

Table 2 4: Human studies reported the relationship between nephrinuria and glomerular injury.

Authors	Clinical condition	Nephrin assay	Findings
Mehta et al. (2012)(256)	Preeclampsia	Eth-Bio ELISA (Philadelphia, PA)	• Urinary NCR is prognostic of PE in the second trimester of pregnancy
Son et al. (2013)[257]	Preeclampsia	ELISA (R&D Systems, Minneapolis, MN, USA)	• Nephrinuria was significantly higher in women with severe PE. • Positive correlation between urinary nephrin and urine protein level
Wang et al. (2007)[258]	Diabetic nephropathy	mRNA RT-PCR	• Nephrinuria elevated in patients with DN compared to control. • Nephrinuria associated with proteinuria
Wang et al.2012[259]	Preeclampsia	Eth-Bio ELISA (Philadelphia, PA)	• Urinary nephrin was significantly increased and highly correlated with PE. • Nephrin was correlated with proteinuria in PE
Jim et al. (2014)[255]	Preeclampsia	Eth-Bio ELISA (Philadelphia, PA)	• Sensitivity and specificity of nephrinuria was 57% and 58%
Kandasamy et al. (2018)[68]	Premature birth	Eth-Bio ELISA (Philadelphia, PA)	• Urinary nephrin: creatinine ratio was elevated in preterm neonates in early postnatal life
Chen et al. (2019)[50]	Glomerular injury	LS-Bio ELISA (Seattle, USA)	• Urinary nephrin is significantly associated with increased risk for AKI and neonatal ICU mortality
Kostovska et al. (2020)[75]	Diabetic nephropathy	Eth-Bio ELISA (Philadelphia, PA)	• Urinary nephrin is a sensitive and specific marker of glomerular injury
Kostovska et al. (2021)[260]	Preeclampsia	Eth-Bio ELISA (Philadelphia, PA)	• Urinary nephrin has a potential diagnostic marker for PE
Devanath et al. (2022)[261]	Hypertension	Elabscience, Biotech Co. Ltd, Wuhan, China	• Urinary nephrin is a potential marker for glomerular injury in untreated hypertensive patients
Kishore et al. (2021)[262]	Diabetic nephropathy	Eth-Bio ELISA (Philadelphia, PA)	• Urinary nephrin is a sensitive and specific marker of glomerular injury
Heimlich et al. (2018)[248]	Glomerulopathy	Eth-Bio ELISA (Philadelphia, PA)	• Urinary nephrin is a sensitive and specific marker of glomerular injury

2.10. Translations of novel biomarkers into clinical practice

The search for a novel biomarker for early glomerular injury is increasing in the modern area of biomedical research. Recently, the conventional urinary markers such as proteinuria and microalbuminuria for glomerular injury (43), UO and SCr (220, 221), and urine microscopic examination for casts have been not sensitive and specific for early prediction of kidney injury. In addition, kidney functional markers such as filtered high molecular weight proteins, tubular enzymes, proteins, inflammatory markers have also shown poor specificity. Animal models and recent human preliminary studies showed nephrin is a novel biomarker for the early detection of glomerular injury in different settings (24, 50, 52, 246, 248).

Currently, there are some renal damage-associated markers for glomerular injury including albuminuria, proteinuria, and casts in microscopic examinations, and for early prediction of AKI, which are accepted by the U.S Food and Drug Administration for clinical practice. Plasma NGAL measurement has a point of care test) (Triage® NGAL device, Biosite Incorporated, San Diego, CA) and urinary NGAL measurement has a rapid assay (ARCHITECT® Analyser, Abbott Diagnostics, IL); these have been endorsed in many settings (263). In addition, KIM-1 dipstick-RenaStick is also approved for KIM-1 detection (128). Further, in 2014, the Food and Drug Administration approved the Nephrocheck urine test for the combination biomarker IGFBP-7 and TIMP-2 for predicting moderate to severe AKI within 12 hours in critically ill patients (264). The role of this combination biomarker has also been assessed for the clinical use of kidney injury risk assessment (237). However, the investigators suggested a need for further clarification, to use these biomarkers for diagnosis, and prognosis of kidney injury.

In general, once a robust biomarker has already been identified and tested in preclinical studies, it might be translated to clinical practice. However, there is evidence that translation for clinical use is not always straightforward. A body of growing evidence in diagnostic test development suggests a basis for evaluating and validating novel biomarkers before translation to clinical use. Recently, there are no point of care tests approved for other biomarkers and there is no indication that any will be available soon. Of late, the measurement of urinary nephrin using immunoassay has gradually become the analytical technique of choice. Indeed, immunoassays will continue to be required for novel biomarker measurement. Therefore, the review in the following sections also tries to explore the critical

steps to assess immunoassay biomarker performance and the clinical relevance of a biomarker through scientific evidence to correctly evaluate and validate nephrin as a biomarker of early glomerular injury for clinical use.

2.10.1. Validation of immunoassay enhance translational research.

The International Organization for Standardization, ISO 15189, and the National Association of Testing Authorities, Australia (NATA) define validation as approval through the provision of unbiased confirmation that a method is fit for purpose, or an application has been fulfilled (265, 266). The organisations recommend defining laboratory tests, whether they will produce accurate and precise results in the context of the proposed use. Moreover, the anticipated use of an assay needs to be carefully balanced between cost, risk, and technical possibilities before validation experiments are performed (267).

Validation studies can be grouped as primary and comparative. Comparative validation compares the performance of two or more methods to yield data within the same or across different studies. The first validated method serves as a gold standard and the new method as a comparator (265). To compare the performance, mean, a one-way analysis of variance (ANOVA), or a paired t-test by sample type and analyte concentration is performed, whereas primary validation is used when comparative validation is not applicable, and validation must be started before introducing the method into clinical practice. In this case, the primary validation becomes an investigative process to institute operational limits and performance features of the new or inadequately characterised method (265).

Immunoassay validation is a wide-ranging experiment that evaluates and documents the quantitative performance of an assay ensuring an appropriate method for its purpose, including specificity/interference study, recovery, parallelism, dilutional linearity, accuracy, precision, the limit of detection (LOD), assay dynamic range and limits of quantitation (LOQ) and stability of the analyte (265, 268). Furthermore, if a method is established in-house, a complete validation, which includes intra-assay and inter-laboratory assessment, should be investigated for all parameters of validation. However, partial validation of a commercially prepared immunoassay may not need to be investigated for robustness, because in most cases it should have been covered by the company during the method development process (265, 267). In addition, partial validation and revalidation are also valid, when a method is

relocated to another laboratory to be run on another apparatus by a different person, and following changes in reagents, instrumentation, and protocol since the precision and LOQ, are more sensitive to changes, while more intrinsic material goods for a method such as dilution of linearity and recovery are not likely to be affected (265, 267).

The validations of assays for different types of analysis can be applied for different analyses, as it depends on the difference in the sample's matrix or analytical technology employed (269). Hence, several investigations supported that all diagnostic methods must be validated before being introduced for clinical studies, to ensure that the values reported will meet clinical expectations with the expected degree of reliability (265, 267, 270).

2.10.2. Parameters considered in immunoassay validation.

An immunoassay method validation, testing the parameters of analytical procedures using performance indicators to establish a protocol comprising well-defined procedures and criteria is required. The validation parameters are dilution of linearity, robustness, recovery experiment, analytical specificity, LOD, LOQ and precision experiments, and sample stability (267).

2.10.2.1. Linearity of dilution

The linearity of dilution is an important parameter for validating ELISA and other analytical techniques, which provides information about the predictability of a sample recovery from a known set of dilution factors in the desired assay range. It governs to what degree the dose-response of the analyte is undeviating in a diluent within a range of the standard curve. At a similar time, the occurrence of the hook effect, meaning suppression of signal at a concentration above the upper LOQ is investigated (267, 271), and the assay recovery is calculated by comparing observed versus expected values.

2.10.2.2. Robustness

Robustness is the extent to which test results remain unaffected by small variations from investigational conditions, including pH, humidity, temperature, incubation time and reagent concentration, reagent lot number, sample type, sample handling, sample quality, and instrument model (272). It tells us the reliability of the methods during a specific purposefully designed challenge or identifies factors that lead to changes in measurements of results at

different times and different facilities (265, 273). In a quantitative method, it is considered a useful prediction of expected precision (272). In the case of an in-house method, it could be examined as a part of the method development process and the effects should be revealed in the assay procedure before other validation considerations are inspected.

2.10.2.3. Recovery experiment

A recovery experiment is carried out to explore if the concentration-response association is comparable in the calibration curve and the samples. It can be determined by adding (that is, spiking) a known concentration of analyte into the sample. Its response is measured by comparison to standard diluent spiked with the same concentration of the analyte. The recovery experiment maximizes the signal-to-noise ratio while achieving a similar response for a concentration of an analyte in a standard diluent (standard curve) and sample matrix (sample + sample diluent). The aim is to determine and evaluate the interfering factors in the sample matrix (271). The percent recovery was calculated by dividing observed concentration of the spiked sample to the expected concentration and multiplied by 100.

2.10.2.4. Analytical interferences

Specificity is the capacity of the test to measure exactly an analyte in the presence of interferences that may be predictable to be existent in the sample matrix. The test aims to confirm that the analyte concerned is identified without cross-reactivity with other closely interrelated molecules (274). To warrant that only the anticipated analyte should be detected, a panel of other substances will be tried for cross-reactivity. Experience from the laboratory work or literature to test the interference that most likely affects measurement methods is valuable. Evidence to date shows that the intracellular portion of *nephrin* has no homology with other proteins, the antibody is specific, but a study by Janech et al. has revealed that the Exocell nephrin ELISA on mouse urine sample appears to cross-react with mouse albumin (275); the authors recommend testing of species-specific albumin cross-reactivity during validation of the Eth-Bio Exocell ELISA kit.

2.10.2.5. Limit of detection (LOD) and limit of quantification (LOQ)

LOD is the lowest concentration of the substance that can be distinguished from negative control, and LOQ is the lowest concentration of analyte in a sample that can be quantitatively

determined with precision and accuracy (276). The determination of LOD or LOQ is necessary for methods proposed to measure the analyte at an amount measured close to zero. Determining LOD or LOQ for methods that will be applied to measure analyte concentrations higher than the LOQ is not mandatory. However, the evaluations have significance for trace and ultra-trace methods where values of concern are often close to the LOD and LOQ. Values reported as 'not detected' may have a substantial impact on regulatory decisions (265, 277). There are different methods for determining the LOD, such as based on visual inspection/ endpoint dilutional analysis, a standard deviation of the blank, the range in which the calibration equation applies, and signal to noise ratio (265).

2.10.2.6. Sample stability

Determining the chemical stability of the analyte, including the effects of sample collection, handling, and storage of the analyte should be documented during validation. Since chemical breakdown may occur during storage, analyte stability would be assessed by the analysis of the sample and known concentration solutions during a pre-selected period, allowing the estimation of the highest interval between sample processing and analysis (265). The stability of the analyte to be measured in the biological matrix can be determined by the properties of the analyte, the matrix, and the storage environments, including the sample bottle, storage temperature, and exposure to light. A worry regarding analytes in a biological matrix is the freeze-thaw cycles (278). The US Food and drug administration endorses that "at least 3 aliquots at each of the low and high values could be stored at the proposed storage temperature for 24 hours and thawed unassisted at room temperature"(279) and the freeze-thaw stability quality control must be compared with recently prepared calibration curve and quality controls.

2.10.3. Reference intervals for biomarkers level in a healthy population

The reference interval (RIs) is the interval between two reference boundaries that includes, usually, 95% of the reference values (280). The RIs are the last step to be studied in the method validation process (281). However, it is not included as a parameter in the decision on method acceptability. It is established after methods performance is acceptable, to support the interpretation of the test results and medical decisions (282).

In consistencies have been reported in clinical laboratory test results from societies in different geographical regions and populations. The variation is frequently driven from genetic, nutritional, demographic, and environmental factors. Despite this, manufacturer manuals and other countries derived RIs are adopted in different settings for clinical trial suitability, and other metabolic abnormality determination. This leads to misinterpretation of laboratory test results. RIs are established from reference populations that meet carefully defined criteria. The approaches for sampling are *a priori* approach (settled predefined criteria before sample collection) and a *posteriori* approach (specimen obtained from the population involved in the analysis established on clinical details or test results, which were not used to define the collection but not all specimens encompassed in the target population for additional analysis) selection process (283).

The International Federation of Clinical Chemistry (IFCC) expert panel on the theory of reference values and the National Committee of Clinical Laboratory and Standards (NCCLS) defined a recommended method for determining the RIs (284), as non-parametric, parametric, and robust methods. Therefore, establishing RIs for coherent interpretation of studies and clinical trials using promising biomarkers for kidney injury is valuable.

2.10.3.1. Non-parametric method to establish RIs

The non-parametric method is a simple way to perform RIs using ranks, and no assumptions as to the underlying distribution of the data needed (282). To calculate the central 95% RIs, the samples are sorted from the least to the highest and ranked using the whole number, requiring the 2.5^{th} and 97.5^{th} percentile to be distinguished. The NCCLS recommends the best way is to establish RIs from reference individuals of at least 120 observations for each partition (284). However, for very expensive tests, samples from the very young and old in the study population of interest (i.e., rare cases), obtaining this sample size is challenging. A study by Friedberg et al. showed that 75% of laboratories utilise less than 100 individuals for each partition while they run the RIs (285). Importantly, in the case of RIs calculated from a small sample size, the non-parametric statistics use extreme values; the RIs are therefore susceptible to influence by outliers. Bjerner et al. showed that non-normally distributed data should warrant the transformation or removal of an outlier (286). However, the IFCC and

NCCLS recommended method for establishing the RIs is non-parametric, which comprises the lowest and highest 2.5% of reference values (287).

2.10.3.2. Parametric method to establish RIs

The parametric method assumes normal Gaussian distributed data, the mean and median of the data set should be similar. However, it is not always applicable, because data are seldom normally distributed. Similarly, for skewed data, the parametric transformed reference interval method can be used. If the parametric method is used to establish RIs, the normality test, a histogram normal quantile plot should check the normal distribution of the data (287, 288), even though the method provides an exact desirable level for RIs, it is not realistic in practice.

2.10.3.3. Robust methods to establish RIs

A robust method uses a small sample size and can resist outlier influence by "down weighting" values farther from the Centre of the sample. Gaussian distribution is not required for this method, transformation to a more symmetric dataset to help better estimate RIs for skewed distributions is recommended, then the Tukey approach is used to identify an outlier cutoff, and the data is trimmed to remove outlier data that are beyond the outlier cutoff. The trimmed set of the data is then used to compute the RIs. In addition, the trimmed sets of data are power transformed to establish the RIs (282). Notably, robust methods allow for the determination of RIs when large samples are not available, as required by non-parametric methods if the population of interest is very young (neonates, preterm births, very low birth weights), invasive measurements, or expensive to obtain. Thus, a robust method is cost-effective and gives a greater degree of confidence in calculating RIs and efficiency in estimating RIs (282).

Most laboratories implement RIs determined by companies rather than developing for a specific population. A study by the College of American Pathology surveyed 500 laboratories (289). They identified about 78% of the laboratories adopted the RIs of the manufacturer's published values. Interestingly, a study intended to evaluate the uptake of RIs endorsed by the Australian Association of Clinical Biochemistry and the Royal College of Pathologists of Australasia for 11 common biochemistry tests in Australia, found that uptake of the suggested

upper and lower limits increased from 40 to 83% over 3 years (290). Moreover, International organisations acclaim population-specific laboratory RIs, because RIs can be affected by many factors such as the instrumentation, reagents, methods used for the determination of an analyte, the geographical locations of the studied population, age, ethnicity, race, diet, and sex of the population being studied. Likewise, the IFCC Committee conducted a global multicentre study on RIs and two RI studies on plasma proteins primarily aimed at developing conjoint RIs for major serum proteins in East and Southeast Asian countries in 2000 and 2004; it revealed apparent between countries variations in many of the analytes, mainly those of inflammatory markers (291-293). Therefore, establishing RIs for a specific population becomes a major concern in many parts of the world.

2.11. Chapter summary

In conclusion, although kidney disease and /or injury have been reported as the fastest-growing public health problem in neonates, young adults, and old age groups of the population, a validated novel biomarker that specifically detects glomerular injury and predicts AKI, has not been well examined. Preclinical and clinical studies on DN, glomerulonephritis, and PE have shown evidence of nephrinuria as a potential marker for glomerular injury. However, neither its diagnostic performance in detecting glomerular injury nor its role in monitoring the prognosis and predicting the outcome of kidney injury has been fully reported in the literature. A more thorough investigation into the contributions of nephrinuria in the early prediction of glomerular injury is required. It is crucial to confirm the specificity and sensitivity of nephrin measurement in patient samples from large cohorts, and for different clinical situations. In addition, developing urinary nephrin RIs within population-specific settings may enhance the accurate interpretation of numerical laboratory test results for glomerular injury in paediatric, as well as the adult population at risk of developing glomerular injury.

The next chapter (**part B of the literature review**) will present a systematic review and meta-analysis on the pooled diagnostic accuracy of urinary nephrin for determining early glomerular injury. The review will also present a subgroup analysis for determining the diagnostic accuracy of urinary nephrin based on clinical reasoning and commonly used methods for analysis of urinary nephrin from the included articles.

Foreword Chapter 2-Part B

This chapter is a published systematic review and meta-analysis article (*Mesfine BB, Vojisavljevic D, Kapoor R, Watson D, Kandasamy Y, Rudd D. Urinary nephrin—a potential marker of early glomerular injury: a systematic review and meta-analysis. Journal of Nephrology. 2023 Feb 20:1-3. Doi: 10.1007/s40620-023-01585-0*) that addresses the diagnostic accuracy of urinary nephrin to predict glomerular injury. It is an exact copy of the journal article, except for the formatting of section subheadings, the figures, tables, and abbreviations, units of measurement, and referencing style which have been modified for the purpose of this thesis. The abstract of the published article is attached in the appendix section of this thesis.

Chapter 2. Part B: Systematic Review and Meta-Analysis

Urinary nephrin - a potential marker of early glomerular injury: a systematic review and meta-analysis

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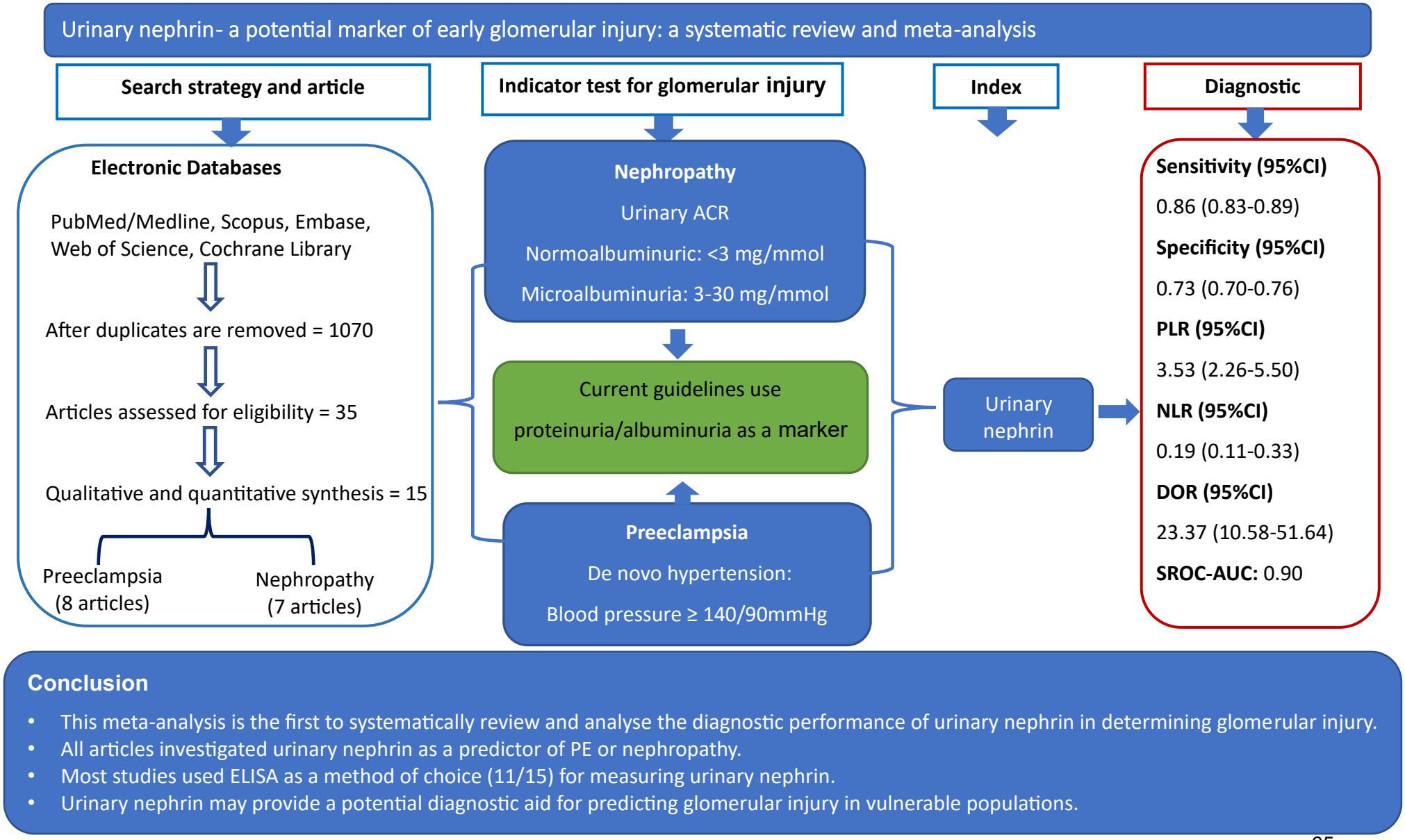

Figure 2B.1. Graphical abstract

2.1. Abstract

Background: Both early recognition of glomerular injury and diagnosis of renal injury remain important problems in clinical settings and current diagnostic biomarkers have limitations. The aim of this review was to determine the diagnostic accuracy of urinary nephrin for detecting early glomerular injury.

Methods: A search was conducted through electronic databases for all relevant studies published until January 31, 2022. The methodological quality was evaluated using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool. Pooled sensitivity, specificity, and other estimates of diagnostic accuracy were determined using a random effect model. The Summary Receiver Operating Characteristics (SROC) was used to pool the data and to estimate the area under the curve (AUC).

Results: The meta-analysis included 15 studies involving 1587 study participants. Overall, the pooled sensitivity of urinary nephrin for detecting glomerular injury was 0.86 (95% CI: 0.83-0.89) and specificity was 0.73 (95% CI: 0.70-0.76). The AUC-SROC to summarise the diagnostic accuracy was 0.90. As a predictor of PE, urinary nephrin showed a sensitivity of 0.78 (95% CI: 0.71-0.84) and specificity of 0.79 (95% CI: 0.75-0.82), and as a predictor of nephropathy the sensitivity was 0.90 (95% CI: 0.87-0.93), and specificity was 0.62 (95% CI: 0.56-0.67). A subgroup analysis using ELISA as a method of diagnosis showed a sensitivity of 0.89 (95% CI: 0.86-0.92), and a specificity of 0.72 (95% CI: 0.69-0.75).

Conclusion: Urinary nephrin may be a promising marker for the detection of early glomerular injury. ELISA assays appear to provide reasonable sensitivity and specificity. Once translated into clinical practice, urinary nephrin could provide an important addition to a panel of novel markers to help in the detection of acute and chronic renal injury.

Keywords: Urinary nephrin, nephrinuria, glomerular Injury

2.2. Introduction

Glomerular injury is structural damage to the glomeruli resulting in declining renal function. Glomerular injury, characterised by moderate to severe proteinuria (294), is well established as a prominent contributor to ESKD worldwide (38, 295). Early glomerular injury has been associated with podocyte loss and the development of proteinuria (296). Early glomerular injury may also contribute to AKI through progressive damage to nephrons (12). Repeated glomerular injury and loss of nephron function, lead to altered renal perfusion and hyperfiltration, leaving the remaining nephrons at greater risk of injury (297, 298).

Acute kidney injury also puts the kidney at risk of long-term damage. AKI is a clinical term that describes a spectrum of injury events that set the scene for further renal damage (12). AKI is complex and has a varied aetiology including, haemodynamic changes, oxidative stress (299), endothelial damage (300), mitochondrial damage, and immune mediated mechanisms (299) about 10% of cases arise from glomerulopathies (301). A number of studies have investigated novel markers for detecting AKI. Choice of these markers reflects the varied aetiologies of AKI, including but not limited to KIM-1, cell cycle arrest markers TIMP-2 /ILGFBP, NGAL and interleukin – 18 (302). These markers are the subjects of numerous excellent reviews and meta-analyses (303, 304). More recently, attention has turned to investigating the appearance of podocyte proteins in urine following AKI in a number of clinical settings, following surgery and ischemia reperfusion injury (50, 53). In order to provide a complete clinical picture of acute and chronic renal damage, the addition of a sensitive indicator of glomerular injury could prove valuable.

Glomerular injury, indicated by the leakage of cells and proteins into the urine (294, 305, 306) is used as a clinical indicator for glomerular damage. There are several well-established biomarkers used for diagnosing and monitoring glomerular damage either alone or in combination (38, 255, 307-309). However, to date, no biomarker has been identified for early detection of acute glomerular injury (310, 311). Recent studies have suggested that podocyte proteins may be a better marker for detection of early glomerular injury (41, 75, 260, 312). A number of studies have found nephrin to be promising early marker for glomerular injury. (21, 24, 61, 75, 313, 314). Nephrin, a 180 KD transmembrane protein is an integral structural component of glomerular podocytes (56), belongs to the immunoglobulin superfamily of cell adhesion receptors, and is expressed in glomerular podocytes (56, 59).

The use of urinary nephrin as an indicator of glomerular damage for the prediction of PE and glomerular nephritis has been well studied. A nationwide cohort study revealed that there is a strong association between PE and later glomerular injury (315) and another study also showed glomerular injury in diabetic nephropathy (75); these studies have revealed that glomerular injury may occur irrespective of proteinuria, and nephrinuria is often detected prior to proteinuria/albuminuria and urinary nephrin levels correlate with disease severity (61, 260, 262).

To date, no study has systematically reviewed and analysed the diagnostic accuracy of urinary nephrin for determining glomerular injury in patients with acute and chronic renal injury. This review aims to systematically explore the literature to determine the pooled sensitivity and specificity of urinary nephrin for determining glomerular injury.

2.3. Materials and Methods

2.3.1. Design and protocol registration

This review was performed in accordance with the Preferred Reporting Item for Systematic Review and Meta-analysis Protocol (PRISMA-P 2020) guideline (316). The review protocol was developed before literature searching and was registered with the International Prospective Register of Systematic Reviews (PROSPERO) database with a registration number CRD42022309659.

2.3.2. Data source and search strategy

This meta-analysis is intended to explore the diagnostic accuracy of urinary nephrin as a biomarker of early glomerular injury. The literature search for eligible studies was performed using an electronic databases PubMed/Medline, SCOPUS, EMBASE, Science Direct, Web of Sciences, and Cochrane Database Library of Systematic Reviews from commencement to January 31, 2022. An updated search on August 26, 2022, yielded no additional articles relevant to the topic. We also performed a manual search using Google, and Google Scholar after retrieving articles from the database.

The database was systematically searched in accordance with the Medical Subject Headings Thesaurus (MeSH) and Boolean operators (AND, OR) terms. The key terms used in searching were “glomerular injury” AND “urinary nephrin” OR “nephrinuria”. To capture more articles

on early glomerular injury and AKI additional search key terms included separately as “Preeclampsia” OR “PE” AND “urinary nephrin” OR “nephrinuria”; “nephropathy” OR “Diabetes nephropathy” AND “urinary nephrin” OR “nephrinuria”; “Acute Kidney Injury” OR “AKI” AND “urinary nephrin” OR “nephrinuria”. The search keywords were searched alone and all possible combinations with other keywords. Moreover, references from retrieved articles were also reviewed to identify cited articles not captured by electronic database searches.

2.3.3. Study selection

Original articles that explored the performance of urinary nephrin in the diagnosis of glomerular injury were included. The authors used EndNote X9 (Thomson Reuters, New York, USA) bibliography manager to check the title and abstracts of the articles and then retrieved and rescreened the selected articles. Duplicated articles were removed electronically, and manually if differences in the citation style of the different journals existed. The reference lists of the eligible articles were checked to find additional relevant articles.

The inclusion and exclusion criteria were systematically applied to studies before they were included in the meta-analysis. Studies eligible for meta-analysis included those that measured urinary nephrin, and studies reporting mandatory data from which the diagnostic accuracy of urinary nephrin could be calculated and which used a reference standard test to classify glomerular injury based on the standardised guidelines. In this regard, PE was classified according to American Congress of Obstetrics and Gynaecology (ACOG) definition, while diabetic nephropathies were defined according to Kidney Disease Improving Global Outcome (KDIGO) guidelines based on measurements of urinary ACR as normoalbuminuria (ACR<3mg/mmol), microalbuminuria (ACR=3-30mg/mmol), and macroalbuminuria (ACR > 30mg/mmol) groups and articles published in English. Studies with duplicate data, review articles, articles’ failure to report necessary information, letter to editors, short communications and conference proceedings were excluded. Initially, 2 authors (BM & DR) independently reviewed the titles and abstracts of all articles to evaluate the eligibility of the articles. For the studies that could not be judged through the abstracts and titles, the full texts of the original articles were retrieved for detailed evaluation.

2.3.4. Outcomes of interest

The main outcome of interest of this meta-analysis was the pooled diagnostic accuracy of urinary nephrin (diagnostic sensitivity, specificity, and other estimates of diagnostic accuracy) for determining glomerular injury. Subgroup analysis was also performed to determine the diagnostic accuracy of urinary nephrin according to clinical conditions, the commonly used assay methodology, study design and reported units.

2.3.5. Data extraction and quality assessment

The data was extracted for all eligible studies. The basic characteristics of the studies were collected using a Microsoft Excel data extraction form, the name of the first author, year of publication, country, study design, sample size, clinical condition, method of analysis, reference test, reported cut-off values of urinary nephrin, the performance of the test including true positives (TP), true negative (TN), false positives (FP), and false negatives (FN), if applicable in the studies. If the studies did not report the mandatory outcome data, the 2x2 table was extracted from the study to calculate the TP/TN/FP/FN values.

The methodological quality of the studies was evaluated using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool (317) which is an improved, redesigned widely accepted, and validated tool to evaluate the source of bias and variation in diagnostic accuracy studies in systematic reviews. The tool includes 4 key realms such as patient selection (PS), index test (IT), reference standard (RS), and flow of patients through the study and timing index test and reference standard (FT). Each domain was assessed for the risk of bias and applicability and classified as “low risk of bias” and “low concern” was considered as having high methodological quality. Any discrepancies in the study selection, data extraction, and/or quality assessment were resolved by discussion with other authors to reach a final consensus. The QUADAS-2 tool scoring criteria was modified according to our aim (**Table 2B.2**).

2.3.6. Data synthesis and statistical analysis

The data were entered into Microsoft Excel and exported to Meta-Disc version 1.4 software (Complutense University of Madrid, Spain) (318) for analysis. The discriminatory power of a diagnostic test is commonly assessed by measuring how well it correctly identifies true

positive and true negative test results in terms of sensitivity and specificity (319). Pooled sensitivities and specificities, positive likelihood ratio (LR), negative likelihood ratio (LR), and diagnostic odds ratio (DOR) with a 95% confidence interval (CI) were obtained using the random-effect model (Dersimonian Laird methods) depending on the heterogeneity of the study group. Forest plots of sensitivities, specificities, positive LR, negative LR, and DOR were presented. Furthermore, the area under the curve-summary receiver operating characteristics (AUC-SROC) values with 95% CI and Cochrane indices (Q) were calculated. The AUC-SROC was calculated, and the value was defined according to the guideline recommended by Swets in 1988 (320) as excellent diagnostic accuracy AUC: 0.9-1.0, very good AUC: 0.8-0.9, good AUC: 0.7-0.8 and sufficient diagnostic accuracy with AUC: 0.6-0.7.

The magnitude of inter-study heterogeneity was assessed using visual inspection of the forest plots of accuracy estimates. If no heterogeneity is present, the estimates from individual studies lie along with a line corresponding to the pooled accuracy estimate, a large deviation from the pooled estimate indicates possible heterogeneity (318). Furthermore, statistically measured by the Cochrane Q test, a significant Q test ($P < 0.05$) suggests the presence of heterogeneity. Further assessment was carried out using the inconsistency index of heterogeneity (I^2 statistics) values of 25%, 50%, and 75% indicated to represent low medium, and high heterogeneity, respectively (321). To further assess the heterogeneity subgroup analysis were conducted based on different parameters including clinical conditions, diagnostic methods, study designs and reported units.

The threshold effect was evaluated by constructing the SROC, to assess for presence of shoulder arm pattern for each data point in the plot. A typical shoulder arm pattern indicates the presence of a threshold effect. Further assessment of the threshold effect was conducted and indicated by the presence of strong positive correlation using a computation of Spearman's correlation coefficient (r^2) between the logit of sensitivity and logit of 1-specificity (318).

2.5. Results

Overall, the initial search identified 1585 relevant articles through various database searches, of which 515 were excluded because of duplication. Of the remaining 1070 studies, 1035 were excluded after screening the titles and abstracts, as the articles are not relevant to the current review. Of these, 35 full-text articles were assessed for eligibility. After screening the full texts for calculable statistics, 15 studies that included 1587 participants, were included in the meta-analysis. All included studies were published between 2011 and January 2022. Flow diagram illustrating the process of the literature screening method is described in **Figure 2B.1**.

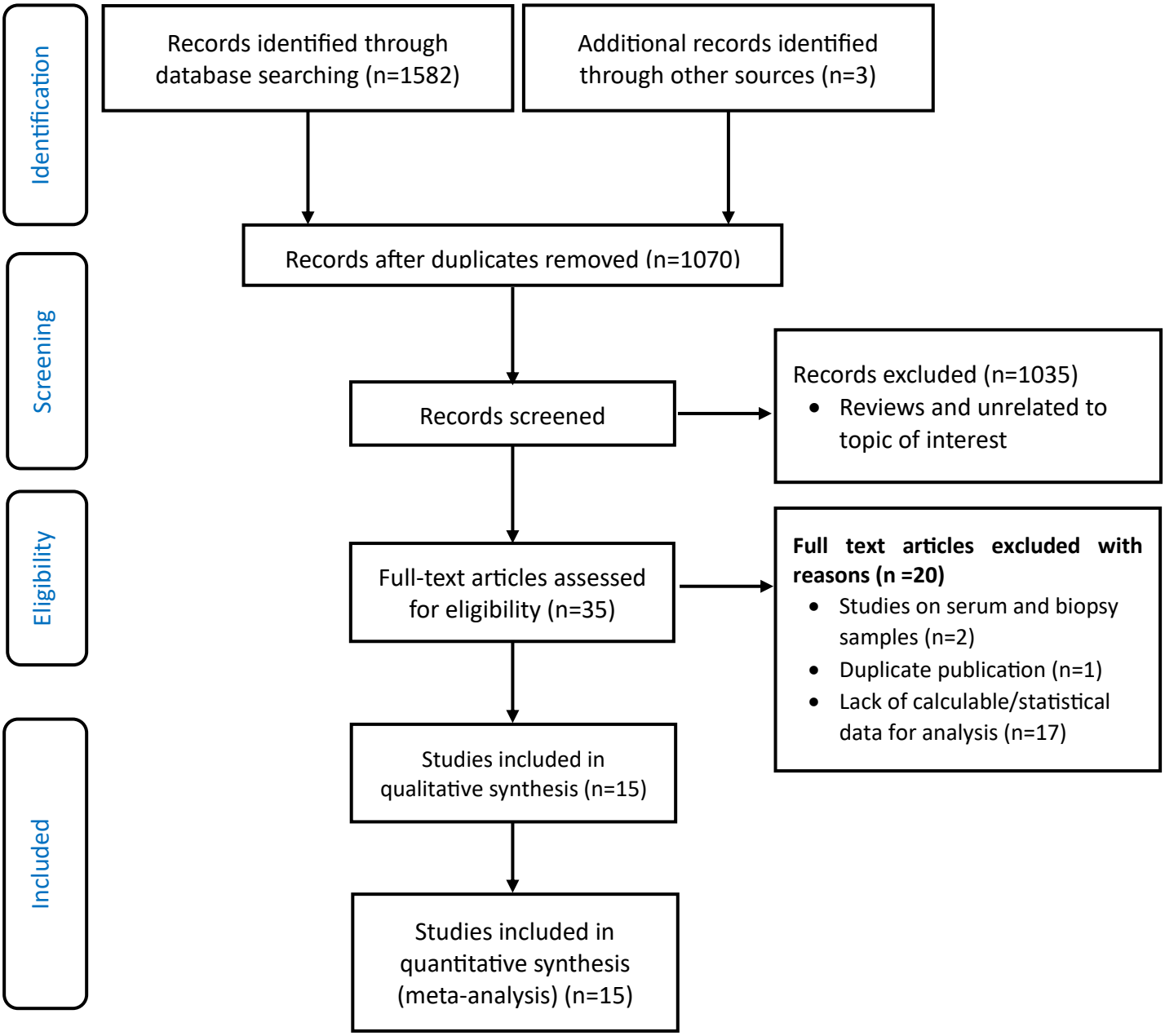

Figure 2B.2. The PRISMA flow diagram illustrates the process of studies reviewed and screened.

2.5.1. Characteristics of studies included for review.

An analysis of the 15 selected studies revealed that all studies aimed to investigate urinary nephrin as an early indicator for glomerular damage in both acute and chronic conditions. Eight studies utilised urinary nephrin for predicting PE (149, 255, 260, 312-314, 322, 323), six studies utilised urinary nephrin for predicting nephropathy (49, 75, 324-327), and one study utilised urinary nephrin for predicting glomerulopathy/glomerular injury (248). Most of the studies used Enzyme Linked Immunosorbent Assay (ELISA) (n=11) and the remaining used Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) (n=3), and Western Blotting (WB) (n=1) to determine the concentration of urinary nephrin. All studies reported urinary ACR and/or hypertension with proteinuria (>300 mg/day) as a reference indicator for glomerular injury. Most articles included in this review report urinary nephrin in two ways: urinary nephrin concentration (n=10) with reported cut-off values for nephrin concentration ranging from 85-850 ng/ml and urinary nephrin corrected by urinary creatinine concentration and reported as urinary nephrin to creatinine ratio (NCR) (n=5), with reported cut-off values ranging from 86.6-622ng/mg. Prospective cohort studies made up 47% of the included studies. The basic characteristics of the eligible studies are summarised in **Table 2B.1**.

Table 2B 1: Characteristics of studies included in the meta-analysis of urinary nephrin to determine early glomerular injury.

Authors name (year)	Country	Study design	Clinical conditions	Sample size	Index test	Methods	Reference test	Cut-off (nephrin)
Jim et al. (2014)[255]	USA	Cohort	Preeclampsia	91	Urinary NCR	ELISA	ACOG guideline	≥ 100 ng/mg
Yang et al. (2013)[149]	South Korea	Case-control	Preeclampsia	83	Urinary nephrin	ELISA	ACOG guideline	85 ng/ml
Kelder et al. (2012)[322]	Netherlands	Case-control	Preeclampsia	81	Urinary nephrin	RT-PCR	ACOG guideline	NR
Son et al. (2011)[312]	South Korea	Case-control	Preeclampsia	45	Urinary nephrin	WB	ACOG guideline	NR
Zhai et al. (2016)[313]	Japan	Cohort	Preeclampsia	89	Urinary NCR	ELISA	ACOG guideline	122 ng/mg
Zhai et al. (2016)[323]	Japan	Cohort	Preeclampsia	254	Urinary NCR	ELISA	ACOG guideline	86.6 ng/mg
Jung et al. (2017)[314]	South Korea	Cohort	Preeclampsia	117	Urinary nephrin	ELISA	ACOG guideline	850 ng/ml
Kostovska et al. (2021)[260]	North Macedonia	Cross-sectional	Preeclampsia	101	Urinary nephrin	ELISA	ACOG guideline	304.6 ng/ml
Kishore et al. (2021)[325]	India	Cross-sectional	Nephropathy	170	Urinary nephrin	ELISA	ACR JCDNP guideline	97.5 ng/ml
Kostovska. (2020)[75]	North Macedonia	Cross-sectional	Nephropathy	120	Urinary nephrin	ELISA	ACR KDIGO guideline	255 ng/ml
Heimlich et al. (2018)[248]	Malawi	Cross-sectional	Glomerulopathy	101	Urinary NCR	ELISA	ACR KDIGO guideline	622 ng/mg
doNascimento et al. (2013)[324]	Brazil	Cohort	Nephropathy	101	Urinary nephrin	RT-PCR	ACR KDIGO guideline	NR
Fayed et al. (2019)[326]	Egypt	Cohort	Nephropathy	80	Urinary nephrin	RT-PCR	ACR KDIGO guideline	≥3.30
Shahid et al. (2017)[327]	Pakistan	Cohort	Nephropathy	78	Urinary nephrin	ELISA	ACR KDIGO guideline	NR
Jim et al.(2012)[49]	USA	Cross-sectional	Nephropathy	76	Urinary NCR	ELISA	ACR KDIGO guideline	≥ 100 ng/mg

ACR: Albumin to Creatinine Ratio; ELISA: Enzyme Linked Immunosorbent Assay; RT-PCR: Reverse Transcriptase Polymerase Chain Reaction; NCR: Nephrin to Creatinine Ratio; WB: Western Blotting; ACOG: American Congress of Obstetrics and Gynaecology; JCDNP: Joint Committee of Diabetes Nephropathy; NR: Not Reported; KDIGO: Kidney Disease Improvement Global Outcome, I^2: Inconsistency Index

The quality and risk of bias of the studies were assessed using the QUADAS2 tool (317). Overall, the studies included in this review were found to be of good quality. While there was a low risk of bias observed in the studies, some studies such as Kelder, et al.2012 (322), Son, et al.2011 (312), do Nascimento, et al.2013 (324), and Shahid, et al.2017 (327) did not provide information on the index test interpretation and did not provide the cut-off value used to interpret the test. Other studies by, Yang, et al. 2013 (149), Kelder, et al.2012 (322), and Son, et al.2011 (312) introduced bias during patient selection (case-control studies) and reported insufficient data to judge the quality based on the criteria. The modified QUADAS-2 quality appraisal criteria checklist and scoring and percentages of each risk category are presented in **Table 2B.2** and supplementary **Figure 2B.3** respectively**.**

Table 2B.2: Modified QUADAS scoring results of the included studies to summarise the risk of bias and applicability concerns.

Studies	**Modified Quality Assessment of Diagnostic Accuracy (QUADAS) criteria**													
First Author (Year)	**Risk of Bias**										**Risk of Applicability**			
	PS	PS	PS	IT	IT	RT	RT	FT	FT	FT	PS	PS	IT	RS
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Jim et al. (2014)[255]	Y	Y	Y	Y	U	Y	Y	Y	Y	U	Y	Y	Y	Y
Yang et al. (2013)[149]	Y	N	Y	Y	U	Y	Y	Y	Y	U	Y	Y	Y	U
Kelder et al. (2012)[322]	Y	N	Y	U	N	Y	Y	Y	Y	U	Y	Y	Y	U
Son et al. (2011)[312]	Y	N	Y	Y	U	Y	Y	Y	Y	Y	Y	Y	Y	U
Zhai et al. (2016)[313]	Y	Y	Y	U	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Zhai et al. (2016)[323]	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Jung et al. (2017)[314]	Y	Y	Y	Y	U	Y	Y	Y	Y	Y	Y	Y	N	Y
Kishore et al. (2021)[325]	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Kostovska. (2020)[75]	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Heimlich et al. (2018)[248]	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
doNascimento et al. (2013)[324]	Y	Y	N	Y	U	Y	Y	Y	Y	U	Y	Y	Y	Y
Kostovska et al. (2021)[260]	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Fayed et al. (2019)[326]	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Shahid et al. (2017)[327]	Y	Y	N	Y	Y	Y	Y	Y	Y	U	Y	Y	Y	Y
Jim et al. (2012)[49]	Y	Y	Y	Y	U	Y	Y	Y	Y	Y	Y	Y	Y	Y

Modified QUADAS criteria CHECKLIST used for assessing the quality of studies included in the meta-analysis

	Yes	**No**	**Unclear**
• **The selection criteria are clearly defined, and a consecutive or random sample of patients enrolled?**			

- Was a case-control study design avoided?
- Were inclusion and exclusion criteria clearly stated?
- Was the diagnosis of glomerular injury made without knowledge of the index test results?

- Was the index test result interpreted in a blinded fashion?
- Was the study described the reference standard to classify the target condition?
- Were the reference standard results interpreted without knowledge of the results of the index test?
- Were results of the index and reference test collected on the same patients at the same time?
- Did all patients receive the same reference standard?
- Were all patients included in the analysis? Or were withdrawals from the study explained?
- Were samples collected from patients with a high risk of developing kidney injury
- Were patients recruited with symptoms consistent with kidney injury?
- Were the methods for testing sufficiently explained?
- Were intermediate test results reported?

Note: PS: Patient selection; IT: Index test; RS: Reference standard; FT: Flow and timing

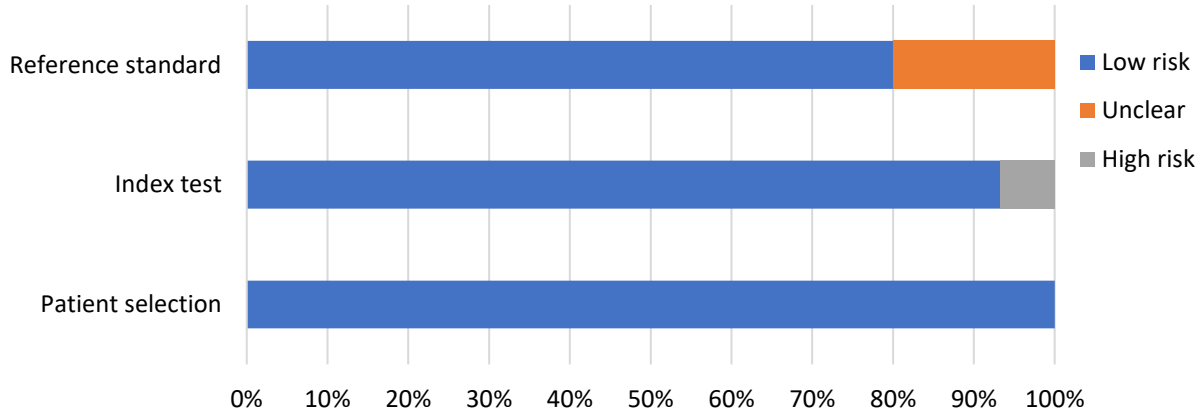

Figure A: Percentage of studies with low, unclear and high risk of applicability

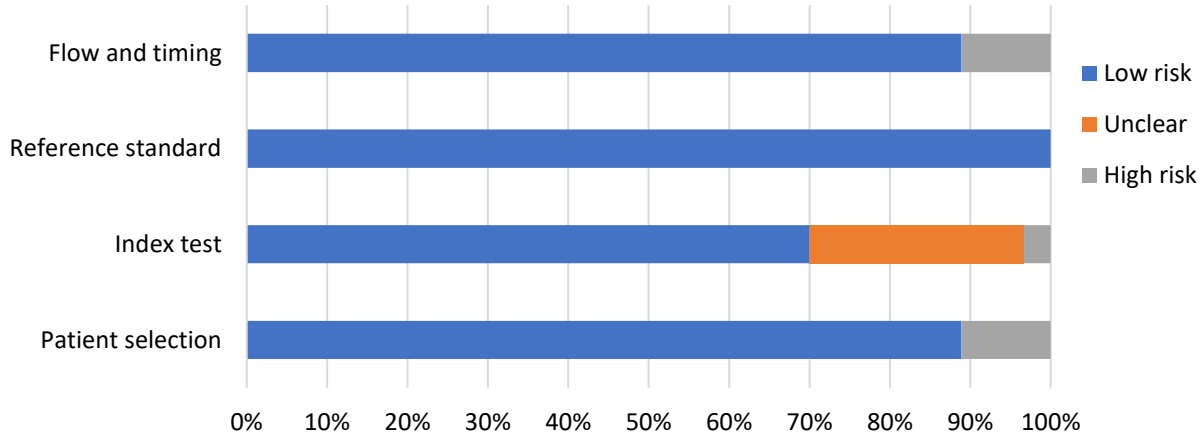

Figure B: Percentage of studies with low, unclear and high risk of bias

Figure 2B.3. Modified Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) quality scores for risk of bias and applicability concerns from included studies.

