Variability in Creatinine and Estimated Glomerular Filtration Rate in diabetic nephropathy

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

Dr Sunil Nair, MBBS, MRCP (UK)

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University of Liverpool, Department of Obesity and Endocrinology, Clinical Sciences Centre, Aintree University Hospital, Liverpool, UK

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Abstract

Introduction

Diabetic nephropathy is a leading cause of end stage renal disease (ESRD) in the world. Accurate screening and staging of chronic kidney disease (CKD) is essential for timely intervention as recommended by national and international guidelines (KDOQI, 2007, RCP, 2006) and to guide dose adjustment of several medicines. Glomerular filtration rate (GFR) is recognised as the best measure of kidney function in health and disease, but measuring it by gold standard techniques such as inulin clearance, and radio-isotopic methods is clinically impractical. Therefore estimation of GFR using serum creatinine and other variables like age, gender race and body size is recommended (Levey, 2003, Levey, 2006). The most widely used formula to estimate GFR is the 4-variable MDRD equation.

Aims & Methods

My thesis contains studies of variation in serum creatinine, estimated glomerular filtration rate and urinary albumin creatinine ratio. There are two main experimental studies and three further studies based on data analyses and validation.

1. Effect of obesity on eGFR in type 2 diabetes: The aim was to estimate the bias between isotopic glomerular filtration rate (GFR) and eGFR in patients with Type 2 diabetes with chronic kidney disease and relate it to their body mass indices (BMI).

GFR was measured using ^{51}Cr EDTA method and was estimated by 4v-MDRD eGFR using IDMS calibrated creatinine in 111 participants.

2. The performance of CKD-EPI formula compared to the MDRD equation in estimating GFR in participants with type 2 diabetes associated CKD: The aim was to use our dataset to compare the effect of obesity on eGFR calculated by the CKD-EPI formula (Levey and Stevens) and by the 4-variable MDRD equation.

3. Derivation of obesity correction equation for 4-variable MDRD: To derive a 'correction factor' for the 4-variable MDRD equation to adjust for the equation's underestimation of true (isotopic) GFR in obese subjects with Type2 diabetes. Linear and non-linear regression analysis were performed to derive equations which reduced the bias between estimated and measured GFR

4. Effect of cooked meat protein on eGFR estimation in type 2 Diabetes related chronic kidney disease: To estimate the biological variation in creatinine levels caused by a standardised cooked meat meal in subjects with diabetes mellitus and various stages of chronic kidney disease, compared to healthy volunteers. 64 participants in chronic kidney disease stages 1-4 and 16 healthy volunteers were fed cooked meat and non-meat protein meals to study the effect of cooked meat protein on creatinine and eGFR.

5. Effect of cooked meat protein on urinary albumin creatinine ratio in patients with type2 Diabetes related chronic kidney disease: To determine the effect of a cooked meat meal on UACR in diabetic patients with diabetes related chronic kidney

disease. 80 participants had their urinary albumin creatinine ratio calculated before and 4 hours after a cooked meat meal.

Results & Conclusions

1. The 4-v MDRD formula underestimates GFR in overweight and obese patients with Type 2 diabetes. The bias between estimated and measured GFR in the obese type 2 diabetic subjects persists across the range of CKD stages. This may have Implications for management of obese patients with Type 2 diabetes, where treatment options for the management of hyperglycaemia, hypertension and other concomitant conditions are often determined by the eGFR.

2. The bias between estimated and measured GFR significantly worsens when eGFR was calculated using the CKD-Epi compared to the 4 variable MDRD formulas in patients with type2 diabetes and chronic kidney disease. There remains a need for better-validated equations to estimate GFR in the obese patients with diabetes.

3. The linear and non-linear equations derived from our study reduce the bias significantly in the external dataset; this improvement being more pronounced in the obese subjects and is best achieved by the ratio model equation which scores consistently well across all three ranges of GFR studied, including a very good positive predictive value in CKD stage 3 in the obese. This simple corrective factor if externally validated can be used when making management decisions in the obese with type2 diabetes based on eGFR.

4. Consumption of a standardised cooked meat meal significantly increased serum creatinine and resulted in significant fall in eGFR in all stages of CKD studied; 6 of 16 CKD 3a patients were misclassified as CKD 3b. This effect of cooked meat on serum creatinine disappears after 12 hours of fasting in all study participants. Creatine in meat is converted to creatinine on cooking. This is absorbed causing significant increase in serum creatinine levels and a consequent drop in eGFR. This could impact management as threshold for commencing and withdrawing certain medications and decisions regarding investigations is defined by eGFR. An eGFR calculated using fasting serum creatinine would be a better reflection of kidney function in these patients.

5. Urine albumin to creatinine ratio falls after a cooked meat meal in patients with diabetes associated chronic kidney disease. This fall in ACR increases with worsening stages of chronic kidney disease. Cooked meat consumption is a major factor leading to variation in ACR values, which should be considered when interpreting results.

PUBLICATIONS AND ABSTRACT PRESENTATIONS FROM THIS WORK

➢ Sunil Nair, Sarah V O'Brien, Katharine Hayden, Bhavna Pandya, Paulo J G Lisboa, Kevin J Hardy, John P H Wilding *Effect of a cooked meat meal on serum creatinine and estimated glomerular filtration rate in diabetes related kidney disease:* Diabetes Care February 2014 vol. 37 no. 2 483-487

➢ S Nair, K J Hardy, J P H Wilding *The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula performs worse than the Modification of Diet in Renal Disease (MDRD) equation in estimating glomerular filtration rate in Type 2 diabetic chronic kidney disease,* Diabetic Medicine October 2011, Volume 28, Issue 10, Page 1279. DOI: 10.1111/j.1464- 5491.2011.03344.x

➢ S Nair, V Mishra, K Hayden, P Lisboa, B Pandya, S Vinjamuri, K J Hardy, J P H Wilding *The 4-variable modification of diet in renal disease formula underestimates glomerular filtration rate in the obese type 2 diabetic subjects with chronic kidney disease*; Diabetologia (2011) Volume 54, Number 6, 1304-1307, DOI: 10.1007/s00125-011-2085-9