(A) Percentage (%) of studies with a low, unclear, and high risk of applicability; (B) Percentage (%) of studies with a low, unclear, and high risk of bias.

2.5.2. Subgroup analysis based on assay methodology.

The subgroup analysis showed that a difference in measurement of urinary nephrin was observed based on the assay methodology. ELISA showed a pooled sensitivity of 0.89 (95% CI: 0.86-0.92, I^2=71.9%) and pooled specificity of 0.72 (95% CI: 0.69-0.75, I^2=92.7%). The pooled positive LR was 3.84 (95% CI: 2.23-6.63), negative LR was 0.16(95% CI: 0.08-0.30), and pooled DOR was 31.55 (95% CI: 12.12-82.14). Urinary nephrin using ELISA showed excellent diagnostic accuracy with an AUC of 0.92 (**Table 2B.3**). Diagnostic accuracy of urinary nephrin observed from three studies using RT-PCR (322, 324, 326) showed a pooled sensitivity of 0.73 (95% CI: 0.64-0.81, I^2= 83.4%) and a pooled specificity of 0.69 (95% CI: 0.59-0.79, I^2=60.1%) and good diagnostic accuracy AUC of 0.77 (Table 2). Higher diagnostic accuracy was observed in a single study (312) using Western blot analysis with a sensitivity of 0.98 (95% CI: 0.83-1.00) and specificity of 0.98 (95% CI: 0.80-1.00).

2.5.2.1. Subgroup analysis based on study designs

Seven of the 15 studies used a prospective cohort study design. The pooled sensitivity of urinary nephrin in prospective cohort studies was 0.86 (95% CI: 0.80-0.91, I^2=73%) and a pooled specificity of 0.71 (95% CI: 0.66-0.74, I^2=94.6%) and the AUC was 0.87 (**Table 2B.3)**. Overall, the diagnostic accuracy of urinary nephrin in all study designs was very good (AUC: 0.8-0.9). However, the high level of heterogeneity following subgroup analysis was still observed across studies.

2.5.2.2. Subgroup analysis based on reporting units of urinary nephrin

In this review, 10 of the 15 studies did not correct urinary nephrin concentration for the urine creatinine concentration. Measurement of urinary nephrin (ng/ml) showed a pooled sensitivity of 0.86 (95% CI: 0.82-0.89) and pooled specificity of 0.77 (95% CI: 0.72-0.80). The normalised urinary nephrin by correction with urine creatinine reported as NCR (ng/mg) showed a pooled sensitivity of 0.89 (95% CI: 0.81-0.94) and specificity of 0.69 (95% CI: 0.64-0.73) (**Table 2B.3**).

***Table 2B 2*: Subgroup -analysis of urinary nephrin as a potential marker of early glomerular injury**

Studies	Sensitivity (95%CI)	Specificity (95%CI)	Positive LR (95%CI)	Negative LR (95%CI)	DOR (95%CI)	AUC
All studies	0.86 (0.83-0.89)	0.73 (0.70-0.76)	3.53 (2.26-5.50)	0.19 (0.11-0.33)	23.37 (10.58-51.64)	0.90
I^2 (%)	79.5	90.8	92.8	79.8	73.3	
Clinical condition						
Preeclampsia	0.78 (0.71-0.84)	0.79 (0.75-0.82)	5.35 (2.72-10.52)	0.24 (0.11-0.52)	18.08 (5.11-64.02)	0.91
I^2 (%)	81.4	89.2	85.8	82	78.4	
Nephropathy	0.90 (0.87-0.93)	0.62 (0.56-0.67)	2.49 (1.44-4.30)	0.16 (0.10-0.26)	22.10 (10.43-46.82)	0.90
I^2 (%)	63.7	89.7	94	35.7	41.5	
Study design						
Cohort	0.86 (0.80-0.91)	0.71 (0.66-0.74)	3.04 (1.64-5.64)	0.28 (0.15-0.53)	13.23 (4.43-39.57)	0.87
I^2 (%)	73	94.6	93.5	56.3	68	
Case-Control	0.72 (0.62-0.81)	0.82 (0.74-0.88)	3.70 (1.59-8.57)	0.32 (0.11-0.95)	15.32 (2.41-97.23)	0.97
I^2 (%)	89.9	65.7	63.3	84.5	79.6	
Cross-sectional	0.92 (0.88-0.95)	0.73 (0.67-0.79)	3.74 (2.07-6.76)	0.10 (0.05-0.19)	53.74 (26.09-110.71)	0.95
I^2 (%)	59.4	86.2	84.9	39	28.2	
Diagnostic Method						
ELISA	0.89 (0.86-0.92)	0.72 (0.69-0.75)	3.84 (2.23-6.63)	0.16 (0.08-0.30)	31.55 (12.12-82.14)	0.92
I^2 (%)	71.9	92.7	94.6	73.2	72.1	
RT-PCR	0.73 (064-0.81)	0.69 (0.59-0.79)	2.16 (1.56-2.99)	0.39 (0.20-0.75)	6.17 (3.19-11.94)	0.77
I^2 (%)	83.4	60.1	4.2	66.7	0.0	
Nephrin reporting methods						
Urinary nephrin (ng/ml)	0.86 (0.82-0.89)	0.77 (0.72-0.80)	4.66 (2.09-10.41)	0.18 (0.09-0.34)	27.36 (10.45-71.69)	0.92
I^2 (%)	86.6	92.4	95.6	82.8	75.1	
Urinary NCR (ng/mg)	0.89 (0.81-0.94)	0.69 (0.64-0.73)	2.48 (1.68-3.64)	0.19 (0.05-0.67)	17.76 (3.71-85.13)	0.86
I^2 (%)	78.3	84.8	75.7	77.5	73.0	

AUC: Area Under the Curve; CI: Confidence Interval; DOR: Diagnostic Odds Ratio; ELISA: Enzyme Linked Immunosorbent Assay; LR: Likelihood Ratio; RT-PCR: Reverse Transcriptase Polymerase Chain Reaction; NCR: Nephrin: Creatinine Ratio

2.5.3. Overall diagnostic accuracy of urinary nephrin

The pooled sensitivity of urinary nephrin for detecting glomerular injury was 0.86 (95% CI: 0.83-0.89) and the pooled specificity was 0.73 (95% CI: 0.70-0.76) (**Figure 2B.4**). The AUC obtained from the SROC was 0.90 (**Figure 2B.5).** This result suggests that urinary nephrin achieved high diagnostic accuracy in diagnosing glomerular injury, due to the observation that AUC > 0.7 is a risk predictor. The pooled positive LR was 3.53 (95% CI: 2.26-5.50) and negative LR was 0.19 (95% CI: 0.11-0.33). Moreover, using a random-effect model, the DOR was 23.37 (95% CI: 10.58-51.64) (**Table 2B.3**). There was no significant heterogeneity found between the studies based on the threshold effect (r^2) =-0.17; P=0.57). A non-threshold effect using estimation of Chi-square test ($p<0.05$) and inconsistency index for pooled sensitivity (I^2 =79.5%) and specificity (I^2 = 90.8%) indicated that there was heterogeneity in the value of urinary nephrin between the 15 studies (**Figure 2B.4**), due to differences between the diagnostic methods, clinical conditions, cut-off values, and study designs of the studies included in the review. Therefore, subgroup analysis was conducted based on the clinical conditions, assay methodology, study designs and units used for reporting of urinary nephrin**.**

2.5.3.1. Subgroup analysis based on clinical condition

The subgroup analysis based on clinical conditions showed that urinary nephrin predicts glomerular injury caused by PE with a pooled sensitivity of 0.78 (95% CI: 0.71-0.84, I^2=81.4%) and a pooled specificity of 0.79 (95% CI: 0.75-0.82, I^2=89.2%) **(Figure 2B.4).** Furthermore, urinary nephrin predicts glomerular injury caused by nephropathy with a pooled sensitivity of 0.90 (95% CI: 0.87-0.93, I^2=63.7%), specificity of 0.62 (95% CI: 0.56-0.67, I^2=89.7%) (**Figure 2B.4**). Urinary nephrin shows excellent diagnostic accuracy for predicting glomerular injury caused by either PE (with AUC of SROC 0.91) or nephropathy with AUC of the SROC 0.90 for predicting nephropathy (**Table 2B.3**).

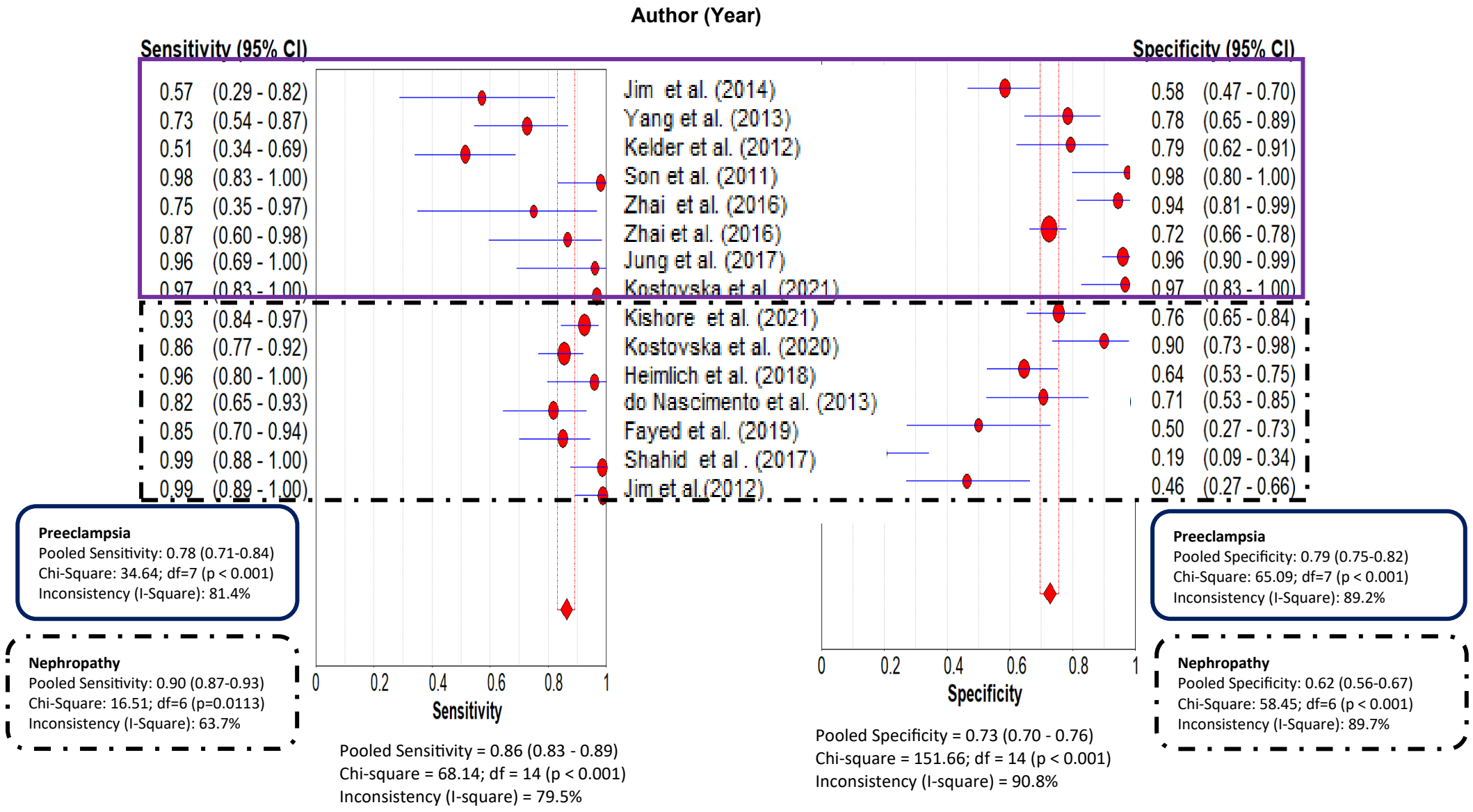

Figure 2B.4. Forest plot of the pooled sensitivity and specificity of urinary nephrin for detecting glomerular injury across all studies.

Subgroup analysis: Preeclampsia (solid line) and Nephropathy (dashed line) shows the pooled sensitivity and specificity of urinary nephrin for detecting these conditions. The red circles and the horizontal lines represent the point estimate and 95% CI, respectively. The circles and the horizontal lines represent the point estimate and 95% CI, respectively. Between the dotted vertical lines represents the pooled estimate, and the diamonds represent the pooled estimate in all studies with 95% CI.

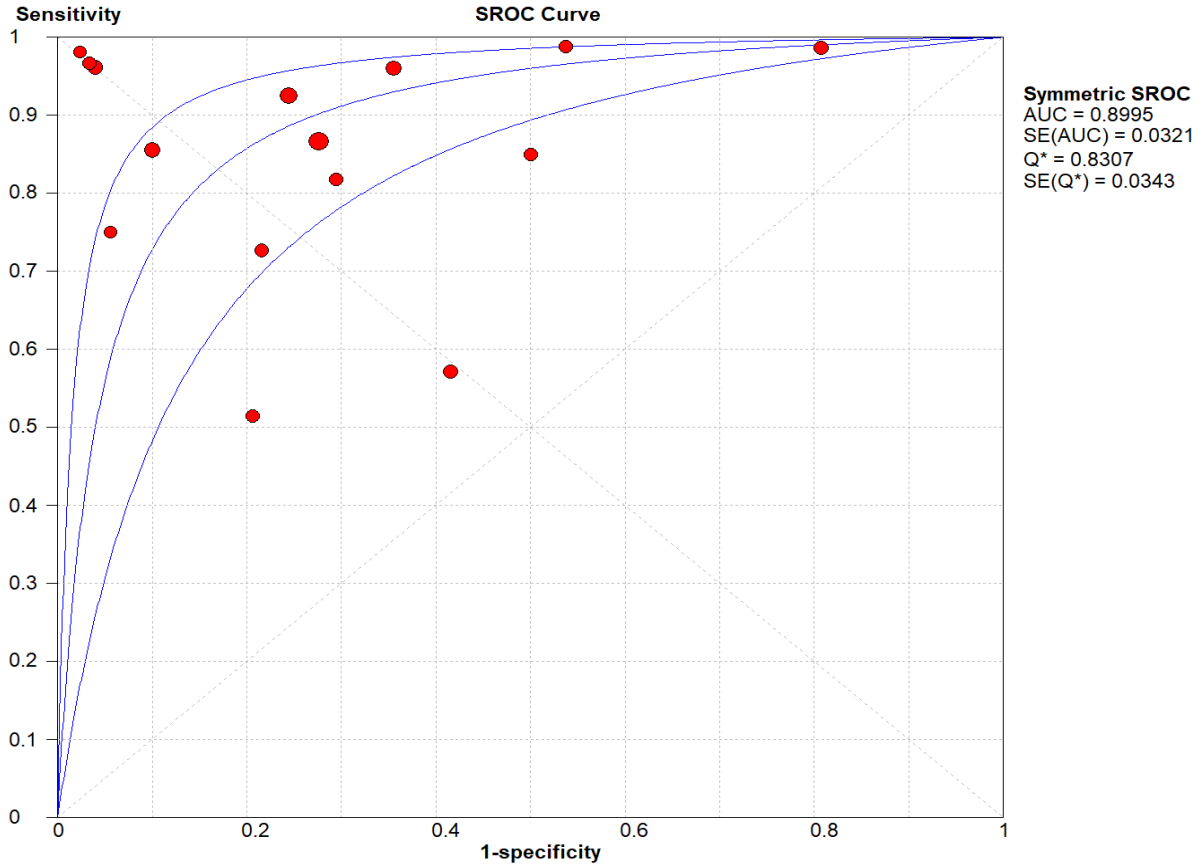

Figure 2B.5. Hierarchical Summary Receiver Operating Characteristics (SROC) plot of urinary nephrin to determine glomerular injury across all settings.

The SROC curve is represented by the middle line; each of the analysed studies is represented by a red circle and the respective 95% CI, by the two upper and lower lines.

2.5.4. Sensitivity analysis

Sensitivity analysis was conducted to assess the impact of each study in the interpretation of the diagnostic accuracy of urinary nephrin on the overall diagnostic accuracy. The sensitivity analysis performed to check heterogeneity was conducted by excluding each study step by step from the analysis. The estimate showed that the excluded study did not lead to significant changes in the overall AUC of the index test (urinary nephrin) (**Table 2B.4**).

Table 2B 3: Sensitivity analysis (pooled diagnostic accuracy with 95 % CI, given the named study, is omitted in the meta-analysis)

First author (Year)	Sensitivity (95%CI)	Specificity (95%CI)	Positive LR (95%CI)	Negative LR (95%CI)	DOR (95%CI)	AUC
All studies	0.86 (0.83-0.89)	0.73 (0.70-0.76)	3.53 (2.26-5.50)	0.19 (0.11-0.33)	23.37 (10.58-51.64)	0.90
Jim B et al. (2014)[255]	0.87 (0.84-0.90)	0.74 (0.71-0.77)	3.86 (2.39-6.24)	0.16 (0.09-0.29)	27.89 (13.09-59.44)	0.91
Yang et al. (2013)[149]	0.87 (0.84-0.90)	0.72 (0.69-0.75)	3.56 (2.23-5.70)	0.17 (0.09-0.32)	26.46 (10.98-63.74)	0.91
Kelder et al. (2012)[322]	0.89 (0.86-0.92)	0.72 (0.69-0.75)	3.65 (2.28-5.84)	0.70 (0.10-0.29)	27.61 (12.06-63.21)	0.92
Son et al. (2011)[312]	0.86 (0.82-0.89)	0.72 (0.69-0.75)	3.32 (2.16-5.10)	0.20 (0.12-0.35)	19.96 (9.28-42.92)	0.89
Zhai et al. (2016)[313]	0.86 (0.83-0.89)	0.72 (0.69-0.75)	3.29 (2.11-5.14)	0.18 (0.10-0.33)	22.42 (9.81-51.23)	0.89
Zhai et al. (2016)(323)	0.86 (0.83-0.89)	0.73 (0.69-0.76)	3.67 (2.22-6.07)	0.19 (0.10-0.33)	24.57 (7.98-42.30)	0.90
Jung et al. (2017)[314]	0.86 (0.83-0.89)	0.70 (0.67-0.73)	3.07 (2.00-4.70)	0.20 (0.11-0.34)	19.84 (9.14-43.09)	0.88
Kishore et al. (2021)[325]	0.85 (0.82-0.88)	0.72 (0.69-0.75)	3.52 (2.20-5.63)	0.20 (0.12-0.36)	22.75 (9.67-53.53)	0.90
Kostovska et al. (2020)[75]	0.86 (0.83-0.90)	0.72 (0.69-0.75)	3.29 (2.13-5.09)	0.18 (0.10-0.34)	21.85 (9.51-50.21)	0.90
Heimlich et al. (2018)[248]	0.86 (0.82-0.89)	0.73 (0.70-0.76)	3.71 (2.25-6.12)	0.20 (0.11-0.35)	22.62 (9.88-51.78)	0.90
doNascimento et al. (2013)[324]	0.87 (0.83-0.90)	0.73 (0.70-0.76)	3.63 (2.26-5.85)	0.18 (0.10-0.33)	25.99 (10.87-62.12)	0.91
Fayed et al. (2019)[326]	0.86 (0.83-0.89)	0.73 (0.70-0.76)	3.82 (2.35-6.23)	0.18 (0.10-0.32)	27.00 (11.57-63.01)	0.91
Shahid et al. (2017)[327]	0.85 (0.82-0.88)	0.75 (0.72-0.78)	3.51 (2.49-4.94)	0.19 (0.11-0.34)	23.98 (10.52-40.47)	0.90
Kostovska et al. (2021)[260]	0.86 (0.82-0.89)	0.72 (0.69-0.75)	3.24 (2.11-4.96)	0.21 (0.12-0.36)	19.06 (8.92-40.71)	0.88
Jim et al. (2012)[49]	0.85 (0.82-0.88)	0.74 (0.70-0.76)	3.84 (2.33-6.33)	0.20 (0.12-0.35)	22.30 (9.88-50.36)	0.90

AUC: Area Under the Curve; CI: Confidence Interval; DOR: Diagnostic Odds Ratio; LR: Likelihood Ratio.

2.6. Discussion

This review shows that urinary nephrin could be a potential indicator of early glomerular injury, demonstrated by a very good diagnostic accuracy in patients with acute and chronic renal injury. Indeed, urinary nephrin has demonstrated potential as a marker for early glomerular injury in several studies (21, 24, 313) and could prove to be a useful routine diagnostic marker used alone or in combination with other novel markers such as NGAL and cell cycle arrest markers (302, 328, 329) for the prediction of early kidney injury. However, appropriate validation of new diagnostic biomarkers requires the demonstration of assay performance against validation and verification criteria set out by professional organisations (265). Progression into clinical use requires an investigation of the diagnostic accuracy and assay's ability to discriminate between diseased and healthy populations. In this regard, this review aims to provide a first step in this process.

Despite demonstrated satisfactory diagnostic accuracy of urinary nephrin (AUC-SROC 0.9), heterogeneity exists across the studies reviewed; this has been documented previously (330) and across other studies (41, 309, 331). The potential source of heterogeneity in this review was evaluated using subgroup analysis by clinical condition, methods of analysis, study design, and reporting units. The analysis also showed heterogeneity existed within subgroups, nevertheless, the diagnostic accuracy of urinary nephrin was considered satisfactory in each group following subgroup analysis.

Laboratories often have to find a balance between diagnostic accuracy and technical complexity when choosing assays to adopt for routine diagnostic use (332). Therefore, an important aspect to include in a meta-analysis such as this is the heterogeneity in diagnostic accuracy of the methods employed by the various studies. ELISA was the method of choice for most studies (n=11) for the detection of nephrinuria in PE and nephropathies (49, 75, 149, 248, 255, 260, 313, 314, 323, 325, 327). Interestingly these assays demonstrated improved sensitivity (0.89) and specificity (0.73) and therefore diagnostic accuracy (AUC-SROC = 0.92) for determining urinary nephrin compared to RT-PCR (322, 324, 326). Additionally, one study demonstrated a sensitivity and specificity of 100% in a single centre trial from 25 women with PE using western blot analysis (312).

Under normal physiological conditions, random urine collections contain varied concentrations of urine biomarkers due to variability in urine volume. Therefore, biomarker concentration is often corrected using urinary creatinine (333). Our result found the pooled sensitivity and specificity of uncorrected urinary nephrin (ng/ml) was 0.86 and 0.77 respectively. In comparison, urinary nephrin normalised by correction for urinary creatinine, NCR (ng/mg), showed a greater pooled sensitivity of 0.89 and lower specificity of 0.69. The difference in reporting methods and lack of consistent cut-off values for urinary nephrin may account for heterogeneity seen across the studies included in this review. Hence, a uniform reporting approach for urinary nephrin is mandatory for ease of interpretation and comparison of results across the literature.

The pooled analysis of the studies investigating urinary nephrin as a diagnostic marker of glomerular injury showed good sensitivity and specificity. Studies investigating urinary nephrin predominantly focused on early detection of PE and diabetic nephropathy. Both conditions rely on the detection of albumin or protein in the urine as an indicator of glomerular damage. PE has an acute presentation associated with endothelial swelling and derangements (306) and has also been associated with podocyte loss and nephrin shedding (306). As is the case with patients suffering AKI, these patients do not always go on to incur further renal impacts and therefore progressive renal decline (155). Conversely, nephropathy develops over time and could be considered an example of the chronic progression of renal disease (334). All studies determining urinary nephrin showed that urinary nephrin increased significantly in patients with increased levels of albumin in urine (75, 325). Likewise, other studies have also shown that the urinary nephrin increased linearly with the progression of the disease, this suggests that quantification of nephrin could be a useful biomarker of glomerular injury progression (49, 260, 326).

Overall, the sensitivity and specificity of the individual studies reviewed for predicting glomerular injury of PE ranged from 51 - 97% and 58 - 97% respectively. Urinary nephrin predicts acute glomerular injury caused by PE with high level of sensitivity (0.78) and specificity (0.79) with SROC of 0.91. Thus, urinary nephrin could be considered a good predictor of the disease, showing an improvement on diagnostic accuracy of albumin (ACR)

with sensitivity of 36% (255) and protein to creatinine ratio with sensitivity of 72% to predict significant proteinuria (335).

There is growing evidence that urinary nephrin may be a superior marker for PE and can achieve better diagnostic accuracy than other podocyte biomarkers. Kerley et al. reported improved diagnostic accuracy of urinary nephrin with sensitivity of 0.81 (95% CI: 0.72–0.88) and specificity of 0.84 (95% CI: 0.79–0.84) when compared to combined podocyte biomarkers (41). Likewise, a previous meta-analysis by Wu et al. investigating the value of biomarkers for the detection of early-stage PE found a low predictive value using single biomarkers (disintegrin and metalloprotease 12, inhibin-A, pregnancy associated plasma protein A, placental growth factor and placental protein 13) with a pooled sensitivity of all single biomarkers 0.40 (95% CI 0.39–0.41) and a pooled specificity of 0.90 (95% CI: 0.90-0.90) in 147 studies of 401 laboratory biomarkers (309). The investigators found increased diagnostic sensitivity and specificity with the use of a panel of biomarkers combined with clinical characteristics; sensitivity of 0.43 (95% CI: 0.41-0.46) and specificity of 0.91 (95% CI: 0.90-0.91). However, unlike our review, the review by Wu et al. was not focused on glomerular-specific biomarkers for determining glomerular injury. A similar systematic review conducted by the World Health Organisation (WHO) in 2004 assessed the usefulness of combined clinical biophysical and biochemical tests for the prediction of PE (331), concluding that there was yet to be a cost-effective or reliable screening test. It has since been demonstrated that urinary nephrin could possibly fill that role. The improved diagnostic accuracy demonstrated by urinary nephrin may warrant its inclusion in these panels to improve early detection of PE.

Identifying nephropathy in the early stages of the disease prior to proteinuria is challenging. Existing guidelines rely on albuminuria as an indicator of glomerular nephropathy (336). However, this has limitations in terms of timing for detection of early nephropathy since glomerular structural damage precedes microalbuminuria (21). In terms of the specificity, ACR is widely accepted for the classification of glomerular injury and chronic kidney disease and while albuminuria has been independently and strongly associated with progression to ESKD (337). The included studies showed that nephrinuria positively correlated with increases in urinary concentrations of albumin and hyperglycaemia status. However, nephrinuria was also detected in a high proportion of diabetic patients with normoalbuminuria, given that, over

time hyperglycaemia is likely to further damage renal vasculature and the glomerular filtration barrier, nephrinuria may provide an early indicator of renal damage. Although not all diabetic patients with nephrinuria progress to kidney disease, nephrinuria can be used as an early indicator of glomerular damage prior to progression to fulminant kidney disease/injury and used to signal the need for interventional strategies in this vulnerable population. In this meta-analysis, the diagnostic accuracy of urinary nephrin for predicting glomerular nephropathy showed good diagnostic sensitivity of 0.90 (95% CI: 0.87-0.93) and specificity of 0.62 (95% CI: 0.56-0.67), SROC= 0.90, suggesting that urinary nephrin may be a promising biomarker of glomerular injury.

Early detection of urinary nephrin before the appearance of protein and albumin in urine could allow for the detection of glomerular injury before the loss of renal function (75). This is important for early diagnosis and intervention. Furthermore, albuminuria may not always be present, a study by An et al. demonstrated more than 30% of patients with kidney disease had undetectable albuminuria despite the presence of severe glomerular damage/renal insufficiency (42). Likewise, previous studies have indicated that podocyte proteins may provide earlier indicators for glomerular nephropathies preceding albuminuria (50, 91, 248, 327). Studies included in this review detected nephrinuria prior to the presence of albuminuria and the urinary nephrin concentration reflects the severity of the disease (313, 325) . This has also been reported in previous studies that found nephrinuria was detected prior to albuminuria during glomerular injury, the study showed that 54 % of diabetes mellitus (DM) patients with normoalbuminuria had nephrinuria and 100% of DM patients with micro-macroalbuminuria had nephrinuria (49). Similarly, another study demonstrated the presence of elevated nephrinuria in 82% of patients with normoalbuminuria, 88% of patients with microalbuminuria, and 100% of patients with macroalbuminuria (75).

The intention of this review was to investigate the role of urinary nephrin as a marker for early glomerular injury for detecting both acute and chronic kidney injury. All studies relate to nephropathy demonstrated that urinary nephrin increased in parallel with albuminuria and correlated with the progression of the severity of nephropathy (49, 75, 248, 324-327), suggesting that nephrinuria is a sensitive indicator for nephropathy. It has been suggested that continued attempts at regeneration and upregulation of nephrin production may be

evidence of podocyte repair following injury (52). Urinary nephrin also negatively correlated with the glomerular filtration rate and increasing level were associated with the progression of injury to other forms of kidney injury/disease (325, 326).

The diagnostic accuracy of urinary nephrin for detecting PE and diabetic nephropathy could therefore be extrapolated into use as a potential predictor of early glomerular injury, particularly in the setting of AKI. Recently studies have emerged investigating the value of urinary nephrin for predicting AKI, particularly in critically ill neonates (50, 338). These studies concluded that urinary nephrin may well provide a marker for predicting AKI, demonstrating a diagnostic sensitivity of 62.5%, 61.5%, and specificity of 82.1%, 76.9% respectively at a cut-off point of NCR = 0.375µg/mg, suggesting that urinary nephrin may give an early indication of podocyte damage as an indicator of those infants at risk of developing AKI. This is an area of intense interest in the literature (21, 50, 338), since a single biomarker may not suffice to define AKI given inherent renal heterogeneity and the disparate settings under which kidney injury occurs (339).

2.7. Strength and limitations

The strength of this meta-analysis is that it is the first to systematically analyse the pooled diagnostic accuracy of urinary nephrin in the diagnosis of glomerular injury. However, the limitations of this meta-analysis cannot be ignored. First, urine ACR and De Novo hypertension were used as a reference standard to stratify cases and controls and to determine the diagnostic accuracy of urinary nephrin as a useful marker for glomerular injury. Second, there was high heterogeneity across the included studies in the meta-analysis. Third, diagnostic cut-off values of urinary nephrin of numerous studies were not consistent, and the included articles used different methods of assay measurement. Fourth, the current guidelines to stratify nephropathies using urine ACR, as a reference standard test, cannot reveal subclinical glomerular damage and might underscore the specificity of urinary nephrin. Fifth, the majority of studies included in this review are cross-sectional studies, hence, the cross-sectional nature of the study design reflects association rather than causality. While nephrin has been demonstrated to play an important role in the slit diaphragm of the glomerulus providing structural stability (340), normal functioning and repair of damaged glomerulus in acute injury (5, 341-343), there is no supportive evidence for nephrin as a causal mechanism

of glomerular injury. Nor is there evidence to support the early detection of nephrinuria as a reliably predictor of consequent glomerular injury and further progression to other forms of kidney injury/disease in the vulnerable populations.

Conclusion

Overall, this meta-analysis found that urinary nephrin could become an effective and robust biomarker for the early prediction of glomerular injury as well as for monitoring disease. Perhaps the addition of urinary nephrin as a marker of early glomerular injury to a panel of promising markers for AKI could provide a fuller clinical picture to help determine renal injury and prognosis in the future.

Author contributions

BM performed the literature search, extracted the data, performed the statistical analysis, and prepare the manuscript. DR extracted the data and checked the quality control aspects of the data. DR, DV, RK, and YK provided input and guidance on the technical and scientific content of the manuscript, to ensure the accuracy of statements made and interpretation of the findings. BM, DR, DV, RK, DW and YK edited subsequent and final drafts of the manuscript. All authors read and approved the final manuscript.

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Declarations

Conflict of Interest

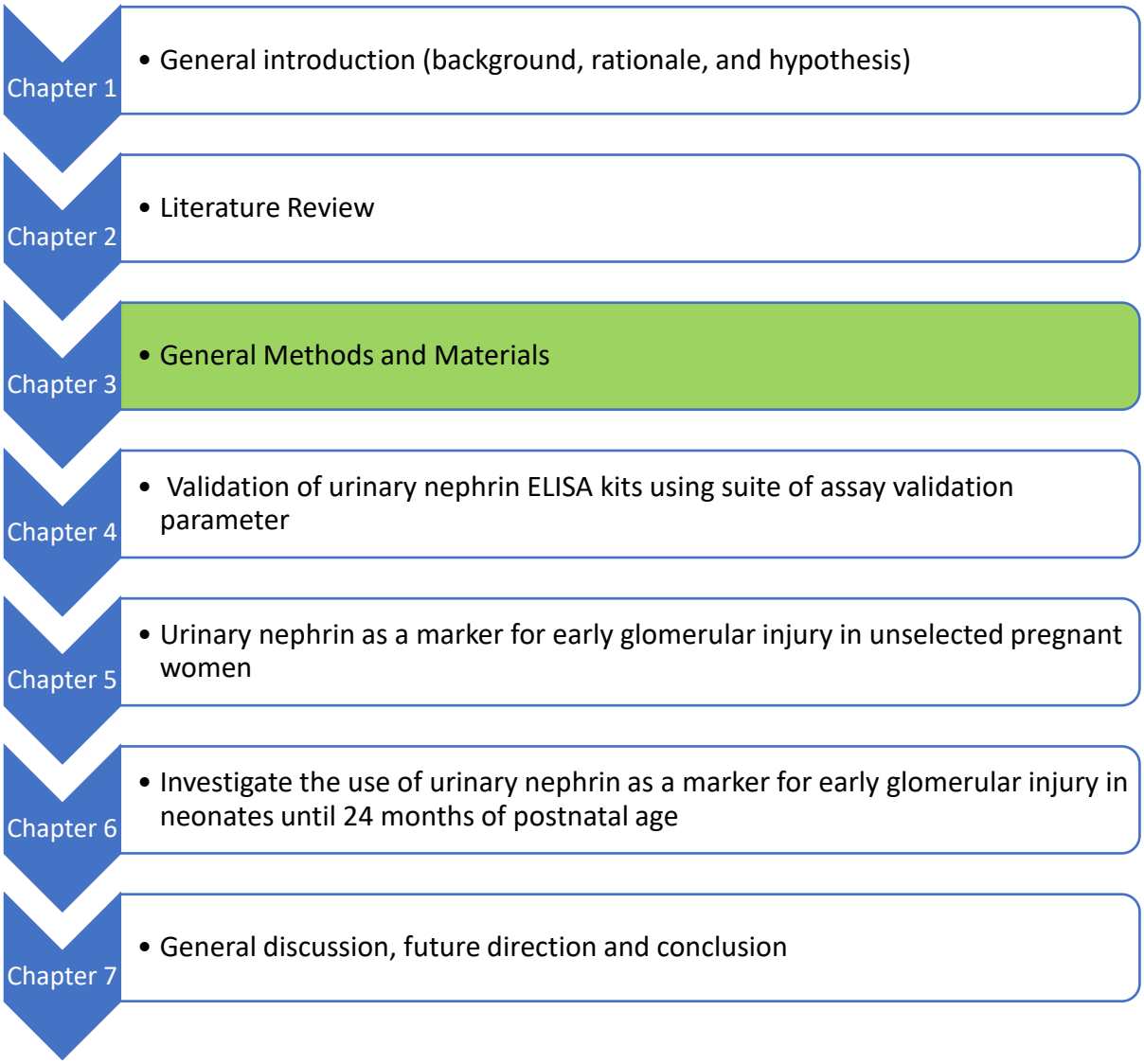
No potential conflict of interest was reported by the authors.

Ethical considerations

This meta-analysis adhered to the Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA-2020) guideline. Institutional approval and patient consent were not necessary.

2.8. Chapter summary

In this chapter, the diagnostic accuracy of urinary nephrin as a marker of glomerular injury described. The next chapter (**chapter 3**) will present the general methods and materials of the thesis.



Foreword Chapter 3

This chapter describes the overall study's methods, materials, and analysis. The two larger longitudinal prospective cohort studies, in which this thesis is embedded, are described. The method section of this chapter has two parts, the first for analytical validation of two commercially available nephrin ELISA kits using a suite of assay validation parameters and the second for clinical validation of urinary NCR along with common renal function markers as a marker for early glomerular injury in cohort of pregnant women and neonates until 24 months of age.

Chapter 3. General Methods and Materials

3.1. Introduction

The validity, reproducibility, and feasibility of immunoassays for determining biomarkers in the preclinical and clinical sciences are of significant importance to translate the biomarker into clinical practice. Findings in animal and human studies are increasingly using nephrin ELISAs as a method of choice for determining glomerular maturity (50), glomerular injury (21), and glomerular injury of PE (260) in a different setup. However, urinary nephrin ELISA has yet to be validated for better translatability into clinical investigations. Therefore, this third chapter of the thesis aims to provide details on the general methods and materials, such as the study population, and study design, and narrate how each chapters compiled together to elucidate the objectives of the thesis. The study design describes the nature of the study, the study population, and the recruitment process, and the method and analysis describe the measurement of biochemical parameters and performance characteristics of urinary NCR to predict glomerular injury and the statistical analysis used for each chapter.

3.2. The process of the research project

The phases of the research process were a review of the scientific literature, laboratory work for the research, clinical and sociodemographic data collection and analysis, and thesis writing. A narrative literature review and systematic review and meta-analysis (**Chapter 2-part A and B**) summarise the existing knowledge of this thesis topic. This encompassed different electronic databases for online literature searches for journal articles, guidelines, and textbooks and systematically analysing the literature findings and identifying research gaps and provide a baseline information for the experimental chapters of this thesis.

To validate the commercially available human nephrin ELISAs, stored de-identified urine samples were analysed. The performance of the assays was evaluated using a suite of assay validation parameters. To determine the performance of the urinary NCR for predicting albuminuria as a marker for glomerular injury, a cohort of pregnant women and neonates were recruited as part of a NHMRC, Australia funded project. The correlation of urinary NCR with clinical characteristics, and other renal function markers was assessed in this cohort. To this end, the sensitivity and specificity of urinary NCR to predict albuminuria as an indicator of glomerular injury was determined in cohorts of pregnant women. For an accurate

interpretation of the result, clinically useful RIs for urinary NCR were determined in a cohort of neonates against postnatal age. This may improve the development of a potential intervention strategy for kidney health through early detection and prediction of glomerular injury and reduce the vulnerability of these cohorts to kidney injury and/or disease later in life. Finally, longitudinal changes in urinary markers of glomerular integrity (urinary NCR and ACR) and renal function markers including SCr, sCysC, and eGFR were investigated in neonates, infants, and children until 24 months of age.

3.3. Methods and Analysis

3.3.1. Study design and setting

This thesis comprises a cross-sectional and longitudinal cohort study as part of NHMRC project, and utilises samples collected during a longitudinal cohort study: ''Relationship *between prematurity, renal volume and retinal vasculature study'',* to validate and describe the analytical performance of two nephrin ELISAs. The findings from the validation study were then be used to analyse urinary nephrin as part of another larger project funded by NHMRC, Australia, ''*The relationship between maternal health and Infant renal development and function*'' (Application identification number: APP1159616), KIDMIN study. This large cohort study will allow as to investigate the diagnostic performance of urinary NCR for the prediction of early glomerular injury during pregnancy and early life. The correlation between urinary NCR with clinical, sociodemographic factors and traditional markers of renal function were determined. From this, the calculation of RIs and clinical cutoff values for urinary NCR were explored. Then longitudinal changes of the urinary NCR in the neonatal cohort up to 24 months postnatal age were also performed. The KIDMIN study was conducted at the Townsville University Hospital and Health Service (THHS), one of the largest non-metropolitan hospitals in Queensland, Australia. The THHS provides a specialist, tertiary referral hospital to a large diverse area across tropical North Queensland. It provides a tertiary perinatal service, which has a catchment area of approximately 750,000 square kilometres and 950,000 people with 10,000 births per year. The THHS covers an area of over 148,000 square kilometres and serves a population of around 240,000, and Townsville University Hospital (TUH) has more than 2400 births per year (344).

3.3.2. Sample size and sampling technique

This project included a larger sample size (240 urine samples) for analytical validation of urinary nephrin ELISAs (**chapter four**). A G*Power analysis for statistical power, effect and sample size was used to determine the sample size of 400 as part of the larger longitudinal prospective cohort study, KIDMIN project considering the difference in kidney volume of 2mls and a SD of 6mls from a previous study (345), power of 90% and a significance level alpha (α) value of 0.05 for the other experimental chapters (**chapters five and six**). In this thesis, the CLSI and IFCC guidelines (346) were followed to determine RIs for urinary NCR and to include an appropriate sample size. The CLSI/IFCC recommends a non-parametric/robust method to establish RIs with 95% reference values with a 90% CIs. The guideline recommends a minimum of 120 reference individuals to determine RIs. A convenient sampling technique was used to achieve the total sample size in both cohorts.

3.3.3. Study population and recruitment

This study included samples collected from pregnant women, neonates, infants, and children recruited to the two independent prospective longitudinal cohort studies (**stated in 3.3.3.1 and 3.3.3.2**) and which were conducted in the Department of Maternal and Neonatology, THHS, Queensland, Australia. The study flow chart is described in **Figure 3.1**.

3.3.3.1. ''Relationship between prematurity, renal volume, and retinal vasculature''

This project aimed at assessing the influence of premature birth on renal development. The study included neonates who were admitted to the NICU at THHS, Australia between August 2014 and October 2016. The study follow-up period was completed in October 2018. Preterm neonates born between 23-28 weeks of gestation, with birth weight centiles appropriate for gestational age between 10^{th}-90^{th}, were included in the study population. These infants were then tracked from a term corrected (37 postmenstrual age) on discharge from the NICU at 6, 12, and 24 months of postnatal age. Data from term infants admitted to the NICU during the same period for minor clinical conditions were enrolled with parents' approval as control. This method section is published in previous publications by project investigators Kandasamy et al. in 2018 (68).

3.3.3.2. "The Relationship between Maternal Health and Infant Renal Development and Function''

This project is a longitudinal cohort study that aims to investigate the determinants of neonatal renal volume and function and following infants to 24 months of postnatal age to determine the impact of these factors on early childhood renal growth and function. The project also aimed to determine these factors' effect on retinal microvasculature and the relationship between infant renal size and retinal vascular development. The study population comprised maternal-infant dyads. Pregnant women who came to the Hospital during the study period (2019-2021) of 24 months were invited to take part in the study and followed the infants until 24 months of postnatal age. The recruitment process was carried out by research midwives/nurses in the antenatal clinic and the birth suite. Information about this study was circulated to all clinicians in the health district and advertised through the newspaper, internet advertisements, and flyers. All data collection was taken place at THHS. Potential pregnant women were asked to sign an informed consent form after the verbal and written description of the study. After consent, the sample for laboratory analysis and sociodemographic and clinical variables were collected.

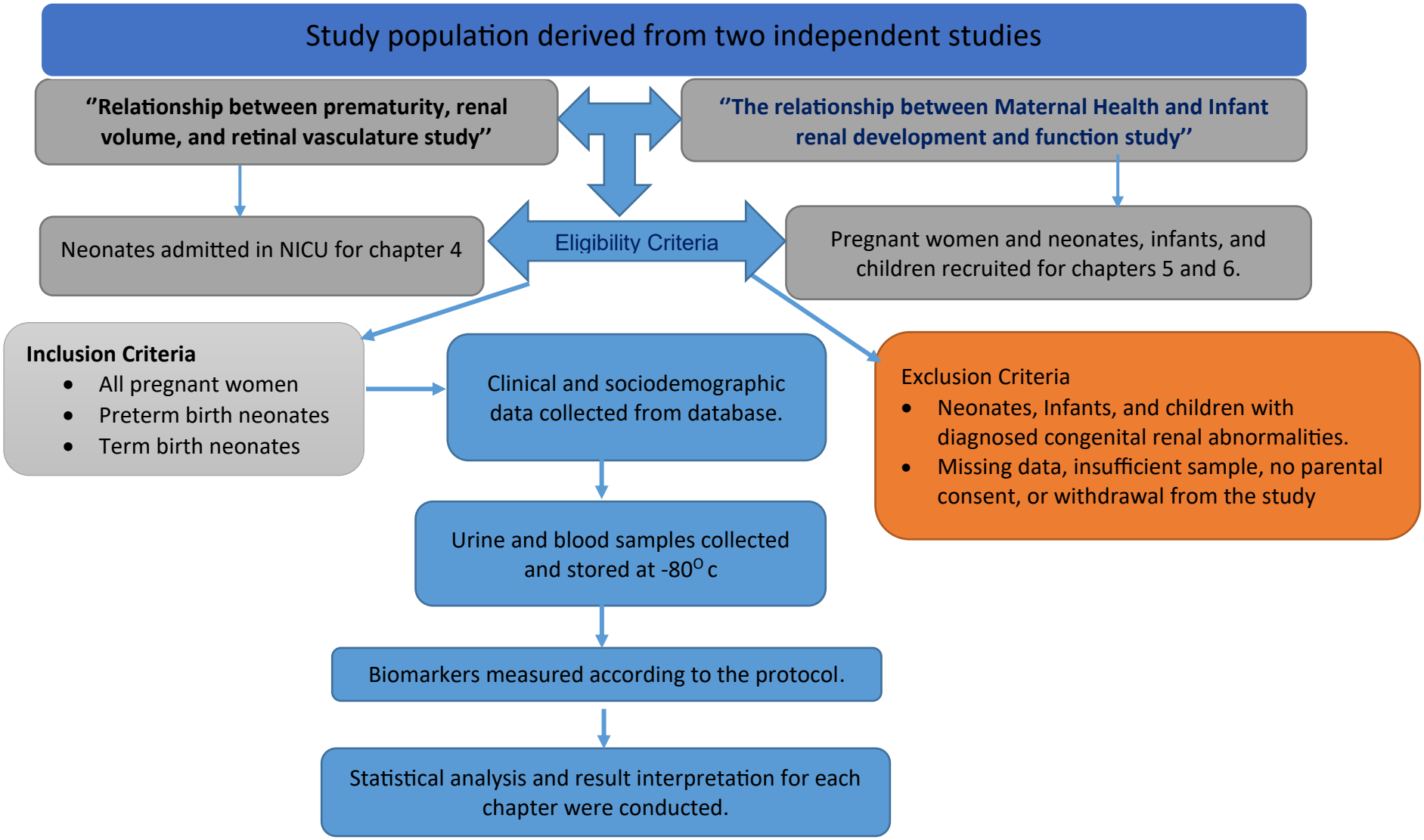


Figure 3.1. Flow chart of the study participants

3.4. Analytical validation experiment

In the validation experiment, the assay precision was determined using mean, SD, and % CV in duplicate measurements. The analytical performance of the urinary nephrin ELISA was determined using a suite of assay validation parameters that included the establishment of the calibration curve, investigation of common interferences, recovery experiment, linearity experiment, assay dynamic range (upper and lower LOQ), the LOD, sample stability and correlation studies. Further investigation was conducted to understand the metrological traceability and accuracy of the standards from the two suppliers. The actual standard concentration supplied by the supplier and the concentrated standards using the Amicon Ultrafiltration technique was used to check the accuracy and metrological traceability of the standards using QuickGel electrophoresis, immunofixation electrophoresis, and size exclusion chromatography **(Figure 3.2)**. The details of each validation parameter are described below.