➢ Sunil Nair, Kevin J Hardy *Dual-blockade may have a role in some patients with diabetic nephropathy. Re: Angiotensin converting enzyme inhibitors and angiotensin receptor blockers in hypertension*. Ritter *342:doi:10.1136/bmj.d1673 (letter to editor)*

ABSTRACTS

> Sunil Nair, Kath Hayden, Bobby Mishra, Sobhan Vinjamuri, Bhavna Pandya, Kevin Hardy and John Wilding The effect of obesity on estimation of glomerular filtration rate in patients with type 2 diabetes associated chronic kidney disease 18th European Congress on Obesity (ECO 2011) May 2011 T5:P.044

> S Nair, Sarah O'Brien, G Jaggard, K Hayden, B Pandya, K Hardy, J P H Wilding, Cooked meat meal decreases albumin creatinine ratio in patients with diabetes, March 2011 Diabetic medicine volume 28 (Suppl.1) P444

Prizes and oral presentations

> First prize Mersey and Cheshire Diabetes and Endocrine Group Research and Audit meeting 2010; The effect of obesity on GFR in type 2 diabetic patients with chronic kidney disease - S Nair, K Hayden, B Mishra, S Vinjamuri, B Pandya, K Hardy, and J Wilding

> DUK Annual Professional conference 2010 oral presentation The Modification of Diet in Renal Disease formula underestimates glomerular filtration rate in obese patients with type 2 diabetes mellitus. Selected for the *YDF travel award competition*

List of abbreviations

ACE	**Angiotensin converting enzyme**
ARB	**Angiotensin receptor blocker**
ACR	**Albumin creatinine ratio**
BMI	**Body mass index**
BSA	**Body surface area**
CAD	**Coronary artery disease**
CC	**Creatinine clearance**
CE	**Capillary electrophoresis**
CG	**Cockcroft-Gault**
^{51}Cr	**Radioactive chromium**
CKD	**Chronic kidney disease**
CV	**Coefficient of variation**
CVD	**Cardiovascular disease**
95% CI	**Confidence interval**
DTPA	**Diethylene triamine penta acetic acid**
EDTA	**Ethylenediaminetetraacetic acid**

EM	Electron microscope
ESRD	End stage renal disease
eGFR	Estimated glomerular filtration rate
GFR	Glomerular filtration rate
HbA1c	Glycated haemoglobin
IBW	Ideal body weight
IDMS	Isotope dilution mass spectrometry
MA	Microalbuminuria
MDRD	Modification of diet in renal disease
MS	Mass spectrometry
PAS	Periodic acid Schiff
RAAS	Renin angiotensin aldosterone system
RR	Relative risk
RRT	Renal replacement therapy
SD	Standard deviation
T2DM	Type 2 diabetes mellitus

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<u>Declaration</u>

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

CHAPTER 1

Glomerular filtration rate

1.2 Glomerular structure and physiology

The two human kidneys contain nearly 2 million glomeruli, each having an average volume of 0.0042 cubic mm. The glomerulus consists of a tuft of capillaries that is supplied by an afferent arteriole. The afferent arteriole breaks into as many as 50 capillary loops, which coalesce into an efferent arteriole. This glomerular tuft is enveloped in a spherical double walled capsule called the Bowman's capsule derived from the renal tubule(Smith, 1951). The glomerular filtration barrier is formed by the fenestrated endothelium, glomerular basement membrane and podocytes and their slit processes.

In each glomerulus about 0.001 cubic mm. of filtrate is formed per second. The total filtration area in the two kidneys has been calculated from autopsy specimen in a child (Book, 1936)and adult kidneys (Vimtrup, 1928) and estimate it to be 0.76 m^2 and 1.76 m^2 respectively or nearly the body surface area of man (1.73m^2).

The average rate of filtration in man corrected to standard body surface area (1.73m^2) is 127 ml/minute: 85% of this is reabsorbed in the proximal tubule (obligatory reabsorption) leaving only about 20 ml/minute to be excreted during maximum water diuresis. During periods of dehydration however, most of this water is reabsorbed (facultative reabsorption) reducing the urine flow to 1 ml/minute (Smith, 1951).
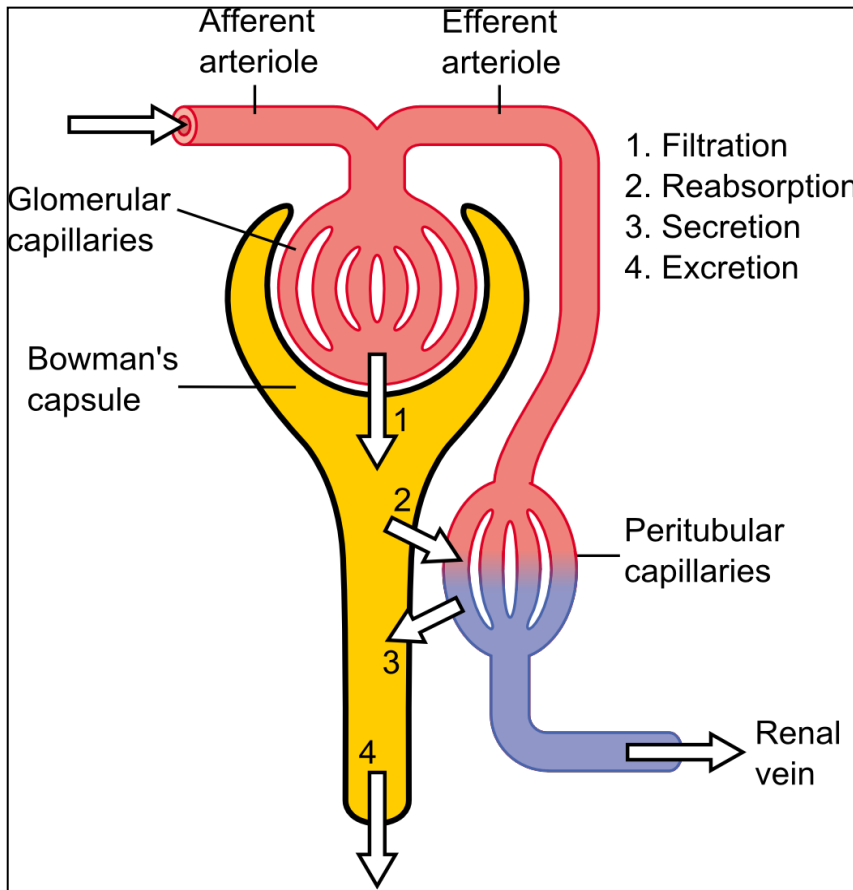
Figure 1: Glomerular physiology

Glomerular filtration rate is the sum of filtration of all nephrons. The determinants of a single nephron GFR are included in the following equation:

GFR = K$_F$ (Ultrafiltration coefficient) X ΔP (Net filtration pressure)

K$_F$ represents the product of glomerular surface area available for filtration and its permeability. **Δ**P is defines as the difference between trans-glomerular hydrostatic pressure and oncotic pressure gradients. The level of renal blood flow and afferent and efferent arteriolar resistances in turn affects the hydrostatic pressure gradients.

The ultrafiltration coefficient can drop owing to a reduction in glomerular surface area as in glomerular injury due to various causes and also as a result of reduced permeability due to damage to glomerular capillary wall as in diabetic nephropathy. Perfusion of previously under perfused capillaries or glomerular hypertrophy may compensate for these changes to maintain K_F. Once ultrafiltration coefficient starts to reduce, increase in trans-glomerular hydrostatic pressure gradient sets in as a compensatory mechanism to maintain glomerular filtration rate. Hence the measurement of GFR may not initially reveal structural damage to the nephrons (Levey, 1990).

1.2 Renal auto-regulation:

Glomerular filtration remains relatively constant over a range of physiological renal blood flow and urine output. This is possible due to renal auto-regulation, which is a process independent of the central nervous system. Auto-regulation is primarily affected by the preferential constriction of afferent and efferent arterioles to maintain a relatively constant glomerular filtration pressure. The renal blood flow remains relatively constant in individuals and this is primarily mediated by the efferent arteriolar tone. Adrenaline infusion causes decreased blood flow but an increase in filtration fraction due to maximal constriction of the efferent arterioles causing increase in filtration pressure. Infusion of a pyrogen such as Typhoid vaccine increases renal blood flow but leads to a fall in filtration pressure due to maximal dilatation of the efferent arterioles. The filtration pressure and filtration fraction remains constant due to the predominance of efferent over afferent arteriolar tone (Chasis et al., 1938).

EXCRETION OF UREA IN GLOMERULONEPHRITIS

FIG. 1. 42 SIMULTANEOUS INULIN (*dots*) AND UREA (*circles*) CLEARANCES ON SUBJECT E. B.
DURING FALLING OR CONSTANT URINE FLOWS
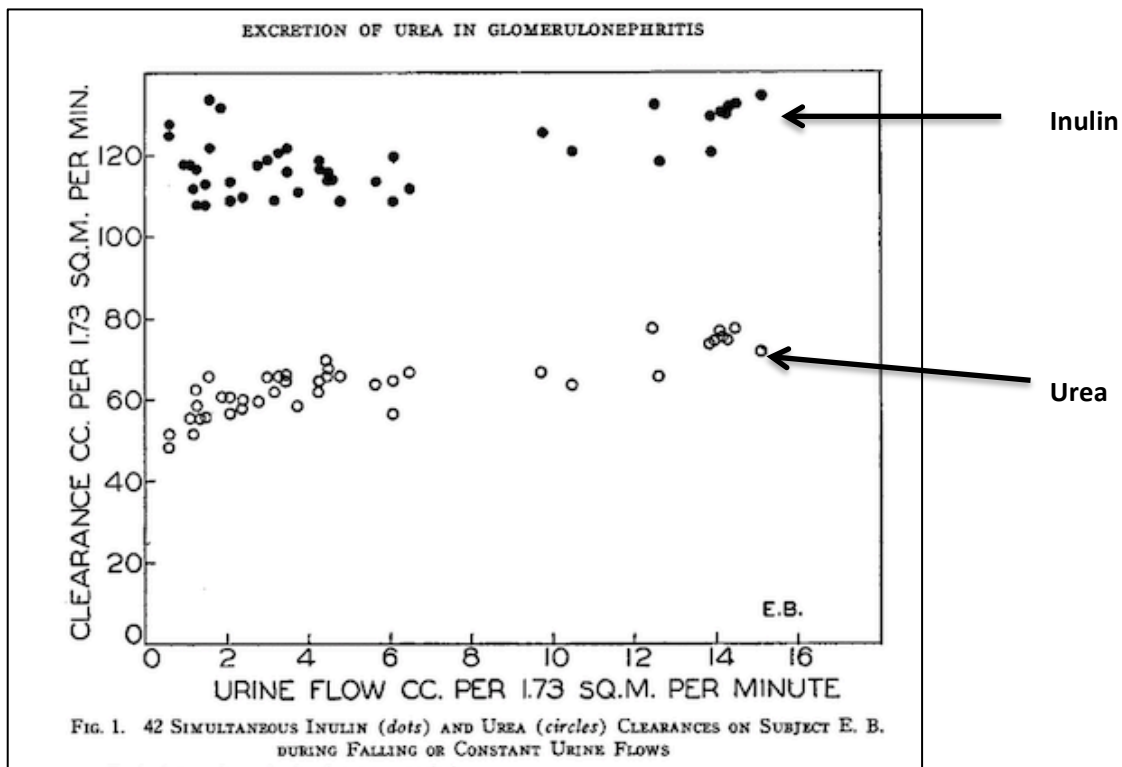
Figure 1.2: Simultaneous inulin and urea clearances in man at constant and decreasing urine flows
Reproduced with permission (Chasis and Smith, 1938). The rate of clearance of urea and inulin
remains relatively constant despite a range of urine flow.