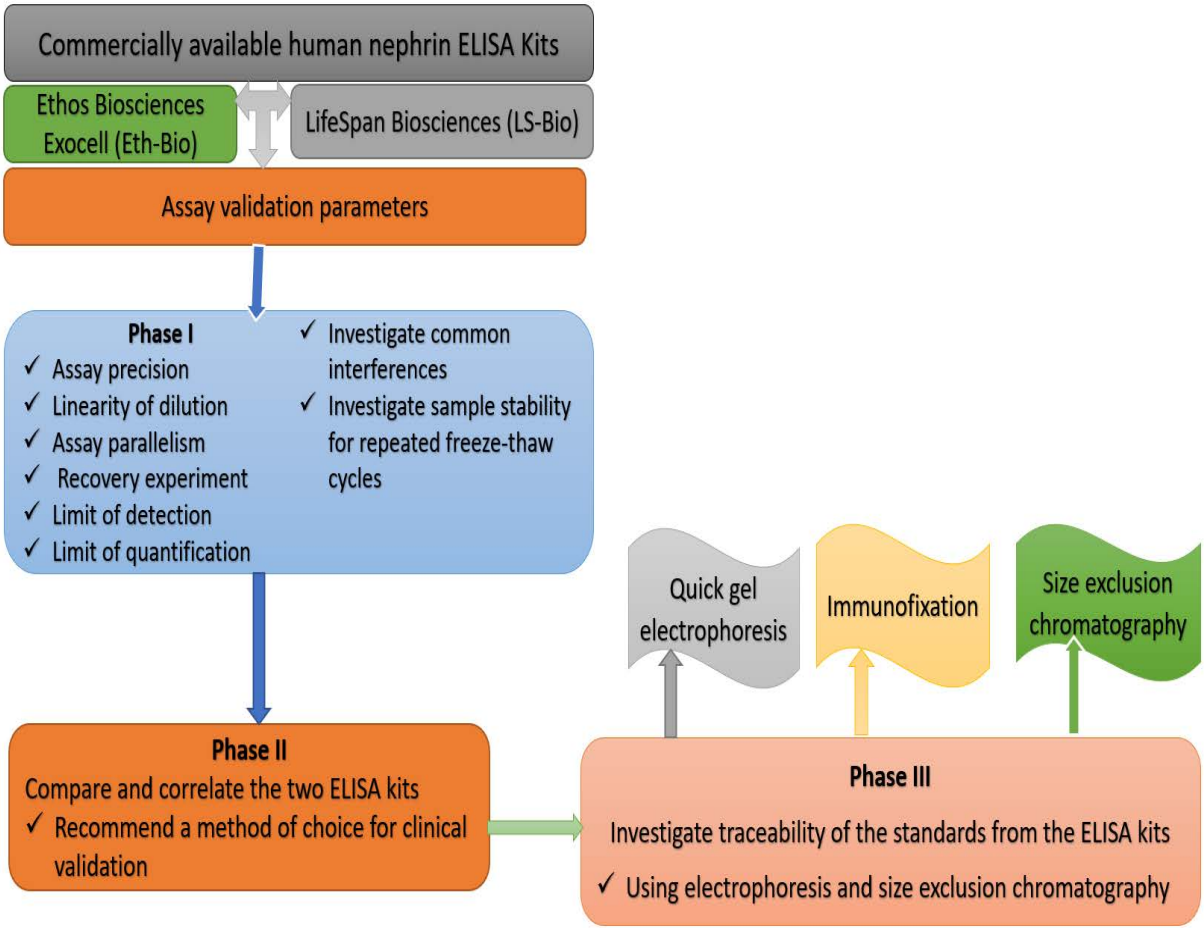


Figure 3.2. Schematic presentation of the validation process

3.4.1. Urine sample collection, preparation, and processing for analytical validation

Urine samples were collected in a clean, leak-proof container without preservatives. The samples were stored at -80 ^{0}c until analysis and avoiding repeated freeze and thaw cycles to maintain the stability of the analyte according to the manufacturer's claim. The previous study by Zhai et al. supported the avoidance of repeated freeze and thaw cycles to maintain the stability of urinary nephrin and the authors did not show significant changes in concentration with an increasing number of days after specimen sampling and time intervals until analysis of nephrin (323). Prior to assay, the samples were brought to room temperature, without the use of extra heating, and completely thawed. The urine samples were centrifuged at 3000 revolutions per minute (RPM) for 10 minutes to remove particulate matter, and the supernatant was used for the analysis. All urine samples were assayed for nephrin concentration using 2 x Human nephrin ELISA kits (Eth-Bio, USA, and LS-Bio, USA).

3.4.2. Reagent preparation for the ELISA experiment

The wash buffer solution was prepared according to the manufacturer's manual for both ELISA kits. The 1-litre solution for Eth-Bio ELISA contained 8.77g of NaCl, 1.21g of Triethanolamine, and 50µl of Tween 20 and made up to 1-litre with distilled water to give a working wash buffer solution of 0.015MNacl, 0.01M Triethanolamine and 0.05% Tween 20. The wash solution for LS-Bio was prepared by diluting 30 ml of 25x concentrated wash buffer, supplied in the kit with distilled water to make 750 ml of working wash buffer and stored at 4 °C. For the LS-Bio ELISA, the 1x Biotinylated Detection Antibody and 1x HRP Conjugate working concentration were prepared by diluting 100x concentrated solution using the Biotinylated Detection Antibody Diluent (1:100), and the HRP Conjugate Diluent (1:100) respectively.

3.4.3. Experimental procedure for Human Nephrin Eth-Bio ELISA (Cat No 1035, USA)

The immunoassay Human nephrin Eth-Bio Exocell ELISA (Ethos Biosciences. Inc., Philadelphia, USA) uses the competitive ELISA principle (**Figure 3.3**). The assay uses human nephrin coated in a 96 well microtiter plate. The nephrin in the urine samples, positive control, negative control, and human nephrin standard were added in the respective wells according to the manufacturer's instructions. The nephrin in the patient sample and nephrin immobilised to the stationary phase was captured by a mouse anti-human nephrin antibody that is specific to the human nephrin immobilised to the stationary phase. The captured nephrin-mouse anti-human nephrin antibody was detected with anti-mouse immunoglobulin G labelled with

Horse Reddish Peroxidase (IgG-HRP). Subsequently, the plate was washed with enzyme immunoassay (EIA) buffer using an automated microplate washer (BioTek 50/^{TS}, USA) to remove unbound nephrin and antibodies. After washing, only the antibody conjugate reacting with the mouse anti-human nephrin antibody bound to the stationary phase nephrin antigen remains in the well, and this was detected by adding a 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The peroxidase-catalysed hydrolysis produces a calorimetric signal; a stop solution was added to stop the hydrolysis. The absorbance was read at a wavelength of 450 nm using a plate reader (BMG LABTECH, Polarstar Omega, Germany). The data were analysed from the logarithmic plot of the standard concentration in the dilution series with the logarithmic [Nephrin] versus the mean absorbance. The colour intensity in nephrin ELISA was inversely proportional to the logarithm of nephrin in the fluid phase. The absorbance of the unknown sample could then be compared to the standard curve to determine its nephrin concentration.

3.4.4. Experimental procedure for the human nephrin LS-Bio ELISA (LS-F21185, USA)

Human NPHS1 / Nephrin ELISA Eth-Bio (Sandwich ELISA) (*LifeSpan Bioscience*, Inc., USA) is based on the sandwich immunoassay principle (**Figure 3.4**). Each well of the supplied 96-well microtiter plate has been pre-coated with a target-specific capture antibody. Standards and samples were added to the wells; therefore, the target antigen binds to the capture antibody. A biotin-conjugated detection antibody was then added, which binds to the captured antigen. The unbound detection antibody was washed away using an automated microplate washer (BioTek 50/^{TS}, USA). An Avidin-HRP conjugate was then added, which binds to the biotin. A TMB substrate was then added, which reacts with the HRP enzyme, resulting in colour development. A sulphuric acid stop solution was added to terminate the colour development reaction. The absorbance of each well was measured using a plate reader (BMG LABTECH, Germany) at a wavelength of 450nm. The absorbance standard curve was generated using nephrin standard concentrations, the colour intensity being directly proportional to the concentration of nephrin in the sample. The absorbance of an unknown sample could then be compared with the standard curve to determine its nephrin concentration.

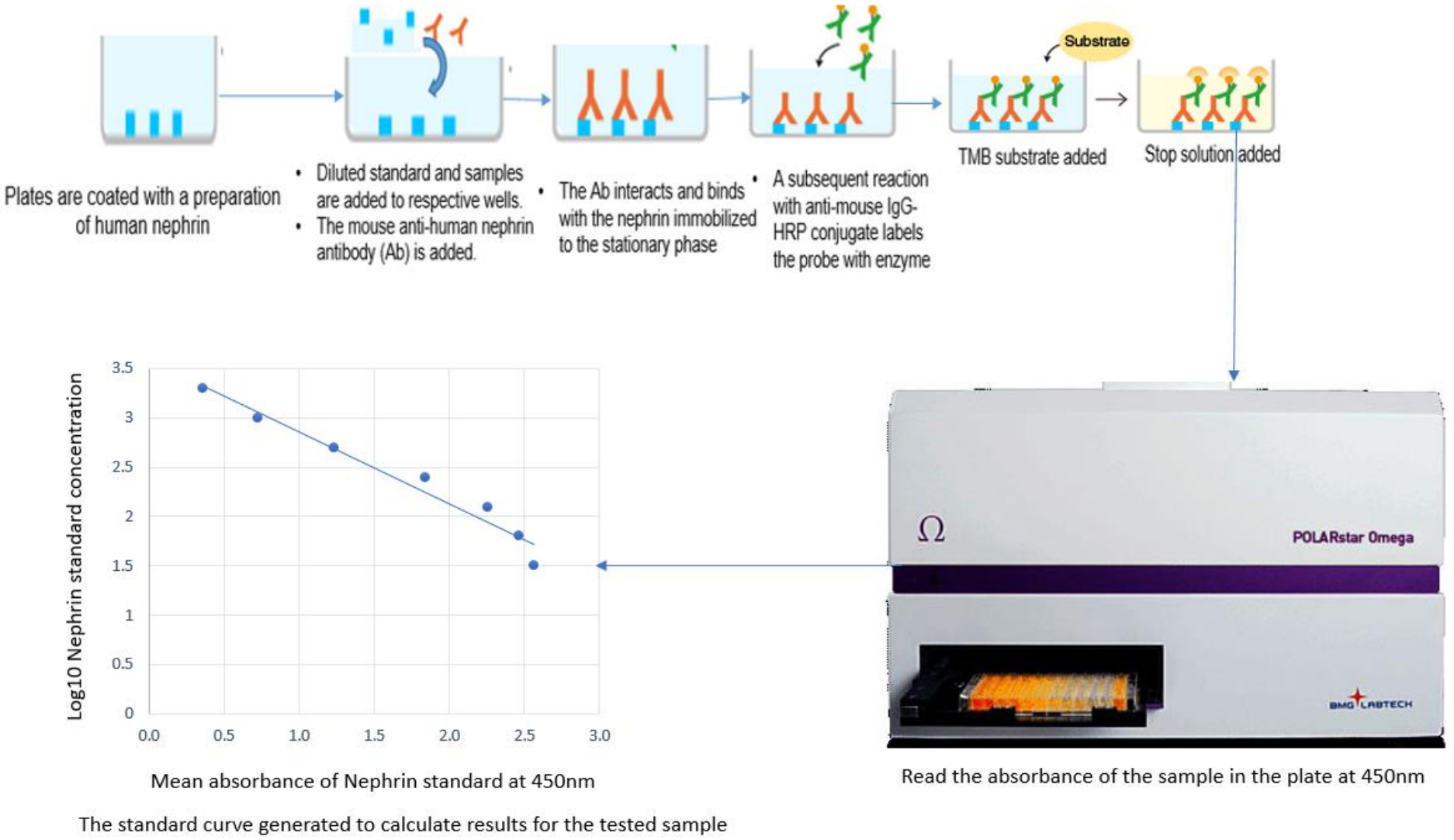


Figure 3.3. The competitive immunoassay principle for human nephrin Ethos Biosciences Exocell (Eth-Bio) ELISA kit

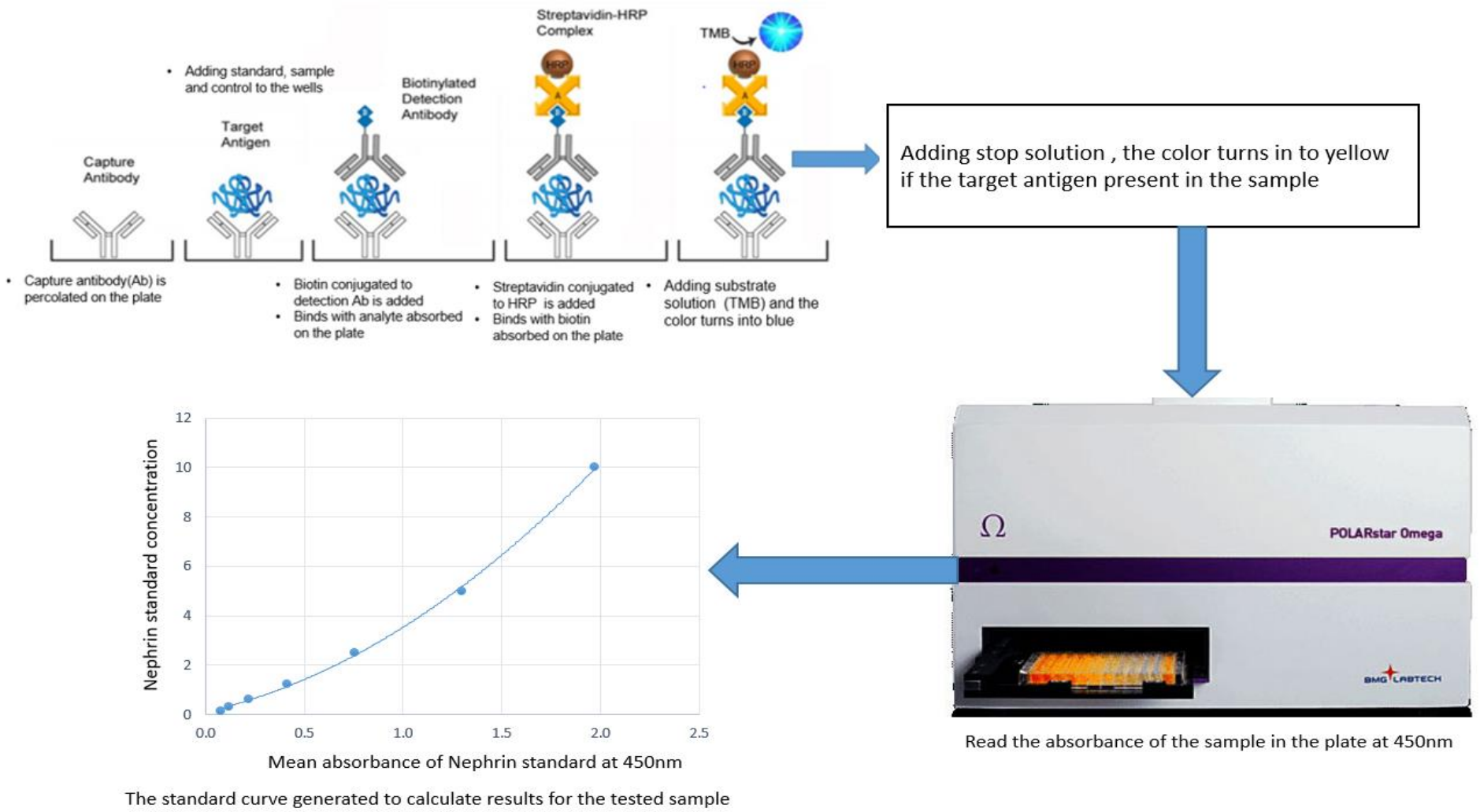


Figure 3.4. The sandwich immunoassay principle for human nephrin LifeSpan Biosciences (LS-Bio (LS-F21185)) ELISA kit

3.4.5. Validation plan

The assay was validated in accordance with the recommendation of CLSI and NATA validation of analytical procedures (265, 347). The human urinary nephrin was analysed in duplicate in both kits. Each assay included a standard curve with serially diluted concentrations, positive (for Eth-Bio only), and negative controls (Blank).

3.4.5.1. Standard curve validation

The calibration curves for both kits were constructed. The goodness of fitness was determined as the estimated squared correlation coefficient (r^2) of the standard data. A coefficient value close to 1.0 is an indicator of a precise fit for the data to the calibration curve. Briefly, blank, and serial dilutions were performed using the recombinant urinary nephrin standard supplied with the kit (31.3-2000ng/ml Eth-Bio, and 0.157-10ng/ml for LS-Bio). All assays were performed in duplicate on at least three different measurements of different days, and mean values of absorbance vs concentration were plotted and a scatter plot on an Excel spreadsheet was constructed (r^2> 0.95 was satisfactory).

3.4.5.2. Formulation of the nephrin standard and antibody solutions

To ensure the accuracy of an assay, it is important to understand the source of the standards included in the commercially available kits; this must be traceable to a known standard. The nephrin standard included in the *Eth-Bio* ELISA is recorded as derived from a recombinant protein (whole protein) that covers most N-terminal extracellular domain (about 1000 amino acids, approximately 111-120Kd) of human nephrin which has 0.05% proclin 300 in the standard as preservative and a mouse anti-human nephrin antibody used as a detection antibody. The standard included in *LS-Bio* is recorded as a recombinant protein produced in Escherichia coli *(E. coli)* that encodes 23-257 amino acids of human nephrin with an approximate molecular weight of 27-29Kd. The capture antibody used in LS-Bio is a mouse monoclonal antibody Protein A purified: immunogen with 23-100 amino acids, and the detection antibody is a rabbit polyclonal antibody immunogen affinity purified: immunogen with 150-257 amino acids.

3.4.5.3. Standard Preparation and Quality Control

Validation parameters were determined using duplicate testing of human urinary nephrin together with positive and negative controls, and a two-fold serially diluted commercially prepared human nephrin standard solution. The preparation of the standard dilution series was used to generate the standard curve, and the standard curve was used to determine the unknown concentration of urinary nephrin in the sample. In the *Eth-Bio* ELISA, the Human nephrin (Hu-Nephrin) standard was supplied as a two-fold concentrate. The nephrin standard was diluted in 7 twofold dilutions of standards using the EIA standard diluent buffer. Seven microfuge tubes with 120µl of EIA diluent per tube were prepared. A 120µl of concentrated (stock 4µg/ml) Hu-Nephrin standard was transferred to a tube. The contents were mixed by aspirating and expelling the fluids 5 times. The 120µl of solution from tube 1 was transferred to tube 2 and mixed and the procedure continued to tube 7 representing serial dilutions 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:28 with the expected nephrin concentration of 2000, 1000, 500, 250, 125, 62.5, 31.3 ng/ml.

In the LS-Bio nephrin ELISA kit, the standard was provided in lyophilised form. The 1 tube of 10ng lyophilised standard was reconstituted in 1.0ml of sample/standard diluent and incubated at room temperature for 10 minutes with gentle agitation. Eight microfuge tubes were labelled, tube 1 containing 500µl of concentrated standard, and tubes 2-7 contained 250µl of sample diluent. The 250µl of concentrated solution from tube 1 was transferred to tube 2, and mixed and the procedure continued to tube 7 representing the serial dilutions as stock, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 with expected concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.157 ng/ml and tube 8 containing sample diluent alone used as a background control (blank).

3.4.5.4. Assay validation experiment

Intra-assay and Inter-assay variability

To determine the precision of the assay, the coefficients of variation (CV) of replicate measurements were calculated from the mean and SD. The intra-assay precision was determined by duplicate measurements of nephrin in urine samples using the same kit. The inter-assay precision was evaluated by analysing a pooled urine sample using batches of nephrin ELISA kits carried out on different days according to NATA (265).

Systematic variation of urinary nephrin measurements

In the ELISA plate, pooled urine sample was added to micro-wells to evaluate variation in urinary nephrin measurements across the ELISA plate. Urinary nephrin was compared between rows and columns. Precision in the measurement of concentrations of nephrin in each row and column of the ELISA plate was described using mean and SD to determine % CV. A student T-test was performed to compare the variations of urinary nephrin concentrations in each row of the ELISA plate with each column. A p-value < 0.05 was considered, showing statistical significance.

Recovery experiment

Recovery of nephrin was determined to check whether analyte detection was affected by differences in sample matrices and sample diluent (265). Aliquots of a well-mixed urine sample and a standard diluent as a control were prepared. Two aliquots of well-mixed urine samples labelled neat and spiked were prepared to generate spiked samples and spiked control. The spiked sample and control contained 1000ng/ml and 500ng/ml, 250ng/ml, 125ng/ml of the nephrin standard (Eth-Bio) and 5ng/ml, 2.5ng/ml, 1.25ng/ml of the nephrin standard (LS-Bio). Recovery was assessed for the measured standards compared to the neat urine sample and the control spike according to the protocol for each kit. The percentage recovery was calculated by dividing the observed by the expected concentration and multiplied by 100.

Linearity experiment

The linearity of dilution was performed to provide evidence to which extent the observed and expected concentration of the urinary nephrin is linear within the range of the standard curve. For the ELISA Eth-Bio, the 2000ng/ml nephrin standard was serially diluted to provide standards of the following values, 1000ng/ml and 500ng/ml, 250ng/ml, 125 ng/ml, 62.5ng/ml, and 31.3ng/ml. For the LS-Bio ELISA, the lyophilised standard of the kit diluted by 1ml of sample diluent to provide 10ng/ml stock nephrin standard and serially diluted to provide 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml, 0.313ng/ml, 0.157ng/ml nephrin standard values were measured in duplicates. The measured and expected concentrations were calculated. The linearity for sample mean recoveries within 75-125% or 80-120% is acceptable (348). The observed and expected concentrations were plotted and then the linear fit with $r^2 > 0.9$ and a

slope of 0.9-1 range was assessed. Likewise, to demonstrate linearity the standard consistency between lots was verified using optical density values obtained for each standard.

Parallelism experiments

The parallelism test gives validation that urine samples containing high nephrin concentration and recombinant standards demonstrate a parallel sample dilution response curve in the standard concentration-response curve after dilution (349). The concentration of nephrin resulting from the validation confirms that the nephrin in the urine sample is recognised in a dose-dependent manner, similar to the standard curve. A previously analysed urine sample with high urinary nephrin concentration was used and diluted at an expected concentration that did not exceed the upper LOQ of the standard curve. Commercially available ELISA kit nephrin standard and the urine sample were serially diluted 2-fold in a standard/sample diluent buffer across the dynamic range of the assay and measured in duplicates according to the protocol. The absorbance at 450 nm of each dilution series was plotted against the human nephrin standard curve and the linearity of urinary nephrin and the standard concentration in the dilution series were compared to ensure that the standard diluted with standard/sample diluent can be used to quantitate nephrin concentration in the urine sample and evaluate the affinity of the antibody to urinary nephrin and the nephrin standard.

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection was evaluated by end-point dilution analysis, which shows the dilution of the known concentration of nephrin in which nephrin is reliably detectable (265). The lowest concentration of the kit standard was serially diluted two folds as 62.5 ng/ml, 31.3 ng/ml, 16 ng/ml, 8 ng/ml, and 4 ng/ml for Eth-Bio and as 0.313 ng/ml, 0.16 ng/ml, 0.08 ng/ml, and 0.04 ng/ml for LS-Bio. The serially diluted standards were measured repeatedly in duplicate. The lowest concentration reliably detected in 99% of the measurement was considered LOD. The LOQ was determined by considering the concentration of the lowest and highest standard on the standard calibration curve. The lower LOQ and the upper LOQ (the upper calibration point) can be measured on the regression curve within the defined range and met the acceptance criteria of mean recovery within the range of accuracy of 80–120% and reproducibility with CV < 20% (348).

3.4.5.5. Analytical specificity (matrix interferences)

Albumin interference in urinary nephrin measurement

Interference from commonly found urinary analytes was tested to confirm that the analyte to be measured is identified without cross-reactivity from other closely associated molecules. Interference from total protein and microalbumin was tested using 2 levels of commercially available urine chemistry controls (Biorad Australia), a total protein with a mean concentration (level 1 control: 132.5 mg/L and level 2 control: 611.5 mg/L kit and the microalbumin level of control level 1: 35.3 mg/L and level 2 control: 113.3 mg/L. Cross-reactivity was also tested for Human albumin (Albumex) (CSL^{TM}, Australia) with a concentration of 40g/L. The 1:1000 and 1:10,000 dilutions were carried out according to the sample preparation protocol of the manufacturer's manual from the concentrated albumin solution. The 40g/ml and 4g/ml of albumin were used to test albumin cross-reactivity with urinary nephrin. Albumin spiked urine samples, albumin diluted with sample diluent as a control, and sample diluent as a background control (blank) were tested according to the protocol.

Biotin interferences in urinary nephrin measurement

Biotin interferences have been reported in the immunoassay. Although sandwich ELISA is considered theoretically less susceptible to interference, evidence showed that in sandwich ELISA (**Figure 4.2**) biotin may falsely reduce the concentration of the analyte of interest (350). However, a recently published article showed that in the sandwich immunoassay biotin falsely elevated the concentration of analyte (351). The suppliers also recommended that the design of the ELISA kit be more robust against biotin interference and that there is no interference. To sort out the controversies, I developed a research question: Does biotin supplementation interfere with urinary nephrin concentrations using an ELISA assay that uses biotin-streptavidin coupling in the design?

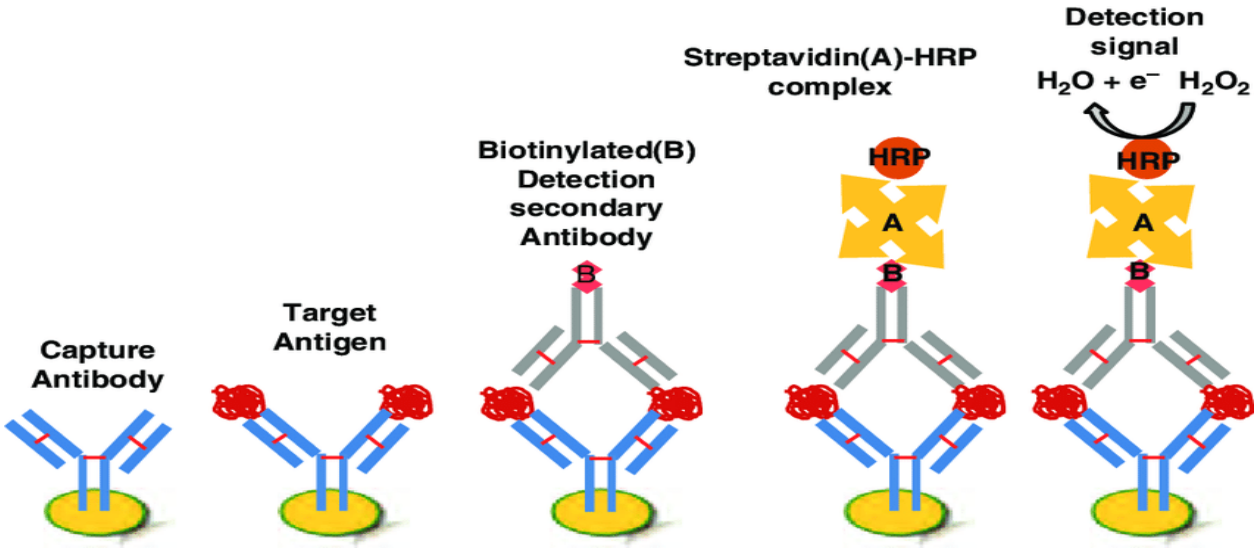


Figure 3.5. Assay design for LS-Bio ELISA (LS-F21185) of the human nephrin sandwich ELISA based on biotin and streptavidin coupling (352).

Experimental procedure for checking biotin interference.

To check biotin interference, the experiment used two approaches. (I) In the sandwich assay, the target antigen (urinary nephrin) and a biotin-conjugated detection antibody, which binds to the captured antigen according to the protocol, were added to each well in duplicates (**Figure 3.5)**. In this experiment, the samples were tested in the presence of a biotin-conjugated detection antibody and without a biotin-conjugated detection antibody to assess the intensity of the signal in each well in the presence or absence of a biotinylated detection antibody. If the wells that do not contain the biotinylated detection antibody produce a signal after the addition of TMB substrate, it shows interference, and conversely, no signal/low indicates that interference does not exist.

(II) Urine samples were collected from volunteers after overnight fasting at three time points: time point 0, directly before biotin containing multivitamin complex intake, to determine the 'baseline level' of urinary nephrin; time point 2, two hours after multivitamin complex intake; and time point 3, six hours after multivitamin complex intake. Biotin (30µg in multivitamin supplements) has a half-life of approximately 2 hours and is completely cleared from the body within 5-6 hours (353). Each urine sample was collected in a leak-proof clean container and labeled with a unique identification number and stored at -80^{0}c until further analysis. The urine samples were thawed at room temperature, promptly centrifuged at 3000 RPM for 10min to remove particulate matter, and then samples were processed for biotin interference

in the measurement of urinary nephrin using human nephrin sandwich ELISA according to the protocol. Finally, the concentration of urinary nephrin was compared with the baseline urinary nephrin concentration to evaluate whether interference existed.

3.4.5.6. Repeated freeze-thaw cycle effects on urinary nephrin measurement

Urine sample handling before laboratory analysis of the analyte of interest has the possibility to influence the test results (267). For this reason, it is imperative to consider whether repeated freeze-thaw cycles contribute to changes in urinary nephrin. This helps us to understand how the urine sample should be stored until laboratory analysis or pending a need for re-analysis.

Experimental procedure

- The urine samples with high concentrations of nephrin were selected during the first analysis. A pooled urine sample was prepared and divided into 6 equal aliquots with equal sample volume in a 1.5 ml Eppendorf tube and the samples were labelled 1 to 6. The aliquots were stored at -80^{0}C.
- Sample numbers 2-6 were thawed at room temperature without any aid and then restored at -80^{0}C after complete thawing.
- Sample numbers 3-6 were again thawed for the second cycle following the above procedure and stored again at -80^{0}C for the second cycle; samples were then subjected to freeze-thaw cycles as below.
- Thaw aliquots number 4–6 and store again at −80°C.
- Thaw aliquot number 5–6 and store again at −80°C.
- Thaw aliquot number 6 and store again at −80°C.
- Finally, after the 7th freeze-thaw cycle, all aliquots were thawed at room temperature and analysed using ELISA in duplicates in the same run to compare the changes if any, in urine nephrin concentration.
- The raw data (replicates of observed concentrations) of the aliquots were entered into Microsoft Excel software. The mean, SD, and % CV of the duplicate measurements were calculated for the observed concentration of urinary nephrin, and the trend in changes in the mean concentration of nephrin was plotted along with the freeze-thaw cycle series.

Note: Each freeze-thaw cycle comprised complete thawing for 2-3 hours at room temperature, followed by storage for at least 12 hours at −80°C.

3.4.5.7. Agarose gel electrophoresis and Immunofixation electrophoresis

Concentrating urinary nephrin standard for electrophoresis

Prior to Agarose gel electrophoresis and immunofixation, the urine sample and nephrin standards of the ELISA kit were concentrated using an Amicon Ultra-4 10k centrifugal filter device (Merck Millipore Ltd. Tullagreen, Carrigtwohill, Co. Cork, IRL, Ireland) that provides fast ultrafiltration, with the capability for high concentration factors and easy concentrate recovery from sample matrices. A 2ml pooled, 4000ng/ml ELISA kit standard (Eth-Bio) and 10ng/ml ELISA Kit standard (LS-Bio) of urinary nephrin were added to the Amicon Ultra filter device (**Figure 3.6**). The sample was centrifuged in a swinging bucket rotator at 4700 RPM for 3 minutes and the concentrate was collected from the filter device sample reservoir using a micropipette, while the ultrafiltrate was collected in the provided centrifuge tube. The final concentration of the recovered concentrate was calculated from the volume of the concentrate and the total original volume, and the original concentration of the standard was multiplied by the dilution correction factor resulting in a 2.5-fold concentrated nephrin standard. The concentrated nephrin was used for QuickGel agarose gel electrophoresis and the leftover concentrated nephrin was transferred to a Nunc tube and stored at -20°C.

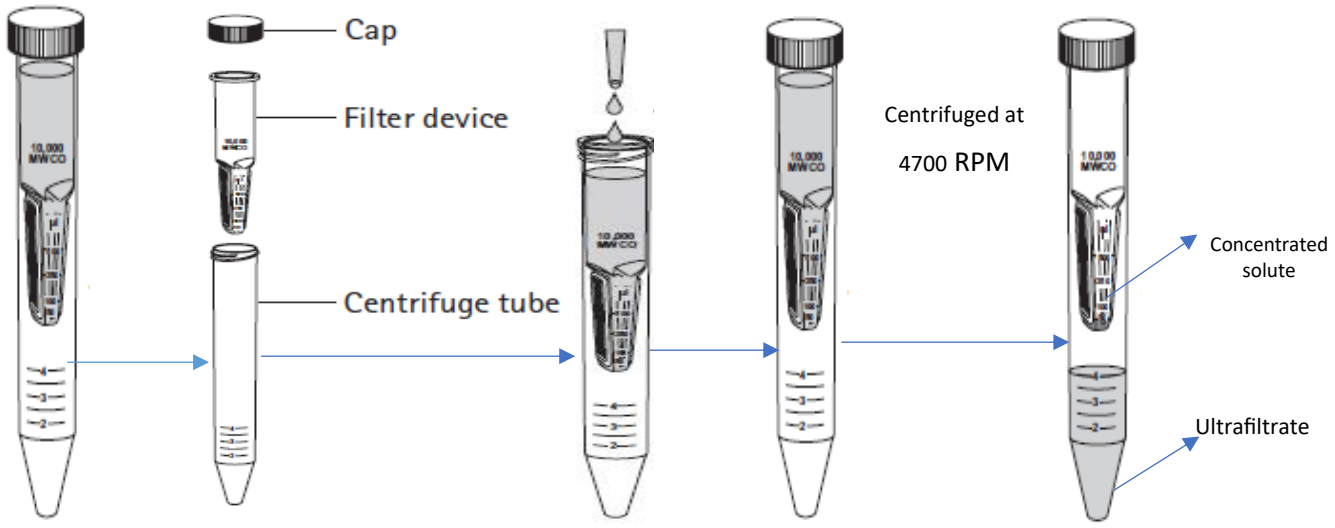


Figure 3.6. Concentrating urinary nephrin standard using Amicon Ultra-4 10K centrifugal filter devices.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed using the QuickGel Split Beta Serum protein electrophoresis (SPE) agarose gel kit according to the manufacturer's, Helena Laboratories manual (Helena Laboratories, Beaumont, TX, USA). Briefly, 15µl of commercially available protein controls and 20µl of filtered concentrated standards and urine samples were applied in duplicates to a numbered sample cup deep well of the sample plate. The samples were applied on the QuickGel SPE using QuickGel modified applicator blades. The gels were electrophoresed at 350 V, 60 A for 8 min at 20 ^{0}C using the QuickGel Chamber containing a cooled electrophoresis platen and built-in carbon electrodes. Then, after electrophoresis was completed, the chamber lid was opened and the gel blocks were removed using gel block remover, the electrodes were replaced on each end of the gel to prevent coiling during drying, and then by changing out the lid of the chamber, and closing the chamber lid, the gel was dried for 15 minutes. After the gel was dry, it was removed from the electrophoresis chamber and stained using a 3% acid blue stain (Helena Laboratories) for 4 minutes to enhance visualisation patterns for ease of interpretation of the band; subsequently, the gel underwent destaining in a 0.3% (W/V) citric acid solution for 2 minutes and dried completely, and then was visualised qualitatively for the presence of the band.

Immunofixation electrophoresis

Immunofixation electrophoresis (IFE) is a two-stage procedure using high-resolution protein electrophoresis on an agarose gel. In the first stage, urinary nephrin standards were resolved by electrophoresis and in the second stage, immunoprecipitation was carried out, (the nephrin standard and its corresponding antibody were allowed to react). The procedure was performed for the detection of urinary nephrin standards using nephrin mouse monoclonal antibody 66970-1-Immunoglobulin (Millennium Sciences, Proteintech, Australia). The standards utilised for this experiment were from Eth-Bio and LS-Bio nephrin ELISA. Prior to electrophoresis, the nephrin standards were concentrated (Amicon Ultra-4 centrifugal filter) about 2.5x according to the instruction manual and refrigerated at -20^{o}C, and then thawed prior to analysis.

The immunofixation electrophoresis was performed using kit techniques (QuickGel Immuno-Fix procedure, Helena Laboratories). A 20µl of concentrated filtered nephrin standards was

applied in triplicates to a numbered sample cup deep well of the sample plate. The electrophoresis procedure was completed. After electrophoresis, gel blocks were removed by a gel blocker, and the edges of the gel were wiped to remove excess moisture. The antisera template was placed on the surface of the gel. We utilised antibodies against nephrin 150ul, 1000µg/ml nephrin mouse monoclonal antibody (Millennium Sciences, Proteintech, Australia). A 140µl commercially obtained mouse monoclonal nephrin antibody and 140 µl of protein fixative were pipetted into the anode end of each channel in the template and antibody absorption was allowed to take place for 2 minutes. The gel was then removed from the electrophoresis chamber. The dried gel was washed with Tris-buffered saline by gentle shaking for 10 minutes, and then stained with 3% acid blue stain for 4 minutes; finally, the gel was destained using a 0.3% (W/V) citric acid solution until the background of the gel became clear. The dried gel was visualised qualitatively for the presence of a band (binding of nephrin-mouse monoclonal nephrin antibody) on the immunofixation pattern.

3.4.5.8. Size exclusion chromatography (SEC)/Gel filtration chromatography

The SEC is stated as a chromatogram (elution profile) that illustrates the difference in concentration of sample components as they elute from the column with respect to their molecular size, with larger molecules elutes first (354). The equipment used was sourced from Cytivia, USA. Instrument maintenance was performed a day before the experiment and the column was washed with 20% ethanol. A 500µl aliquot of either Eth-Bio and LS-Bio ELISA diluent alone or Eth-Bio and LS-Bio ELISA nephrin standards resuspended in diluent were loaded onto a Superdex 200 increase 10/300 GL prepacked glass column using AKTA pure fast protein liquid chromatography (FPLC) system and UNICORN^{TM} 4.0 control software and eluted in 500µl fractions in phosphate buffer saline (PBS) (0.05M phosphate buffer pH=7.2, 0.15M NaCl, 1mM $CaCl_2.2H_2O$, 0.5mM $MgCl_2.6H_2O$) at a flow rate of 0.6 ml/min at 8^{o}C. The LS-Bio ELISA of lyophilised standard vials was diluted with 150µl of PBS to minimise interferences from proteins present in the diluent as a preservative and carrier protein, and a 100µl of the concentrated standard loaded on Superdex 200 increases 10/300 GL following the procedure above. The elution was monitored at 280nm absorbance (UNICORN^{TM} 4.0 control software (Cytivia, USA) to determine the protein presence.

3.4.5.9. Data analysis and interpretation

Data were analysed using Excel software (Microsoft Corp., Redmond, USA). The mean, SD, intra-assay, and inter-assay CV were estimated using descriptive statistics. Intra and inter-assay precision were determined as CV derived from the mean and SD of replicate measurements. The chromatogram of the nephrin standards was drawn by an Excel spreadsheet using the data generated from the software. The correlation between the kits was determined using Bland and Altman plot (355), the difference and the mean of the difference in urinary nephrin (ng/ml) Eth-Bio and LS-Bio ELISA were plotted on the Y-axis and the X-axis, respectively.

3.5. Clinical validation experiment

Urine and blood samples were collected by research midwife nurses and coded with a unique registration number. Each sample is designated a unique specimen identification barcode (laboratory number) to which the test requests are assigned in the system, and there is also a unique record number for each neonate, infant, child, and pregnant woman, so this number remains unchanged throughout the follow-up period. In this validation experiment, first a prospective cross-sectional study that utilised samples collected as part of the ongoing project conducted at THHS, Australia was used. The study was conducted between 2019 and June 2021. All pregnant women attending THHS for routine antenatal service were invited to take part to explore the association of urinary NCR with the development of pregnancy-associated complications known to involve the kidney in a cohort of pregnant women **(Chapter 5).** Second, a longitudinal cohort study to determine clinically relevant RIs for urinary NCR against postnatal age in term neonates **(Chapter 6)** and investigate longitudinal changes in glomerular integrity markers (urinary NCR and ACR) and renal function markers (SCr, sCysC, and eGFR) in term and preterm neonates until 24 months of postnatal age was conducted **(Chapter 6).**

3.5.1. Validation of urinary NCR as a marker for early glomerular injury during pregnancy

The Townsville Hospital and Health services have more than 2,400 births per year, and the region has 10,000 births annually. Approximately 20-25% of babies born in this hospital are from the Indigenous Community. This study was open to all pregnant women who presented to the TUH, during the recruitment period (August 2019 to August 2021), obstetric history and other pre-existing medical conditions, such as diabetes, hypertension and kidney disease

were collected. Convenience sampling method at the time of their routine antenatal visit was employed. There were no exclusion criteria. Maternal blood and urine were collected for renal function testing serum creatinine (SCr), serum cystatin C (sCysC), urinary albumin, ACR, urinary nephrin and NCR at the antenatal visit. The samples were then processed and stored until analysis at -80 degrees. The demographic data was collected from the participant's antenatal records including maternal health during the pregnancy including history of hypertension (HTN), PE, pre-pregnancy diabetes (DM) or gestational diabetes (GDM). All data and biomarker results were entered into a Research Electronic Data Capture (REDCap) database and exported de-identified for analysis.

3.5.1.1. Association of serum and urine markers of renal function between women with normo, micro and macroalbuminuria

Once the data was collated, the women (N=273) were classified into three groups based on the ACR mg/mmol values according to KDIGO guidelines (242). Normoalbuminuria (Normo: ACR<3 mg/mmol, N=225)); Microalbuminuria (Micro: ACR 3-30 mg/mmol, N=38); Macroalbuminuria (Macro: ACR≥30 mg/mmol, N=10). The SCr, sCysC, urinary nephrin and NCR were compared between the three groups. The normoalbuminuric group was further divided for analysis of nephrinuria into a NORM group: women with ACR <3mg/mmol and no reported comorbidities and a NCOM group: women with ACR<3 mg/mmol but with reported comorbidities.

3.5.1.2. Association of urinary NCR with a diagnosis of Diabetes or PE during pregnancy

Women were classified into two groups according to their clinical notes, Group 1: hypertensive group (PE/HTN) including women reported to have PE and/or hypertension, and Group 2: Diabetes in pregnancy (DIP), women reported to have Gestational Diabetes (GDM), Type 1 diabetes (T1DM), Type 2 diabetes (T2DM). Due to small numbers in the HTN and T1DM and T2DM groups, these groups were combined with others for analysis, and these groups were compared to a NORM group: women with ACR <3mg/mmol and no reported comorbidities and a NCOM group: women with no reported comorbidities.

3.5.1.3. Comparison of urinary nephrin, NCR and ACR for the prediction of PE and DIP

The diagnostic accuracy of urinary NCR for predicting elevated ACR levels was determined by comparing the urinary NCR for women in the complete cohort and between those with

normal albuminuria (Normo) and a combined group micro-macroalbuminuria. To determine the predictive potential of urinary NCR and ACR for glomerular injury of PE, the women were also classified into the PE group (N=26) and compared to the NORM group: women with ACR <3mg/mmol and no reported comorbidities (N=135). The PE group were clinically confirmed cases of PE with denovo hypertension, blood pressure >140/90 mmHg and with /without proteinuria >300 mg/day, and other pathological changes after 20 weeks of gestation according to the definition of the American College of Obstetrics and Gynaecology (ACOG) definition (356). The majority of the women were > 20 weeks of gestation at the time of sample collection, the women included were in the second and third trimesters. Women with clinically confirmed PE were used to estimate the predictive potential of urinary nephrin, NCR and ACR for glomerular injury of PE.

3.5.2. Longitudinal changes in biomarker levels in neonatal cohort against postnatal age

This is a longitudinal cohort study conducted in the framework of the Kidney of Mother Infants and Neonates (KIDMIN) project entitled "*The Relationship between Maternal Health and Infant Renal Development and Function*". The data for this study were collected from a cohort of mother-infant dyads, recruited at THHS, in North Queensland over 24 months period (August 2019 to August 2021) and followed up for 24 months (total study duration of 48 months), between 2019 and 2023. For determining RIs **(Chapter 6)** for urinary NCR, apparently healthy-term neonates (gestation ≥37 completed weeks) born at THHS, and the surrounding health institutes were recruited and followed until 24 months of age and were included for this aim using *a posteriori* approach. This approach selected the reference individuals after collecting the relevant information and testing in the laboratory based on the clinical characteristics (**Figure 3.7**). The approaches in selecting the reference population and the relationship between defined terms and the establishment of RIs for urinary nephrin were according to the CLSI and IFCC documents. Inclusion criteria were apparently healthy neonates born at gestational age ≥ 37 weeks. Exclusion criteria were the refusal of parents to allow blood and urine sampling and preterm neonates. Data with missing demographic information and laboratory test results were also excluded.