The current view is that renal autoregulation is based mainly on two adaptive mechanisms acting on the pre-glomerular resistance vessels (arcuate, interlobular and afferent):

1. Myogenic response: it is a function of smooth muscle to contract in response to stretching force. The exact mechanism remains unclear, but it is known to involve a stretch dependent depolarisation of cell membranes and influx of calcium ions leading to contraction. This is a rapid response occurring within 3-10 seconds.

Myogenic response has not been observed in efferent arterioles probably because of lack of voltage gated calcium channels.

2. Tubulo-glomerular feedback (TGF): TGF leads to constriction of afferent arterioles in response to increase in sodium chloride at the maculo-densa in the early distal tubules. Increased arterial pressure increases tubular flow by increasing GFR which leads to rise in tubular sodium chloride concentration(Just, 2007).

NSAIDs and COX 2 inhibit afferent arteriolar vasodilatation by inhibiting prostaglandin E2 production. ACE-inhibitors / Angiotensin receptor blockers inhibit efferent arteriolar vasoconstriction by inhibiting the effect of Angiotensin II. These medications severely limit the ability of renal autoregulation to maintain kidney function.

Inulin clearance

1.3 Renal Clearance

Renal clearance is a longstanding concept that came into being before the concept of glomerular filtration rate. It is defined as the rate at which the kidney excretes a substance in relation to its plasma concentration. Renal clearance is expressed by the equation $U_x V_x / P_x$ where U_x is the urinary concentration of x, V is the rate of urine formation, and P_x is the plasma concentration. It is the volume of plasma completely cleared of that substance in a minute(Smith, 1951). Renal clearance however does not inform us of the mechanism of clearance of a substance; whether by filtration and tubular reabsorption, filtration alone, or filtration plus tubular secretion.

Homer Smith and co-workers undertook to find a substance that can be used to measure glomerular filtration. The observation of aglomerular kidneys in marine fishes particularly goosefish and toadfish was very useful for some of the subsequent glomerular physiological studies. The substances which were previously shown to be excreted by aglomerular kidneys, like magnesium, sulphate, chloride, creatinine, uric acid, phenol red, etc. could be excluded straight away. The fact that aglomerular kidney is unable to excrete glucose was an interesting observation and made glucose a potential candidate, but experiments with phlorizin had already established that glucose is reabsorbed from the tubules. They therefore focussed attention on metabolically inert carbohydrates that may not be reabsorbed by the renal tubules (Smith, 1951).

Inulin is a starch like polymer consisting of fructose with a molecular weight of about 5000 Daltons, but having an elongated structure its diffusion coefficient is similar to a substance

with a molecular weight of 15000. The limit of glomerular permeability has been shown to be around 60000 (Smith, 1951). Inulin is completely filterable from plasma. It is not excreted by aglomerular fish kidney. After intravenous administration in 7 volunteers with normal kidney function inulin was rapidly and completely excreted in the urine and was shown to have a linear relationship between its rate of excretion and plasma concentration. This could only be achieved by a substance, which is not reabsorbed or excreted by the renal tubules, as both these processes have a tubular maximum. Inulin clearance was thus considered a good measure of glomerular filtration rate. The actual method of measuring inulin clearance has remained a subject of discussion and controversy.

1.4 Urine collection method

The classical method of inulin clearance involved infusing inulin and measuring inulin in timed urine and plasma samples. These values are then used to calculate inulin clearance using the clearance equation mentioned above. The following method is as described by Shannon and Smith in their classical experiment published in 1935.

Inulin is dissolved in sterile sodium chloride by heating to a temperature of 85 degree Celsius and then cooled. 100 grams of inulin yield a blood concentration of 300-400 mg percent and fall to 100 mg percent in 1.5 to 3 hours. Inulin infusion is completed in 30 minutes. A urinary catheter is inserted at 80 minutes and a preliminary washout of bladder performed. The first period of urine collection is started at 90 minutes; each collection period varies between 10 to 20 minutes in duration and 3 to 12 in number. Blood samples were drawn as close to the middle of the periods of urine collection as possible, one sample

for each urine collection. Glucose was removed by treating with yeast, as it is necessary to make the sample starch free before measuring inulin. Inulin was hydrolysed in sugar tubes by adding 0.5cc of sulphuric acid, heating in a water bath for 15 minutes and cooling and neutralising with potassium hydroxide. Inulin concentration was expressed as the total reducing power expressed as the amount of glucose after hydrolysis. Once the urine and plasma concentrations of inulin are measured, inulin clearance is calculated by the clearance equation UV/P (Shannon and Smith, 1935).

1.5 Constant infusion technique:

Several investigators introduced a constant infusion technique to obviate the need for timed urine collections to measure urinary clearance (Earle and Berliner, 1946, Berger et al., 1948). Earle et al. demonstrated that if a substance is completely excreted through the kidneys and if the plasma level were constant, then the rate of renal excretion would be equal to the rate of infusion. Therefore in the clearance equation UV/P, UV can be replaced by the rate of infusion. Earle et al compared renal clearance measured by serial urine collection and constant infusion technique. The methodology used was described as follows. A priming dose of inulin was given intravenously to achieve a plasma level of 5 to 20 mg per cent. 2grams of inulin priming injection resulted in plasma levels of 10 to 15 per cent. Maintenance infusion was delivered by a pump at a rate calculated according to estimated renal function of the subject. If the estimated filtration rate was 100mls/min and if the plasma concentration desired and achieved by the priming was 5mg per cent, then each minute 100 x 0.005 or 5 mg would be excreted in urine. The maintenance infusion should

therefore be 5mg per minute. Generally, infusion equilibrium is achieved at the end of 1 hour.

They performed 45 experiments in 26 participants and found the results comparable. Also the variation in clearance was much less with the infusion method compare to the timed urine collection method

1.6 Single injection technique:

The intravenous infusion and timed urine measurement being labour intensive, several studies were conducted to investigate the possibility of single injection techniques.

The filtration rate or plasma flow of a substance can be calculated from the rate of change of plasma concentration of a substance. This however assumes that the volume of distribution (V) of the substance is constant, mixing throughout of the substance is instantaneous and so its concentration is uniform, and the substance is cleared from the plasma at a constant rate (C). The concentration P_2 at any time t (in minutes) after P_1 will be:

Log P_2=log P_1-Ct/V

The rate of clearance is expressed as:

C=V (log P_1- log P_2/t)

The assumptions made were shown to be not valid by several investigators. Schachter *et al*. showed that the mean concentration of inulin in the extracellular fluid is identical to that in plasma at only one moment. They pointed out that after a single injection of inulin,

equilibrium between plasma and extravascular component of the volume of distribution is never reached(Schachter et al., 1950). Hence for inulin clearance the single injection technique was not frequently employed. There have been some subsequent studies demonstrating good agreement between single shot inulin injections and constant infusion techniques over wide range of GFR. The coefficient of variation for the plasma clearance by constant infusion and single shot injection was shown to be much lower than the urinary clearance methods in a study(Florijn et al., 1994).

1.7 Limitations of inulin as a marker of renal clearance:

Inulin is most extensively investigated from a physiological point of view and hence considered the gold standard, but it has several limitations. Its high molecular weight and viscosity doesn't allow it to reach volume of distribution. This may be the reason why constant infusion with timed urinary collection is the only methodology with good accuracy. Inulin is also expensive and not easily available on the market. There is also significant variability in the different methodologies involved in its measurement in urine and plasma (Delanaye et al., 2012). Most inulin measurement methods are based on fructose estimation, as it is a fructose polymer. Methods other than the enzymatic ones can be subject to assay interference by glucose leading to overestimation of inulin concentration, which makes it not an ideal marker for patients with diabetes. Lentjes et al. described a method to measure glucose simultaneously to make correction for it which could be helpful in diabetes(Lentjes G W M, 1994). Autonomic neuropathy in patients with diabetes could contribute to incomplete urinary bladder clearance which makes timed urine collection very challenging (Sambataro et al., 1996).

1.8 Single injection technique with Radiolabelled markers:

Nosslin in 1965 demonstrated plasma clearance using the single injection technique. If a substance mixes instantaneously with plasma, its initial concentration is the dose administered divided by plasma volume (V). This is taken as 1 and subsequent values represent the proportion of initial dose remaining at the time of sampling. The clearance is calculated by the equation: C= V/A, where A is the total area under the plasma disappearance curve. Since renal clearance occurs from the arterial blood, ideally arterial or capillary samples should be used to construct the plasma disappearance curve. As time elapses after injection at some point the arterial and venous concentrations become equal and then the venous concentration starts to rise. The other caveat explained by Nosslin was that some substances might require a long time to reach its true final slope. If sampling ceases before reaching the final slope and the curve is extrapolated to infinity, the area under the curve becomes too small and the clearance too large. The shorter the plasma disappearance curve is followed the greater the error. It is better to sample at long intervals for a long time than short intervals for a short period of time(Nosslin, 1969). The single shot technique is based on bi-exponential function of time. The initial part of the plasma disappearance curve is rapid and it is due to the redistribution of tracer into the extracellular space. The second portion, called the second exponential represents the much slower elimination of the tracer by glomerular filtration once the extracellular compartment is saturated (Peters, 1991). The figure below represents a 2-compartment model assuming fixed flow rates between the intra and extra vascular volumes. A third compartment is

considered when a patient has significant third space fluid such as oedema, pleural effusion and ascites (Murray et al., 2013).

Two- and 3-compartment models with respective rate constants, kij. Anthony W. Murray et al. J. Nucl. Med. Technol. 2013;41:67-75
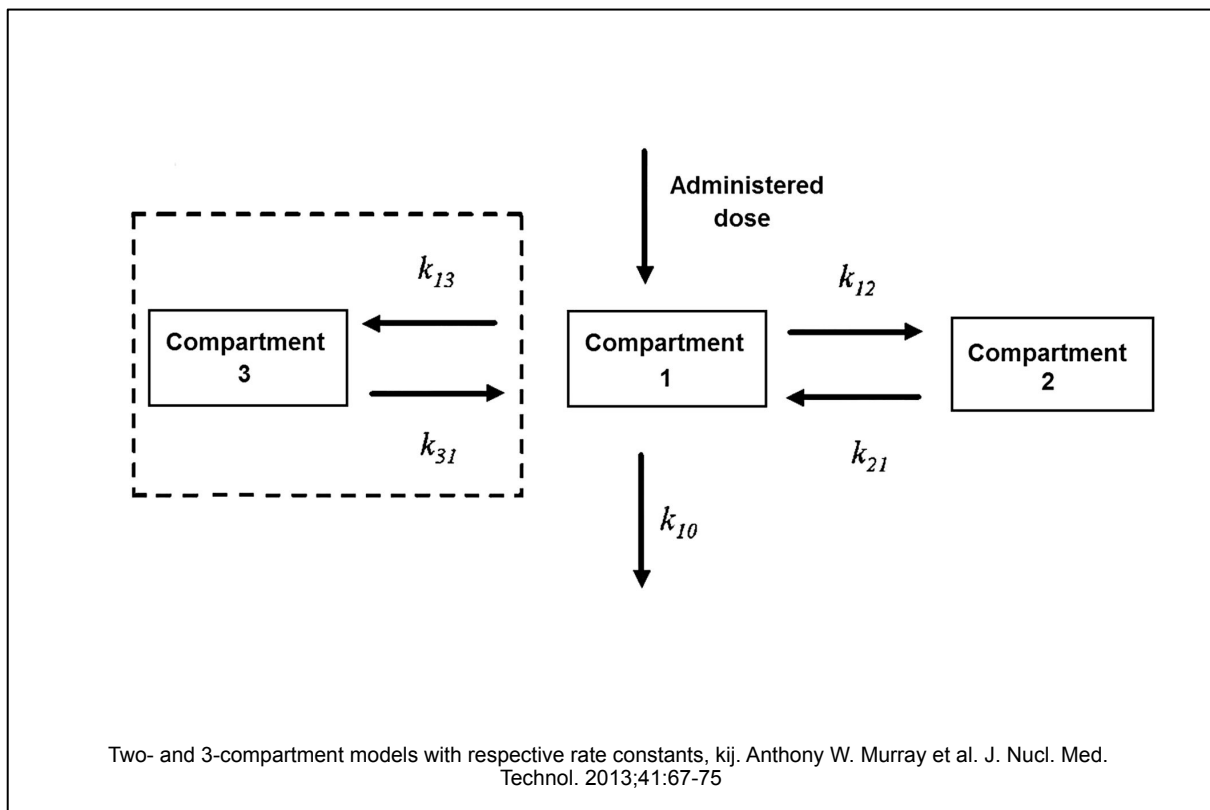
Figure 1.3: 2 and 3-compartment models with respective rate constants. K_{10} is taken to be GFR clearance. Compartment 1 is intravascular volume and compartment 2 is the extravascular volume. Compartment 3 is considered to be present in patients with known third fluid compartments.