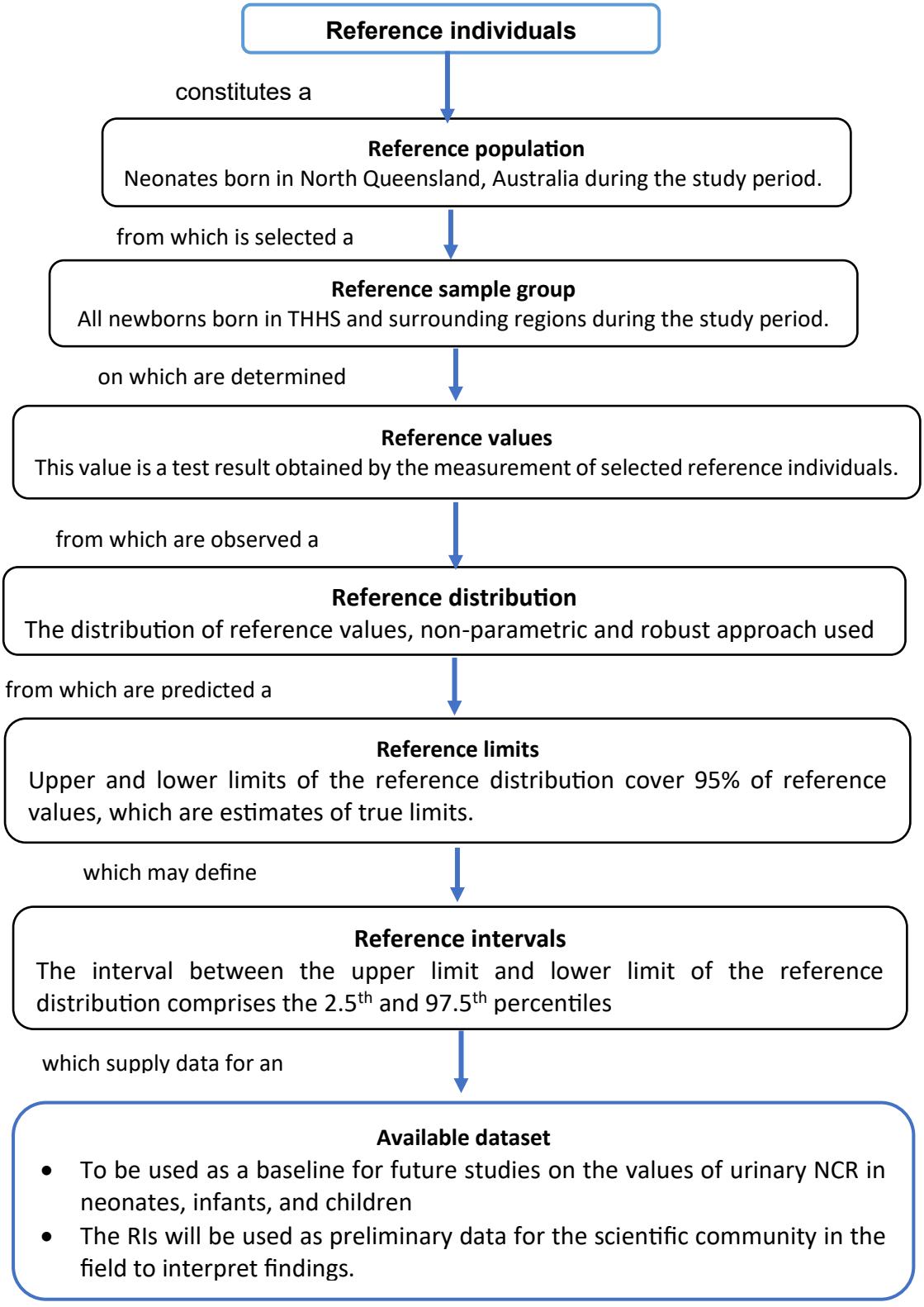


Figure 3.7. Flow chart showing the relationship between defined terms and establishment of RIs for urinary NCR in neonates, infants, and children. Adapted from Higgins C. 2012 (357).

To investigate the longitudinal changes of the biomarkers, in **Chapter 6**, all neonates who were delivered during the study period were eligible to take part in this study. Postnatal age, sex, gestational age at birth, birthweight, renal function markers, and maternal clinical characteristics such as hypertension, DM, GDM, and PE were collected. The z-score and centile for the given estimated foetal weight were calculated using International Foetal Growth Standards-Estimated Fetal Weight (Version 2.0) beta version calculator according to foetal biometry (gestational age in weeks + days and birthweight in grams). The centile for birthweight in grams was categorised for descriptive purposes. A neonate whose birthweight was less than the 10th percentile for gestational age was classified as small for gestational age, appropriate for gestational age (between the 10th and 90th percentile), and large for gestational age (greater than the 90th percentile) (358). Neonates with birth weight <2500 grams, regardless of gestational age at birth, were described as having low birth weight (358). Preterm neonates and term neonates as comparator groups were categorised. All neonates included in the study were followed until 24 months of age. Once enrolled, the neonates undertook the first assessment/measurement at birth and a second assessment at 12 months, and the third assessment at 24 months postnatal age **(Figure 3.8)** and during each assessment, the neonate undertook laboratory examinations.

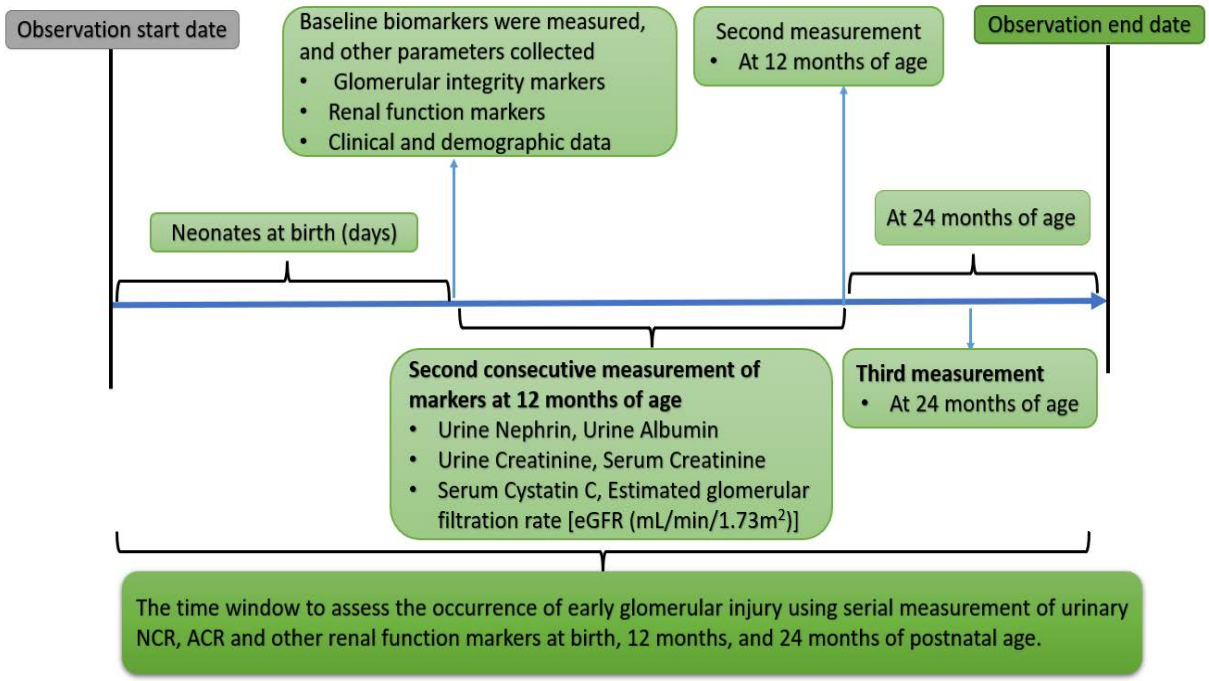


Figure 3.8. Study timeline flow diagram

3.5.3. Sample collection and biomarker measurement

A venous blood sample was collected using a plain tube for the measurement of SCr and sCysC. Urine samples were collected into a clean, leak-proof container without any preservatives. All samples were stored at -80^{o}c until analysis. Once thawed at room temperature, samples were centrifuged at 300 (RPM) for 10 minutes. Urinary nephrin concentration was measured using a human nephrin sandwich ELISA assay (Human NPHS1/Nephrin, LS-Bio [LS-F21185], Inc, USA) measurement is described in section **3.4.4.(Figure 3.4).** The urine samples were analysed in duplicate, the limit of detection of the kit was 0.16ng/ml and the samples with higher concentrations (>10ng/ml) were diluted from 1:10 to 1:100 using a sample/standard diluent supplied by the manufacturer. The concentration of urinary nephrin was calculated from the standard curve and reported as ng/ml. The precision of the duplicate measurements was calculated in our experimental data, and the inter-assay and intra-assay %CV were ≤10. Urinary nephrin and albumin were adjusted by dividing to urine creatinine concentration and described as albumin-to-creatinine ratio (ACR) mg/mmol, and nephrin-to-creatinine ratio (NCR) ng/mg. The clinical and demographic characteristics of the study participants were collected as part of the routine follow-up.

3.5.3.1. Urine albumin measurement

Urinary albumin was measured using Beckman Coulter AU urine CSF/Albumin immunoturbidometric method using the automated Beckman coulter Biochemistry analyser (AU480, Beckman Australia). In the reaction, anti-human serum albumin antibodies react with albumin from the sample to form immune complexes that scatter light in proportion to their size, shape, and concentration. The urine albumin calibrator (B38859), where values are traceable to IFCC Certified Reference Material CRM470 was used to calibrate the system. Prior to conducting the test sample procedure, two levels of quality control material were tested according to the manufacturer's manual, and deionised water was used for performing reagent blank. The absorbance of the sample was measured at 380 nm with a subtraction of the reference wavelength at 800 nm. The absorbance of the sample is directly proportional to the urine albumin concentration, and the concentration of albumin in the sample was

reported as mg/L. The albumin concentration in urine was corrected by urine creatinine and described as urinary ACR (mg/mmol).

3.5.3.2. Urine and serum creatinine measurement

The creatinine in the sample was measured using Beckman Creatinine, a kinetic modification of the Jaffe reaction method at alkaline pH (359) using the automated Beckman Coulter Biochemistry analyser (AU480, Australia). Creatinine in the sample reacts with picric acid at alkaline pH to form a creatinine-picrate complex. The rate of change in absorbance was measured at 520/800nm. The absorbance was directly proportional to the concentration of creatinine in the sample **(Figure 3.9).** The calibration for SCr measurement was performed by use of the chemistry calibrator, catalogue number: DR0070, which is traceable to an isotope dilution procedure mass spectrometry (IDMS) reference method using the National Institute of Standards and Technology standard reference material 967. The urine creatinine measurement urine calibrator with catalogue number, DR0091, was used. Quality control was run in each procedure according to the manufacturer's manual. The linearity range for SCr was 0.2-25mg/dl and for creatinine in the urine, 1300 mg/dl, and the sensitivity of the analyser to measure creatinine was 1mg/dl of absorbance change. Samples with a concentration higher than the upper limit of linearity were diluted and repeated; the corrected concentration was reported as the final concentration.

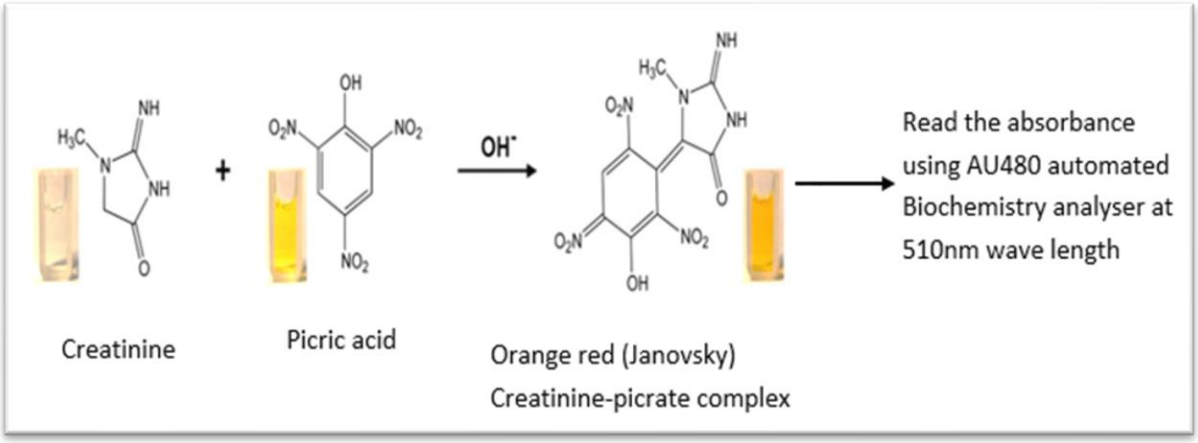


Figure 3.9. Principle of the modified Jaffe reaction to determine creatinine concentrations.

3.5.3.3. Measurement of sCysC concentration and calculation of eGFR

The sCysC measurement was performed by using gentian cystatin C Immunoassay on Beckman Coulter AU Systems (Beckman Coulter AU480, Australia). The method employed was a particle-enhanced turbidimetric immunoassay. The cystatin C in the serum sample reacts with the anti-cystatin C antibodies from the immunoparticle solution to form aggregates that increase the turbidity of the solution. The degree of turbidity was proportional to the concentration of cystatin C, which can be determined from the standard calibration curve. The gentian Cystatin C calibrator was standardised against the international calibrator standard ERM-DA471/IFCC. The eGFR was calculated from sCysC concentration using the Zappitelli sCysC formula (eGFR [mL/min/1.73 m^2] = 75.94/ [$sCysC^{1.17}$]) (212).

3.6. Data analysis and interpretation

Statistical analyses were performed using Excel software (Microsoft Corp., Redmond, USA), IBM SPSS Statistics 28 (Armonk, NY, USA), MedCalc (Ostend, Belgium), and GraphPad Prism 9 (Version 9, GraphPad Software Inc, San Diego, CA, USA) for each chapter as necessary.

The normality of the data was visually checked using a QQ plot and histogram, and statistically using the Kolmogorov-Smirnov test and Lilliefors correction of the central limit theorem and the Skewness and Kurtosis Z scores of the data distribution divided by their standard errors, and the central limit theorem was used as appropriate if the sample size was ≥30 in each group. The data were presented as mean± SD for normally distributed data and as the median and interquartile range (IQR) for non-normally distributed data or percentages as appropriate. Kruskal-Wallis H test with post-hoc Bonferroni correction, and Mann-Whitney *U* nonparametric test were performed to determine the difference in continuous variables between groups in pregnant women, and the longitudinal changes in biomarker levels across postnatal ages from birth to 24 months and comparison of biomarker levels in term and preterm neonates, respectively.

The predictive potential of urinary NCR for early glomerular injury was tested and described by binary logistic regression with ROC analysis. The optimal cutoff value for urinary NCR (ng/mg) was estimated at a maximum value of the Youden J index (sensitivity + specificity - 1) and the AUC with its 95% CI. Diagnostic estimates, such as sensitivity, specificity, and predictive values, were calculated for the optimal cutoff values of the biomarker in predicting

early glomerular injury. The diagnostic performance of urinary nephrin, NCR and ACR in predicting adverse pregnancy outcomes-PE were described by ROC analysis and AUC. Correlation was performed to determine the association of urinary NCR with other biochemical parameters.

For determining RIs normality was also checked by D'Agostino-Pearson (DAP) test. In non-normally distributed data, Box-Cox transformation was used to transform the data to a normal distribution or symmetrical distribution. Box-Whisker plot was used to investigate the outliers using Tukey's method (360). The 25^{th}, 50^{th}, and 75^{th} percentiles were determined, and then IQR was calculated. Data lower than 1.5 x IQR of the first quartile or greater than 1.5 x IQR of the third quartile was considered outliers and removed from the analysis either before or after the transformation of the data at either side. A nonparametric and a robust method were used (361) to calculate the RIs as the data was found non-parametric following the assumption of normality, and small sample size in the groupings, respectively. In the robust approach, the CIs for the reference limits are estimated using the bootstrapping method with 10,000 iterations and a random number seed. The RIs were described as the lower 2.5^{th} and upper 97.5^{th} percentiles (covering 95% of the reference values), with their respective 90% CIs for lower and upper limits according to CLSI C28-A3 guidelines for estimating percentiles (362). Harris and Boyd's rules were used to determine the need for partitioning of the RIs (363) regarding the postnatal age and gender of the neonates. The upper limit of normal (ULN) value of urinary NCR was considered a clinically relevant cutoff to define early glomerular injury and/or glomerular immaturity. A P-value < 0.05 was considered statistically significant in all cases.

3.7. Data management and storage

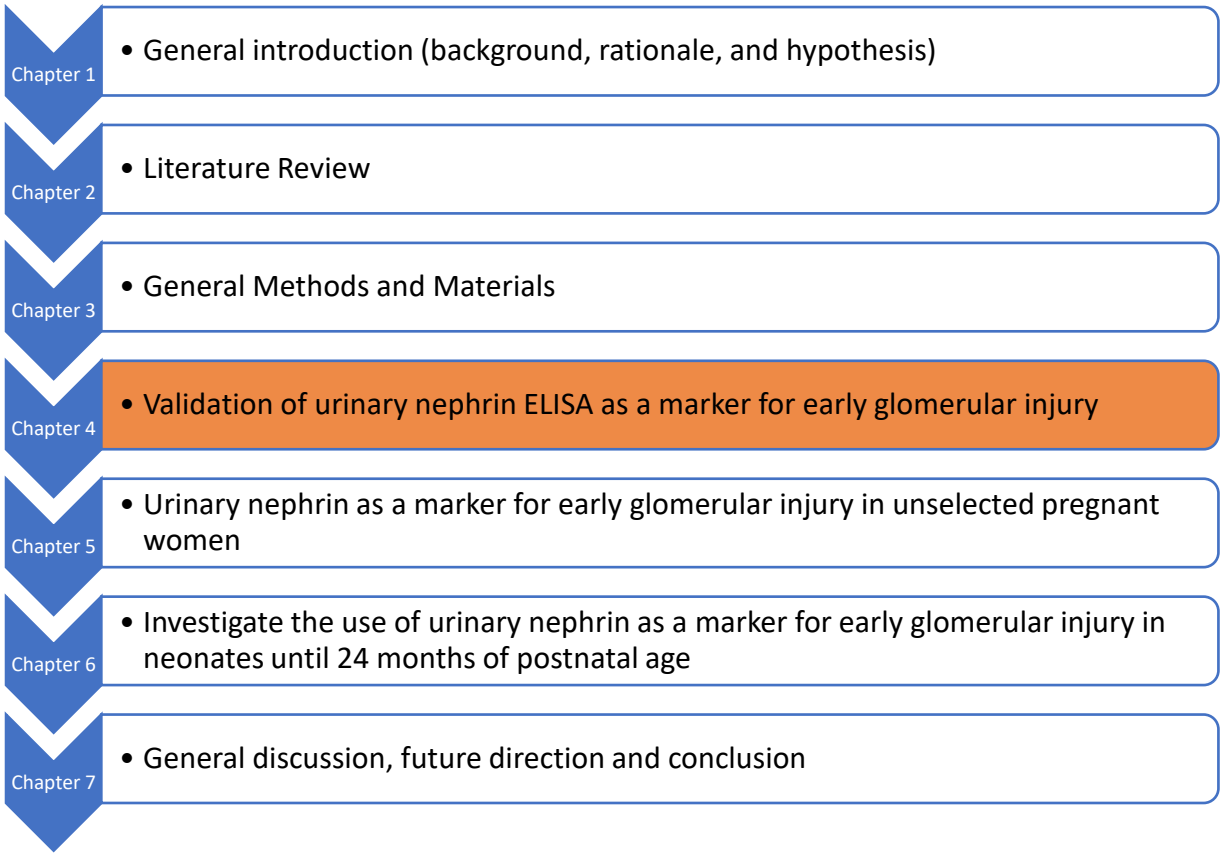
The data are managed using the REDCap system at THHS and all data collected for this study were stored in the THHS database and kept confidential. All data were de-identified before analysis and labelled with a unique laboratory identification number code to ensure the confidentiality of the study participants. All laboratory data collected for this study was stored in the THHS database with clinical data. Only the principal investigator and the members of the research project team had access to the data. For data analysis and publication arising from this study, no personal identifier will be released, only looks at the participants as a collective cohort, and never individually.

3.8. Human research ethics and other approvals

Ethics approval was obtained from the THHS Human Research Ethics Committee (HREC Reference number: HREC/2018/QTHS/48326, and the JCU human research ethics committee (JCU/H7684) according to the tenets of the Declaration of Helsinki and its amendments on ethical principles for medical research involving human subjects. The objectives and expected outcomes of the project were explained, and all the questions raised were clarified for the parents. Written informed parental consent was secured for all neonates following verbal explanation of the study aims by research nurses and midwives before enrolment in the study. Moreover, the biosafety application (JCU-IBC-200831) and the risk assessment (Ref Number: 10654) have been approved.

3.9. Chapter summary

In this chapter, the detailed methods and study design have been described. Study participants' recruitment procedures have been addressed. The experimental analysis of biomarkers, methods of collection, and analysis of clinical and sociodemographic data have been stated. The statistical analysis for quantitative data in each experimental chapter has been described and used the appropriate statistics relevant to chapter four to six of the thesis. The next chapter (**Chapter 4**) will present validation of urinary nephrin ELISA using a suite of assay validation parameters, compare the analytical performance and correlate measurement of urinary nephrin between the two kits. Finally, the chapter will investigate the metrological traceability of the standards of the two ELISA kits.



Foreword Chapter 4

The studies included in this chapter represent the validation of two commercially available human-specific urinary nephrin ELISAs. This validation process was undertaken to provide consumer confidence and scientific rigor to an assay used for the determination of urinary nephrin. This urinary biomarker has shown promise as a marker of early glomerular injury (21, 50). Ensuring the robustness and clinical utility of this assay is the first step required to translate the urinary nephrin ELISA into clinical practice for determining early glomerular injury. The urinary nephrin may become a useful tool for determining and predicting early glomerular injury. In this study, a suite of assay validation parameters was used to evaluate the analytical performance of the two ELISA kits. To date, the two ELISA kits have been used in the scientific community to determine glomerular maturity and glomerular injury and to predict AKI. However, there are huge variations in the assay dynamic range, source, and formulation of the standard material between the two kits. An important part of the validation study was to test the correlation between urinary nephrin concentrations determined using the two kits and to test whether the two kits can be used interchangeably to determine glomerular injury.

Chapter 4. Validation of human urinary nephrin ELISA as a marker for early glomerular injury

4.1. Abstract

Introduction: Urinary nephrin has been demonstrated to be a non-invasive biomarker of glomerular injury. However, it is yet to be translated into clinical practice. This study aimed to validate the analytical performance of urinary nephrin ELISA for the diagnosis of early glomerular injury.

Methods: This study used stored deidentified urine samples to validate Eth-Bio and LS-Bio ELISAs for measuring human urinary nephrin. The analytical performance of the two kits was investigated using a suite of assay validation parameters including linearity of dilution, recovery, parallelism, precision, the limit of detection, assay dynamic range, and analytical interferences. The stability of nephrin was investigated for repeated freeze and thaw cycles. Measurements for urinary nephrin between the two kits was correlated. A p-value <0.05 was considered statistically significant.

Results: Of the 244 neonatal urine samples collected as part of a previous study, 240 samples were analysed using Eth-Bio ELISA, 191 using *LS-Bio ELISA,* and from these, 187 using both kits. *Eth-Bio* and *LS-Bio* ELISA demonstrated an intraassay CV of 13 and 10%, respectively. Both kits had an interassay CV less than 10%. The batch-to-batch interassay variation of the ELISA kits was <10%. The detection limit for Eth-Bio ELISA was 31.3ng/ml and for LS-Bio ELISA, it was 0.157ng/ml. This study showed acceptable results in the linearity of dilution for both kits, but it was not linear at the lowest dilutions for the Eth-Bio ELISA kit. The ELISA kit used for the measurement of nephrin showed no interferences with albumin and biotin. Following a kit-to-kit correlation study, we found a statistically significant difference in the measurement of nephrin between the two kits ($p < 0.05$).

Conclusions: Urinary nephrin can be quantified reliably using human nephrin-specific ELISA tests. The LS-Bio nephrin ELISA demonstrated satisfactory analytical performance in most assay validation parameters, which suggests it may have the potential to be a diagnostic tool for early glomerular injury. However, the accuracy of the ELISA kits warrants further investigation, as there was no agreement between the urinary nephrin measurements performed using the two kits.

4.2. Introduction

Glomerular injury is a common element in the pathogenesis of both AKI (50, 364, 365) and CKD (71). Neonates born prematurely and admitted to the NICU are at increased risk for AKI due to arrested kidney development and reduced nephron endowment (366). The glomerular injury occurs in neonates after hemodynamic insults, including perinatal hypoxia-ischemia events, as well as inflammation and sepsis (12, 14, 15). It may also occur as a result of lifesaving but potentially nephrotoxic treatments in the NICU such as the administration of nephrotoxic drugs including nonsteroidal anti-inflammatory drugs or aminoglycosides (193, 364, 367). Glomerular injury from nephrotoxicity and hypoperfusion leads to structural and functional changes over time, which is unnoticed and lead to long-term impacts on kidney function (31, 366). This leaves preterm infants at greater risk of the development of CKD later in life (50, 368, 369). Early detection of glomerular injury and mitigating the progression of injury could avoid further complications (370, 371).

Although great improvements have been made in the diagnosis and management, kidney disease continues to be a major global public health problem, affecting 750 million people worldwide (79). Kidney disease and/or injury biomarkers are needed for diagnosis and monitoring, perhaps for early detection of glomerular injury (ideally before progression to other forms of kidney injury and/or disease). Given the importance of early detection of glomerular injury; there is a pressing need to validate an assay method for a novel biomarker of glomerular injury.

Importantly, before an assay can be brought into routine use for diagnostic and prognostic purposes, it is important to determine the performance and trustworthiness of the assay. Method validation is mandated by guidelines that define clinical and laboratory standards such as Clinical and Laboratory Standard Institutes (CLSI) (347), and in Australia is covered by the National Pathology Accreditation Advisory Council (NPAAC) (372) guidelines for laboratory accreditation before the translation of the method for clinical application.

Traditionally, methods for determining glomerular injury involve the determination of urinary protein levels. Different analytical methods are utilised for determining urinary protein. The traditional methods for detecting proteinuria include chemical examination (e.g., Biuret method) and colorimetric (turbidimetric, dye-binding) methods. However, these

methods have limitations in specificity, lack clear agreement for measuring the low level of proteinuria between laboratories, and have missed early diagnosis of injury (373, 374). In the past decade, microalbumin has increasingly become a protein biomarker, and due to the increased sensitivity and specificity of ELISA measurement, is often used for the measurement of urinary protein biomarkers for the diagnosis of glomerular injury and AKI in clinical settings (21, 43, 50, 75).

The translation of biomarkers for clinical use is not always simple and the quality of the assay methods varies, due to the wide variety of ELISA kits available from different suppliers, which may lead to errors in biomarker measurement (375). Guidelines, including CLSI (347), and the NATA (265) recommend validation of the assay and meeting the specific assay performance characteristics requirements before the translation of the novel biomarker into clinical practice.

Nephrin is a 180 Kd integral transmembrane protein of the immunoglobulin superfamily, and a structural component of the Sd of the glomerular podocyte, which is involved in various aspects of podocyte biology (5, 376). It forms an intricate mesh at the tips of the podocytes, providing a strong negative charge to repel proteins from the Bowman's capsule. Therefore, nephrin, together with the fenestrated endothelium, and GBM forms a GFB (5, 377).

The importance of nephrin was initially discovered in the late 1990s by Kestilia *et al.* when mutations in NPHS1 were detected in children with congenital nephrotic syndrome of the Finnish type (56). Since then, nephrin has been detected in diseased glomerular (21, 43) and urine in the presence of podocyte injury. Therefore, it has been suggested that nephrinuria may reflect early glomerular injury (21, 50, 68). Human and animal studies have shown that nephrinuria may be a non-invasive biomarker of early glomerular injury preceding other podocyte proteins (23, 55, 251), albuminuria, and these studies have further shown a correlation between nephrinuria and the severity of glomerular injury (21, 50, 149, 239). However, these studies are yet to be validated and the analytical performance of the immunoassays compared for translation into clinical use. Another important aspect has been the lack of a cutoff for urinary nephrin concentrations to determine early glomerular injury. Thus, analysis of urinary nephrin and demonstrating its potential as a diagnostic,

prognostic, and monitoring tool for glomerular injury may help to develop intervention strategies for short and long-term kidney health consequences in vulnerable populations.

To the best of our knowledge, there have not been studies on the validation of urinary nephrin ELISA for the detection of early glomerular injury. Therefore, this experimental chapter aimed to validate the analytical performance of two commercially available human specific nephrin ELISA kits to confirm the analytical performance using a suite of immunoassay method validation parameters, as a first step toward clinical validation. The following specific objectives were investigated to achieve this aim:

- Establish the standard calibration curve and confirm the accuracy and precision of the kits for measuring urinary nephrin.
- Confirm the level of interference from commonly encountered interferences (albumin and biotin) for measuring urinary nephrin.
- Correlate and compare the two methods based on their analytical performance using the validation parameters and to recommend a method of choice for clinical validation of the urinary nephrin ELISA.
- Investigate the metrological traceability and accuracy of the standard concentrations of both ELISA kits using electrophoresis and size-exclusion chromatography.

4.3. Results

This study utilised 244 stored de-identified urine samples from a longitudinal cohort study *"Relationship between prematurity, renal volume, and retinal vasculature study*". Urine samples were analysed using the *Eth-Bio ELISA* (n=240), *LS-Bio* (n=191); and both kits (n=187). The individual performance of each kit was acceptable using several parameters (**Table 4.3**). However, no correlation was found between the results of the two kits in the measurement of urinary nephrin (**Figure 4.11**). The standards from the Eth-Bio ELISA test when replicated, yielded reproducible results, and showed a good fit to the scatter plot on the regression model (**Figure 4.4A**), likewise, in the LS-Bio ELISA test, the standards also yielded a reproducible result and a good fit to regression model (**Figure 4.4 B**).

4.3.1. Immunoassay standard calibration curve

The calibration curves for both kits are displayed below (**Figures 4.1A and B**). Briefly, serial dilutions were made using the recombinant urinary nephrin standard supplied with the kit (31.3-2000ng/ml Eth-Bio and 0.157-10ng/ml, LS-Bio). Sample data were generated during the validation of the assay for both kits described in **Tables 4.1 and 4.2** to evaluate the measured nephrin standard in the defined range that met the acceptance criteria for mean recovery within the range of 80–120% and precision < 20%. In the Eth-Bio ELISA test, the concentrations of the standards yield reproducible results and are a good fit to the scatter plot on the regression model (**Figure 4.1 A).** However, the lowest dilution deviates from the regression line; likewise, in the LS-Bio ELISA test, the standards yielded a reproducible result and a good fit to the regression model (**Figure 4.1 B**).

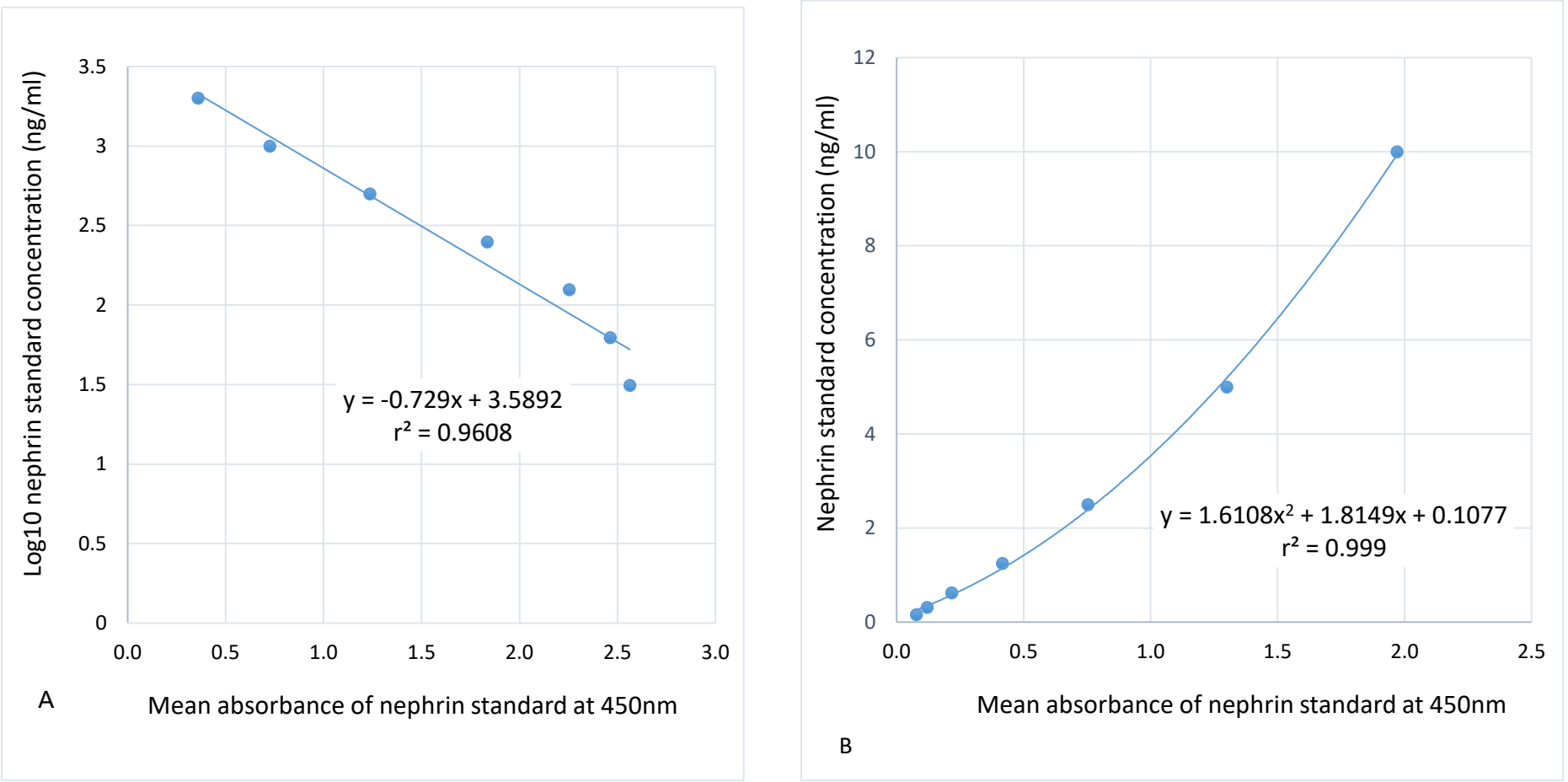


Figure 4.1. A standard curve validation of **A**) An Eth-Bio ELISA test (r^2=0.9608) and **B**) The LS-Bio ELISA test (r^2=0.999).

4.3.2. Assay dynamic range and limits of detection and quantification.

In this experiment, the calibration curves for both ELISA kits were based on dilutions of the recombinant urinary nephrin standard. The measured concentration of the standard in the defined range could meet the acceptance criteria of mean recovery within the range of 80–120% and reproducibility with CV less than 20% (348). In this experiment for ELISA Eth-Bio, the standard recovery values are shown in **Table 4.1**. Calibration values for the nephrin standard were between 2000ng/ml and 31.3ng/ml. The recovery was between 74-173% for ELISA Eth-Bio. The CV was less than 20% between the observed and expected concentrations of the standards. This variability was present across the concentration range, as stated in **Table 4.1.** The CV at the lowest cutoff/decision-making point of the kit standard was 8.8%. At the highest concentration points of the calibration curve, the CV was less than 20% and the accuracy/recovery was 99% and complied with the recommended criteria.

For test Eth-Bio, the assay dynamic range was 62.5-2000ng/ml, the lower LOQ for this kit did not fulfill the criteria of recovery (80-120%) but the assay showed a good precision of <20% at the lowest concentration of the kit standard (31.3ng/ml). At the lower end of the dynamic range, the regression of the calibration curve is not linear and is not used as the lower LOQ, at this point falsely high confidence may be placed in the lowest standard of the curve where a high nonlinear relationship is observed between measured and observed concentration and the assay could not accurately detect /distinguish nephrin concentration between the two lowest points on the calibration curve. The measured concentration is higher than the expected concentration at the lowest concentration of the kit standard, resulting in higher recovery (>120%), which did not fit the recommended criteria. However, the precision of the replicate measurements was acceptable (<20%). The next point of the curve was deemed to be the lower LOQ at 62.5ng/ml (**Table 4.1**). This was contrary to the manufacturer's claims.

Using endpoint dilution analysis to determine the LOD, the Eth-Bio kit does not accurately detect nephrin concentration below 31.3ng/ml. In the dilution series from 62.5ng/ml to 4ng/ml, dilution containing the expected concentration of 31.3ng/ml, the measured concentration was ≥31ng/ml in repeated measurements. The mean measured concentration was relatively close to the lower LOQ; the calibration curve is not linear (**Figure 4.1A)** and the linearity of the dilution curve is flat at the lowest two dilutions (**Figure 4.2**). We can detect

nephrin at this dilution but not accurately (**Table 4.1**). Therefore, the LOD for Eth-Bio is at dilution with a concentration of 31.3ng/ml but the lower LOQ (62.5ng/ml) is the decision-making point in this kit.

Table 4 1: Data generated from the standard calibration curve to determine the lower and upper limit of quantifications for the Eth-Bio ELISA (N=3)

Nephrin (ng/ml)	Mean nephrin (ng/ml)	Standard Deviation	Coefficient of variation (% CV)	Recovery (%)
2000	1986	57.0	2.87	99.3
1000	1215	49.8	4.10	121.5
500	479	42.2	8.80	95.9
250	184	17.0	9.23	73.5
125	94	7.8	8.31	75.0
62.5	57.7	5.0	8.71	92.3
31.3	54.1	4.8	8.84	172.8

The standard curve was based on serial dilutions of recombinant nephrin with a concentration range of 31.3-2000ng/ml. Recovery= Obs/Exp x100: Observed (Measured)/ Expected concentration.

In this experiment, the LS-Bio ELISA test, the standard recovery, and the assay dynamic range, are presented in **Table 4.2**. The overall recovery of the measured concentration of nephrin standard ranges from 98-102% and the precision of replicate measurements is 0.9-17%. This varied over the concentration range from the highest to the lowest concentration of the standard. In this kit, both the recovery and the CV are within the range of the recommended criteria. At the lower end of the dynamic range, this kit could accurately detect an observed mean concentration of nephrin 0.16ng/ml (manufacturer's claims 0.157ng/ml). At the upper end of the dynamic range, the test could also detect a measured mean concentration of 9.98ng/ml. Therefore, the lower and upper LOQ for this kit fulfills the criteria of recovery (80-120%) and the CV of < 20%. Taking all measurements into account, the lower LOQ of this assay is defined as 0.157ng/ml, and the upper LOQ is defined as 10ng/ml in the LS-Bio ELISA test. The assay dynamic range is presented as 0.16-10ng/ml (**Table 4.2**).

Using endpoint dilution analysis to determine LOD, the LS-Bio ELISA accurately detects a mean nephrin concentration of 0.16ng/ml. In the dilution series from 0.625ng/ml to 0.04ng/ml. The 4-parametric logistic regression analysis curve does not detect nephrin below 0.16ng/ml and

is reported as below the standard range. In repeated measurements, the mean nephrin concentration detected was 0.16ng/ml. Therefore, the LOD for LS-Bio is 0.16ng/ml.

Overall, the results suggested that the mean absorbance at 450nm of the sample/standard dilutions that fall within the linear portion of the standard calibration curve and within the recovery range would provide an accurate measurement of the nephrin concentration in the urine sample.

***Table 4 2*: Data generated from the standard calibration curve to determine the lower and upper limit of quantifications for the LS-Bio ELISA (N=8)**

Nephrin (ng/ml)	Mean nephrin (ng/ml)	Standard Deviation	Coefficient of variation (%CV)	Recovery (%)
10	9.98	0.09	0.92	99.8
5	5.04	0.09	1.74	100.8
2.5	2.48	0.07	2.76	99.2
1.25	1.26	0.04	3.07	100.6
0.625	0.64	0.03	5.05	101.7
0.313	0.31	0.02	5.34	98.3
0.157	0.16	0.03	17.32	98.2

The standard curve was based on six serial dilutions of recombinant nephrin with a concentration range of 0.157-10ng/ml. Recovery = Obs/Exp X100: Observed (Measured)/ Expected concentration.

4.3.3. Assay precision experiment

The assay precision for urinary nephrin ELISA was evaluated using a replicate experiment. The *Eth-Bio ELISA test* (n=240) and LS-Bio ELISA test (n=191) demonstrated intraassay precision of 13% and 10%, respectively. To determine the interassay precision, a pooled urine sample was run with each assay similar to a quality control sample. The interassay precision for both ELISA kits were found to be <10% (**Table 4.3**); overall, these results show an acceptable precision between wells of the same plate as well as plate-to-plate precision.

To determine batch-to-batch precision, duplicate measurements of urine samples were performed on ELISA kits with different lots and received at different time points during the study. The batch-to-batch variation was calculated from the mean of duplicate measurements of urinary nephrin and the corresponding SD for the batches. The back calculated concentrations of the first 4 dilution series of the standards of each kit were also used to assess intraassay variations of duplicate measurements in the same batch and different

batches of the ELISA kits, and the CV of the standards of duplicate measurement was < 10%. Pooled urine sample was used to evaluate the batch-to-batch interassay CV, and at least 10 duplicate measurements were conducted in each kit resulting in an interassay precision of ≤10% (ranging from 7 to 10% in each analysis) for both kits (**Table 4.3**).

Table 4 3: Summary of the assay precision and characteristics of ELISA kits

Assay precision	Eth-Bio ELISA		LS-Bio ELISA	
	Tested	Package insert	Tested	Package insert
Intraassay precision	13%	N/A	10%	<6.25%
Interassay precision	<10%	N/A	<10%	< 5.77%
Limit of detection	31.3ng/ml	N/A	0.16ng/ml	0.16ng/ml
Batch to batch interassay variation	<10%	N/A	<10%	N/A
Minimum sample required	100 µL	100 µL	100 µL	100 µL
Number of standards	7	7	7	7
The turnaround time to results	1.5 days	1.5 days	3.25 hours	3.25 hours
Assay platform	manual	manual	manual	manual
Technical expertise	High	High	High	High

N/A: Not stated in the package insert.

4.3.4. Systematic variations

This experiment was aimed to check whether there is a variation in the concentration of nephrin due to technical and other factors. A pooled urine sample was used for this analysis. Row by row, column by column investigation was conducted to determine if there were any differences across the plate. This would rule out issues with incubation, washing and pipetting during the run. The urinary nephrin concentrations were compared within rows (n=16) and columns (n=16) using the Eth-Bio ELISA test, and rows (n= 35) and columns (n=32) using LS-Bio ELISA test in duplicate measurements. The CV was ≤10% in both ELISA kits **(Table 4.4**).

Table 4 4: Variations in urinary nephrin measurement across row and column of ELISA wells

ELISA Kits	Rows			Columns		
	Mean (ng/ml)	SD	CV%	Mean (ng/ml)	SD	CV%
Eth-Bio ELISA (N=16)	280	0.02	8	280	0.03	10
LS-Bio ELISA (N=35)	3.0	0.27	9.2	2.99	0.28	9.3

4.3.5. Linearity of dilution experiment

Linearity was confirmed for the standard curve pertaining to each kit. The linearity was good for both kits. However, in ELISA Eth-Bio, there deviates from the slope of the line at the second and third dilutions. The two lowest dilutions of Eth-Bio are flat at baseline and the concentrations are close to each other; this showed that the measured nephrin concentration is slightly higher than the manufacturer's claims. Furthermore, LS-Bio showed a linear relationship between the observed and expected concentrations and linear across the range of dilutions in the regression equation (**Figure 4.2**). Overall, the linearity is good for a wide range of dilutions and may provide flexibility for urine samples with different concentrations of nephrin. However, LS-Bio ELISA demonstrates good dilutional linearity relative to Eth-Bio. The mean recovery (%) for both kits was within the recommended criteria except for the lowest dilution of Eth-Bio, which is greater than 120%.

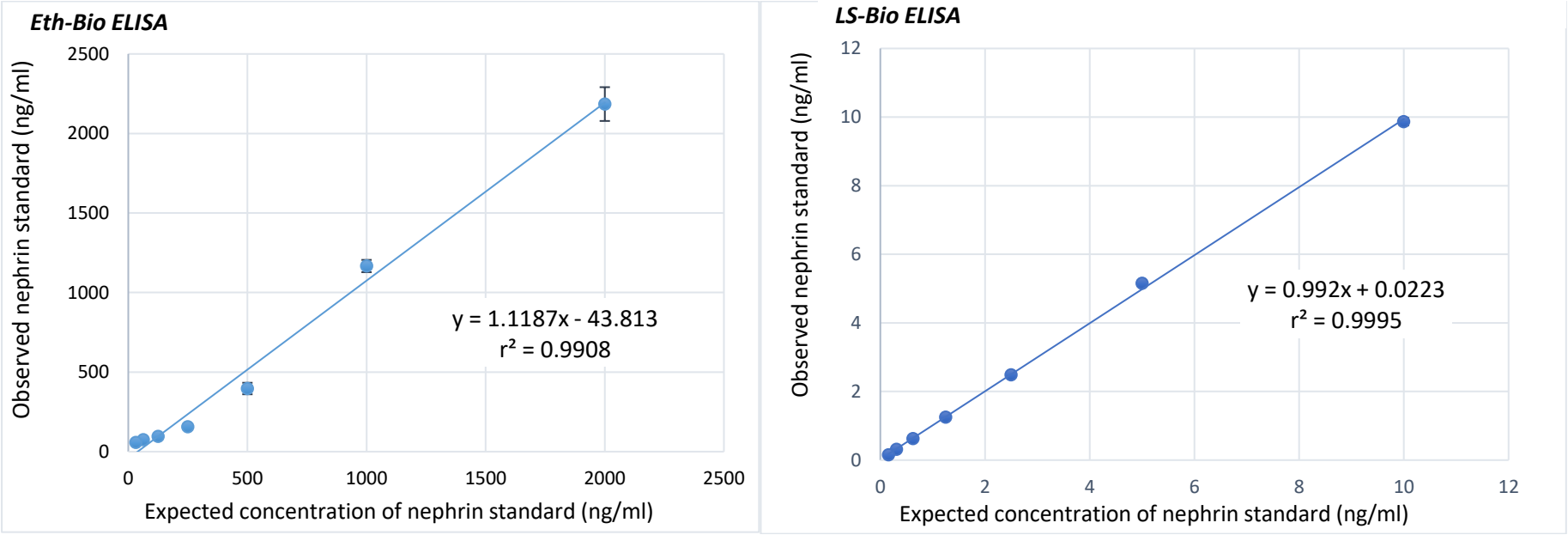


Figure 4.2. Investigation of linearity of dilution for ELISA Kits through a dilution of nephrin standard.
The values are expressed as the mean ± standard error of the mean (SEM).

4.3.6. Assay parallelism experiment

To verify parallelism, the LS-Bio ELISA standard and a urine sample diluted across the assay dynamic range were measured in duplicates. For this experiment, the dilution series from 0.157-5ng/ml of the kit standard were used. By calculating the urine nephrin concentrations at each dilution, it was found that the mean of the duplicate wells fell within the quantitative range of the assay, resulting in a mean of 1.43ng/ml. The CV of the mean urinary nephrin in the dilution series was <5%. **Figure 4.3A and B** show that the experiment has a good assay linearity of dilution and parallelism. The urine sample dilution response curve and the recombinant nephrin standard concentration response curve show relatively similar (**Figure 4.3 A**), and the dilution response curve (dilution adjusted concentration) is linear over the measuring range (**Figure 4.3 B**). The data shows the standard could accurately reflect the concentration of nephrin in the human urine sample and the assay demonstrates the affinity of the antibodies to nephrin in the standard and a patient sample found to be similar.

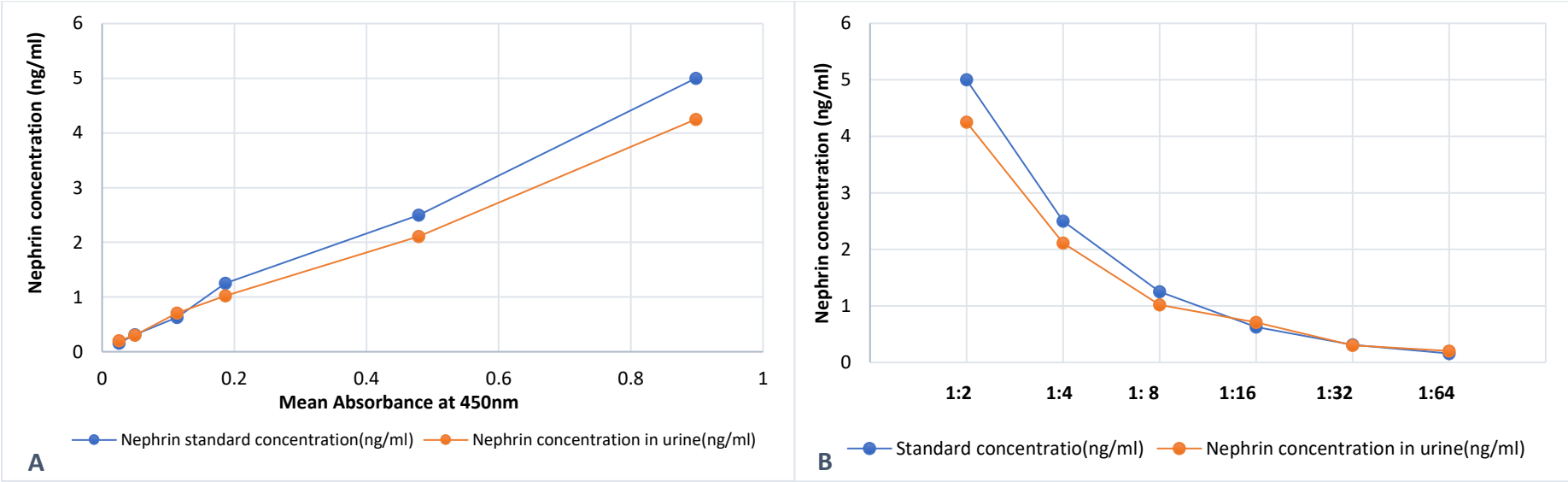


Figure 4.3. Evaluation of the parallelism of the nephrin ELISA assay with commercially available nephrin standard and urine sample

4.3.7. Recovery experiment ELISA test kits.

This experiment aimed to determine the recovery nephrin between the urine sample and the standard diluent. The results from the recovery experiment in the diluted standards spiked into urine samples containing 222ng/ml of nephrin (Eth-Bio) and 2.4ng/ml (LS-Bio) are shown in **Table 4.5**. These results at the dilution series of the assay dynamic range of the kit show the tested matrices have no obvious interferences with human nephrin in the ELISA kits. The percent recoveries for each highest dilution were within the recommended criteria (80-120%) and suggest confidence in ELISA compatibility with the diluent and urine sample (**Table 4.5**).

Table 4 5: Recovery experiment of recombinant nephrin standards spiked in a urine sample containing 222ng/ml of nephrin (Eth-Bio) and 2.4ng/ml of nephrin (LS-Bio)

Eth-Bio ELISA	**Actual amount Spiked (ng/ml)**	**Observed value (ng/ml)**	**Recovery (%)**
Diluted nephrin standards	1000	1274	127.4
	500	425	84.2
	250	221	88.4
	125	129	103.2
Standards spiked in a urine sample	1000	1450	118.7
	500	810	112.2
	250	470	99.6
	125	395	113.8
LS-Bio ELISA	**Actual amount spiked (ng/ml)**	**Observed value (ng/ml)**	**Recovery (%)**
Diluted nephrin standards	5	4.18	83.6
	2.5	2.54	101.6
	1.25	1.58	126.4
Standards spiked in a urine sample	5	6.4	86.5
	2.5	5.6	114.3
	1.25	4.15	113.7

*Recovery (%) calculated from the observed value/ (actual spiked + urinary nephrin) *100.*

4.3.8. Interferences experiment

Albumin interference experiment

Nephrin was measured in urine samples after spiking human albumin at varying concentrations and sample matrix. The mean urinary nephrin concentration (**Figure 4.4 A**) and the absorbance at 450nm of each sample (**Figure 4.4 B**) show not much difference in nephrin concentrations between the urine sample spiked with different concentrations of albumin, this shows that no cross-reactivity was detected in this assay.

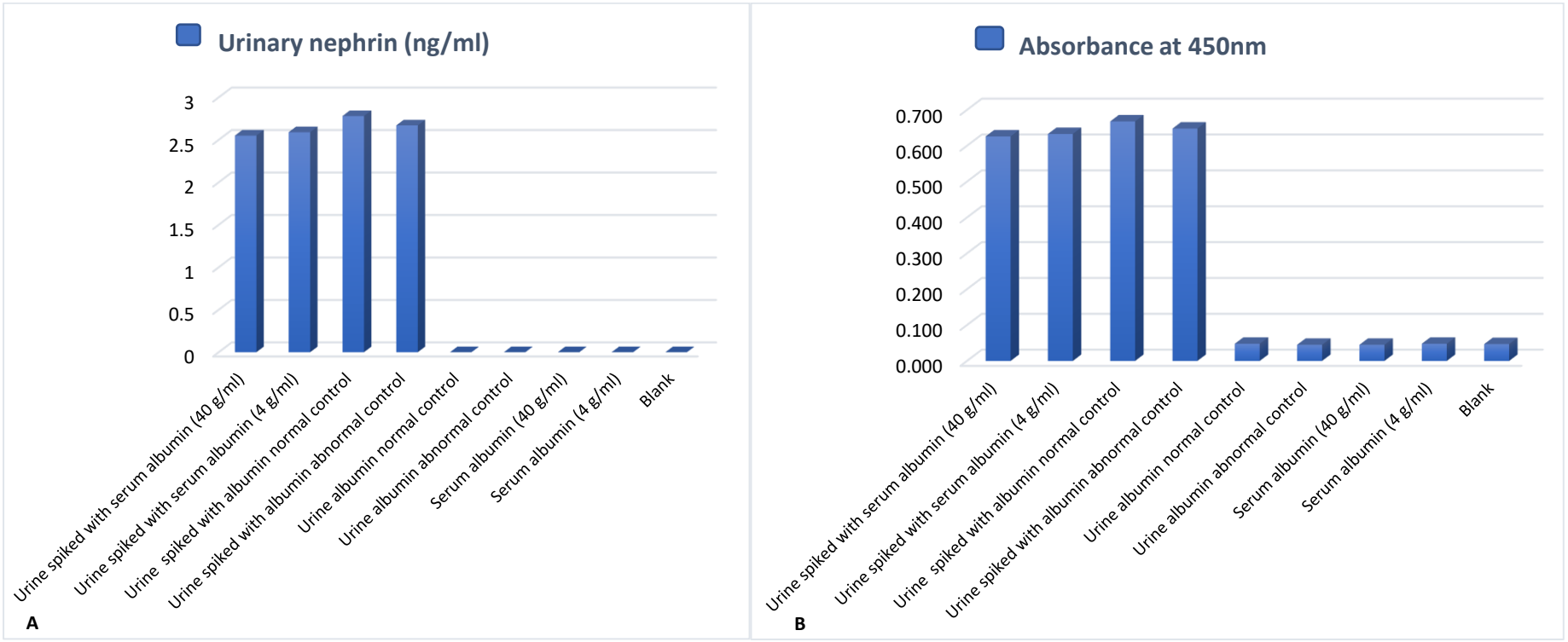


Figure 4.4. Human albumin interference with the urinary nephrin LS-Bio ELISA assay.

Biotin interference experiment

In the LS-Bio ELISA test, biotin interference was investigated for urinary nephrin measurement. Samples were tested in the presence of a biotin-conjugated detection antibody and without a biotin-conjugated detection antibody (**Figure 4.5**), hypothesising if biotin in a urine sample can produce a signal in the absence of biotin conjugated antibody. The result shows that the sample without a biotin-conjugated antibody did not produce a signal after the addition of the substrate.

Interference was also checked by measuring the concentration of nephrin in urine collected before biotin containing multivitamin intake, 2 hours after multivitamin intake, and 6 hours after multivitamin intake. The result showed that the mean concentration of nephrin in each measurement was 6ng/ml and the CV between the measurements was <10%. This shows that there is no difference in the concentration of nephrin before and after biotin supplementation. Hence, the result showed that biotin interference was not observed in nephrin measurement in this ELISA kit.

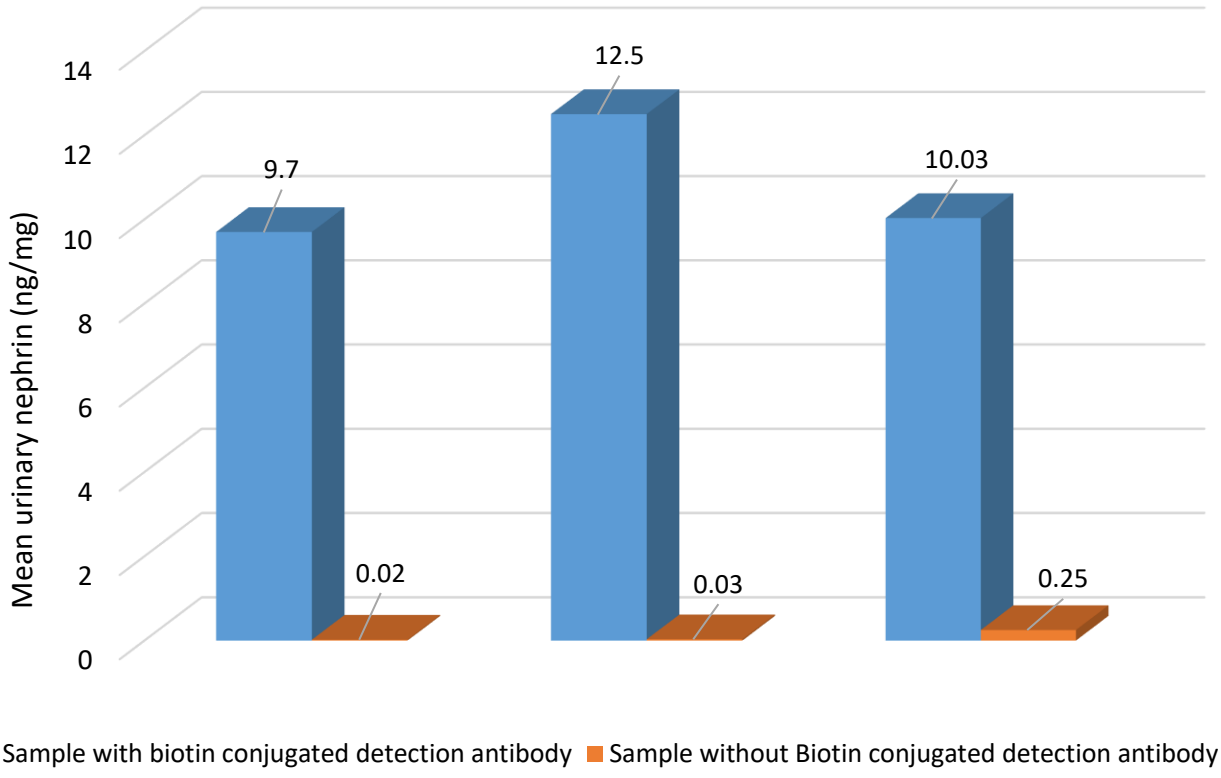


Figure 4.5. Urine biotin interference with urinary nephrin measurement in LS-Bio ELISA.

4.3.9. The effects of repeated freezing and thawing on urinary nephrin

The study examined the influence of freeze-thaw cycles on the measurement of urinary nephrin. The urinary nephrin was stable for the first two additional freeze and thaw cycles. The result shows that the concentration of nephrin decreased at the fourth freeze-thaw cycle and was consistent until the fifth cycle, but a sharp decline in the concentration of nephrin was observed after additional freeze and thaw cycles (**Figure 4.8**). Therefore, urinary nephrin stability affected during repeated freeze-thaw cycling.

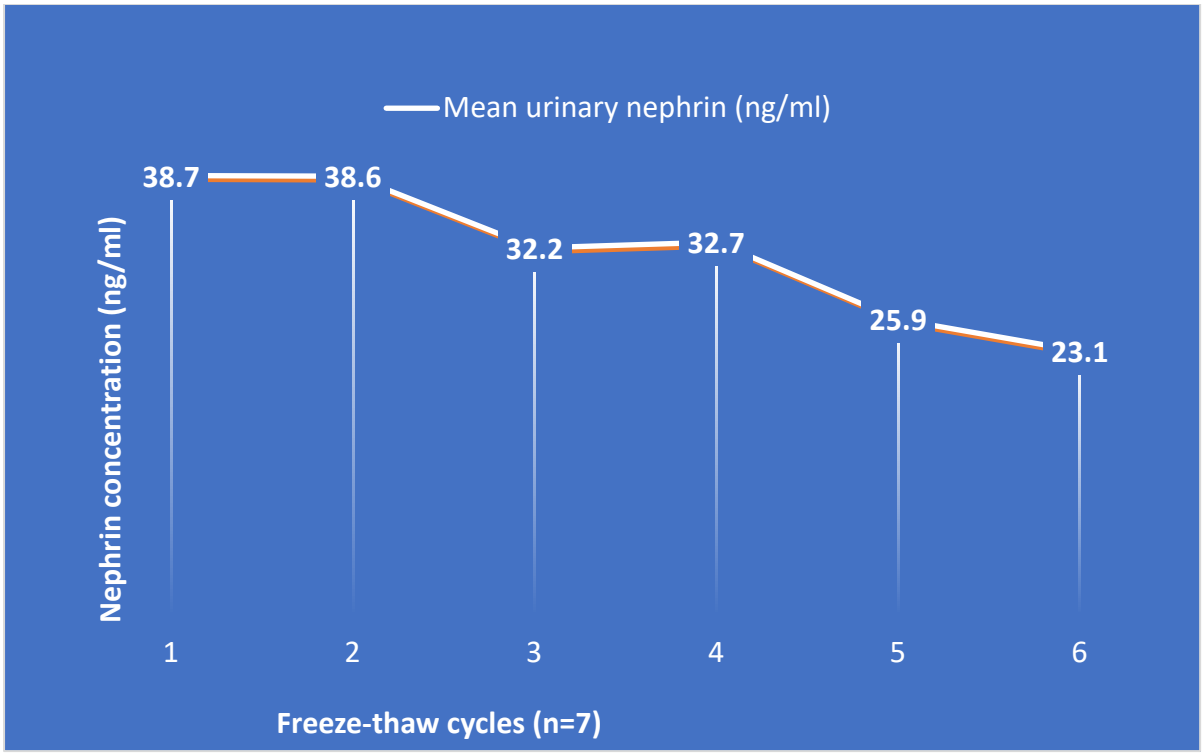


Figure 4.6. The effect of freeze and thaw cycling on the stability of urinary nephrin.

4.3.10. Correlation between ELISA kits in measuring urinary nephrin.

The results of the spearman rank correlation showed that there was no significant correlation between the measurements of urinary nephrin between Eth-Bio and LS-Bio ELISA kit, rho (185) = 0.034, p = 0.65, and a simple linear regression analysis also showed no significant linear relationship in urinary nephrin concentration measured by the two methods, LS-Bio (Y) = 0.04035 Ethos-bio (X)+3.997, and p= 0.88.

The Bland-Altman plot also showed that there was no agreement between the two human nephrin ELISA kits in measuring urinary nephrin. In fact, there was a 100-fold difference between the two kits' nephrin standard concentration. The mean of the differences in the measurement was 665.3ng/ml with a SD of 1008; $p < 0.001$. The difference in values of each measured value shifted to the values of the ELISA Eth-Bio and most values are outside the interval between the lower and upper limit of 95% agreement (mean ± 1.96 SD) (**Figure 4.7**). The linear regression analysis (difference in concentration as dependent and mean as the independent variable) showed proportional bias (Beta (β) coefficient = 2) and the $p < 0.001$.

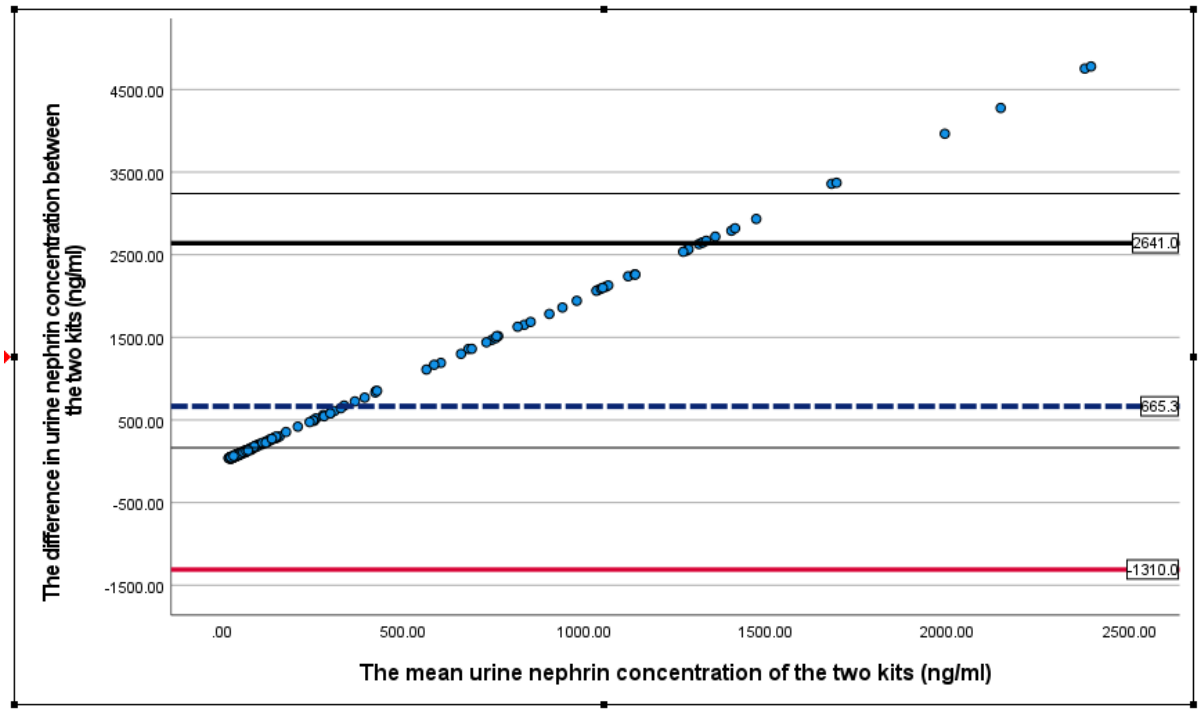


Figure 4.7. Bland–Altman plot for urinary nephrin obtained from 187 paired human urine samples using Eth-Bio and LS-Bio ELISA.
The solid black line represents the mean difference. The two upper and lower lines show the limits of agreement (i.e., mean ±1.96 × SD of the difference).

4.3.11. Accuracy and metrological traceability of the standards of the two ELISA kits.

4.3.11.1. Agarose gel electrophoresis

The result of the protein electrophoresis of the two ELISA kit standards is shown below (**Figure 4.8**). The electropherogram result shows there is a band detected for nephrin standards for both kits and travels an equal distance with the protein control on the gel. In this experiment, we expect different bands depending on the size of the standards in each kit, unfortunately; the band detected here was not nephrin, and rather it was albumin supplemented in the kit as a stabilising protein.

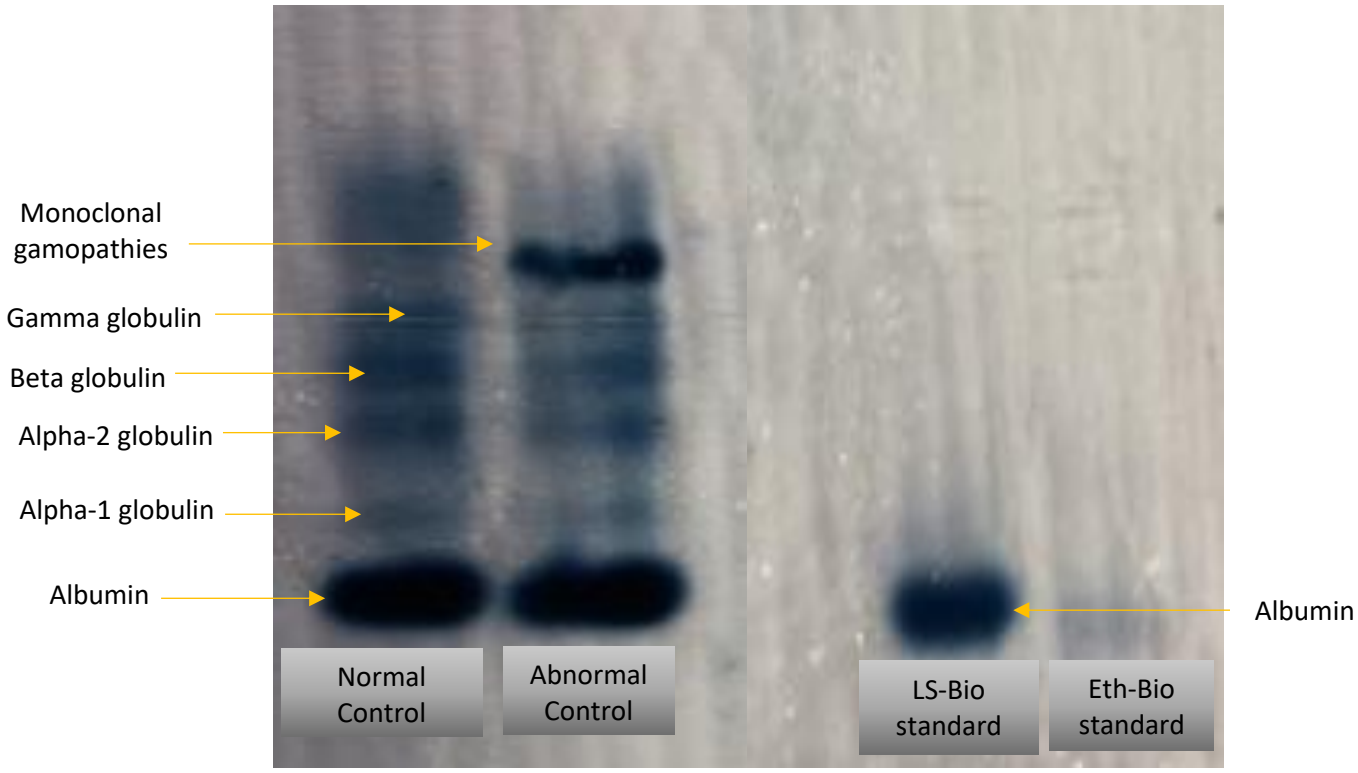


Figure 4.8. QuickGel agarose gel electrophoresis using the reference controls.
Lane 1: Abnormal control, lane 2: Normal control, Lane 3: LS-Bio standard and Lane 4: Eth-Bio standard.

4.3.11.2. Immunofixation electrophoresis

Immunofixation procedure was conducted to test if the standard of the two kits was traceable/detected by another supplier's anti-nephrin antibody. The result showed that the nephrin standard of both ELISA kits was not detected by the commercially available mouse nephrin monoclonal antibody. No band was detected on the electropherogram. The figure not shown here.

4.3.11.3. Size exclusion chromatography

The calibration curve of the column was performed with chicken serum albumin (CSA) with an approximate molecular weight of 43Kd. The 4µg/ml CSA as a control result on the Superdex 200 increase 10/300 GL column showed protein peak size of 14.9 ml, showing a molecular weight of 43Kd (**Figure 4.9**).

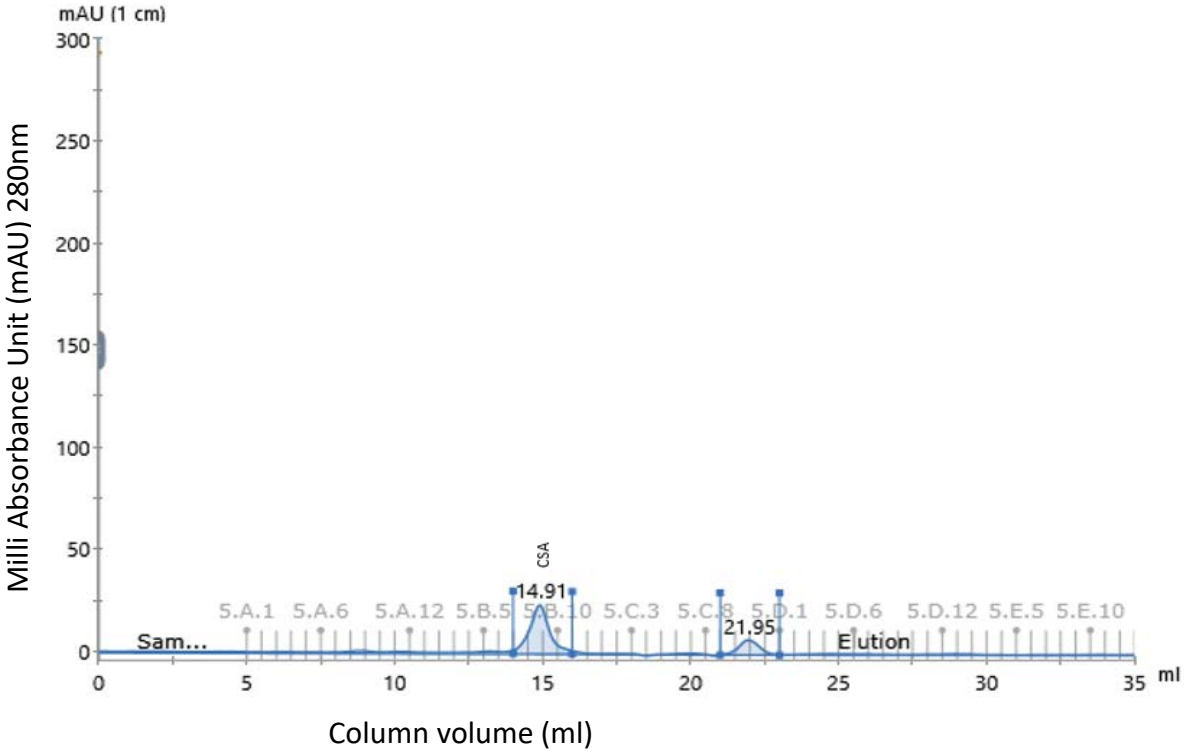


Figure 4.9. Chromatogram showing size exclusion chromatography of CSA as a control on Superdex 200 increase 10/300 GL column.

Human nephrin ELISA test Eth-Bio standard

In this aim, the comparisons of protein separation were performed using different concentrations and sample volumes. The chromatogram shows the same peak on the diluent (500µl load of diluent alone) as well as the standard (500µl undiluted standard (4µg/ml) with an approximate molecular weight of 120Kd nephrin standard) loaded and eluted in 500µl of PBS, showing nephrin was not detected (**Figure 4.10**).

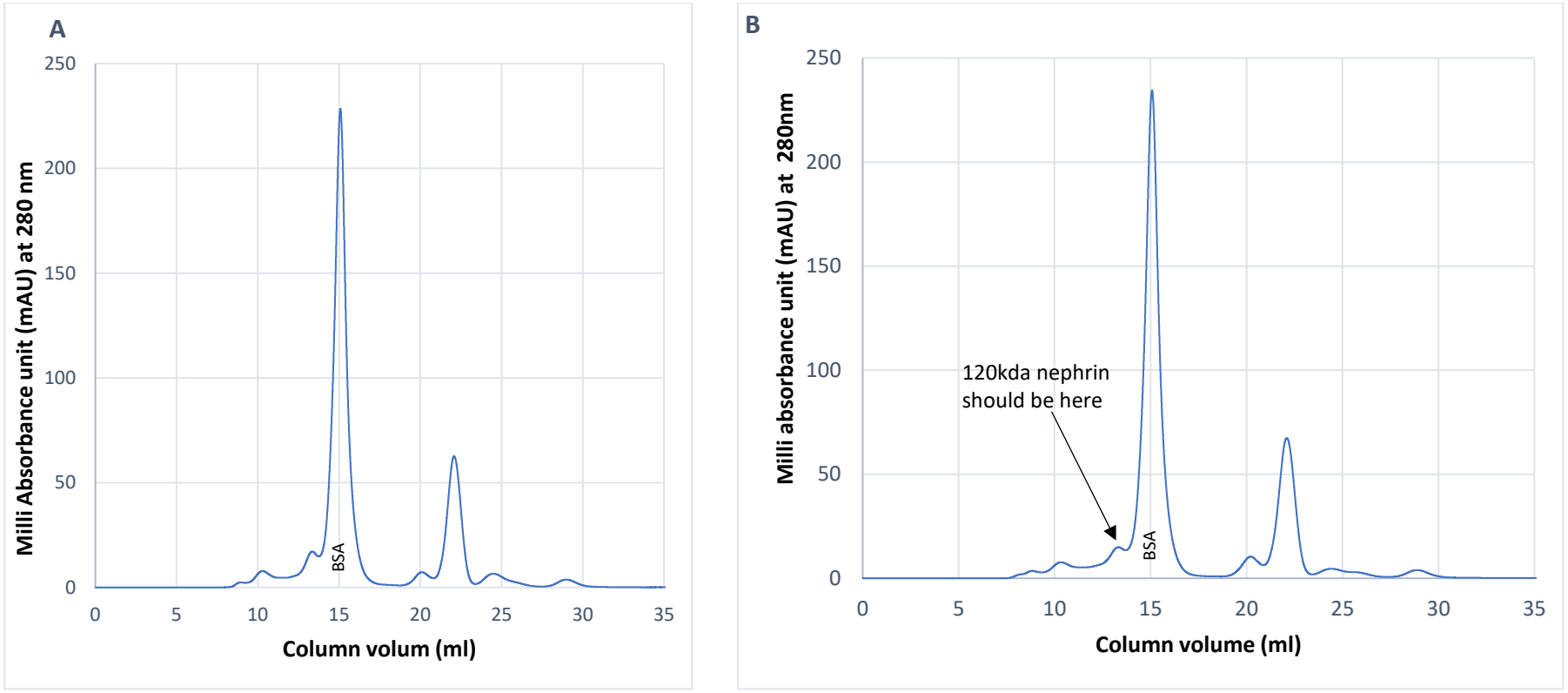


Figure 4.10. Chromatograms showing size exclusion chromatography of Eth-Bio human nephrin standard on Superdex 200 increase 10/300 GL column.

*Note: **A**) Eth-Bio standard diluent. **B**) Eth-Bio nephrin standard.*

Human nephrin LS-Bio ELISA Standard

In this experiment, the one vial of the lyophilised standard was diluted by its diluent and 500µl of either diluent alone or standards suspended by the kit diluent were loaded on the Superdex 200 increase 10/300 GL, unfortunately, the clear peak for nephrin was not detected although difference exists in peak size on the chromatogram (**Figure 4.11 & 4.12**). Standard diluted in PBS and 100µl of concentrated standard loaded on Superdex 200 increase showed a small shoulder at 16ml that would be the location of the 27Kd LS-Bio ELISA standard nephrin protein (**Figure 4.12 A&B)** based upon the protein standard curve. However, the substantial bovine serum albumin (BSA) peak makes the clear determination of the nephrin uncertain.

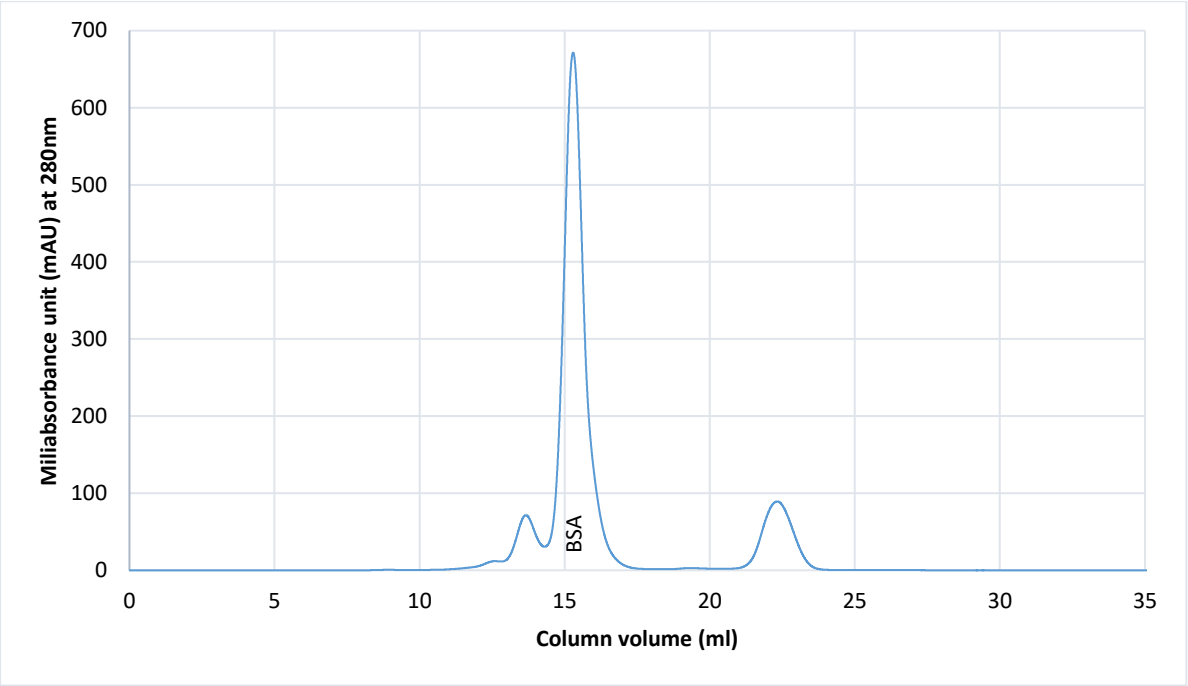


Figure 4.11. Chromatograms showing SEC of LS-Bio ELISA nephrin diluent alone on Superdex 200 increase 10/300 GL column.

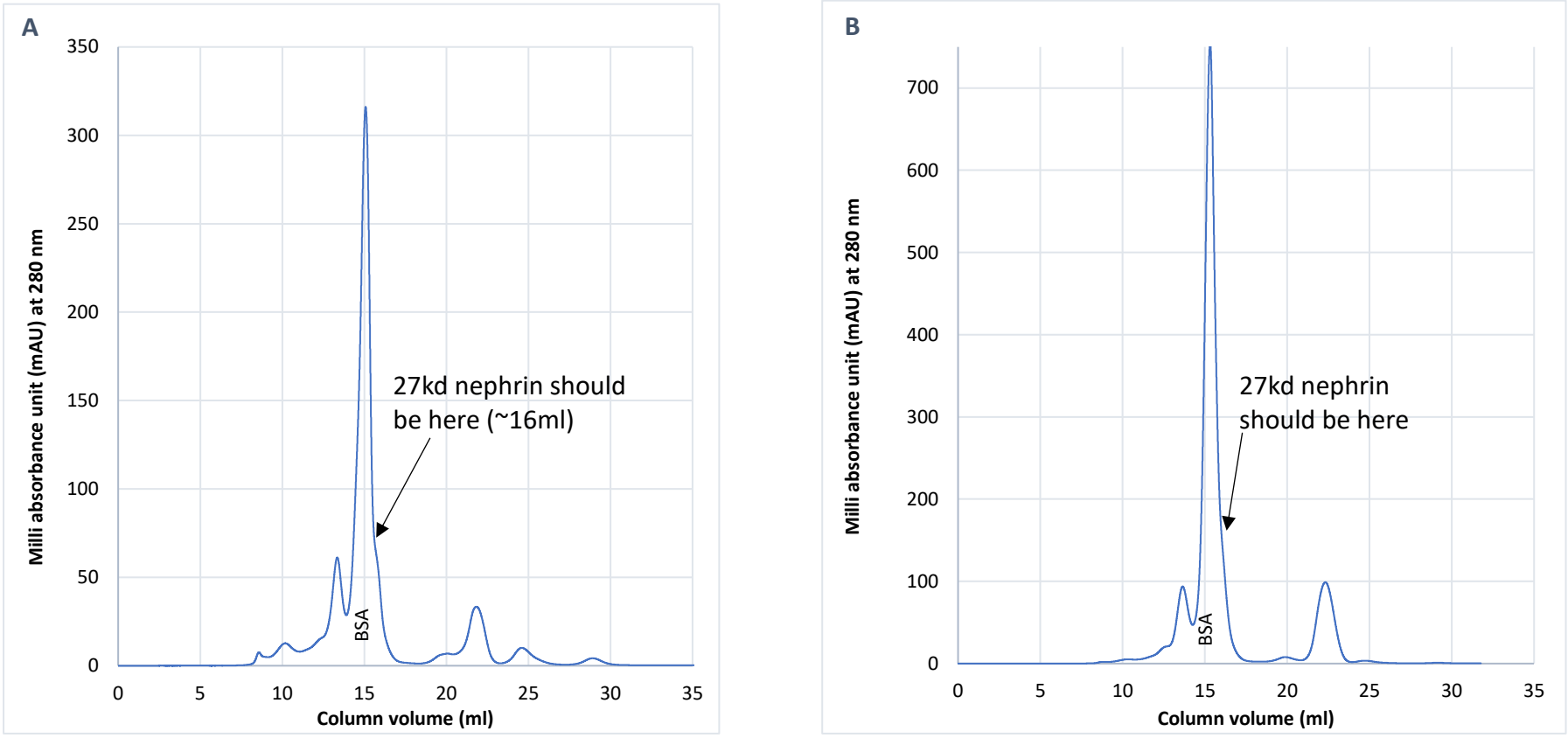


Figure 4.12. Chromatograms showing size exclusion chromatography of LS-Bio human nephrin standard on Superdex 200 increase 10/300 GL column.

Note: ***A****) Nephrin standard diluted with PBS.* ***B****) Nephrin standard diluted with its diluent supplied by the manufacturer.*

4.4. Discussion

To ensure patient safety, all assays used in diagnostic laboratories must undergo strict validation and performance checks. These are enforced by internationally and nationally determined standards (372, 378), and the results need to be reported. To our knowledge, this study is the first to validate commercially available urinary nephrin ELISA kits for use in the determination of nephrin in human urine samples. Commercially available urinary nephrin ELISA assays are available, mostly Eth-Bio ELISA. A review of the literature determined that the most commonly used ELISA kits are those purchased from Eth-Bio ELISA but more recently LS-Bio ELISA has been introduced to measure urinary nephrin as a marker for glomerular maturation, injury, and a predictor for AKI (50).

In this study, the analytical performance of the Eth-Bio and LS-Bio ELISA was investigated using a suite of assay validation experiments including precision, assay dynamic range, LOD, linearity of dilution, parallelism, recovery, and analytical specificity (matrix interferences), and sample stability for the freeze-thaw cycles. Individual performance of each kit was acceptable for different validation parameters. However, there is no correlation between the measurements of urinary nephrin concentration between the two ELISA kits, in fact, there was a 100-fold difference between the measured concentrations. To understand this difference, quick-gel electrophoresis, immunofixation electrophoresis and size exclusion chromatography were performed.

4.4.1. Assay dynamic range and limit of quantification.

In this study, the calibration curve was fitted for the best description of the experimental data. The model to a standard data set was presented by the closeness of the agreement of actual standard concentration with those back calculated from the model (379). The standard calibration curve for the Eth-Bio and LS-Bio ELISA test appeared to give reproducible results and is a good fit for the scatter plot in the regression model. The performance of the model was verified by calculating the recovery/accuracy of the kit standards, using the cumulative data generated during the validation study. Recovery (80-120%) and CV <20% of the calculated standard concentration was used as a criterion to determine assay dynamic range and LOQs (348).

Despite the manufacturer's claim that the assay has a dynamic range covering the concentration of the calibration curve, we found that the Eth-Bio ELISA, whilst precise at the lower levels; the assay lacked accuracy (80-120%) particularly the lowest standards below 62.5ng/ml of Eth-Bio kit. The data points at the lowest two dilutions of the curve are close to each other. The measured concentration of the lowest standard is higher than the expected concentration, resulting in higher recovery beyond the recommended criteria. As Eth-Bio works with the principle of competitive principle, down the dilution series the intensity of signal increased and concentration decreased but at this dilution series the analytical response is not linear and deviated from the expected. There is no clear explanation for this discrepancy but might be due to a problem in defining the minimum required dilution during the formulation in a concentration of the standard by the manufacturer. Evidence by Armbruster et al. recommended that adequate analyte concentration must be present to generate an analytical signal that can consistently and reproducibly be distinguished from "analytical noise" (277). The signal generated in the analyte's absence and delineating the limits of an assay at a low concentration is associated with its assay dynamic range (380). For instance, assay dynamic range might be influenced by incubation times and incubation temperatures, poor variations between replicate measurements, and the method for interpolating the absorbance reading to concentration.

4.4.2. Assay precision of the ELISA kits

The finding showed that performance parameters and precision meet the acceptance criteria for the ELISA validation parameters (348). The finding of the precision of the assays showed both kits present an acceptable characteristic and are comparable to the CLSI specified assay precision (381) and CLSI document EP05-A2, which describes the protocols for determining the precision of a method (21). Our study supported by the previous study stated that the acceptance criteria for intra- and interassay precision for ELISA to be ≤10% and 15% respectively (347, 375, 382), and the Interassay and intraassay precision of Eth-Bio were <10% in previous studies in pregnancy with PE and premature neonates (68, 149). Although precision was acceptable, LS-Bio ELISA resulted in lower precision compared to those reported by the manufacturer's claim (Intra-assay: 10% vs. <6.25%; inter-assay: <10% vs. <5.77%). The practical reason which will give a better estimate of the variability includes human technical error such as inter-individual variations in pipetting technique, the

difference in sample preparation and processing, the use of different filter settings to start, the use of different methods for calculating the concentration of urinary nephrin including software, using different plate washer and plate reader for measuring urinary nephrin by the manufacturer and our laboratory may account for these non-significant variations in precision. Likewise, the precision of the assay is also influenced by moderate changes in incubation temperature and incubation time (267, 383, 384).

4.4.3. Assay linearity and percent recovery

The results revealed that the linearity range of each ELISA kit was acceptable, whereas linearity is inadequate in the lower range of Eth-Bio ELISA. However, the regression equation of the line showed satisfactory in both kits. This is supported by a study that showed the acceptance criteria for dilution of linearity $R^2 \geq 0.850$ (375, 385), and dilution of samples should not affect the accuracy and precision of the assay (267). The failure of linearity at the lowest standard could that be possibly that the standard to be detected is not a full-length protein but low molecular weight peptides and exhibits translational modifications which differ from the original protein found in the filtration barrier of the kidney. Hence, the protein affinity to bound to the antibody or the specificity of the interaction between antibodies to a specific antigen maybe affected perhaps due to steric hindrance and other technical factors resulting in a low signal. This may cause slightly higher concentration as the kit uses a competitive principle. The linearity may be affected by some components in the formulation of buffer, interfering salts, organic, inorganic molecules, and changes in pH of detergents (375).

Further, the study showed a recovery experiment in an assay to evaluate the detector response from an amount of the analyte spiked into a neat urine sample compared to the actual concentration of the kit standard according to a guideline (386). We used different spiking concentrations of each kit standard to explore the dependency on the quantity of standard added and to evaluate interferences in the sample matrix and the diluent. The recovery shows acceptable recovery for the highest spike standards and a slightly high recovery for low spike standards.

Evidence revealed that accuracy should normally be within 80-120% over the working range of the assay, but the lower or higher intensity of accuracy may be satisfactory if it is

maintained throughout the working limit of the ELISA assay (267, 382). However, in this study, there seems to be a slight difference between the percentage recovery of the lowest standard of LS-Bio in the spiked and controls. The slight deviation in linearity and recovery in ELISA assays is not a distinctive event. A study on the analytical performance of ELISA assay in urine showed poor performance for most tests performed (375). Similarly, another study evaluates the analytical performance of the ELISA assay for NGAL (387). The authors found poor performance in linearity and recovery experiments. This reveals variation in immunoassay performance that needs careful consideration in sample analysis. The other probable factor for variations might be the imprecision in the measurement of these very low concentrations during spiking. The high recovery may be anticipated to the non-specific binding of proteins to the antibody immobilised on the ELISA assay plate that causes a slight change in absorbance or the cumulative effect of absorbance of a urine sample and the spiked standard may cause a change in concentration resulting in higher than the expected concentration.

4.4.4. Interference experiment

Interferences in immunoassays confounds the interpretation of test results and results in poor patient outcomes (388). Badrick et al. advises to assess common interferents during assay validation process, and testing interference is an important component of assay validation (388). Due to the complex nature of interactions of antigen and antibody, ELSA are susceptible to interferences (388). This study investigates interferences of urine albumin and biotin for urinary nephrin measurement. The finding did not show significant changes in urinary nephrin concentration between samples collected before and after multivitamin (containing biotin) intake. No differences in nephrin concentration were detected after spiking different concentrations of albumin into a neat urine sample. However, in this study interference for other analytes/non-protein organic and inorganic ions was not evaluated.

In immunoassay validation, evidence showed that assay performance may be related to matrix elements binding to antibody, non-specificity of analyte of interest and detection antibody reaction or analyte epitopes (389). Taylor et al. to verify matrix interference in protein biomarker measurement in the urine sample, and standards of proteins were spiked in urine samples from kidney disease patients. The authors observed a significant variation

in protein percentage recovery in urine samples among assays, showing that matrix elements differ among urine samples and highlighting their potential to variably hinder accurate protein measurement (388). Likewise, Lamb et al. reported sample-to-sample variation in the concentrations and composition of urinary proteins and non-protein interfering elements, including inorganic ions, are high relative to the protein concentration (373). Chatziharalambous et al. also showed challenges in the analytical performance of ELISA assays in urine (375). The authors described the difficulties in developing urine-based ELISA assays of adequate analytical performance for translation into clinical practice. The authors justified the explanations for this bottleneck, which are likely ascribed to the urine matrix itself and/or biomarkers in various isoforms in urine. More relevant to this study, Wood et al. comparably encouraged the use of dilution to reduce matrix interferences and measure analytes in urine (390). Standard addition, as described in our study, is a well-supported approach for conquering interference effects (391).

All these aspects alter the performance of the test by fluctuating the response to the signal. Hence, urine is likewise a dense fluid, with a substantial variation in matrix components other than albumin and biotin, which may affect the accurate measurement of urinary nephrin in ELISA assays. To overcome interference the manufacturer's instruction, and previous studies recommend the use of a diluent representing the biological sample in which the standard and the native protein give the analogous detector signals all along with the measuring range (267, 386).

4.4.5. Correlations of ELISA test kits in the measurement of urinary nephrin.

The finding showed, there was no significant correlation between the two kits for measuring urinary nephrin. The currently available assays refer to metrological traceability to the manufacturer's claim, but the assays do not produce comparable results (91, 314). Studies showed discrepant results in urinary nephrin concentration in normal pregnant women in the third trimester who reported 86 ng/ml of nephrin using the Eth-Bio ELISA (91) and 0.26µg/ml using the USCN Life Sciences ELISA kit (314). The reason explained by the authors for this high discrepancy was the usage of differences in commercial immunoassay kits. This is probably the manufacturer's standard is not commutable. However, the standard of each kit behaves differently for measuring nephrin from the patient sample, so the concentrations

of nephrin are not within a meaningful dynamic range by different measurement procedures, like our ELISA kits, which have a huge gap in assay dynamic range.