1.9 Slope-intercept technique:

Although simpler than inulin infusion, the bi-exponential techniques are still cumbersome, as they require multiple blood sampling over several hours. Chantler et al demonstrated that the plasma clearance technique correlated well with urinary clearance methods using ^{51}Cr-EDTA and restricting blood sampling to the second of the two exponential components of clearance. This is called the slope intercept method (Chantler, 1969) which is demonstrated in the figure below. The logarithm of plasma activity is plotted as a function of time. The zero time plasma activity (P_0) is determined by extrapolation of linear part of the curve to the y-axis. The half period ($T_{1/2,}$ minutes) of the linear part of the plasma disappearance was determined from the graph and the slope calculated as: C-slope= $(0.693/T_{1/2})$ X Volume of distribution.

First exponential

Second exponential

P

P_0
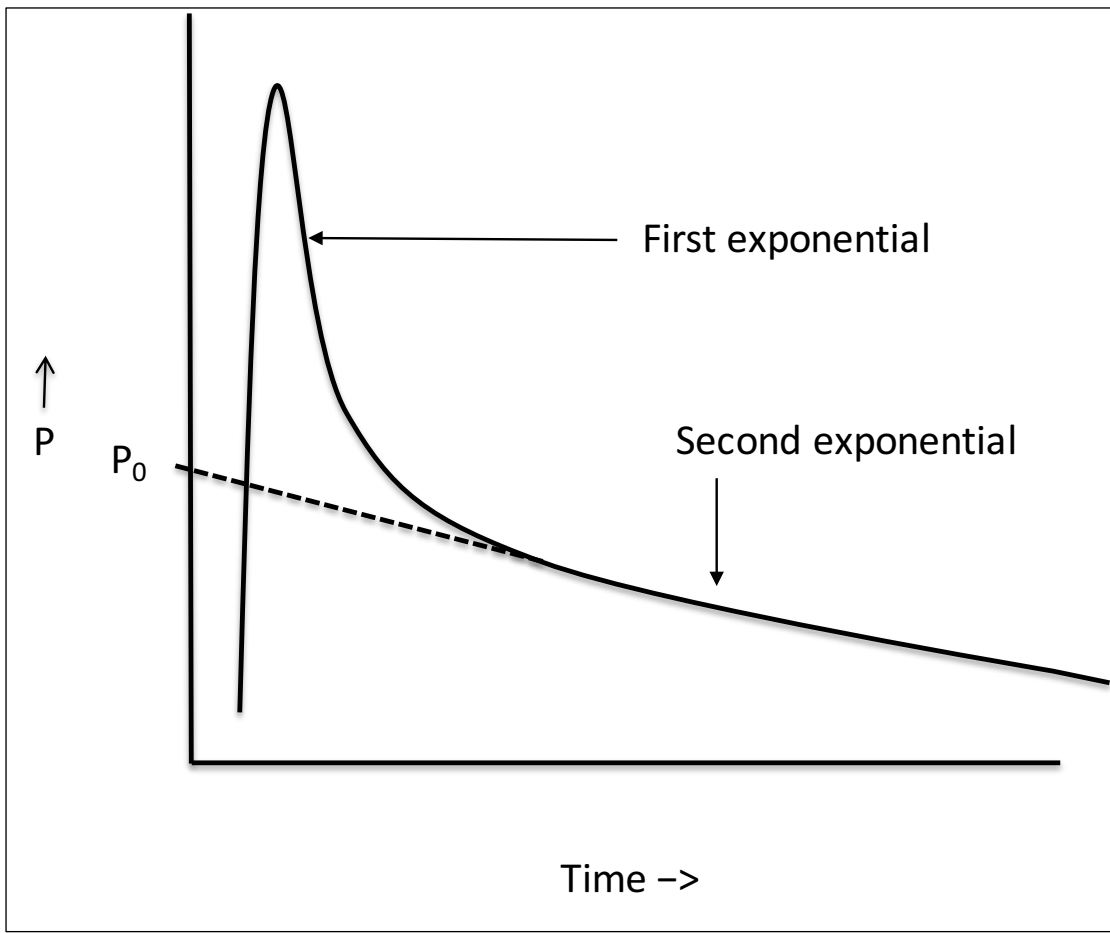
Time ->

Figure 1.4: Plasma disappearance curve showing the bi-exponential curve with time on the x-axis and plasma concentration on the y-axis. The logarithm of plasma activity is plotted as a function of time

1.10 Correction factors for the first exponential:

The slope intercept method is based on only the second exponential and as it neglects the first exponential, leads to overestimation of clearance.

Chantler correction: Chantler et al. in 1972 estimated GFR using ^{51}Cr-EDTA with both the slope intercept and urinary clearance methods. The slope clearance was shown to consistently overestimate GFR measured by urinary clearance. They conclude that a correction factor of 0.87 (i.e. clearance by slope intercept method X 0.87) provides a reasonable estimate of the GFR (Chantler and Barratt, 1972).