A study by Badric et al. showed the challenges in investigating interferences due to patient specific components when comparing two assays. The author recommends using a reference method in the method validation process and to compare the performance of methods using Bland Altman plots to translate the method to routine laboratory use (388). However, for this study, there was no validated reference method to quantify urinary nephrin that resulted in a clear interpretation of the inconsistency in measurement of urinary nephrin uncertain. In this study, we hypothesised that patient specific factors as well as the characteristic of the standard may be attributed to inconsistent measurement between the kits, probably by alterations of the matrix during preparations and formulation of the standard. This leads to the standard reacting differently to different measurement procedures.

In this study, the standard of other kit was not detected by the other ELISA Kit, this is also confirmed by the manufacturer's claim as it is not detected by any other ELISA kit supplier except the standard supplied by the supplier, and this shows that the two kits may detect urinary nephrin differently. This discrepancy might probably be due to the difference in formulation of the standards. For example, the Ethos-Bio Exocell kit used whole human nephrin (most extracellular domain) while the LS-Bio ELISA kit used a recombinant protein produced in *E. coli* that encodes 23-257 amino acids. For instance, the different suppliers use different fragments of protein, expression techniques, and purification methods. Therefore, the two ELISA kits use different antibodies with different epitopes (as evidenced by the lack of standard detection in the two kits). The manufacturers have defined their standards differently and the kits have differences in specificity of capture and detection antibodies. This inconsistency in the measurement of urinary protein is supported by a previous study that showed variations in the relative amount of urinary protein forms depending on the assay and antibody configurations in the assay developed for other biomarkers (392).

Traceability and commutability are important concepts in laboratory medicine (393). Understanding these concepts in the measurement of urinary nephrin using the two ELISA kits needs further investigation. To understand this, protein electrophoresis was undertaken

if a difference in concentration of the standards exists. However, the experiment had interfered with the presence of stabilising protein BSA in the standard solutions of both kits.

Immunofixation electrophoresis was conducted to determine the metrological traceability of the standards using mouse monoclonal antibodies from different suppliers; still, the monoclonal nephrin antibody did not detect nephrin in the experiment. The probable explanation for this might be that suppliers might use different fragments of nephrin, expression systems, and purification techniques and may use a specific fragment of antibody binding sites that cannot be detected by any anti-nephrin antibodies except antibodies supplied as part of their ELISA kit. Overall, the kit manufacturers have defined their standards differently. This occurs because there is no certified reference method for the measurand. So, metrological traceability can only be to the manufacturer's master calibration standard. Under these circumstances, metrological traceability depends on the manufacturing process and the long-term consistency of the calibrator value assignment that makes the detection of the standards traceability difficult using different methods.

Likewise, size exclusion chromatography using a Superdex 200 increase 10/300GL column was also carried out to size the standards of the two kits. The results showed that similar observation with the gel electrophoresis and nephrin was not accurately sized because of the substantial BSA peak, which makes the clear determination of the nephrin uncertain. There is no scientific justification for the undetectability of the standards of both suppliers using recently available techniques. The LS-Bio supplier recommends and determined the concentration of the kit standard from high concentration of primary standard through serial dilution rather than using electrophoresis and size-exclusion chromatography and justified that the standard concentration is very low (10ng/ml), may be below the detection limit and the reconstituted standard may adhere on the column of the chromatography that results in an undetectable peak on the chromatogram.

In general, the validation of commercially available ELISA following the standards of CLSI, and NATA guidelines showed that the individual performance of these assays using a suite assay validation parameter can be used reliably and reproducibly for determining urinary nephrin in most parameters. However, addressing the inconsistencies in the presentation of analytical performance data of validation parameters by companies would be of great value.

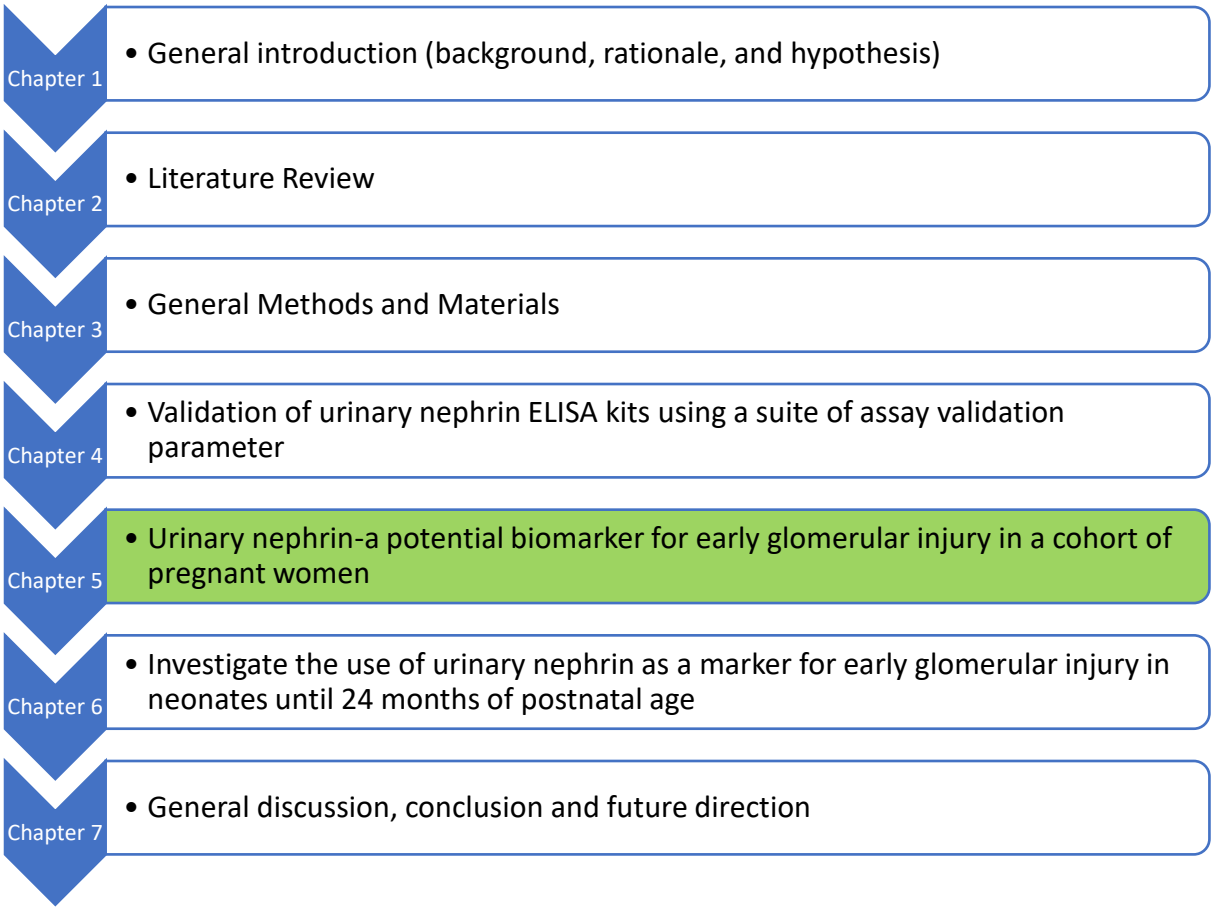
4.5. Strengths and limitations

The strength of this study is it is the first to validate the two urinary nephrin ELISAs and determined the performances of the assay using a suite of assay validation parameters to be used for clinical application. The study attempted to assess the metrological traceability of the ELISA kit standards of both suppliers through various methods. The study's constraints need to be acknowledged. For example, being the measured concentrations concerning the methods bias appropriate, the measurement of precision by the observable calibrator using an endogenous standard for nephrin could have been performed. However, there was a lack of availability of endogenously synthesised nephrin standards on the market.

4.6. Chapter summary

To the best of our knowledge, this study is the first to present an analytical validation of commercially available human nephrin ELISA kits to detect urinary nephrin as a novel marker of early glomerular injury. The data overall support the kits meet most of the requirements for utilisation of these assays in clinical practice for diagnosing early glomerular injury, although inconsistency in urinary immunoassay performance parameters that needs to be taken into attention in experimental sample analysis. As there are discrepancies in the quantitative measurement of urinary nephrin, the two kits cannot be reliably used interchangeably for the measurement of urinary nephrin to predict glomerular injury.

Additional endeavours would be essential for enhancing the standardisation of laboratory findings between commercially available ELISAs. Moreover, during the analytical validation of an ELISA test kit the cost, ease of use and equipment, practicality (example need for -80C freezer), the stability of nephrin during the collection and transportation process and staff training are recommended to translate urinary nephrin ELSA test into clinical practice. The next chapter (**chapter 5**) will investigate urinary nephrin as a potential biomarker for early glomerular injury in unselected pregnant women attending TUH for routine antenatal care service.



Foreword Chapter 5

This chapter describes the investigation of urinary nephrin and NCR as markers for the detection of early glomerular injury in pregnant women. The potential of urinary nephrin as a marker for glomerular injury has been confirmed by several studies in high-risk populations including DN and PE (21, 75, 260, 261). However, despite this, urinary nephrin has not yet translated into routine use. For a new biomarker assay to be translated into clinical practice, it needs to be clinically validated and tested in a confounding population to understand its diagnostic predictive potential. This chapter aims to explore the association of urinary nephrin and NCR with the development of pregnancy-associated complications known to involve the kidney in pregnant women. The performance of urinary nephrin and NCR for predicting early glomerular injury associated with PE and diabetes in pregnancy was determined in comparison with urinary ACR. Another important aim of the studies contained in this chapter was to determine the correlation of urinary nephrin and NCR with other parameters measuring renal function in different clinical presentations.

Chapter 5. Urinary nephrin-a potential biomarker for early glomerular injury in a cohort of pregnant women attending routine antenatal care services

5.1. Abstract

Introduction: Glomerular injury may occur during pregnancy as a consequence of systemic disease and pregnancy-related medical complications such as gestational diabetes and PE. While urinary nephrin has been shown to provide early identification of PE in high-risk pregnancies, the role of urinary nephrin for determining early glomerular injury in a group of pregnant women is yet to be explored. This study aimed to investigate the use of urinary nephrin as a predictor for early glomerular injury in a large cohort study (KIDMIN) conducted at the Townville University Hospital.

Methods and Materials: A prospective cross-sectional study was conducted. All pregnant women with a full dataset (n=273) were classified into 3 categories according to their urinary albumin to creatinine ratio (ACR): normoalbuminuria, microalbuminuria, and macroalbuminuria. Continuous variables were compared between groups and the cutoff value for the urinary nephrin to creatinine ratio (NCR) was determined to predict albuminuria as an indirect indicator of early glomerular injury. The percentages of pregnant women who had elevated nephrinuria were calculated for each of the ACR categories.

Results: Urinary NCR showed a positive correlation with urinary ACR ($r=0.29$, $p<0.0001$). Urinary nephrin and NCR increased comparably in women categorised as having normoalbuminuria, microalbuminuria, and macroalbuminuria. Using a cutoff value of 14ng/mg, nephrinuria was detected in 64.9% of women with normoalbuminuria, 94.7% with microalbuminuria, and 100% with macroalbuminuria. Of the women with normoalbuminuria 77.8% demonstrating nephrinuria were diagnosed with a hypertensive disorder and 62.7% demonstrating nephrinuria were diagnosed with diabetes in pregnancy. In women with PE, urinary NCR and ACR were markedly higher than in women who did not develop PE. The AUC of the ROC for urinary NCR was 0.74 (95% CI: 0.650-0.824), with a sensitivity of 97% and a specificity of 36% to predict glomerular injury, and a sensitivity of 93% and specificity of 42% to predict glomerular injury of PE.

Conclusion: The study found that urinary nephrin and NCR were elevated in women with micro-macro albuminuria but also in pregnant women with normoalbuminuria. Increased urinary NCR without increased urinary albumin may be associated with early glomerular injury. Urinary NCR may be a more sensitive marker than microalbuminuria to detect early glomerular injury and may be a useful tool for monitoring glomerular injury in women with systemic disease and adverse pregnancy outcomes.

5.2. Introduction

Kidney disease is an important and increasing public health burden. Of concern, it is often under recognised particularly in remote communities and low socioeconomic populations (142, 394). Kidney injury prior to or during pregnancy can accelerate the decline in maternal renal function and lead to adverse pregnancy outcomes (395). While globally, the incidence of AKI in pregnancy has declined, concern remains for women in both developing and developed nations (396, 397).

Pregnancy-induced changes in glomerular filtration which occur in normal pregnancy such as renal vasodilation, hyperfiltration, and enhanced glomerular permeability can lead to a reduction in maternal SCr (398) making early diagnosis of renal damage during pregnancy difficult, and pre-existing renal disease often under-diagnosed. Up to 5% of Australian women of childbearing age have increased markers of kidney disease (142), secondary to systemic disorders, including DM and / or primary hypertension (HTN). This preexisting undiagnosed kidney disease coupled with damage secondary to pregnancy-related disorders (hypertensive or microangiopathic disorders) can lead to pregnancy-associated AKI (pr-AKI) (155). In particular, PE, a disease of the glomerulus during pregnancy, may further damage the glomerulus (399), and increase the risk of AKI (397). Pr-AKI has been associated with a chronic decline in renal function with a recent case report of nephropathy during pregnancy progressing to ESKD within one year postpartum (400).

Given the consequence of glomerular damage prior to and during pregnancy, and accompanying complications, early detection and management of glomerular injury remains vital. To date, routine chemical urinalysis and measurement of proteinuria remain the main tools for diagnosis and monitoring of kidney disease progression (401). The KDIGO guidelines for kidney disease assessment during pregnancy, are often incorporated into local guidelines, and recommend urinary protein loss be monitored by measuring urinary ACR, as an indicator of progressive glomerular injury (401). Measurement of urine albumin concentration using 24-hour urine samples is recommended, but not feasible in clinical practice, therefore, a spot urine sample for ACR analysis is commonly used (401). KIDIGO guidelines recommend an ACR level of ≥3 mg/mmol to classify patients as at a higher risk of glomerular injury and this is used for monitoring the progression and staging of CKD (401). New-onset hypertension and

proteinuria predicted by an ACR > 8 mg/mmol after 20 weeks of gestation correlated with maternal kidney damage from PE (395).

While both proteinuria and albuminuria are important for detecting patients with severe glomerular damage. However, a specific and sensitive diagnostic marker for identifying early glomerular injury may be of value. There has been increased interest to evaluate biomarkers for early detection of glomerular damage (75, 402, 403).

Podocyte proteins have been shown as useful biomarkers for predicting glomerular injury (22, 307). In the past decade, it has been shown that urinary nephrin may provide a sensitive and specific marker of early glomerular injury, often preceding albuminuria (21, 75). Nephrin is a structural and functional component of glomerular podocytes (56), and together with glomerular endothelial cells, the GBM forms the GFB, that allows the passing of the ultrafiltrate by restricting macromolecules. Preclinical and clinical studies have shown that nephrin expression decreased in proteinuric disease (404) and samples obtained from pregnant women with PE (25, 405). An increased concentration of nephrin in urine is reported in glomerular injury (21), showing damage to the glomerular podocytes. Early diagnosis and identification of new-onset maternal glomerular injury, and management of pregnancy-related complications in a patient-centred approach could provide potential intervention strategies for the kidney health of the mother and foetus. As urinary ACR does not become elevated until there is substantial glomerular damage, monitoring with ACR may delay early diagnosis of the glomerular damage.

To our knowledge, there is limited evidence for the use of urinary nephrin as a potential diagnostic marker for early glomerular injury in a cohort of unselected pregnant women. Therefore, the overarching aim of this chapter was: To explore the association of urinary nephrin concentrations with the development of pregnancy associated complications known to involve the kidney. To address this, the following specific aims were considered to:

- Explore the association of urinary nephrin and NCR with ACR and other parameters of maternal renal function.
- Explore the association of urinary nephrin and NCR with diagnosis of diabetes (GDM, T1DM and T2DM) during pregnancy.

- Explore the association of urinary nephrin and NCR with diagnosis of PE during pregnancy.
- Investigate the diagnostic accuracy of urinary nephrin and NCR for the prediction of albuminuria as an indirect indicator of glomerular injury.

5.3. Results

5.3.1. Maternal characteristics

The maternal characteristics of the study population are summarised in **Table 5.1**. Of the 401 women consented for this study, 56 women withdrew from the study, 54 did not attend antenatal clinics due to Coronavirus (COVID-19) restrictions and 18 did not have a full dataset. Overall, 273 pregnant women took part in this study, the mean age was 30 ± 6 years and the gestational age (GA) at the time of sample collection was 33.2 ± 5.5 weeks. One hundred and fifty (55%) women had no reported comorbidities. The remaining 123 (45%) women were found to have pregnancy-associated comorbidities with hypertensive disorder of pregnancy 25/123 (20.3%), diabetes in pregnancy 69/123 (56.1%) and diabetes-hypertensive disorder of pregnancy accounting for 23/123 (18.7%).

Table 5.1: Maternal clinical characteristics and pregnancy outcomes

Variables	Count/Mean ± SD	Percent (%)
Maternal age at sample collection time (years)	30.0 ± 6.0	
GA at sample collection time (weeks)	33.2 ±5.5	
No reported comorbidities (NCOM)	**150**	**55.0**
Reported comorbidities (COM)	**123**	**45.0**
Hypertensive disorder of pregnancy		
Preeclampsia (PE)	16	13.0
Hypertension (HTN)	9	7.3
Diabetes in pregnancy		
Gestational Diabetes (GDM)	56	45.5
Type 2 Diabetes mellitus (T2DM)	9	7.3
Type 1 Diabetes Mellitus (T1DM)	4	3.3
Diabetes-hypertensive disorder in pregnancy		
T2DM-HTN	4	3.3
GDM-HTN	9	7.3
PE-GDM	7	5.7
PE-T2DM	3	2.4
Chorioamnionitis	1	0.8
History of renal problem	5	4.1

Data are given as count or mean ± SD, or percent (%); GA: Gestational age, SD: standard deviation.

5.3.2. Association of serum and urine markers of renal function between women with normo, micro and macroalbuminuria

This study compared clinical and biochemical parameters between women categorised into normo, micro and macroalbuminuric groups (**Table 5.2**). Despite there being no significant difference in SCr levels between the three groups, there was a significant difference in sCysC (mg/L) between women with normo and microalbuminuria and normo and macroalbuminuria ($p<0.05$). There was no significant difference between the urine creatinine levels of the women with normo, micro and macroalbuminuria. There was, however, a significant difference in urinary nephrin and NCR levels of between these three groups ($p<0.001$). The urinary nephrin and NCR increased with increasing urinary ACR levels, and this was significant ($p<0.05$) (**Table 5.2**). There was a significant correlation between urinary nephrin, NCR and ACR across the whole (r) = 0.29, $p<0.0001$, N= 273) and when compared to urine albumin ($r=0.24$, $p<0.0001$, N=273), and the combined micro-macroalbuminuria group (ACR ≥3 mg/mmol) ($r = 0.39$; $p = 0.006$, N=48).

Table 5.2: Maternal urinary and serum indices

The table shows association of maternal urinary and serum indices with albuminuria categories. Urinary nephrin and NCR increased significantly and comparably across the three categories of albuminuria. There was a significant difference between women with normoalbuminuria and micro and macro albuminuria for urinary nephrin, NCR, urinary albumin, and serum cystatin C.

Variables	Normo (N=225)	Micro (N=38)	Macro (N= 10)	p-value
Urinary markers (n=273)				
Urinary nephrin (ng/ml)	9.7 (62.7) *	34.4 (77.3) *	82.1 (122.2) ǂ	< 0.001
Urinary NCR (ng/mg)	27.7 (54.9) *	44.7 (88.7) *	86.3 (65.5) ǂ	< 0.001
Urine albumin (mg/L)	5.8 (8.2) *	63.8 (127.8) *	525 (675.0) ǂ	< 0.001
Urine creatinine (mmol/L)	6.4 (7.8)	7.4 (8.6)	6.5 (7.1)	0.508
Serum markers (n=271)				
Serum cystatin C (mg/L)	0.96 (0.4) *	1.12 (0.8) *	1.34 (1.2) ǂ	0.007
Serum creatinine (µmol/L)	44.0 (12.0)	43.0 (13.0)	52.0 (41.0)	0.085

Values are reported as median (IQR). Normo (<3.0 mg/mmol), Micro (3.0 – 30 mg/mmol) and Macroalbuminuria (> 30 mg/mmol) for urinary nephrin (ng/ml) ($\chi^2 = 17.97$), urinary NCR (ng/mg) ($\chi^2 = 21.88$) and sCysC (mg/L) ($\chi^2 = 9.9$) (Kruskal Wallis H test with Bonferroni post-hoc analysis; $p < 0.05$).

**: Significant difference between normoalbuminuria vs microalbuminuria ($p<0.05$)*

ǂ: Significant difference between normoalbuminuria vs. macroalbuminuria ($p<0.05$)

Association of urinary NCR with the diagnosis Diabetes or PE during pregnancy.

5.3.3. Percentage of pregnant women with elevated nephrinuria

The normal value for urinary nephrin (nephrinuria) in different sub-groupings of pregnant women was determined using the ROC-generated cutoff values (**Table 5.3**). There was a high proportion of pregnant women showing elevated nephrinuria (NCR >14ng/mg). In the full cohort, nephrinuria was detected in 146/225 (64.9%) of women with normoalbuminuria, 36/38 (94.7 %) of women with microalbuminuria and 10/10 (100%) of women with macroalbuminuria at a cutoff value of 14 ng/mg (**Figure 5.1**).

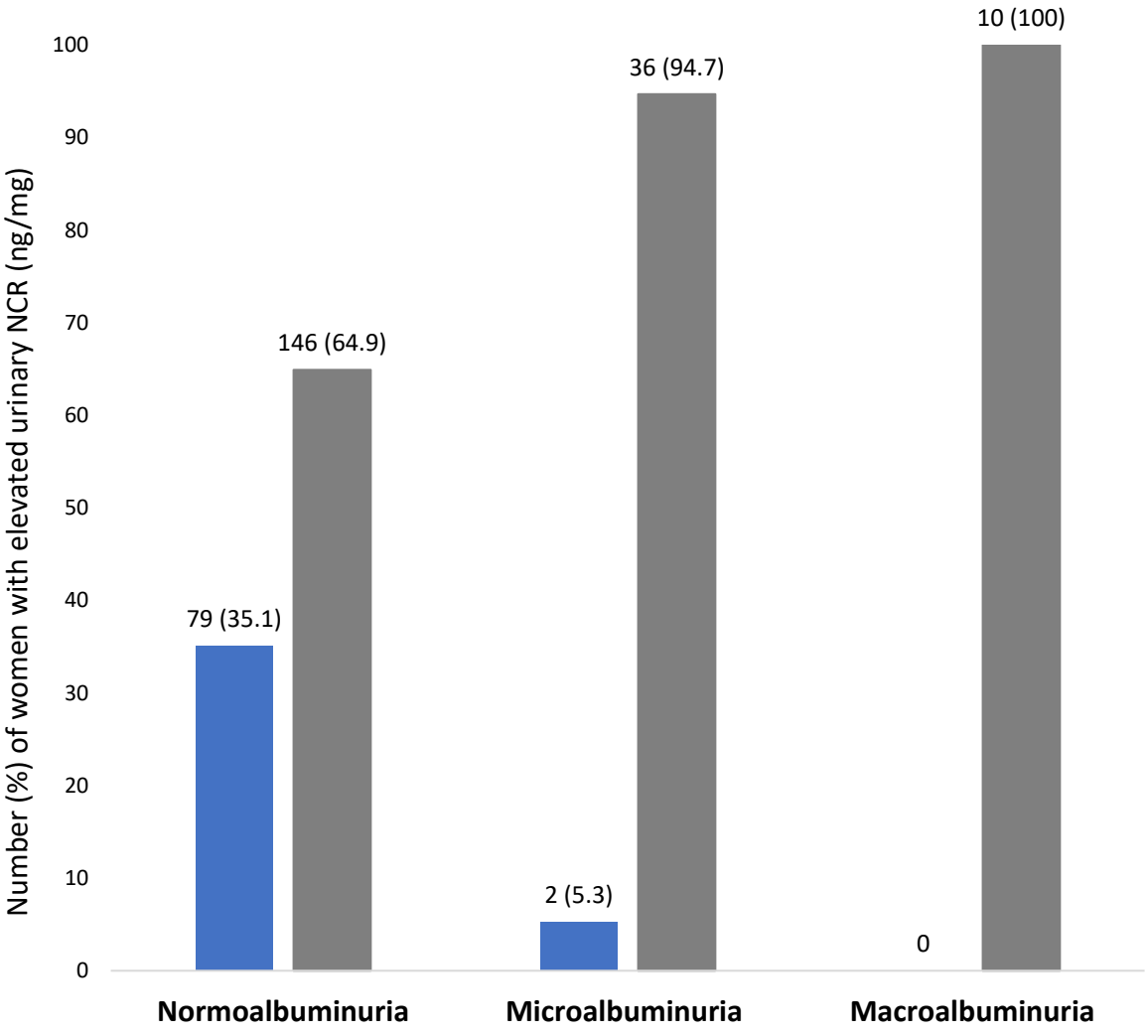


Figure 5.1. Number(percentage) of women with elevated urinary NCR in each of the urinary ACR Groups (Normo: <3mg/mmol; Micro: 3 – 30mg/mmol and Macro: >30mg/mmol.

Using urinary NCR cutoff value of <14ng/mg for normal nephrinuria; blue bars), and ≥14ng/mg for elevated nephrinuria; grey bars).

Within the group of women with normoalbuminuria demonstrating elevated nephrinuria 85/225 (37.8%) showed heterogeneity in their clinical characteristics and comorbidities, 5/225 (2.2%) had a history of renal disorders and 135/225 (60%) had no reported comorbidities (**Figure 5.1**). This group of normoalbuminuric women was further explored in (**Figure 5.2).** Of the women with normoalbuminuria who also had elevated nephrinuria [NCR≥14 (ng/mg)], 14/18 (77.8%) were diagnosed with a hypertensive disorder (PE-HTN) with mean ± SEM (47.1±6.9) of urinary NCR, 42/67 (62.7%) were diagnosed with DIP with mean ± SEM (62.8±5.9) of urinary NCR, and 2/5 (40%) of nephrinuria women had history of kidney disease.

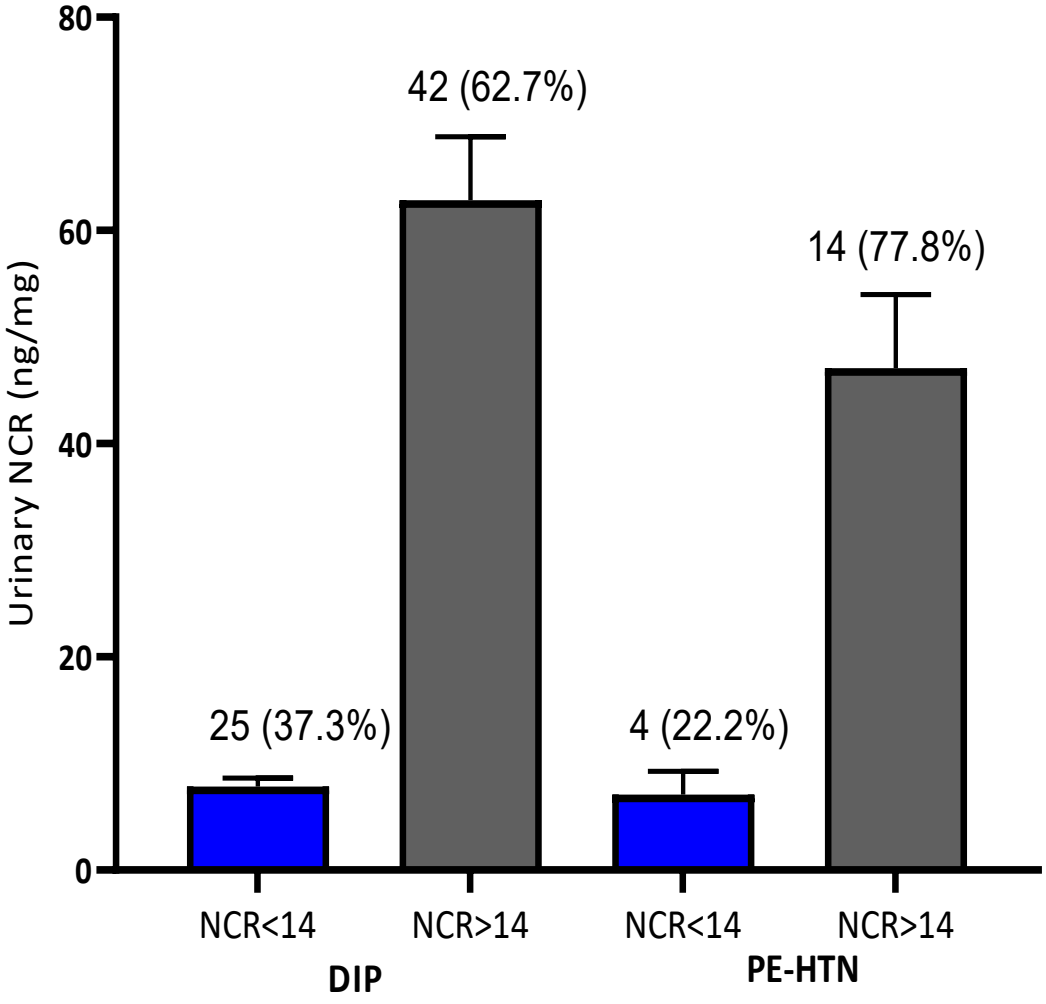


Figure 5.2. The mean (SEM) levels of urinary NCR and number (percentage) at the top of the bar for women within the normoalbuminuric group who were diagnosed with either DIP or hypertensive disorder (PE-HTN).

These are categorised using urinary NCR cutoff value of <14ng/mg for normal nephrinuria, blue bars); and ≥14ng/mg for elevated nephrinuria, grey bars).

5.3.4. Predictive characteristics of urinary NCR for determining early glomerular injury

The predictive characteristics of urinary NCR for significant albuminuria as an indirect indicator of glomerular injury were explored. The women were grouped according to ACR as the main determinant (normoalbuminuria (<3mg/mmol) and micro-macroalbuminuria (≥3mg/mmol). A ROC analysis showed that the diagnostic accuracy of urinary NCR for predicting glomerular injury appears satisfactory (AUC > 0.7) in the entire cohort. The result showed a higher sensitivity of 83.3% but a lower specificity of 48% (**Table 5.3 A**). An improved predictive probability is observed when stratifying to different cutoff values (**Tables 5.3 B and C**). In all cases, the sensitivities are higher than the specificities. Overall, urinary nephrin and NCR show moderate predictive probability (AUC: 0.69-0.75). Urinary nephrin and NCR exhibited low positive predictive value (PPV), but higher negative predictive value (NPV).

Table 5.3: Predictive characteristics of urinary nephrin for determining early glomerular injury.

A. Predictive characteristics of urinary nephrin for predicting albuminuria in the full cohort (ACR ≥3 mg/mmol; N=48 vs ACR <3mg/mmol; N=225).

Biomarker	Sensitivity (%)	Specificity (%)	Negative PV (%)	Positive PV (%)	Cutoff	AUC (95% CI)	p-value
Urinary NCR	83.3	48.0	93.1	25.5	24.0	0.71 (0.63-0.78)	<0.001
Urinary nephrin	79.2	57.3	92.8	28.4	16.7	0.69 (0.61-0.77)	<0.001

B. Predictive characteristics of urinary nephrin for predicting albuminuria in the normal control group (ACR ≥3 mg/mmol; N=33 vs ACR <3mg/mmol; N=135).

Biomarker	Sensitivity (%)	Specificity (%)	Negative PV (%)	Positive PV (%)	Cutoff	AUC (95% CI)	p-value
Urinary NCR	97.0	36.0	98.0	27.0	14.0	0.74 (0.65-0.82)	<0.001
Urinary nephrin	82.4	60.2	92.9	35.3	16.4	0.74 (0.65-0.83)	<0.001

C. Predictive characteristics of urinary nephrin for predicting albuminuria in women with comorbidities (ACR ≥3 mg/mmol; N=33 vs ACR <3mg/mmol; N=90)

Biomarker	Sensitivity (%)	Specificity (%)	Negative PV (%)	Positive PV (%)	Cutoff	AUC (95% CI)	p-value
Urinary NCR	88.2	48.0	91.8	37.1	22.1	0.75 (0.65-0.84)	<0.001
Urinary nephrin	85.3	54.2	91.2	40.0	16.7	0.72 (0.63-0.82)	<0.001

5.3.6. Predictive characteristics of urinary nephrin, NCR and ACR for determining PE

Using the AUC-ROC analysis, the cutoff value for urinary NCR was 14 ng/mg and significant differences were observed between women who developed PE and who did not develop PE (p=0.038). The cutoff value for urinary ACR calculated using ROC analysis for this cohort was ≥3.7 mg/mmol, and this compared well with the KDIGO recommended cutoff value for urinary ACR was ≥3 mg/mmol. The sensitivity of urinary NCR was found to be higher (92.3%) than the specificity of 32.4% and this was comparable with urinary nephrin (92% and 42%) respectively. The urinary nephrin and NCR exhibited poor PPV (<15%) but higher NPV (>97%). Urinary ACR showed 50% sensitivity and 88.7% specificity to predict glomerular injury of PE. A comparison of the performance of these markers in the entire cohort (n=247) is described in **Table 5.4**.

Table 5.4*:* Predictive performance of urinary nephrin and ACR for PE (PE: N = 26 vs non-PE: N = 247)

Diagnostic performance	Urinary NCR	Urinary nephrin	Urinary ACR
Sensitivity (%)	92.3	92.0	50.0
Specificity (%)	32.4	42.0	88.7
Negative likelihood ratio	0.2	0.2	0.6
Positive likelihood ratio	1.4	1.6	4.4
Negative predictive value (%)	97.6	98.0	94.4
Positive predictive value (%)	12.6	14.0	31.8
Optimal Cutoff value	14.0	8.8	3.7
AUC (95% CI)	0.63 (0.52-0.73)	0.68 (0.58-0.78)	0.71 (0.59-0.82)
p-value	0.038	0.003	0.001

AUC: Area under the curve; CI: Confidence Interval

5.3.5. Association of urinary nephrin, NCR and ACR with diagnosis of PE and diabetes during pregnancy.

Markers of renal function were compared between pregnant women who developed PE and DIP and women with normal ACR (3 mg/mmol) and no reported comorbidities (NORM) in the full cohort. There was no significant difference between the three groups for the urine creatinine and albumin, or SCr and sCysC between the three groups ($p>0.05$). There was a significant difference between the mean urinary NCR [61.9 (9.2) vs. 56.4 (7.7)] and 42.7 (3.9)] ng/mg, urinary nephrin [81.9 (18.2) vs 57.8 (9.2)] and 36.7 (4.3)] ng/ml and ACR [14.9 (5.4) vs 9.4 (3.7)] and 1.04 (0.05)] mg/mmol. A statistically significant difference was observed between women who developed PE and the normal group NORM ($p < 0.05$) (**Figure 5.3 A and B**). There was no significant difference in urinary NCR between the women who developed DIP and the NORM group.

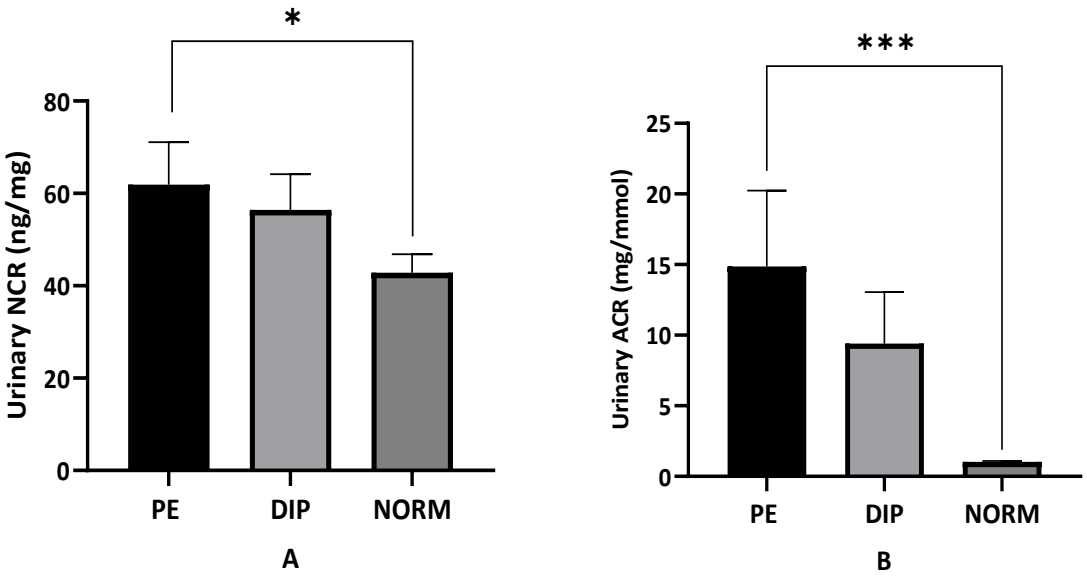


Figure 5.3. Urinary NCR ng/mg values in pregnant women who developed a pregnancy complication, PE, DIP compared with women with ACR<3mg/mmol and no reported comorbidities (NORM).

*Values are described as mean (SEM). *p<0.05, ***p<0.001.*

5.3.6. Comparison of urinary nephrin and NCR for the prediction of PE and DIP with ACR levels

Number (percentage) of women who developed PE and DIP were compared to a group of women with normal albuminuria and no comorbidities using NCR <14 ng/mg as a cutoff. Elevated nephrinuria was detected in 92.3% of women who developed PE **(Figure 4A)**, while using the cutoff values of urinary ACR obtained from ROC (similar to the KDIGO guideline provided cutoff value) **(Figure 4B)** only 13/26 (50%) of women with PE identified with high albuminuria.

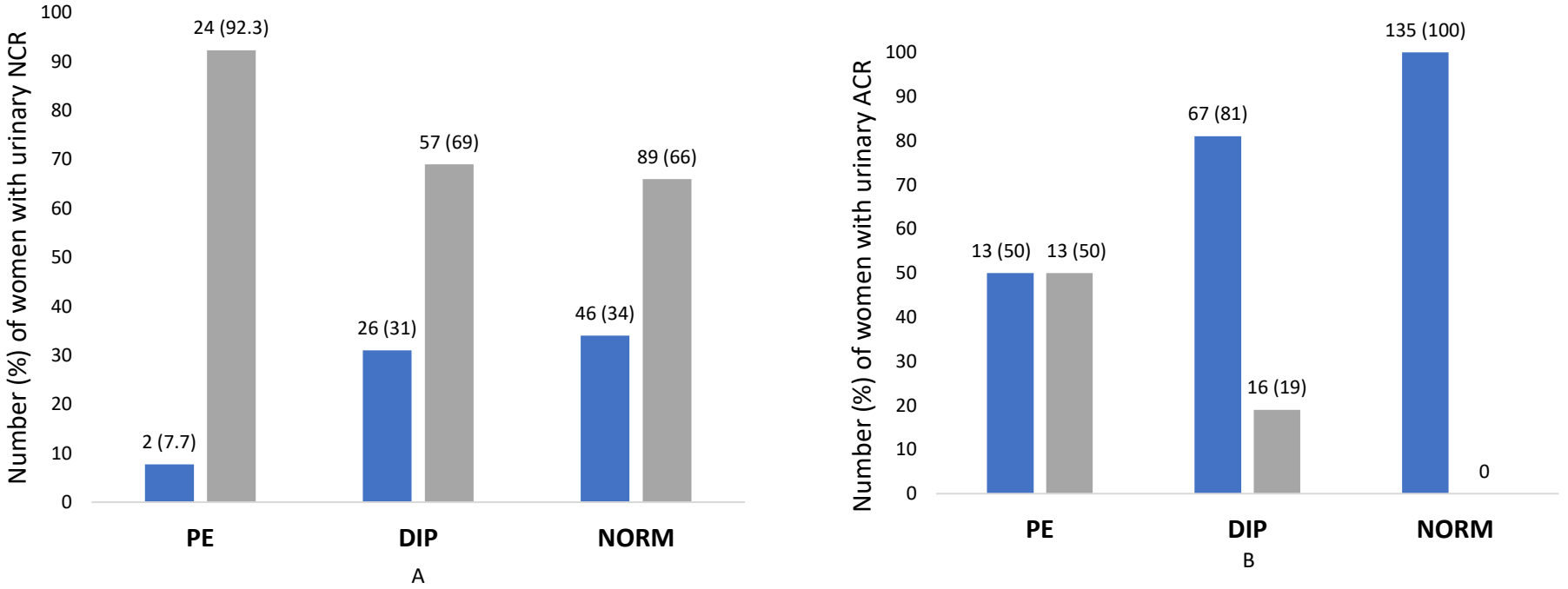


Figure 5.4. The percentage of women from the full cohort identified using either NCR (A) or ACR (B) who developed PE, women who had DIP and women who did not have reported comorbidity with normoalbuminuria the full cohort.

(A) Normal urinary NCR (blue bars) and high urinary NCR (grey bars) at 14ng/mg cutoff value, (B) Normal ACR (blue bars), high ACR (grey bars) at 3.7mg/mmol cutoff value in the full cohort.

5.4. Discussion

This study investigated the use of urinary nephrin and NCR as a marker for determining early glomerular injury in a cohort of pregnant women. Existing guidelines recommend urinary ACR as a standard reference test for the diagnosis and monitoring of glomerular damage (242)**.** While an elevated ACR provides a strong indicator of advanced glomerular damage, ACR is a less sensitive marker of early glomerular injury. Albuminuria may also be detected in urine of patients secondary to other pathological conditions (406). In the context of early glomerular injury, it has been hypothesised that structural damage precedes albumin leakage through the filtration barrier (75). Therefore, it is thought that the excretion of the structural protein nephrin might precede microalbuminuria providing any earlier indicator of glomerular damage (75).

Nephrinuria correlated well with albuminuria and other markers of renal function in this cohort of women. Our study found that urinary nephrin, NCR, and sCysC significantly increased with an increase in urinary ACR. Urinary nephrin, NCR, and sCysC but not serum or urinary creatinine were significantly elevated in women who had micro-macroalbuminuria (≥3mg/mmol), when compared to women with normoalbuminuria (<3mg/mmol) with and without comorbidities (**Table 5.2**). While the percentage of pregnant women in the microalbuminuria and macroalbuminuria group with elevated nephrinuria was high, 94.7% and 100% respectively. There was a high proportion of pregnant women who were normoalbuminuric that demonstrated elevated nephrinuria (64.9%) (**Figure 5.1**). The presence of diabetes and/or hypertensive disorders during pregnancy may increase the risk of glomerular damage and AKI. Glomerular injury is associated with podocyte protein loss (407), and previous studies have suggested that podocyte damage is associated with increased sCysC, urinary nephrin and urinary albumin in patients with diabetes (49, 75, 325, 327, 408). Hence, an increased urinary NCR and sCysC may be a risk predictor of glomerular injury, indicatingsubclinical damage to the glomerular podocytes preceding leakage of albumin in the urine. To further investigate this finding, the normoalbuminuric group was evaluated for comorbidities that may be involved in kidney injury (**Figure 5.2**). Interestingly, within the normoalbuminuric group 14/18 (78%) of women who had a hypertensive disorder and 42/67 (63%) of women who had diabetes in pregnancy had elevated nephrinuria. Thus, urinary NCR may be a more sensitive indicator than urinary ACR for predicting early

glomerular injury.

A recent study conducted by Kostovska et al. in 90 patients with T2DM determined that 82% of normoalbuminuria patients had nephrinuria, 88% of patients with microalbuminuria, and 100% of patients with macroalbuminuria had elevated nephrinuria (75). Numerous studies have demonstrated that nephrinuria may be a sensitive marker for determining glomerular injury in diabetic patients. Jim et al. found that 54% of DM patients with normoalbuminuria demonstrated an elevated nephrinuria and 100% of patients with micro-macroalbuminuria had nephrinuria (49). Shahid et al. using ELISA (Eth-Bio, USA) to quantitate urinary nephrin demonstrated an elevated nephrinuria in 81.4% of DM patients with normoalbuminuria and 100% of patients with macroalbuminuria (327). Interestingly, Kishore et al. also found that nephrin excretion was significantly higher in DM patients with normoalbuminuria (325), suggesting that urinary nephrin may prove to be a more sensitive indicator of early renal dysfunction.

5.4.1. Urinary NCR as a predictive marker of glomerular injury in PE and DIP

Proteinuria is a common symptom of glomerular injury during pregnancy and as consequence women are routinely monitored for the development of proteinuria. However, its presence is not required for a clinical diagnosis of PE (409). In the current study, an elevated urinary NCR (nephrinuria) was detected in 92.3% of women who developed PE, 69% of women identified with DIP and 66% of women with an ACR (<3.0 mg/mmol) and no reported comorbidities. This is in contrast with those reported to have micro-macroalbuminuria (>3.7 mg/mmol); detected in 13/26 (50%) of women who developed PE and 15/247 (18%) of women identified with DIP. Urinary nephrin, NCR and ACR were significantly higher in women who developed PE when compared with women who did not develop PE. Urinary nephrin and NCR were significantly correlated with urinary ACR in PE ($r = 0.50$, $p= 0.02$). Several studies have found urinary nephrin levels to be significantly higher in women who develop PE when compared to their counterparts (255, 259, 260, 410).

A recent study among pregnant women revealed the association of elevated urinary nephrin with PE. The finding showed a 9-fold increase in urinary nephrin in PE compared to normotensive women (411). Likewise, a recent review by Kandasamy et al. described the critical role of nephrinuria in the pathogenesis of proteinuria during PE (24) and the authors

suggested nephrinuria as an indicator of glomerular injury. In the past, studies have suggested podocyturia occurs prior to albuminuria, showing that podocyturia can be detected earlier in PE (314, 407), therefore nephrinuria may be a more sensitive marker than angiogenic markers particularly for asymptomatic women (323). Recently an observational cohort study investigated the association between PE and long-term kidney outcomes (412), and found that women with PE during pregnancy were at increased risk of later developing chronic HTN, declined glomerular filtration rate (GFR: <60ml/min/1.73 m^2) and increased risk of albuminuria when compared with women who did not develop PE. Early detection of renal injury in PE could be a valuable opportunity to diagnose AKI during pregnancy and therefore identify women at risk of developing CKD and may aid in the development of early intervention strategies and reduce kidney disease in later life.

5.4.2. Predictive performance of urinary NCR for the detection of glomerular injury in PE and DIP

The predictive characteristics of urinary NCR in this study using a cutoff 14ng/mg showed a sensitivity of 92.3% and specificity of 32.4% for predicting glomerular injury associated with PE. Others have also found urinary nephrin to have a high sensitivity and specificity for predicting renal injury in PE in different cohorts. Kostovska et al. found that urinary nephrin had a sensitivity and specificity of 96.7% comparing patients with T2DM and DN (260). Jim et al. demonstrated a sensitivity and specificity of 57% and 58%, and Yang et al. a sensitivity and specificity of 67% and 83% respectively for the prediction of PE(26, 255).

Previous studies investigating the sensitivity and specificity of urinary nephrin for predicting glomerular injury in patients with DN found that the sensitivity (92.5%) and specificity (76.7%) of urinary nephrin for the detection of glomerular nephropathy (325). Kostovska et al. demonstrated, in 90 patients with T2DM (30 known DN and 60 without diagnosed DN) and 30 healthy controls, that urinary nephrin had a higher predictive probability of 96% for patients with DN (75). Similarly, Jim et al. demonstrated a comparable finding to our study using an ELISA to quantitate urinary nephrin, with a sensitivity of 99% and 46% specificity for the prediction of glomerular nephropathy (49). Differences in reported sensitivity and specificity of results between our findings and those reported by others possibly relates to variation in the recruitment of participants with previous studies using strict inclusion and exclusion criteria to allocate high-risk groups and frequency matched controls and our

prospective study including all women presenting for antenatal screening. Other studies included various stages of patients with DN with a longer duration of illness and have comparable sample sizes between groups to determine the predictive probability of urinary nephrin. However, our study recruited pregnant women with no exclusion criteria. Together, these studies support the idea that urinary nephrin may be a more sensitive marker for detection of early glomerular injury than albuminuria.

5.4.3. Predictive performance of urinary NCR for the detection of early glomerular injury

This study investigated the use of urinary nephrin and NCR as a marker for determining early glomerular injury in a cohort of pregnant women. Existing guidelines recommend urinary ACR as a standard reference test for the diagnosis and monitoring of glomerular damage (242). While an elevated ACR provides a strong indicator of advanced glomerular damage, ACR is a less sensitive marker of early glomerular injury. Albuminuria may also be detected in urine of patients secondary to other pathological conditions (406). In the context of early glomerular injury, it has been hypothesised that structural damage precedes albumin leakage through the filtration barrier (75). Therefore, it is thought that the excretion of the structural protein nephrin might precede microalbuminuria providing any earlier indicator of glomerular damage (75).

Urinary NCR appears to have a high sensitivity for the prediction of early glomerular injury of PE relative to urinary ACR. The majority (82%) of the participants in our study were normoalbuminuric, of these 52% demonstrated had an elevated nephrinuria, this group had heterogeneous clinical characteristics and comorbidities, all of which may contribute to an increased risk of podocyte damage. Urinary ACR >3.7mg/mmol, demonstrated a 50% sensitivity and 88.7% specificity in the prediction of PE in this cohort only 50% women who developed PE had albuminuria. Similar observations have been reported by Jim et al. in 91 pregnant women, of whom 78 were in a high-risk group, found the sensitivity of albuminuria found to be 36% and specificity of 96% to predict PE. However, the authors revealed that none of the low-risk women exhibited albuminuria. A recent study by Devanath et al. demonstrated urinary nephrin as a biomarker of early glomerular injury in newly diagnosed hypertensive patients (261). The urinary nephrin was quantified using human nephrin ELISA Kit (Elabscience Biotech Co. Ltd., Wuhan, Hubei Province, China). The authors reported that

significantly high urinary NCR in hypertensive patients with normoalbuminuria, and the investigators concluded that urinary nephrin can be used as a marker for early glomerular injury preceding albuminuria (261). This might lead to speculation that early glomerular injury can occur without proteinuria in the early stages of PE and that early clinical diagnosis and management of PE could prevent glomerular damage associated with hypertensive disorders. Moreover, mild glomerular endotheliosis has been reported in women with pregnancy-associated hypertension without proteinuria (413). Glomerular damage associated with PE often reverses following delivery, coinciding with hypertension management, non-progressive glomerular damage.

5.5. Strengths and limitations of the study

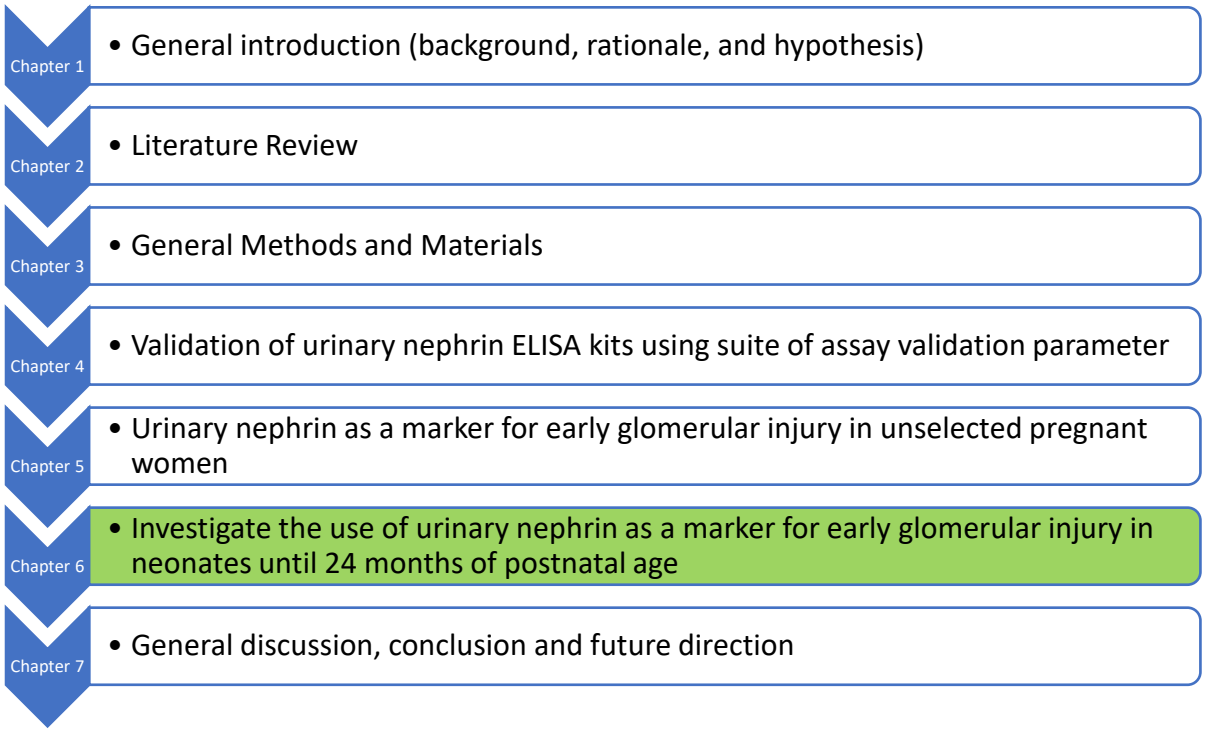
The strengths of this study are that it is a prospective cross-sectional study that includes a clinically heterogeneous group of pregnant women who may also present with nephrinuria, such as women with HTN, women with PE, and GDM. The study also compares urinary NCR with ACR as a comparison marker to predict glomerular injury, since current guidelines used ACR as a marker for glomerular damage and monitoring and management of CKD. Compared to all other studies on nephrinuria to date, the study had a larger sample size of pregnant women, which is another strength. The limitation of this study is that the cross-sectional nature of the study design provides the basis for association, rather than causality. We do not know if nephrinuria is the causal mechanism or if early detection of nephrinuria will reliably predict consequent glomerular injury in women with normoalbuminuria. Finally, the reference test urinary ACR may not reveal subclinical glomerular damage and might underscore the specificity of urinary NCR in this study.

5.6. Chapter summary

In conclusion, our findings showed that urinary nephrin and NCR could be used for detection of early glomerular injury in the general population of pregnant women. Urinary NCR was detected in a high percentage of women with normoalbuminuria, suggesting this may have a role as a marker of early glomerular injury. There was a significant increase in urinary nephrin, NCR and sCysC in pregnant women with micro-macroalbuminuria compared with those women with normoalbuminuria. There was a significant positive correlation between urinary NCR and ACR. There was a statistically significant difference in urinary nephrin, NCR and ACR

between women who developed PE and women who did not develop PE. Our results suggest that urinary NCR holds discriminatory power in identifying women at risk of significant albuminuria as well as those that develop PE among pregnant women.

This study recommends further longitudinal prospective studies aimed at translation of urinary NCR into clinical practice. Also, to investigate the clinical utility of NCR for identifying glomerular injury prior to albumin appearing in the urine for these women. Cutoff values for urinary NCR vary across the literature making studies difficult to compare. There is currently no recommended cutoff value for abnormal urinary NCR excretion in different populations and for the prediction glomerular injury in low and high-risk pregnant women. Therefore, establishing clinically useful RIs for urinary NCR among healthy pregnant women would be of considerable clinical value. The next chapter (**Chapter 6**) will investigate the use of urinary NCR in infantes aged 0-2 years.



Foreword Chapter 6

This chapter addresses aims 3 and 4: to investigate the use of urinary nephrin in infants. Biomarker that detects early recognition of kidney injury and initiation of preventive measures helps to mitigate further complications. To our knowledge, only two studies have shown the potential use of urinary NCR in predicting glomerular injury (68) and predicting glomerular injury and AKI in neonates (50, 338). However, the longitudinal changes of urinary NCR, along with the renal function markers, have not been investigated. A biomarker to be used for clinical use, first, the biomarker should be tested for physiological dynamic changes across postnatal ages. The purpose of this chapter was to investigate the longitudinal changes of biomarkers in neonates from birth to 24 months of postnatal age with measurement of the glomerular integrity markers (urinary NCR and ACR) and renal function markers (SCr, sCysC, and eGFR) regardless of any congenital kidney anomalies and other medical complications. This chapter also compared the biomarker levels between term and preterm neonates. The other important part of this chapter was determining a clinically useful cutoff and RIs for urinary NCR in neonates against postnatal age and gender. Finally using the upper limit of normal (ULN) of the RIs, the percentages of neonates with elevated nephrinuria were determined. In addition, the levels of renal function markers were compared between neonates with elevated nephrinuria and normal nephrinuria at birth.

Chapter 6. Investigation of the use of urinary nephrin in infants for determining early glomerular injury.

6.1. Abstract

Introduction: The discovery of nephrin has given attention to the ongoing need to recognise early glomerular injury in various clinical conditions. However, the use of urinary nephrin has not been validated in infants. To achieve this a harmonised cutoff for urinary nephrin is required to monitor both healthy infants and infants at risk of glomerular injury. This study aimed to investigate the use of urinary NCR in infants and establish a working cutoff and RIs for urinary NCR in neonates against postnatal age to detect early glomerular injury.

Methods and Materials: A prospective longitudinal cohort study was conducted from August 2019 until August 2021 and follow-up completed in August 2023, involving neonates born at term and preterm. The study included neonates at birth (N=190) and examined at 12 months (N=74) and 24 months (N=93); and preterm neonates at birth (N=74), 12 months (N=36) and 24 months (N=39) of postnatal age. A urine sample was collected for the measurement of urinary nephrin, and albumin corrected with urine creatinine, and the blood sample was collected for measuring SCr and sCysC. The differences in biochemical and clinical parameters between the groupings were compared. After Box-Cox transformation and removal of outliers the nonparametric and robust methods were used to calculate 95% RIs with related 90% CIs against postnatal age and gender.

Results: In this study the median gestational age of term neonates was 38.5 (1.3) weeks with a mean birthweight of 3340±512 grams. The median gestational age of preterm neonates was 34.8 (3.6) weeks, with a mean birthweight of 2145±707.6 grams. There was a statistically significant decline in median urinary NCR [13(39)] to [8.5(20)] ng/mg, p=0.046 and urinary ACR [8.43 (11.7)] to [1.38 (3.1)] mg/mmol, p<0.001 from birth to 24 months age in preterm neonates. However, there was no significant difference in median urinary NCR [8.4 (21.4)] to [10.3 (22.4)] ng/mg and remains stable from birth to 24 months of age in term neonates, p=0.326. A statistically significant difference was observed in urinary NCR [8.4 (21.4)] vs [13 (39)] ng/mg between term and preterm neonates at birth, p=0.006. The nonparametric RIs of the upper limit of normal (ULN) for urinary NCR with 90% CI was 69 (62.5-87) ng/mg at birth. The robust RIs of the ULN for urinary NCR with 90% CI at 12 months were 64 (47-84) ng/mg

and at 24 months were 67 (53-83) ng/mg. The study revealed no significant difference in urinary NCR (ng/mg) across gender and postnatal age, $p>0.05$.

Conclusion: This study showed that urinary NCR could be used as a potential non-invasive biomarker for the diagnosis of early glomerular injury and/or immaturity in neonates. Urinary NCR is influenced neither by postnatal age nor by gender**.** Hence, these RIs could be reliable and applicable preliminary values for healthy-term neonates and children. Moreover, it will provide a tool to assist clinicians in diagnosis and monitoring of glomerular injury in neonates and children if translated into clinical practice.

6.2. Introduction

Nephrin, a transmembrane protein, is the main structural and functional component of the GFB (56). Since it was first described in 1998 in children with congenital nephrotic syndrome of the Finnish type resulting from mutation of the gene NPHS1, it has been broadly researched and is reported as a novel promising marker of glomerular injury (21) in patients with DN (75), and PE (314). It has also played a crucial role in kidney development and organising the molecular structure of the Sd and is served as a molecular sieve of the GFB (56). Moreover, nephrin has a role in the podocyte signaling cascade through tyrosine phosphorylation, which preserves glomerular function and promotes anti-apoptotic signals (10, 47, 377), nephrin may play a key role in identifying early glomerular injury.

Glomerular injury is damage to the glomeruli of the kidney. Over time, the injury may progress to AKI and/or CKD (414). Regardless of aetiology progressive glomerular injury is characterised by elevated proteinuria, which is the sign of glomerular damage (415, 416). Diverse clinical conditions affect the GFB of the nephrons and lead to glomerular and/or glomerular-tubular pathologies via glomerular-tubular crosstalk signaling from the tubules (341). However, in proteinuric kidney disease, the glomerular injury is probably a forerunner of other forms of kidney injury and/or disease. In this context, glomerular specific biomarker for detecting early glomerular injury is essential for developing potential intervention strategies.

Conventionally, SCr and urinary ACR are used for screening and management of kidney disease (417), but these markers are influenced by non-renal factors and less sensitive in the early stage of kidney damage. The increase in proteinuria/albuminuria in neonates associated with glomerular immaturity, impaired permeability, and /or glomerular injury remains unclear and the cutoff urinary ACR in neonates and children under 24 months of age is not yet validated, whilst the improved sensitivity of urinary nephrin has been described in the literature and can be detected in urine during early glomerular damage (21, 50, 68, 75, 418) and was suggested to be a possible predictor of AKI (50). However, scientific literature did show a variation in urinary nephrin cutoff values among general populations with different clinical conditions (260, 313, 314).

Establishing cutoff values for the identification of healthy individiauls and reference Intervals (RIs) are an integral part of decision-making tools (419) and help with interpreting laboratory test results (420). Saris and *Gräsbeck* introduced the concept of statistics at a Congress in 1969 (421), and the reference values become the main discussion point in clinical laboratory medicine to be used as a tool for clinical decision-making (421). Later, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCCLM) and the CLSI guidelines produced general recommendations on the principle and terminology of RIs (282, 422). The guidelines recommended a 90% CI with lower 2.5^{th} and upper 97.5^{th} percentile limits in a nonparametric method that represents 95% of the reference population and 95% CI for parametric methods in at least 120 healthy populations (282, 422).

Good laboratory practice is vital for research and clinical practice (423), in which almost 70-80% of medical decisions are made using laboratory test results (424). However, a laboratory test result by itself does not provide relevant information unless reported with valid clinically related interpretation (425). For instance, many clinical laboratories used RIs from textbooks and package inserts of the reagent suppliers (422). However, these values are derived from different populations and might vary from population to population, lack of metrological traceability and would lead to inappropriate clinical decision-making that raises questions to investigate the use of urinary nephrin and determine clinically useful RIs in infants and children.

The RIs for most biochemical parameters vary among the general population (426). The difference in diagnostic methods and reagent formulations can also affect the accuracy of RIs. Determining RIs in neonates and children is also a challenging task due to sample size and required sample volume and natural physiological changes of biomarkers with age (427). Likewise, many factors impact foetal kidney development such as maternal health, infection during gestation, premature birth, and low birth weight which impact the developmental programming of the kidney (428), all these factors have a deleterious effect on kidney vasculature and impair the GFB of the kidney and leads to glomerular injury in neonates and later in life (428).

To our knowledge the use of urinary nephrin in neonates, infants, and children as a marker of glomerular injury, has not been well investigated. To address the existing gap and better

utilise urinary nephrin in clinical settings, this study aimed to investigate the use of urinary nephrin in a cohort of infants from birth until 24 months of age for detecting early glomerular injury. To address this, the specific aims of this study are:

- To investigate urinary NCR and renal function markers in neonates longitudinally from birth to 24 months of age
- To establish a cutoff value and RIs for urinary NCR in neonates against postnatal age and gender
- To determine the percentages of neonates with elevated nephrinuria as a marker of early glomerular injury
- To compare renal function markers between neonates with elevated nephrinuria and normal nephrinuria

6.3. Results

6.3.1. Study population

In this study, 401 mother-infant dyads consented. In terms of birth outcomes, there were 26 sets of twins, and 1 set of triplets. Of these, 56 mother-infant dyads withdrew from the study, and the remaining neonates were not included in this study, due to congenital kidney anomalies and other medical problems (n=22), and either the maternal-infant dyad did not attend clinic due to COVID-19 restrictions/ maternal factors (n=86). At birth, 190 term neonates, at 12 months of age 74 term born infants, and at 24 months of postnatal age 93 children were included in this study. Moreover, from 79 preterm neonates, 74 preterm born neonates included for final analysis (n=4 excluded due to congenital kidney anomalies and other medical problems), 36 attended the clinic at 12 months and 39 attended at 24 months of postnatal age provided urine and blood samples. It is to be noted that missing data exists for SCr and sCysC results due to difficulties in obtaining samples/insufficient samples from infants. Eight missing serum samples for analysis of SCr and sCysC at birth, 6 serum samples at 12 months, and 8 serum sample at 24 months were recorded as missing in term neonates, and 2 serum samples at birth, 5 samples at 12 months, and 5 samples at 24 months were recorded as missing sample in preterm neonates.

6.3.2. Clinical characteristics of the study population

Demographic and clinical characteristics of the study population are shown in **Table 6.1**. Overall, males accounts for 97 (51%) in term neonates and 26 (35%) in preterm neonates. The median postnatal age at the sample collection time point of term neonates was 6 (30.3) days and preterm neonates was 24 (53) days. The median gestational age of the term neonates was 38.5 (1.3) weeks, and preterm neonates was 35.1 (3.6) weeks. In terms of birthweight percentiles for gestational age, 13 (6.8%) and 14 (18.9%) are small for gestational age for term and preterm neonates respectively.

Table 6 1.Demographic and clinical characteristics of term and preterm neonates at birth

Birth demographics	**Term neonates (n=190)**	**Preterm neonates (n=74)**
Male, n (%)	97(51%)	26 (35%)
Female, n (%)	93 (49%)	48 (65%)
Postnatal age (days)	6 (30.3) *	24 (53) *
Gestational age (wks.)	38.5 (1.3)	35.1 (3.6)
Birth weight (g)	3340±512	2145±708
Birth weight centiles		
Small for gestational age	13 (6.8)	14 (18.9)
Appropriate for gestational age	125 (65.8)	51 (68.9)
Large for gestational age	52 (27.4)	9 (12.2)

*Data are presented as the number with the percentage given in parentheses, *the median with the IQR given in parentheses, and the mean ± SD as appropriate.*

6.3.3. Comparison of urinary NCR and biomarkers of renal function across postnatal age

The longitudinal changes in biomarker levels were compared according to postnatal ages **(Table 6.2**). There was no statistically significant difference in urinary NCR across postnatal age in term neonates. However, there was a statistically significant decrease in urinary NCR in preterm neonates between birth and 24 months of postnatal age, 13 (39), 11 (22), and 8.5 (20) ng/mg; p = 0. 046. In term infants, there was a statistically significant decrease in urinary ACR from birth to 24 months of postnatal age 7.4 (10), 2.6 (3.9), and 1.5 (1.4) mg/mmol; p<0.001. Similarly, in preterm infants there was a statistically significant decrease in urinary ACR across postnatal age 8.43 (11.7), 2.51 (1.8), and 1.38 (3.1) mg/mmol; p<0.001. The levels

of sCysC declined and eGFR (mL/min/1.73 m^2) was gradually increased with age in term and preterm neonates; p<0.001.

Table 6 2: Comparison of longitudinal changes in biomarker levels in term and preterm neonates

Term Neonates	**At birth (N=190)**	**12 months (N=74)**	**24 months (N=93)**	**p-value**
Urinary NCR (ng/mg)	8.4 (21.4)	9.1 (24.8)	10.3 (22.4)	0.326
Urinary ACR (mg/mmol)	7.4 (10)	2.6 (3.9) #	1.5 (1.4) *	<0.001
Urine albumin (mg/L)	10.3 (30.5)	5.2(6.2) #	5.6 (6.9) *	<0.001
Urine creatinine (mmol/L)	1.4 (2.5)	2.06 (2.3) #	3.41 (2.8) *	<0.001
Serum Cystatin C (mg/L)	1.6 (0.38)	0.93 (0.26) #	0.81 (0.3) *	<0.001
Serum creatinine (µmol/L)	47 (30)	21 (33.8) #	20 (11.5) *	<0.001
eGFR (mL/min/1.73 m^2)	45.1 (13)	82.7 (26.4) #	97.2 (31) *	<0.001
Preterm neonates	**At birth (N=74)**	**12 months (N=36)**	**24 months (N=39)**	**p-value**
Urinary NCR (ng/mg)	13 (39.0)	11 (22.0)	8.5 (20.0) *	0.046
Urinary ACR (mg/mmol)	8.4 (11.7)	2.5 (1.8) #	1.4 (3.1) *	<0.001
Urine albumin (mg/L)	7.6 (9.0)	6.2 (6.0) #	4.4 (7.0) *	0.002
Urine creatinine (mmol/L)	0.9 (1.06)	2.2 (2.2) #	2.9 (5.9) *	<0.001
Serum Cystatin C (mg/L)	1.6 (0.4)	0.9 (0.2) #	0.9 (0.3) *	<0.001
Serum creatinine (µmol/L)	27 (26)	25 (53) #	18 (13) *	0.0042
eGFR (mL/min/1.73 m2)	43.2 (13)	91 (25) #	91 (35) *	<0.001

Values are reported as median (IQR).

Kruskal Wallis H test with Bonferroni post-hoc analysis; p <0.05.

*: Statistically significant difference exists between at birth and 24 months

#: Statistically significant difference exists between at birth and 12 months

6.3.4. Comparison of biomarkers of renal functions between term and preterm neonates

The biomarker levels were compared between term and preterm neonates at the corresponding postnatal ages (**Table 6.3**). There was a statistically significant increase in urinary NCR in preterm neonates 13 (39) compared to the term neonates 8.4 (21) ng/mg at birth; p=0.006. However, no statistically significant difference was observed in urinary ACR, sCysC or eGFR between term and preterm neonates at any postnatal age.

Table 6 3: Comparison of biomarkers between term and preterm neonates with respect to ages

Biomarkers	Postnatal age	Term neonates	Preterm neonates	p-value
Urinary NCR (ng/mg)	At birth	8.4 (21.4)	13 (39)	0.006*
	12 months	9.1 (24.8)	11 (22)	0.976
	24 months	10.3 (22.4)	8.5 (20)	0.417
Urinary ACR (mg/mmol)	At birth	7.4 (10)	8.4 (11.7)	0.326
	12 months	2.6 (3.9)	2.5 (1.8)	0.624
	24 months	1.5 (1.4)	1.4 (3.1)	0.922
Urine albumin (mg/L)	At birth	10.3 (30.5)	7.6 (9.0)	0.019*
	12 months	5.2 (6.2)	6.2 (6.0)	0.414
	24 months	5.6 (6.9)	4.4 (7.0)	0.281
Urine creatinine (mmol/L)	At birth	1.4 (2.5)	0.9 (1.06)	<0.001*
	12 months	2.06 (2.3)	2.23 (2.2)	0.219
	24 months	3.41 (2.8)	2.86 (5.9)	0.181
Serum Cystatin C (mg/L)	At birth	1.60 (0.38)	1.62 (0.4)	0.245
	12 months	0.93 (0.26)	0.86 (0.2)	0.231
	24 months	0.81 (0.3)	0.93 (0.3)	0.302
eGFR (mL/min/1.73 m^2)	At birth	45.1 (13)	43.2 (13)	0.244
	12 months	82.7 (26.4)	91 (25)	0.157
	24 months	97.2 (31)	91 (35)	0.215

Values are reported as median (IQR).

Mann-Whitney U test; p <0.05.

* Statistically significant difference exists in biomarker levels between term and preterm groups

6.3.5. Reference intervals of urinary NCR in term neonates against postnatal age and gender

The biochemical markers of the kidney were compared between groups. There was no statistically significant difference in urinary NCR between males (median: 6.4; IQR: 21.1) and females (median: 10.9; IQR: 25.1) at birth, p=0.403. Males (Median: 6.81; IQR: 11.7) and females (median: 12.25, IQR: 28.7) at 12 months, p=0.11. Males (Median: 10.23; IQR: 20.6) and females (median:14.7; IQR: 23.3) at 24 months of age, p=0.319. A Kruskal-Wallis H test did not show a statistically significant difference in median urinary NCR between the birth (median: 8.4; IQR: 21.3); 12 months (median: 9.1; IQR: 24.8) and 24 months (median: 10.3; IQR: 22.4) of postnatal age (p=0.326) (**Table 6.1**). The urinary ACR (mg/mmol) showed a significant difference between males and females and significantly declined from birth to 12 months of age ($p<0.05$), but no significant difference was observed at 24 months of age (p=0.267). There was no significant difference in postnatal age 6.0 (28.5) vs 8(31), p=0.441; gestational age 38.6 (1.1) vs 38.5 (1.6), p=0.905; birthweight 3397±469 vs 3280±550, p=0.119 between males and females respectively. Although no significant difference, there was gradual decline in urine albumin, creatinine, SCr, sCysC, and gradual increment in eGFR in males and females across postnatal ages, $p>0.05$.

6.3.6. Data distribution and transformation

The skewness-kurtosis tests were used to assess the normality of the data. The urinary NCR showed a skewed distribution, and the degree of symmetry was shown by the Coefficient of skewness (CS) and tailedness by Coefficient of Kurtosis (CK) with respective p-values. After Box-Cox transformation, the distribution seems approximately symmetrical ($p>0.05$) but not normally distributed in the data at birth subgroups ($p<0.05$). Extreme outliers were removed before analysis and all the data were within the 25^{th} percentile minus 1.5 x IQR, and the 75^{th} percentile plus 1.5 x IQR in the box-whisker plot. The data distribution of gender and age-specific sub-groupings are described in **Table 6.4.**

Table 6.4: Skewness-Kurtosis test for the data before or after transformation in subgroups

Variables	**Before Box-Cox transformation**					**After outliers deleted and Box-Cox transformation**				
Urinary NCR (ng/mg) [a]	**N**	**CS**	**P-value**	**CK**	**p-value**	**N**	**CS**	**P-value**	**CK**	**p-value**
Male	97	3.7239	<0.0001	15.2034	<0.0001	89	0.01439	0.9535	-0.3591	0.4909
Female	93	2.1575	<0.0001	4.4236	<0.0001	86	-0.0333	0.8942	-0.9891	0.0007
Combined	190	3.8634	<0.0001	18.6787	<0.0001	176	0.0051	0.9773	-0.7363	0.0022
Urinary NCR (ng/mg) [b]	**N**	**CS**	**P-value**	**CK**	**p-value**	**N**	**CS**	**P-value**	**CK**	**p-value**
Male	43	2.7367	<0.0001	8.0310	0.0001	41	-0.01303	0.9703	-0.5848	0.6858
Female	31	4.9893	<0.0001	26.3886	<0.0001	29	-0.08159	0.8418	-0.8921	0.1752
Combined	74	6.8911	<0.0001	53.5514	<0.0001	68	-0.04122	0.8825	-0.8369	0.0318
Urinary NCR (ng/mg) [c]	**N**	**CS**	**P-value**	**CK**	**p-value**	**N**	**CS**	**P-value**	**CK**	**p-value**
Male	52	1.1924	0.0013	0.4852	0.3654	51	1.1820	0.00015	0.4468	0.3933
Female	41	1.366	<0.0016	1.1801	<0.0022	38	-0.110	0.9760	-0.6955	0.2771
Combined	93	3.4333	<0.0001	18.4979	<0.0001	89	-0.0332	0.8929	-0.7113	0.0526

a: data at birth; b: data at 12 months; c: data at 24 months; CS: Coefficient of skewness; CK: Coefficient of kurtosis

6.3.7. Reference intervals of urinary NCR in term neonates against postnatal age

Fourteen suspected far outliers from the total sample at the birth, six far outliers from data of 12 months, and four far outliers from data of 24 months of postnatal age groups were excluded from the analysis using Tukey's method. The lower 2.5th and the upper 97.5th percentiles with corresponding 90% CIs show an overlap in CIs across age groups and gender (**Table 6.5**). The combined (male and female) RIs of the upper limit of normal (ULN) for urinary NCR (ng/mg) using the nonparametric percentile method was 69 (62.5-87) at birth, using the robust method, the common RIs for the ULN was 64 (47-84) and 67 (53-82.3) at 12 and 24 months of postnatal age respectively.

Table 6.5: Upper Limits of Normal (95th percentiles) and associated 90% CIs for urinary NCR for neonates against postnatal age.

Analyte	Postnatal age	Gender	Sample size	Outlier (Tukey)	Min. value	Max. value	Mean (SD)	Median	95% CI for median	Lower limit (90% CI)	Upper limit (90% CI)	DAP test
Urinary NCR (ng/mg)	Birth	Male	97	8	0.52	73.80	13 (17)	6.20	4.6-8.6	0.82 (0.64-1.08)	76.0 (50-113)	P=0.7874
		Female	93	7	0.49	74.85	16 (17)	8.80	5.2-15.0	0.58 (0.37-0.86)	85.0 (64-114)	P=0.003
		Combined	**190**	**14**	**0.49**	**86.95**	**15 (18)**	**6.40**	**5.4-9.9**	**0.72 (0.49-1.05)**	**69.0 (63-87)**	**P=0.009**
Urinary NCR (ng/mg	12 months	Male	43	2	0.42	56.50	11 (13)	6.40	4.0-11.0	0.45 (0.26-0.81)	63.0 (39-100)	P=0.686
		Female	31	2	0.50	70.40	19 (14)	12.0	5.6-28.0	0.39 (0.11-1.24)	95.0 (62-146)	P=0.391
		Combined	**74**	**6**	**0.42**	**56.50**	**13 (14)**	**8.0**	**4.4-11.7**	**0.45 (0.27-0.78)**	**64.0 (47-84)**	**P=0.099**
Urinary NCR (ng/mg)	24 months	Male	52	1	0.90	55.30	16 (15)	10.3	7.5-14.8	0.84 (0.46-1.49)	67.8 (49-87)	P=0.004
		Female	41	3	1.30	58.40	17 (16)	10.0	7.2-17.3	1.49 (0.97-2.36)	77.0 (50-110)	P=0.554
		Combined	**93**	**4**	**0.90**	**58.40**	**16 (15)**	**10.2**	**7.9-14.8**	**1.12 (0.76-1.62)**	**67.2 (53-83)**	**P=0.152**

CI: *Confidence interval****; DAP****: D'Agostino-Pearson test****; Outliers were identified and removed from analysis using the Tukey method and visual inspections.; SD: standard deviation. All CIs are both left- and right-inclusive.*

6.3.8. Comparison of parametric and non-parametric methods for determining RIs

The parametric 95% RIs, double sided and non-parametric/robust (2.5th and 97.5th) percentiles were compared for urinary NCR to determine the RIs and clinically useful decision limit. The upper limit of normal (ULN) was comparable across ages and gender. However, at birth the data was not normally distributed after transformation (DAP test $p<0.05$) and the combined ULN for the RIs of males and females were 69 (62.5-87) with narrow CIs compared to the parametric method 81.8 (62.8-106.6) (**Table 6.6**). The ULN of the RIs in the age groups and gender were closely related to each other, 64-69 (ng/mg), although sample size was small and slightly wider CIs. Since there is no significant difference in urinary NCR across age and gender, the larger sample size with narrow CIs could be clinically useful ULN for urinary NCR in neonates and children.

Table 6 6: The 95% RIs of urinary NCR using the common methods across postnatal age.

		Parametric (normal distribution)		**Non-parametric /Robust (CLSI C28-A3)**		
Postnatal age	Gender	Lower limit (90% CI)	Upper limit (90% CI)	Lower limit (90% CI)	Upper limit (90% CI)	Remarks
Birth	Male	0.87 (0.65-1.16)	72.2 (48.4-109.3)	0.82 (0.64-1.08)	76 (50-113)	The RIs are comparable
	Female	0.61 (0.38-0.97)	80.9 (58.5-111)	0.58 (0.37-0.86)	85 (64-114)	The RIs are comparable
	Combined	**0.75 (0.58-0.97)**	**81.8 (62.8-106.6)**	**0.72 (0.49-1.05)**	**69 (62.5-87)**	The CLSI C28-A3 provide more precise 95% RIs (the CIs were narrower than parametric method)
12 months	Male	0.51 (0.28-0.92)	57.0 (34.8-92.4)	0.45 (0.26-0.81)	63 (39-100)	The RIs are comparable
	Female	0.52 (0.14-1.48)	84.0 (52.6-129)	0.39 (0.11-1.24)	95 (62-146)	The parametric provide more precise 95% RIs (the CIs were narrower than CLSI C28-A3)
	Combined	**0.51 (0.28-0.87)**	**60.1 (43-83)**	**0.45 (0.27-0.78)**	**64 (47-84)**	The RIs are comparable
24 months	Male	0.93 (0.49-1.69)	63.6 (46.1-86.2)	0.84 (0.46-1.49)	68 (49.3-87.2)	The RIs are comparable
	Female	1.69 (1.1-2.72)	68.9 (45.6-108.4)	1.49 (0.97-2.36)	77 (50-109.5)	The RIs are comparable
	Combined	**1.19 (0.79-1.77)**	**64.4 (50.4-81.5)**	**1.12 (0.76-1.62)**	**67 (53-82.7)**	The RIs are comparable

6.3.9. Percentage of the study participants with elevated urinary NCR at birth

The study used the urinary NCR value of 69 (ng/mg) as clinical decision limit or ULN for detecting risk of early glomerular injury. The percentage of study participants with elevated urinary NCR (≥69 ng/mg) in term and preterm neonates described in (**Figure 6.1**). The urinary NCR was elevated in 18/190 (9.5%) of term neonates and 12/74 (16.2%) of preterm neonates at birth. At 12 months of age 4/74 (5.4%) and at 24 months of age 1/93 (1.1%) of infants had elevated nephrinuria. Moreover, at 12 months of age 2/36 (5.6%) of preterm born infants had elevated nephrinuria.

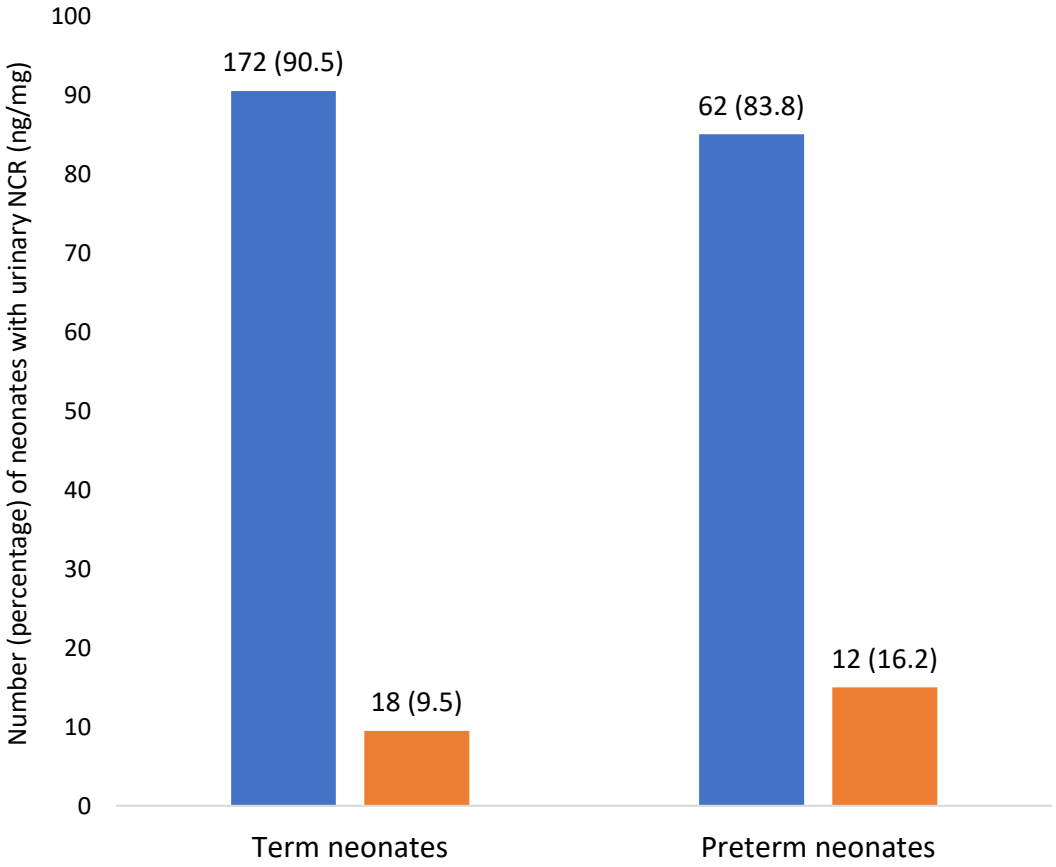


Figure 6.1. Number (percentage) of neonates who had elevated nephrinuria at birth.

Using urinary NCR upper limit of normal of <69ng/mg for normal nephrinuria; blue bars, and ≥69ng/mg for elevated nephrinuria; orange bars.

6.3.10. Comparison of biochemical and clinical parameters in neonates at birth.

The biochemical parameters were compared between neonates who had elevated nephrinuria and normal nephrinuria. The urinary ACR was significantly higher in neonates who had elevated nephrinuria (p=0.035), while the sCysC and SCr values were comparable in neonates between the two groups, as no statistically significant differences were observed, p>0.05. A chi-square (χ^2) test revealed that there is no linear association of levels of nephrinuria and neonate's birth weight centiles, χ^2 (1) = 0.375, p=0.540. Of neonates who had elevated nephrinuria (n=30), 16.7% (n=5) had small for gestational age, 60% (n=18) had appropriate for gestational age, 23.3% (n=7) large for gestational age birth weight centiles. This was not significantly different from those with normal nephrinuria (n=234); 9.4% (n=22) who had small for gestational age, 68% (n=159) who had appropriate for gestational age, or 22.7% (n=53) who had large for gestational age (**Table 6.7**).

Table 6.7: Comparison of biochemical parameters in neonates at birth.

Variables (N=264)	**Elevated nephrinuria (n=30)**	**Normal nephrinuria (n=234)**	**p-value**
Urinary ACR (mg/mmol)	9.7 (12.2)	7.3 (11.4)	0.035*
Urine albumin (mg/L)	7.1 (69.8)	9.1 (19.8)	0.835
Urine creatinine (µmol/L)	0.7 (1.24)	1.22 (1.97)	0.064
Serum Cystatin C (mg/L)	1.59 (0.37)	1.56 (0.39)	0.184
Serum creatinine (mmol/L)	42 (36)	40 (32)	0.362
eGFR (ml/min per 1.73m^2)	44.1 (11.8)	45.1 (13.5)	0.179
Birth weight (g)	3050 (1570)	3065 (1035)	0.630
Birth weight centiles	**Elevated nephrinuria**	**Normal nephrinuria**	**p-value**
Small for gestational age	5 (16.7%)	22 (9.4%)	0.540
Appropriate for gestational age	18 (60%)	159 (68%)	
Large for gestational age	7 (23.3%)	53 (22.7%)	

*Values are reported as median (IQR) and number (%) for birthweight centiles. Mann-Whitney U test and chi-square used to test statistical significance between groupings, respectively. *: significance difference between the groups (p<0.05).*

6.4. Discussion

The main aim of this study was to investigate the use of urinary nephrin in infants for detecting glomerular injury. First, to verify the accuracy and precision of urinary nephrin measurement, the ELISA kit was validated using a suite of assay validation parameters, and the urine creatinine was analysed with a reference method traceable to the IDMS and urinary nephrin and albumin corrected for urine creatinine excretion. Second, urinary nephrin and renal function markers were compared across postnatal age and gender. Finally, RIs were established for urinary NCR in cohort of neonates against postnatal age and percentage of neonates with elevated nephrinuria was determined.

Little evidence is available for the detection of early glomerular injury in neonates (50, 68). Early preventive measures and follow-up are advised for preterm neonates (429) through the detection of markers of early kidney injury (170). To our knowledge, this study is the first to investigate the use of urinary nephrin in neonates and children longitudinally across postnatal ages.

6.4.1. Comparisons of urinary NCR and ACR in neonates across ages

In this study, there was a significant difference in urinary NCR along with ACR in preterm neonates, and a significant difference was observed in urinary NCR between term and preterm neonates during the early neonatal period (birth) but remained non-significant at 12 and 24 months of postnatal age. The decline in urinary NCR across postnatal age in preterm neonates might be partly due to glomerular maturity. However, the rise in the concentration of urinary NCR along with urinary ACR in neonates might signal glomerular injury and /or structural immaturity.

The neonatal kidney is immature at birth and the renal function is influenced by glomerular as well as tubular structural and functional maturity (90). In this study, urinary NCR loss was limited to the early neonatal period (birth) in preterm neonates. Although this study followed up neonates until 24 months of postnatal age, it is not clear whether persistent urinary NCR loss would continue until adulthood. A study in the animal model showed podocyturia was reported in preterm rats from 3 weeks to 12 months of age (430). The authors speculated persistent podocyte loss in preterm birth could venture future risk of CKD. Prematurity reduces the nephron number as nephrogenesis is incomplete during premature birth (431). This puts preterm neonates on a trajectory for kidney disease due to hyperfiltration of remaining nephrons to compensate for normal glomerular filtration function (432).

The clinical utility of urinary nephrin has been described in the literature. Previous work by Kandasamy et al. analysed data on 53 premature neonates and 31 term neonates and undertook assessments at 28, 32 and 37 weeks postmenstrual age (68). The authors found a significant decline in urinary NCR and ACR from 32 to 37 weeks, and no significant difference in eGFR was observed between preterm and term neonates. The authors conclude that premature neonates show evidence of glomerular injury, this is congruent with our study finding. Other studies by Chen and colleagues published the clinical utility of urinary NCR in predicting glomerular immaturity/injury and AKI in a cohort of critically ill neonates (50, 338). Likewise, the measurement of urinary nephrin excretion has been also investigated in adults as a diagnostic marker for glomerular injury in different clinical conditions (76, 246).

Evidence of increasing podocyturia was identified in preterm infants (430, 433). In this study, while comparing term and preterm neonates, urinary NCR was significantly higher in preterm neonates compared to term neonates at birth. The significantly higher concentration of urinary NCR in preterm neonates might show podocyte injury and/or immaturity at birth. From this, the study hypothesises that increased nephrinuria in the absence of significant albuminuria in preterm neonates during early neonatal life might be caused by podocyte immaturity and/or a decrease in the number of nephrons/podocytes per glomerulus, this might be related to increased single nephron hyperfiltration, resulting in early shedding of urinary nephrin, which might be an indicator of early glomerular injury.

A previous study confirmed the effect of prematurity on nephron numbers by measuring kidney volume as surrogates of nephron numbers (68). The authors found lower nephron numbers in preterm birth. In another study, the researcher analysed kidney tissue samples collected at autopsy from preterm neonates (64, 98). The authors revealed morphologically abnormal glomeruli from preterm kidneys. The abnormal glomerular development may be caused by ischemia, and exposure to low oxygen tension that triggers ischaemic glomerular injury (68). A human study by Gao et al. showed a 5-fold increase in urinary podocyte excretion and explained a significantly higher concentration of podocyturia in 35-36 weeks preterm neonates than full-term neonates (433). Ding et al. also showed increasing podocyte excretion and progressive podocyte loss in premature neonates (430), and podocyte excretion over time leads to progressive decline in renal function. Hence, preterm infants should be monitored for intervention and management of kidney health.

Traditionally, albuminuria used as a marker for glomerular injury, and evidence of significant correlation with severity of injury was reported (21, 248). The presence of higher albuminuria could show either gradual decline in podocyte function and glomerular injury/immaturity and/or decreased tubular reabsorption of filtered proteins/tubular functional immaturity (434, 435). This study showed a fluctuation in albuminuria across postnatal age in the term and preterm neonates, which may signify glomerular maturation, but fluctuation raises questions about the validity of urinary ACR for the follow-up of the glomerular damage in neonates and no data tying childhood urinary ACR to future clinical events.

A study by Kandasamy et al. using data from premature neonates admitted to the neonatal department to investigate extra-uterine development of preterm kidney showed declining urinary ACR from 28 to 37 weeks but the elevation of ACR in premature neonates at 37 weeks postmenstrual age compared with term neonates (68). The authors suggested that premature neonates may have a residual glomerular injury. Evidence exhibited that kidney function was impaired at birth in term and preterm neonates but gradually corrected by the first 3 days of life (436). However, correction of glomerular function is slow in preterm neonates with underlined disease conditions and results in a slow gradual decline in urinary ACR. Another study found sustained elevated levels of albuminuria in preterm neonates, while in term neonates, the albuminuria slowly declined in the first 28 days of postnatal age (437). The authors revealed that sustained elevated albuminuria in premature neonates is described as due to increased glomerular permeability and decreased tubular handling of albumin. Overall, maturation of renal function precedes in term birth than preterm birth neonates. However, albuminuria is used as a marker for glomerular damage and a measure of the progression of CKD (438), it has not been validated in neonates less than 24 months of age. In summary, this study showed an elevated urinary ACR in neonates with elevated nephrinuria and a fluctuation in the levels of albuminuria across postnatal age in the term and preterm neonates, which may signify glomerular immaturity and/or injury but instability raises questions about the validity of urinary ACR for the follow-up of the glomerular damage in neonates and no data tying childhood urinary ACR to future clinical events.

6.4.2. Changes in renal function markers in neonates across age

In this study, renal function marker, sCysC was investigated. CysC is freely filtered in the kidney glomerulus and entirely reabsorbed by the proximal convoluted tubule and totally catabolised in the tubule, and little concentration of CysC is eliminated in urine. However, the degree of kidney

tubular damage can be confirmed by the detection of elevated sCysC (439). In our cohort, there was a significant decline in sCysC observed from birth to 24 months of postnatal age, which implies glomerular maturity in term and preterm neonates. However, there was no significant difference in sCysC between term and preterm neonates.

Previous study by Kandasamy et al. analysed data from 45 premature neonates admitted to NICU without diagnosed AKI (440). The authors did not detect a significant difference in sCysC across 28, 32, and 37 weeks of postnatal age and followed until 24 months of age, and the concentration was higher in the neonatal period and gradually decreased at 24 months of postnatal age. They also compared sCysC between preterm and term neonates and did not detect a significant difference in sCysC concentration. Another study analysed data from 261 preterm neonates with no recognised kidney injury (441). The authors measured sCysC at 6 days to 1 month, 7 to 9 months, and 12 to 14 months after birth. The authors stated that the median sCysC concentration was significantly decreased with postnatal age up to 12-14 months, these trends are exactly congruent with our findings. In addition, a similar study supported the decrease in the concentration of sCysC in the first 24 months of life (442). The decrease in the concentration of sCysC across age is associated with increased renal maturity in neonates without recognised kidney disease. However, increased concentrations of urinary NCR along elevated concentrations of sCysC have been reported in critically ill neonates (50). Moreover, eGFR calculated using sCysC concentration was gradually increased with glomerular function maturation. This is partially due to the maturation of the GFR contingent on the development of renal blood flow through time, and an upsurge in blood pressure and decline in renal vascular resistance which results in an increased renal blood flow and subsequent increase in eGFR after birth.