Brochner-Mortensen correction: Brochner-Mortensen compared ^{51}Cr-EDTA plasma clearance (Cl) with ^{51}Cr-EDTA clearance from a one-pool system (Cl_1) in 74 adult patients with renal dysfunction. Plasma clearance was calculated as a ratio of injected dose of tracer and the total area under the plasma disappearance curve. This was always exceeded by ^{51}Cr-EDTA clearance by one pool system and the correlation between them can be expressed as:

Cl=0.990778 Cl_1 – 0.001218 $(Cl_1)^2$

This correction is widely used to improve the accuracy of the slope-intercept GFR measurements (Brochner-Mortensen, 1972).

A study by De Sadeleer et al. compared the Chantler's linear correction formula and Brochner-Mortensen quadratic equation using data from 47 adults with normal renal function with the bi-exponential fit method. In the normal clearance range of 120 to 140 mls/min. both corrections led to slightly lower results than the bi-exponential method.

However, in all values less than 140mls/minute the difference between the 2 correction formulae was clinically insignificant (De Sadeleer et al., 2006).

Radioisotope labelled renal clearance markers

Radiolabelling allows detection of minute quantities of the filtration marker in urine or plasma. Radiation exposure is minimal with all commonly used isotopes and the choice is primarily based on ease of obtaining, administering and counting the radioisotopes (Levey, 1990). One of the main disadvantages of radiolabelled techniques is the requirement for storage and disposal of radioactive substances and the need to perform these investigations in nuclear medicine laboratories. The most commonly used radiolabelled isotopic markers worldwide are ^{51}Cr-EDTA, ^{99}Tc-DTPA, and ^{125}I Iothalamate, which are discussed below. Iothalamate and iohexol are non-ionic contrast markers and can be measured using high performance liquid chromatography (HPLC).

1.11 ^{51}Cr-EDTA (Ethylene diamine tetra acetic acid):

^{51}Cr-EDTA is a radionuclide marker with low molecular weight of about 292 Dalton and does not bind to plasma proteins, so it is freely filtered through the glomerulus. The measurement of ^{51}Cr-EDTA by nuclear count is easy because of its long half-life of 27 days. The radiation dose received by the patient is relatively small and also the dose of EDTA is 100 times less than the dose considered to be safe (Chantler, 1969). Its clearance has been

shown to be closest to that of inulin and is considered the radionuclide agent of choice for GFR measurement, particularly in Europe (Blaufox et al., 1996). Garnett *et al*. in 1967 first described measurement of GFR using ^{51}Chromium conjugated to EDTA. They compared inulin clearance with ^{51}Cr EDTA using both infusion and urine collection techniques. ^{51}Cr EDTA showed very good correlation (r=0.995) to inulin clearance. Since equilibrium with extravascular compartment takes less than 2 hours and there is no significant extra-renal excretion, the slope-intercept method based on 2-4 hours plasma activity can be used as a simple procedure. The authors emphasise that this method is not accurate in the oedematous patients (Garnett et al., 1967).

The main limitations of ^{51}Cr EDTA as a marker for renal clearance are that it needs to be performed in a nuclear medicine department and also that it is not FDA approved hence not studied in USA

1.12 ^{99}Technetium-DTPA (Diethylenetriaminepenta-acetic acid):

^{99}Tc-DTPA is another widely used radionuclide marker for GFR measurement, particularly in the United States. It has a low molecular weight of 393 Dalton. Klopper 1972 studied the use of ^{99}Tc-DTPA-clearance in dogs and humans and found the clearance to be lower than inulin clearance primarily due to protein binding of ^{99}Technetium-DTPA. Protein binding was much lower when a single injection technique was used compared to continuous infusion (Klopper et al., 1972). This study, which is one of the few physiological studies with this marker, showed no tubular secretion or reabsorption for ^{99}Technetium-DTPA.

The main limitation of [99]Technetium-DTPA is its protein binding. It also has a shorter half-life compared to ^{51}Cr EDTA, which means GFR measurements have to be done soon after sampling and can cause some practical difficulties. Simultaneous measurement of [99]Technetium-DTPA, ^{51}Cr EDTA and inulin in patients with decreased renal function showed that plasma clearance of inulin exceeded that of ^{99}Tc-DTPA, however clearance of the two radio isotopic markers were comparable(Rehling et al., 1984).

1.13 Iothalamate:

[125]I Iothalamate is one of the most used radiolabelled markers in USA and notably, the modification of diet in renal disease (MDRD) study used this marker. There is however evidence to suggest that Iothalamate is secreted by renal tubules and it also has some extra renal clearance. These are its major limitations as a marker and lead to slight overestimation of GFR. Iothalamate can also be measured without radiolabelling, but the limiting issues are the need for larger doses with the risk of nephrotoxicity and allergies, and the need for expensive assays such as high performance liquid chromatography (HPLC) methods.

Elwood and Sigman in 1967 compared GFR in 21 patients with various levels of renal function using inulin and [125]Iodine Iothalamate by constant infusion technique. They determined the activity of ^{125}I in a scintillation (gamma) counter and demonstrated the ratio of inulin to Iothalamate clearance to range from 0.93 to 1.09 with a mean of 1.00 (Elwood and Sigman, 1967).

1.14 Iohexol:

Iohexol is a non-ionic contrast agent that has been relatively popular in Scandinavia for GFR measurement in adults and children (Krutzen et al., 1990). Like Iothalamate iohexol can be measure using HPLC or by X-ray fluorescence.