The traditional marker for AKI, SCr increased at birth in term neonates and declined at 12 and 24 months of postnatal age. In the early neonatal period, the levels of SCr are higher in the first 7 days and are affected by maternal SCr (443). The SCr levels are also affected by birth weight (221). In this study, most term neonates blood samples were collected at birth with median postnatal age of 6 days, and SCr possibly shows maternal SCr concentration in the first few days (≈14 days) of life and declines gradually with advancing age to reach a steady state neonatal level and is consequently not a trustworthy index of kidney function at birth (161, 444).

6.4.3. Reference intervals of urinary NCR for neonates against age and gender

To the best of our knowledge, there are no published RIs in the literature for urinary NCR in paediatric populations. In this study, the ULN for urinary NCR was established using methods recommended by the CLSI C28-A3 guidelines (282) and compared with parametric methods. The CLSI EP28-A3 recommends 39 samples in each group using robust methods and a minimum of sample size of 120-126 for non-parametric methods (445, 446).

Evidence demonstrated that Box-Cox power transformation used to transform the data to normal distribution for parametric calculation of the RIs and to determine the 2.5^{th} and 97.5^{th} percentiles for non-parametric percentiles/robust methods (447, 448). The author recommends a validated RIs using samples from a healthy reference population. Another study by Coskun et al. compare parametric and non-parametric methods for computing RIs in small sample size (257). The authors demonstrated that in normally distributed data the parametric method provided more precise RIs while in the non-normally distributed data, the non-parametric method provide precise RIs (257).

In this study, the RIs and ULN for urinary NCR was closely related in parametric and non-parametric percentile/robust methods. In a sample of 190 neonates with non-normally distributed data, the non-parametric percentile method revealed a narrower CIs relative to parametric method. In a sample of small sample size, the robust bootstrapping method and parametric method showed comparable 95% RIs. However, the robust bootstrap method revealed a slight loss of precision in the upper limits, revealed by width of CIs. In congruent to this study, Coskun et al. and Ozarda reported that the method using a small sample size for computing RIs leads to increased uncertainty around the upper limits (257, 448). The authors also showed that CIs estimated using the bootstrap method are wider than the non-parametric percentile methods estimation but the RIs can be comparable. Although there are many reasons for differences in RIs, a survey of RIs by the Australasian Association of Clinical Biochemists found differences in RIs, even using the same analytical methods and reagents (449).

Dynamic physiological variation is linked with many factors in laboratory medicine and forms a base for setting quality controls and external quality assurances limits, and RIs (450, 451). A study showed that the biological variation indicates the changes in analyte concentrations within person or between persons (450). To understand this variation, our study demonstrated that urinary NCR had no significant difference across age and between male and female neonates ($p>0.05$). A similar study by Kandasamy et al. in 38 Indigenous preterm neonates at term-corrected dates found no significant

difference in renal function between male and female neonates (368). This showing that the urinary NCR excretion is consistent across age and gender, which is one of the ideal characteristics of a biomarker (452).

Theoretically, there is no clear evidence of the likelihood of shedding nephrin in urine of healthy people. To date, there is lacking evidence of the normal concentration of urinary nephrin in a healthy population with intact glomerular structure and function. However, what is clear is the association of nephrin with the slit diaphragm (Sd) and its importance in providing structure, signaling, and repair within the glomerulus (5). Evidence revealed that little or elevated nephrin is excreted in the urine of people recruited as a healthy control in previous literature. A study by Kostovska *et al.* (75) reported that 10% of healthy control subjects have increased nephrinuria, whereas another study by Kishore et al. (325) reported that none of the control subjects had nephrinuria using the same ELISA test kit from the same supplier to detect and quantitate urinary nephrin. Another recent study by van Duijl et al. (453) used an in-house multiplex mass spectrometry method aimed to establish RIs in the Dutch middle-aged population for urinary kidney injury biomarkers and revealed that urinary nephrin was below the detection limit of the method in the urine samples.

Interestingly, the different value for urinary nephrin has been pointed out in adults (75, 260, 313, 325). For instance, a study by Kostovska et al. determined reference values of urinary nephrin to be >255ng/ml (75). Jim et al. also defined the urinary NCR (ng/mg) ≥ 100ng/mg in 10 healthy control groups (49, 255). Another study by Zhai et al. also determined the 95th percentile urinary NCR from healthy women found to be >122 ng/mg (313) and Zhai et al. from different cohort also determined the cutoff for urinary NCR to be 86.6 ng/mg in pregnant women (323). Li and colleagues (338), also reported the value of urinary NCR of 160 ng/mg in neonates who did not develop AKI. In another publication, Chen and colleagues also reported the cutoff urinary NCR to distinguish neonates who develop AKI and did not develop AKI and the reported cutoff for initial urinary NCR was 375ng/mg (50). Furthermore, Heimlich et al. measured urinary NCR in children and determined the cutoff to predict glomerular injury to be 622ng/mg (248), which is higher than the ULN of the RIs of our study.

6.4.3.1. Applicability and transferability of the RIs of urinary NCR for clinical use

RIs are an integral part of the clinical validation of a method to translate the biomarker into clinical practice. However, establishing and transferring RIs to use in clinical laboratories remains challenging due to variations in the assay methods used by the scientific community, source and

formulations of standards, and lack of consensus in the ways of reporting units for urinary nephrin. For instance, to support the interpretation of urinary nephrin test results, it has been proposed to report normalised urinary nephrin concentrations by urine creatinine (454). Normalising biomarkers by dividing to urine creatinine are deemed to be the default parameters for urinary biomarkers within the discipline of the clinical laboratory to inspect the variations in urine flow rate. The hypothesis for normalisation is that urine creatinine excretion is constant across and within the study individuals. Over time, it is less variable, such that the variations in the ratio can reflect changes in marker excretion (454).

The variations in the study populations are also considered for the applicability of the RIs. The population of this study was designed *a posteriori,* and the participants did not have clinically reported kidney abnormalities. Apart from the study population, different clinical conditions which have different pathophysiological mechanisms for glomerular injury are also an issue and may reduce the validity of the cutoff values to use for interpreting the test result. To date, the scientific community used its own ROC generated cutoff values to stratify patients and healthy controls in different clinical conditions (21, 75, 260). Nevertheless, the cutoff values varied across literature and varied from method to method and ELISA kit to kit dependent (50, 75). This discrepancy in cutoff value of urinary nephrin across the literature rises a question that leads to further investigation to fill the gap and establish this clinically useful RIs. Thus, the RIs of urinary NCR in neonates and children will provide the foundation for integrating urinary nephrin into clinical practice for non-invasive diagnosis of early glomerular injury and provide baseline information for scientific literature. Overall, the RIs should be considered the most reliable of those published to date using the ROC curve to discriminate healthy people from glomerular injury of different clinical conditions. Our study believes that these RIs may allow a better classification of early glomerular injury in neonates, infants, and children and are essential for early diagnosis and prognosticate kidney injury, by extension, the most useful step forward to translate urinary nephrin into clinical practice.

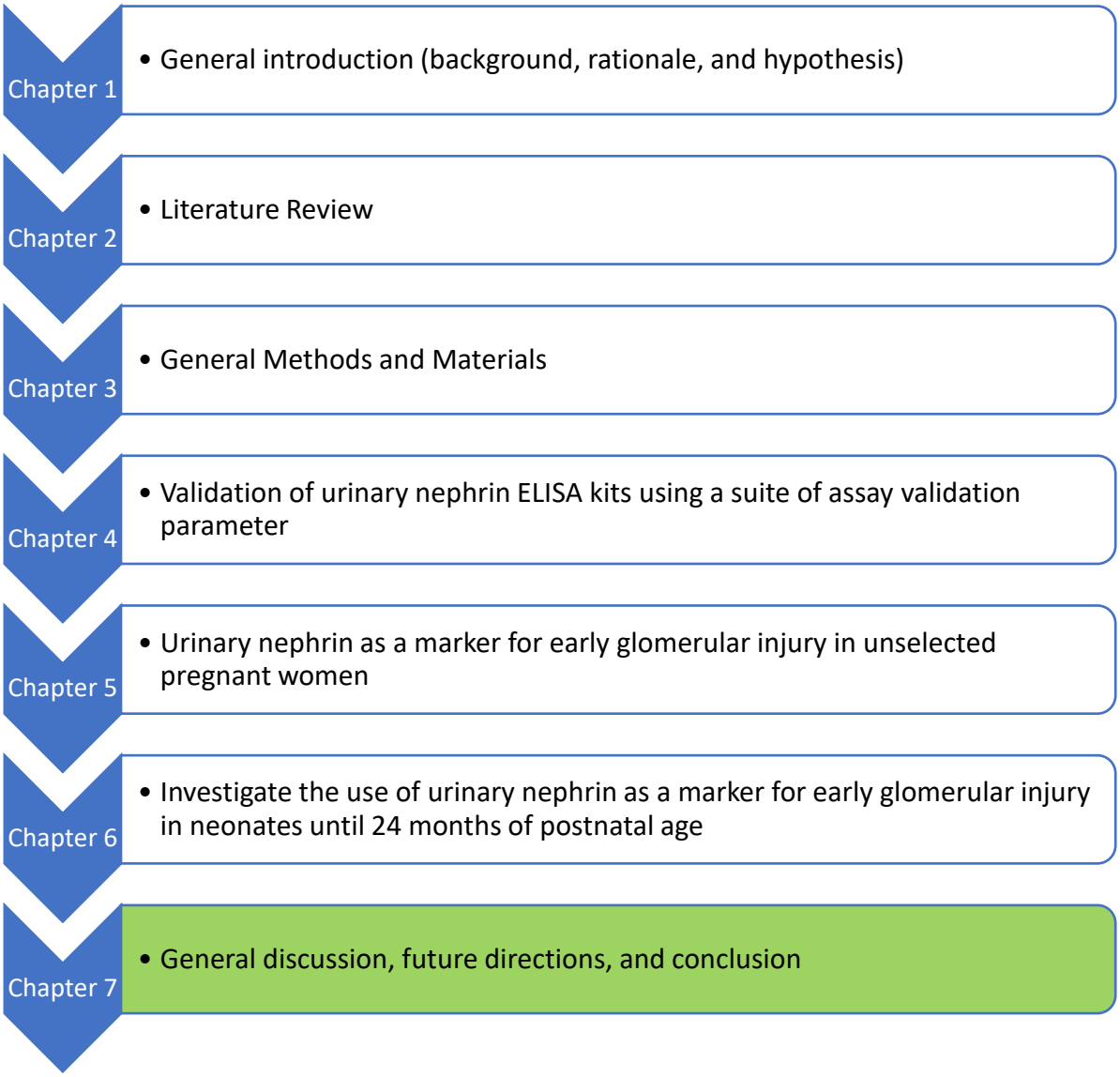
6.5. Strengths and limitations of the study

This study shows clinically important data, but there are strengths and limitations. The strength of this study is the prospective longitudinal cohort nature of the study design by measuring glomerular integrity markers along with the renal function markers, which allows a better understanding of the markers with postnatal age. This study is the first study to establish RIs for urinary NCR in healthy term neonates, infants, and children. The urine samples are analysed by validated human nephrin

ELISA kit and from one supplier throughout the study. The limitation of this study cannot be ignored. First, the higher proportion of neonates lost to follow-up due to the COVID-19 pandemic and closure of the health facility, barriers to in-person contact in enrolment, and follow-up assessment during the study period limits the availability of the participants for full participation in the study. Second, this is a single-centre study with a healthy cohort, which may hinder the generalisability of this finding to neonates with underlined disease conditions in the clinical setting. Third, preterm neonates were not adjusted at term postmenstrual age, because most of the preterm neonates provide samples after 1-month postnatal age (median age at sample collection time 24 days). This study recommends further longitudinal cohort study with controlled trials, including neonates with renal disease/glomerular lesions and randomising neonatal renal function patterns.

6.6. Chapter summary

The findings showed that urinary NCR and ACR along sCysC are important markers for glomerular and tubular function in healthy neonatal cohorts. Of concern, preterm neonates had significantly higher urinary NCR at the early neonatal period (birth) compared to the term neonates. However, there was no significant difference in urinary ACR and sCysC levels between term and preterm neonates at birth. Given, the immaturity of preterm kidney, preterm neonates have no difference in eGFR, possibly through single nephron hyperfiltration and it is important to highlight that, a possibility of shedding of nephrin in preterm neonates as an indicator of the risk for early glomerular injury preceding albuminuria and abnormality in renal function markers. The next chapter (**Chapter 7**) will discuss the findings from this thesis and provide future directions and conclusions of the thesis.



Foreword Chapter 7

Kidney disease is becoming a global public health problem. The best possible way to prevent the problem is early screening of susceptible cohorts. This chapter is the synthesis, where findings from all chapters are brought together in discussion and highlight avenues for future research direction. The thesis raises issues that need further investigation but also recommends opportunities to improve kidney health and reduce kidney injury. The chapter describes a brief introduction, significance, and main outcomes of the project. Finally, the future research directions and conclusions are summarised.

Chapter 7. General Discussion, Future Direction, and Conclusion

7.1. Introduction

The general aim of this thesis was to validate a biomarker for early glomerular injury. Precision Health depends on validated markers to better stratify patients based on the risk of developing kidney disease (455). Urine and blood-based biomarkers are widely used to diagnose and manage the progression of kidney disease (456). During pathological conditions, the biomarkers originate from tissue, blood cells, metabolic products, and specific organs. A biomarker specific to the tissue and organ could reflect better sensitivity and specificity for a specific disease process. Since glomerular injury has multifactorial causes involving different mechanisms, we hypothesised that a glomerular-specific biomarker that shows pathologic changes in glomerular structure may provide a practical picture of the injury. As such, this thesis focused on validating a glomerular-specific protein as a marker of glomerular injury. In urine, there are potential biomarkers of glomerular injury. The most widely reported are podocin, podocalyxin, and nephrin (21, 457). However, the research team reviewed the literature (21), and recently published a systematic review and meta-analysis (76) relevant to the thesis and found evidence that nephrin is detected in urine before other podocyte proteins and has the potential to determine early glomerular injury.

The research team used the Eth-Bio nephrin ELISA kit for a previous longitudinal cohort study. However, the formulation of this kit has changed over time, the kit previously used a nephrin standard based on rat nephrin and recently used a human nephrin standard, which causes differences in precision in measuring urinary nephrin, and this raises the question to validate the nephrin ELISA kit in comparison with another supplier's nephrin ELISA kit. More recently, the LS-Bio nephrin ELISA was introduced into the literature in 2019 (50). Since then, validation of nephrin ELISA kits has been proposed for the larger longitudinal cohort study where this thesis is embedded.

This thesis describes a coherent stage of studies to investigate urinary nephrin as a novel marker of early glomerular injury in combination with other renal function markers. First, the validation of the method was performed using a suite of assay validation parameters for the first time. The ELISA kit, which has better analytical performance and meets Australia's Therapeutic Goods Administration customs clearance criteria, was then further used for clinical validation in the two cohorts. Second, increased urinary nephrin was used to reflect early glomerular injury using urinary ACR as an indirect indicator of glomerular injury in unselected pregnant women and evaluated the predictive potential of urinary nephrin for glomerular injury of PE. Third, longitudinal changes in urinary NCR along with

urinary ACR, sCysC, SCr, and eGFR were investigated. RIs for urinary NCR were determined against postnatal age in the neonatal cohort, and deviation from the ULN of urinary NCR also reflects glomerular injury and/or immaturity. This chapter will summarise the overall findings of the thesis and provide suggestions for future research.

7.2. Significance and main outcomes

The first 1000 days between a woman's pregnancy and the child's 2 year birthday provide a typical window of opportunity for later development (77). Increasing evidence shows that a significant proportion of kidney diseases arise in utero during foetal renal programming during pregnancy (78). For instance, incomplete nephrogenesis (low nephron number at birth), and impaired renal programming that leads to defects in urethral Budd formation, branching morphogenesis, kidney tubule branching, formation of kidney and collecting system increases the risk of postnatal renal insults and glomerular injury. A decreased nephron number does not directly relate to expected kidney damage or future CKD. However, the remaining nephrons will have an increased demand due to hyperfiltration (458), getting the kidneys more vulnerable to kidney injury. To reduce the burden of the disease, early diagnosis using a validated diagnostic tool that reduces the time lag between early glomerular damage to the progression of other forms of kidney disease and/or injury is valuable. The principal aim of this thesis was to validate urinary nephrin ELISA as a novel biomarker for determining early glomerular injury and to investigate the diagnostic performance of urinary NCR in the cohort of pregnant women-infant dyads. Specifically, the general hypothesis of this thesis is validating the urinary nephrin ELISA test can help explore the clinical utility of the assay for use in humans as a test for diagnosis of early glomerular injury. By combining evidence from a narrative review, systematic review and meta-analysis, validation study, cross-sectional, and longitudinal cohort study, this thesis tested the above hypotheses.

In **Chapter 2,** a review of the literature was carried out to explore the existing knowledge and gaps relevant to the thesis. The scientific literature revealed that urinary nephrin is a potential marker for glomerular injury**.** The literature used different assay methodologies to detect and quantitate nephrin, including ELISA, WB, immunohistochemistry and RT-PCR to detect and quantify nephrin specific messenger RNA (49, 50). These methods showed a diagnostic value in determining progressive proteinuric glomerular disease most widely in DN patients. To further our understanding, a systematic review and meta-analysis were carried out to determine the diagnostic accuracy of urinary nephrin to predict glomerular injury and the different methods used by the

reviewed literature. The findings showed that urinary nephrin has a high diagnostic accuracy and ELISA was used as a method of choice and showed higher sensitivity and specificity compared to other methods (76).

The main finding relevant to the analytical performance of the nephrin ELISA is presented and showed the LS-Bio ELISA kit chosen for clinical validation (**Chapter 4**), Elevated nephrinuria observed even in normoalbuminuric pregnant women, urinary NCR comparably increased across albuminuria stages, a statistically significant difference in urinary NCR was observed in women who developed PE and those who did not develop PE (**Chapter 5**). RIs established for urinary NCR with ULN of ≥69ng/mg in term neonates at birth (**Chapter 6**), and longitudinally declined in urinary NCR and urinary ACR, sCysC, and SCr were reported in preterm neonates. A statistically significant difference in urinary NCR was observed between term and preterm neonates at birth but no significant difference in eGFR and other markers between the groups (**Chapter 6**). The findings address current knowledge gaps and add to a growing body of evidence aimed at determining early glomerular injury in vulnerable populations.

7.2.1. Validation of human-specific nephrin ELISA kits

Chapter 4 aimed to validate the analytical performance of human nephrin ELISA, as the first step toward its clinical validation. Validation of urine biomarkers is a challenging task with various pitfalls. The road map for a biomarker to become a potential novel marker to use for clinical practice is long. Nephrin after being reported in patients with congenital nephrotic syndrome of Finnish type (57) was identified as a potential biomarker in glomerular nephropathy (21, 75) and PE (260). The translation of urinary nephrin has been brought to attention in different ways. However, it has yet to be translated into clinical practice.

First, Chapter 4 of this thesis validated urinary nephrin ELISAs from different suppliers to confirm whether the analytical performance is comparable to each other, can the nephrin ELISAs pass the performance checks using a suite of assay validation parameters, and can be used interchangeably for quantitating urinary nephrin. The individual analytical performance of the two kits is relatively good in most parameters. The assays had satisfactory intraassay and interassay reproducibility. The Eth-Bio ELISA kit, which uses a competitive assay principle, although successful in the validation of the standard curve for the first five highest dilution series, performance failed in recovery and linearity studies at the lowest standard. There was no significant correlation between the two kits in measuring urinary nephrin. Although the two kits have a 100-fold difference in the upper limit of

quantification, the concentration determined using the same urine sample in both kits was different. Practically, such a difference in concentration is unacceptable, and then we further investigated the accuracy and metrological traceability of the standards of both kits using electrophoresis and size exclusion chromatography. However, none of the methods clearly identified nephrin standards due to the very low concentration of the standards and the presence of other stabilising proteins and preservatives that make the visualisation of the bands uncertain. Most importantly, the standard of the LS-Bio ELISA kit was not detected by the Eth-Bio ELISA kit and vice versa. The standards of both kits were not detected by anti-nephrin antibodies from a different supplier using the immunofixation electrophoresis technique. Although various studies used electrophoresis for determining proteins, due to the low concentration of nephrin and the method is visually subjective to variation in band clarity, band size and migration through the gel, the quantification of nephrin using electrophoresis was not possible. The differences in the specific fragments of nephrin standard formulated by the suppliers, expression systems, and purification techniques may also a factor for variations measuring urinary nephrin between the two kits. This was confirmed by the LS-Bio supplier insert sheet reported that due to the antigen specificity of the antibodies used in the assay, users may not be able to detect native or recombinant proteins from other suppliers using the LS-Bio ELISA kit.

7.2.1.1. The relevance of the findings and the ELISA method for quantitating urinary nephrin

The main pathological indicators that are linked to glomerular injury are the shedding of podocyte proteins, blood cells, and macromolecules. Our findings and previous studies have shown that the detection of podocyte proteins such as nephrin shows early glomerular injury preceding albuminuria. The guidelines also recommend validation of the ELISA method before applying it for research and clinical use. The ELISA method is a commonly used ligand-binding assay originally developed in the 1970s (459). It is used in laboratories with automated techniques to wash and read plates, which increases sensitivity, specificity, adaptability, and reliability compared to other techniques. Hence, laboratories often should find a balance between diagnostic accuracy and technical complexity when choosing assays to move into routine diagnostic use. To date, diagnostic laboratories utilise automated ELISA platforms and therefore translate into routine diagnostic use due to factors such as ease of use, automation, cost, and availability at the point of care, becoming important. While ELISA is the method of choice for measuring urinary nephrin, kit-related

differences, including differences in sources and formulation of the standards and antibodies used, can lead to discrepancies in the accuracy and precision of the urinary nephrin ELISA test.

Although validation of immunoassays is an important step ahead of clinical validation, validating an immunoassay method using a urine sample is challenging since urine is a metabolic product that contains a complex mixture of cells, cellular debris, inorganic ions, protein metabolites, and organic molecules (375, 460). In addition to the nature of the standard and antibodies used in different suppliers of ELISA kits, the chemical properties of urine and changes in chemical compositions after collection can impact the efficient binding of proteins to the binding antibody used in an ELISA (461). Chatziharalambous et al. showed the performance of ELISA tests in urine samples to measure potential markers for bladder cancer (375). The authors found low performance for most of the tests performed and only 3 assays passed the accuracy threshold. The authors reflect on the problems in developing urine-based ELISA assays with adequate analytical performance for clinical application. Taylor et al. determined matrix interference in urine. The authors found high variability in protein recovery even between assays, demonstrating that matrix elements vary between urine samples and highlighting their ability to affect protein measurement (462). Another study on the analytical performance of NGAL ELISA assays reported poor recovery and linearity (387), and the authors showed variability in the performance of urinary immunoassays that requires to be considered in clinical sample testing. In general, the validation of a biomarker for the diagnosis and prediction of kidney injury is valuable and should be validated using a suite of assay validation parameters in samples collected according to standard operating procedures before it is translated to clinical practice.

7.2.2. Urinary nephrin as a predictor of glomerular injury during pregnancy

Second, clinical validation of urinary nephrin ELISA was conducted in pregnant women-infant dyads (**Chapters 5 and 6**). The chance of succeeding in diagnosing kidney injury is dependent on how early the biomarker detects kidney injury. It is well known that urinary nephrin is a potential marker of glomerular injury (76). In the clinical context, SCr is used for the diagnosis of AKI, and urinary ACR is used for the diagnosis of glomerular injury and the management of CKD. However, these markers are not sensitive and specific for early kidney injury and did not provide earlier information on the degree of damage, the location of the injury, and the progression of the injury. One strategy for early detection of injury could be the use of a validated novel biomarker to detect early glomerular injury.

In the past decade, several authors have argued that urinary nephrin is a potential marker for glomerular injury. The main arguments in the literature can be grouped into (1) those that propose nephrin as a sensitive and specific marker of glomerular injury mainly in glomerular nephropathy patients, and pregnant women with PE, (2) recently emerged arguments about nephrin as a marker for glomerular maturation, glomerular injury and predicting AKI in neonates, and those that discuss nephrin as a marker to understand podocyte biology and pathophysiology of glomerular injury, (3) another important area of research is the translatability of urinary nephrin into clinical practice to determine early glomerular injury and predict other forms of kidney injury/disease.

The research presented in **Chapters 5 and 6** directly addresses the 2nd and 3rd research concerns. A marker to be translated into clinical practice needs to be clinically validated and tested in a confounding population for its predictive diagnostic potential. **Chapter 5** investigated whether urinary NCR can be translated into clinical practice in a cohort of unselected pregnant women. In our cohort, most pregnant women have documented evidence of comorbidities, including GDM, DM, HTN, and PE. The main findings were (1) a significant proportion of pregnant women with normoalbuminuria showed elevated nephrinuria and nephrinuria proportionally increased from normoalbuminuria to macroalbuminuria stages, suggesting its role as an early marker. (2) The discriminatory power of urinary NCR is acceptable (AUC=0.71) to discriminate against women with significant albuminuria with normoalbuminuria and to discriminate against women who develop PE and who did not develop PE (AUC=0.65). The observation that urinary NCR increased in women with comorbidities and women who developed PE is related to an early glomerular injury.

7.2.2.1. Diagnostic Implications

It is important to highlight that the findings obtained from this chapter focus on unselected pregnant women for the diagnosis of early glomerular injury when symptoms of kidney disease are not recognised. Previous studies have studied the value of urinary nephrin in high-risk women (260). The findings provide insight into the predictive power of urinary nephrin for PE. While it is true that all diabetic and hypertensive patients showing nephrinuria will not develop kidney disease/injury, the appearance of nephrin in urine may show damage to the GFB. A study in animal models of renal disease reviewed in 2018 by Clare Martin and Nina Jones has demonstrated the importance of intact nephrin for normal functioning and repair of damaged glomerulus even in acute injury (5). Although the aetiology of glomerular damage is heterogeneous and is likely to be so in DN and PE, the presence of nephrin in Sd is well documented (340), therefore, the presence of nephrin in urine,

either due to damage to the Sd or through up-regulation stimulated during repair or down-regulated in damaged nephrons seems to provide an indicator of glomerular damage in several causes of nephropathy, including DN and hypertensive nephropathy (463, 464).

The study in this chapter agrees with previous investigations of urinary nephrin in high-risk pregnant women who developed PE and healthy controls (260), high-risk pregnant women and low-risk pregnant women (49), DM, and healthy controls (75). The authors of these studies measured urinary nephrin and found significantly higher urinary nephrin in cases compared to control groups, and they reported a significant positive correlation with albuminuria, and the authors also reported that nephrinuria gradually increased in the albuminuria stages. Similarly, elevated nephrinuria was observed in normoalbuminuric patients and women who did not develop PE. This observation argues that nephrinuria precedes albuminuria and is used as a marker of early glomerular injury.

During the same study period, when our study was under investigation, a study measuring urinary nephrin in patients with 40 newly diagnosed untreated hypertensive patients and 40 healthy controls was published in 2022 (261). The authors found urinary nephrin was significantly elevated in hypertensive patients with normoalbuminuria and observed significantly higher urinary nephrin compared to normotensive controls. This observation is consistent with our study, suggesting that nephrinuria may play an important role in the pathogenesis of hypertensive nephropathy, preceded microalbuminuria, and loss of glomerular function.

Together, **Chapter 5** addresses a knowledge gap and provides supportive evidence on the role of urinary nephrin in glomerular injury. Limitations in this chapter are the current guidelines for stratifying glomerular injury using urinary ACR, as a reference standard test (imperfect gold standard), cannot reveal subclinical glomerular damage and underscore the specificity of urinary NCR. The other limitation of this chapter is the cross-sectional nature of the study design that provides the basis for association, rather than causality. It is not clearly understood if nephrinuria is the causal mechanism or if early detection of nephrinuria will reliably predict consequent glomerular injury in women with normoalbuminuria and nephrinuria will be used to prognosticate glomerular injury.

7.2.3. Longitudinal changes in urinary nephrin in the neonatal cohort

An important question raised in **Chapter 5** involved determining whether urinary nephrin precedes micro- and macroalbuminuria and can be used to predict early glomerular injury in unselected populations. This question is more important because increased nephrinuria may contribute to the

foundations of the pathophysiology observed in glomerulopathy. If nephrinuria is detected prior to albuminuria, it might be an indicator of the shedding of podocyte proteins, which is suggestive of glomerular injury. This question informed the necessity of a longitudinal cohort study to understand if nephrinuria is the causal mechanism or if early detection of nephrinuria will predict consequent glomerular injury in patients with normoalbuminuria, which is not addressed in **Chapter 5**.

The pathophysiological mechanism of glomerular injury varies in adults and neonates, and the urinary ACR to define albuminuria status is not validated in children under 24 months of postnatal age. However, the questions inform us to conduct a longitudinal study for this project addressed in **Chapter 6** in neonates and the child up to 24 months of age to look at the dynamic physiological changes of the biomarkers in healthy cohorts.

The aim was to determine RIs and longitudinal changes in biomarkers across postnatal age in term and preterm neonates. Establishing RIs for urinary NCR helps with early diagnosis and clinical decision-making for kidney injury. The RIs were determined using the nonparametric and robust method. To the best of our knowledge, reliable RIs for urinary NCR have not been established. To establish RIs, first, the pre-analytical, analytical factors and demographic characteristics that impact the biomarker levels of the study participants were considered. In this study, the method precision was investigated, and the pre-analytical and analytical phases of analysis of the biomarker were performed following the standard operating procedures. The key questions to answer in this chapter were, what are the RIs for urinary NCR, and do urinary NCR, ACR, SCr, sCysC, and eGFR vary across postnatal age in neonates at birth, 12 months, and 24 months? To answer the questions, 190 healthy term neonates at baseline and 74 infants at 12 months, and 93 children at 24 months were included for determining RIs. RIs were calculated using robust and non-parametric methods, as per the CLSI guidelines. The findings have the potential to be used for clinical decision-making and increase understanding of maternal and other clinical characteristics on nephrin concentration in healthy term neonates, infants, and children.

There are active arguments in the scientific communities about the advent of the RIs concept. In the past decades, there has been an improvement in the methodological and statistical power of RIs in the paediatric population (280). Large sample sizes of healthy populations are the gold standard for developing RIs. RIs with large sample sizes are available for renal function markers due to statistical and computing power advances that adapt variations to datasets (465). The methods and statistical analysis for RIs vary depending on the choice of establishing discrete or continuous RIs. There are arguments to establish separate RIs for neonates and paediatric populations and for the usage of

statistical methods in the estimation of the RIs. The CLSI recommends partitioning data by age group following the assumptions of the data distribution as parametric and non-parametric to determine percentile values (445). In 1988, Cole proposed an option to estimate discrete age-specific RIs (466). Moreover, in 1991, Royston suggested continuous age-specific RIs using flexible parametric curves (467). Over the past two decades, the generalised additive model for location shape and scale and quantile regression were also suggested by the scientific community for any distribution to estimate continuous age-specific RIs (468). These approaches have been used in child growth charts and kidney function to determine the centile for continuous age (469).

In summary, this study is the first to establish RIs in this cohort and could be used for interpreting laboratory test results. Deviation from ULN could be considered clinically relevant for decision-making. The urinary NCR remains stable with increasing postnatal age. This finding correlates with the hypothesis that nephrogenesis is complete in neonates born at 37 completed weeks of gestation (98). However, to translate into clinical use, the RIs need to be validated according to CLSI guidelines for assessing the validity of discrete RIs in similar cohorts. Therefore, due to the differences in RIs derived from different populations, analytical principles, and assay methodologies, diagnostic laboratories have the responsibility of reporting results with valid RIs.

Finally, **Chapter 6** investigates the longitudinal changes in glomerular integrity markers such as urinary NCR, ACR, and renal function markers such as sCysC, SCr, and eGFR across postnatal age in preterm neonates and term neonates as a control. Recognition of kidney injury and initiation of preventive measures helps to mitigate further complications, and showing the longitudinal changes of biomarkers through time helps to better understand kidney disease and predict the outcome of the disease (470).

In Chapter 6, the concentration of urinary NCR, ACR, sCysC, and SCr decreased significantly and the values of eGFR increased significantly across postnatal age. However, urinary NCR remains stable, and no significant difference exists in term neonates, suggesting glomerular and renal functional maturity. While comparing the biomarkers in preterm and term neonates, urinary NCR significantly increased in preterm neonates. Despite this, there was no significant difference in urinary ACR and eGFR, we hypothesise that preterm neonates have glomeruli immaturity and/or early glomerular injury in the early neonatal period, resulting in shedding of nephrin in urine preceding albumin.

In Australia alone, CKD contributes to 11% of all deaths in 2020 (471). Antecedents of increased risk of CKD in people born preterm may originate early in the neonatal and /or period during pregnancy.

Maternal clinical characteristics and foetal environment contributed to the development and function of the foetus's kidney (472). In Australia, a study has shown an effect of maternal renal impairment on the development and function of the foetal kidney (473). Another study also showed maternal comorbidity during pregnancy can have consequences for newborn kidney function (474), and leads to kidney disease that originates in early life, particularly in preterm neonates (475). Worldwide, an estimated 11% of neonates are born preterm (476), and in Australia, nearly 8.2% of neonates are born preterm (477).

The pathogenesis of kidney disease in preterm neonates has not been well understood, but a body of evidence revealed that nephrogenesis is incomplete in preterm neonates and the renal function is immature (431, 478). Prematurity results in a reduction in the number of functioning nephrons and is an independent risk factor for kidney injury in early as well as later childhood life (475). Supporting evidence showed an increase in podocyturia in preterm neonates compared to the term neonates (433). Urinary NCR was significantly associated with AKI and may be an independent predictor of AKI (50). Another study in humans showed significantly thinner renal parenchyma in premature neonates, likely due to low nephron number and compensatory hyperfiltration (478).

In summary, it is important to highlight that the possibility of shedding of nephrin in the early neonatal period of preterm neonates could be an indicator of the risk for early glomerular injury preceding albuminuria and abnormality in renal function markers. Preterm birth may contribute to an increased risk of glomerular injury and CKD in later life. Hence, regular follow-up of renal function should be considered for preterm neonates. The study showed clinically important data, using prospective longitudinal cohort measurements by measuring glomerular integrity markers along with renal function markers. Although longitudinal changes in biomarkers have been used to predict the outcome of glomerular injury and the change in biomarker level within an individual level improves predictive accuracy, this study compares collective groups of neonates at postnatal ages. The higher proportion of neonates lost to follow-up due to the COVID-19 pandemic and closure of the health facility, barriers for in-person contact in enrolment, and follow-up assessment during the study period limit the availability of the participants for full participation in the study.

7.3. Limitations of the study

This thesis has comprised validation study, observational cross sectional, and longitudinal studies. The validation study was a partial validation study and did not include robustness of different nephrin ELISA kits in different laboratories to confirm and evaluate the precision when variations that most likely impact the assay's analytical performance are introduced. There was a lack of availability of endogenously synthesised observable calibrator to check performance of assay validation parameters and a high concentration of primary nephrin standard to investigate metrological traceability and accuracy nephrin standard of the ELISA test kits. Although urine is a metabolic product that contains a complex mixture of cells, cellular debris, inorganic ions, protein metabolites, and organic molecules, this study investigated only albumin and biotin interferences for urinary nephrin measurement.

This was an observational study carried out in a single centre in unselected pregnant women and healthy neonatal cohort, which may hinder the generalisability of this finding to all pregnant women and neonates with underlined disease conditions in the clinical setting. A high proportion of pregnant women and neonates lost to follow-up due to the COVID-19 pandemic and closure of the health facility, barriers to in-person contact in enrolment, and follow-up assessment during the study period limits the availability of the participants for full participation in the study.

Another limitation was the inability to get the infants and children to attend at the same postnatal age from birth to 24 months, and result in missing data and, unable to see whether the marker or injury is likely to progress individually in neonates until 24 months of age.

7.4. Future directions

This thesis reported part of a larger NHMRC, Australia-funded project to study (''*The Relationship between Maternal Health and Infant Renal Development and Function*''. It is the study to understand the impact of maternal environmental factors on foetal renal development and function. The study will contribute to the step forward to close the gap in the occurrence of kidney disease in early childhood. Several other clinical markers, urinary and blood markers, were also investigated as part of this large longitudinal study and will be published as part of the project. However, this thesis is confined to the validation of urinary nephrin assay as a novel marker of early glomerular injury in a cohort of pregnant women-infant dyads.

ELISA has a variety of applications in research and diagnostic settings. The advancement in technology allows the measurement of antigens and antibodies as a biomarker for kidney disease and/or injury. The technology used to measure urinary nephrin in this thesis is ELISA, which shows good sensitivity and specificity in measuring urinary nephrin, as confirmed by our literature review. Other technologies used for measuring nephrin are RT-PCR, electrophoresis and WB, immunohistochemistry, and mass spectrometry, but these are not suited for clinical practice. Although ELISA technologies are currently used as a choice for measuring urinary nephrin, suppliers used different assay principles, different antibodies, and different fragments of nephrin amino acid sequences for the design and development of the immunoassay. This may cause variations in assay performance in measuring nephrin from different suppliers. Hence, validation of urinary nephrin ELISA (**Chapter 4**) showed a need for further study to investigate if nephrin ELISAs from different suppliers can be used interchangeably for quantifying urinary nephrin and to address the lack of uniformity in the analytical performance of the different supplier's ELISA kits.

- Further validation of urinary nephrin ELISA test kits should be performed from suppliers with similar nephrin assay dynamic ranges and assay techniques for enhancing the standardisation of laboratory findings between the ELISA assays.
- The robustness of the assay should be further investigated to evaluate the precision when variations that most likely impact the assay's analytical performance are introduced.
- The metrological traceability and accuracy of the standards of the ELISA test kits warrant further investigation using a high concentration of primary nephrin standards.

Biomarkers that show a risk for early glomerular injury and renal functional defects can help with patient stratification and development for clinical trials. Since kidney biomarkers are detected in asymptomatic patients and injury is ongoing before symptom onset, diagnosing patients after developing clinical signs and symptoms for kidney injury is likely to be too late and the injury might progress to irreversible damage. Stratifying pregnant women who have an increased risk of early glomerular injury with longitudinal follow-up study allows us to test whether the marker used as an early screening tool and used to prognosticate the injury. Since the marker is used to monitor the prognosis of the injury, we can match apparently healthy women and women with increased risk of injury or clinically known glomerular injury groups to determine the predictive performance of the biomarker. For instance, during the work of this thesis, previous literature on different clinical conditions has reported similar observations (**Chapter 5**), which encourage further studies and

strengthen our study findings. Future work involving the clinical validation of urinary nephrin to study the hypothesis that consistent urinary nephrin excretion in women with normoalbuminuria, to assess the prognostic value of urinary nephrin for glomerular injury and to determine the clinically useful cutoff for urinary NCR:

- This study was carried out in a single centre at TUH, North Queensland, Australia that may not represent general pregnant women population in different regions. A longitudinal comparative prospective cohort study in patients with known glomerular lesions in comparison with healthy control would be of interest to answer the hypothesis that nephrinuria is consistently detected in women with normoalbuminuria and nephrin is involved in the prognosis of glomerular injury in women with increased albuminuria that may better describe and confirm this finding before being translated into clinical practice.
- Establishing clinically useful RIs for urinary NCR among healthy women selected using standard recruitment criteria may help interpret the test result and appropriate clinical decision-making if translated into clinical practice.

Early diagnosis and management of kidney disease/injury during the neonatal period may contribute to decreasing the burden of CKD in childhood as well as adulthood life. To enhance this, a clinically validated novel marker of kidney injury is required and applicable in clinical settings. A crucial step toward clinical application is determining the cutoff values of markers as part of clinical validation. The fundamental path for the validation process is the pre-analytical stage. Hence, it is also of great importance to evaluate the dynamic physiological changes of a biomarker and understand whether partitioning is required according to age for a better interpretation of the test result. To this end, the cutoff value of urinary NCR was determined to identify patients with an increased risk of early glomerular injury and to determine if the injury is likely to progress or not. In **Chapter 6**, we did not observe dynamic physiological changes across age and gender, and the RIs for urinary NCR were determined in neonates against postnatal age. However, the RIs need to be:

- Validated according to CLSI guidelines to assess the validity of discrete RIs using the same analysis method (LS-Bio ELISA) before translating for clinical use.

Finally, **Chapter 6** investigates longitudinal changes of biomarkers across postnatal age and compared term and preterm neonates. In preterm neonates, urinary NCR was significantly elevated in the neonatal period and decreased at 12 and 24 months of postnatal age. However, due to the

high loss to follow-up of the study participants, unable to see whether the marker or injury is likely to progress individually or not individually in neonates until 24 months of age.

- To assess the diagnostic and prognostic value of urinary NCR, a similar further longitudinal cohort study with controlled trials should be conducted for individuals instead of group averages, including neonates with kidney injury and healthy control neonates.

7.5. Conclusion

In conclusion, urinary NCR showed a reasonable sensitivity to predict glomerular injury. Longitudinally, ULN values for urinary NCR were determined, and significantly higher urinary NCR was observed in the early neonatal period in preterm neonates. To date, there is no validated single biomarker for glomerular injury that will meet the clinical needs of different cohorts due to the complex pathophysiology of glomerular injury. It is not realistic that urinary NCR alone will be used for clinical use for early glomerular injury at this stage, but it will hopefully be translated to clinical use after further validation in different clinical conditions. Given the multifactorial causes and complex pathophysiology of glomerular injury and glomerular injury of PE together with the inconsistency of biomarker's cutoff values and diagnostic performance, it is questionable for a single biomarker to provide an adequate clinical picture of glomerular injury/immaturity at this stage. Instead, a panel of novel injury-associated biomarkers of glomerular injury that could detect glomerular damage can provide an accurate diagnosis and prognosis of the injury. We hope that including a marker of early glomerular injury with urinary ACR alongside other markers of renal injury such as NGAL, cell cycle arrest markers, and inflammatory markers will provide a screening panel for early detection and follow-up of acute and chronic renal injury. Taken together, the work in this thesis will be used as a baseline for others who work on early glomerular injury.

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APPENDICES

Appendix I

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SYSTEMATIC REVIEWS

Urinary nephrin—a potential marker of early glomerular injury: a systematic review and meta-analysis

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Abstract

Background Both early recognition of glomerular injury and diagnosis of renal injury remain important problems in clinical settings, and current diagnostic biomarkers have limitations. The aim of this review was to determine the diagnostic accuracy of urinary nephrin for detecting early glomerular injury.

Methods A search was conducted through electronic databases for all relevant studies published until January 31, 2022. The methodological quality was evaluated using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool. Pooled sensitivity, specificity, and other estimates of diagnostic accuracy were determined using a random effect model. The Summary Receiver Operating Characteristics (SROC) was used to pool the data and to estimate the area under the curve (AUC).

Results The meta-analysis included 15 studies involving 1587 participants. Overall, the pooled sensitivity of urinary nephrin for detecting glomerular injury was 0.86 (95% CI 0.83–0.89) and specificity was 0.73 (95% CI 0.70–0.76). The AUC-SROC to summarise the diagnostic accuracy was 0.90. As a predictor of preeclampsia, urinary nephrin showed a sensitivity of 0.78 (95% CI 0.71–0.84) and specificity of 0.79 (95% CI 0.75–0.82), and as a predictor of nephropathy the sensitivity was 0.90 (95% CI 0.87–0.93), and specificity was 0.62 (95% CI 0.56–0.67). A subgroup analysis using ELISA as a method of diagnosis showed a sensitivity of 0.89 (95% CI 0.86–0.92), and a specificity of 0.72 (95% CI 0.69–0.75).

Conclusion Urinary nephrin may be a promising marker for the detection of early glomerular injury. ELISA assays appear to provide reasonable sensitivity and specificity. Once translated into clinical practice, urinary nephrin could provide an important addition to a panel of novel markers to help in the detection of acute and chronic renal injury.

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Appendix II

Urinary nephrin is a potential biomarker of early glomerular injury in a cohort of pregnant women attending routine antenatal care services.

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Highlights

- Urinary nephrin and NCR increased significantly and comparably across the three categories of albuminuria, normoalbuminuria, microalbuminuria, and macroalbuminuria.
- Women with micro/macro albuminuria also had elevated urinary nephrin, NCR, albumin and serum cystatin C when compared to normoalbuminuric women.
- Urinary NCR was elevated in 64.9% of women with normoalbuminuria, 94.7% of women with microalbuminuria, and 100% of women with macroalbuminuria.
- Of the normoalbuminuric women with a NCR>14 ng/mg, 77.8% were diagnosed with a hypertensive disorder and 62.7% were diagnosed with diabetes in pregnancy.
- Urinary nephrin, NCR, and ACR levels were higher in women who developed PE than those who did not develop PE.
- The sensitivity of urinary NCR for the prediction of PE was 93% and specificity was 42%.
- Urinary nephrin may provide a more sensitive indicator for the detection of early glomerular injury during antenatal screening.

Abstract

Introduction: Glomerular injury may occur during pregnancy as a consequence of systemic disease and pregnancy-related medical complications such as gestational diabetes and preeclampsia (PE). While urinary nephrin has been shown to provide early identification of PE in high-risk pregnancies, the role of urinary nephrin for determining early glomerular injury in a group of pregnant women is yet to be explored. This study aimed to investigate the use of urinary nephrin as a predictor for early glomerular injury in a large cohort study (KIDMIN) conducted at the Townville University Hospital.

Methods and Materials: A prospective cross-sectional study was conducted. All pregnant women with a full dataset (n=273) were classified into 3 categories according to their urinary albumin to creatinine ratio (ACR): normoalbuminuria, microalbuminuria, and macroalbuminuria. Continuous variables were compared between groups and the cutoff value for the urinary nephrin to creatinine ratio (NCR) was determined to predict albuminuria as indirect indicator of early glomerular injury. The percentages of pregnant women who had elevated nephrinuria were calculated for each of the ACR categories.

Results: Urinary NCR showed a positive correlation with urinary ACR ($r=0.29$, $p<0.0001$). Urinary nephrin and NCR increased comparably in women categorised as having normoalbuminuria, microalbuminuria, and macroalbuminuria. Using a cutoff value of 14ng/mg, nephrinuria was detected in 64.9% of women with normoalbuminuria, 94.7% with microalbuminuria, and 100% with macroalbuminuria. Of the normoalbuminuric women who had an elevated urinary NCR (>14 ng/mg), 77.8% were diagnosed with a hypertensive disorder and 62.7% were diagnosed with diabetes in pregnancy. In women with PE, urinary NCR and ACR were significantly higher when compared to women who did not develop PE. The AUC of the ROC for urinary NCR was 0.74 (95% CI: 0.650-0.824), with a sensitivity of 97% and a specificity of 36% to predict glomerular injury, and a sensitivity of 93% and specificity of 42% to predict glomerular injury of PE.

Conclusion: The study found that urinary nephrin and NCR were elevated in women with micro-macro albuminuria but also in pregnant women with normoalbuminuria. Increased urinary NCR without increased urinary albumin may be associated with early glomerular injury. Urinary NCR may be a more sensitive marker than microalbuminuria to detect early glomerular injury and may be a useful tool for monitoring glomerular injury in women with systemic disease and adverse pregnancy outcomes.