Renal clearance of inulin and plasma clearance of iohexol (HPLC assay) using the two-compartment model showed highly significant correlation across a range of renal function (Gaspari et al., 1995). Iohexol clearance also compared very well with radioactive labelled markers like ^{51}Cr-EDTA (Olsson et al., 1983)

Schwarz et al. investigated how plasma disappearance curves could be used in children and adolescents with mild to moderate chronic kidney disease by iohexol method assayed by HPLC. They compared GFR using a 9 data point (10 minutes to 360 minutes following injection) plasma disappearance curve method with 4 data points curve, which showed excellent correlation (r=0.999). One compartment GFR with two data points at 120 and 300 minutes after injection using Brochner-Mortensen quadratic equation also showed good correlation (r=0.986) (Schwartz et al., 2006). The iohexol method has been used extensively in adults, children and adolescents in several cohort and experimental studies. Iohexol as a contrast agent compared to other markers is less expensive. The main limitation seems to be that there are only few studies comparing iohexol with inulin and also very few physiological studies on its renal tubular handling.

1.15 Creatinine biosynthesis and physiology

Creatine and Creatine phosphate from which creatinine is formed exists in muscle tissue and serve as a rapid source of high-energy phosphate to produce ATP. Thus the rate of endogenous production of creatinine depends on the muscle mass; it averages 20mg/Kg/24 hours for normal subjects. Once formed, creatinine distributes rapidly throughout the body. It is normally produced at a constant rate and eliminated at a rate proportional to serum creatinine; thereby achieving a relatively steady state in the blood(Lott and Hayton, 1978).

Up to 94% of the Creatine in the body is found in muscular tissues. Muscle does not synthesise creatine and thus has to take in large quantities of it through creatine transporter on the plasma membrane. Creatine in the body is either of dietary origin or synthesised de novo. The first step of Creatine biosynthesis probably occurs mainly in the kidney, whereas the liver is likely to be the principal organ accomplishing the subsequent methylation of guanidinoacetic acid (GAA) to Creatine. The contribution of other tissues in creatine generation remains unclear. Creatine is phosphorylated enzymatically by creatine kinase to Phospho-Creatinine, which is the source for immediate generation of ATP. The muscular Creatine and Phospho-Creatine are nonenzymatically converted at an almost steady rate (~2% of total Cr per day) to creatinine, which diffuses out of the cells and is excreted by the kidneys into the urine (Wyss and Kaddurah-Daouk, 2000).

Major routes of Cr metabolism in the mammalian body.
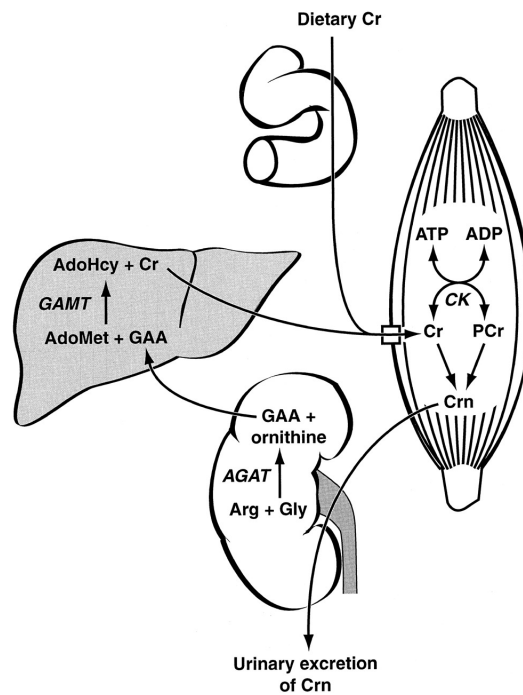
Markus Wyss, and Rima Kaddurah-Daouk Physiol Rev
2000;80:1107-1213

Physiological Reviews

Figure 1.5: *(permission sought, but none required for reproduction in thesis)*: **shows synthesis of creatine in the body; formation of guanidinoacetic acid in the kidneys and subsequent conversion into creatine in the liver. Biosynthesised and dietary creatine is then transported into muscle tissue (Wyss and Kaddurah-Daouk, 2000).**

Rehberg as part of his investigation of the two prevailing theories of kidney function; the secretion theory and the filtration-reabsorption theory described the use of creatinine as an "exogenous filtration marker" as early as 1926 (Rehberg, 1926). In his experiment on himself, Rehberg ingested 5g of creatinine dissolved in 200mls of water; blood samples were drawn hourly by finger prick and urine collected as per diuresis. Creatinine in plasma and urine was assayed using the picric acid method described as follows; 1 ml of the sample is mixed with 5ml-saturated solution of picric acid and at the same time 1 ml of a standard solution of creatinine is treated in the same way in another tube. To both tubes a 10% solution of picric acid is added and then they are compared using a colorimetric technique. A plasma creatinine against hours after ingestion of creatinine curve was drawn and the plasma creatinine corresponding to the timing of urine samples were used to calculate the filtration rate. He concluded that the amount of creatinine in the urine after ingestion is so large that filtration of up to 200 mls per minute needs to happen to explain it and that the filtration reabsorption theory was more tenable than the secretion theory.

1.16 Tubular secretion of creatinine

Creatinine is not present in the urine of aglomerular fishes, but when injected they excrete creatinine in their urine. It has also been shown that the rate of excretion does not increase in proportion to the rate at which creatinine is injected. For a substance that is freely filtered through the glomerulus, this should not be the case. Several subsequent studies showed that tubular secretion, which has a tubular maximum, is responsible for this curvilinear relationship. As the plasma concentration of creatinine rises above 10mg per

cent, there is reduction in creatinine clearance. To investigate if this was due to reduction in filtration Shannon in 1935 compared creatinine clearance with inulin clearance, which had been shown to be equivalent to glomerular filtration. They showed that at low concentration of creatinine, the creatinine clearance was 30 to 45% higher than inulin clearance. As the plasma concentration of creatinine rises, the creatinine clearance falls in comparison to that of inulin to about 12%. This phenomenon could only be explained by tubular secretion of creatinine. As the next step, they administered high dose of intravenous phlorizin (100mg/Kg) and repeated the experiment. This brought creatinine clearance very close to that of inulin, which the author concluded was due to specific inhibition of tubular secretion of creatinine by phlorizin(Shannon, 1935).

Shemesh et al. compared creatinine clearance with that of inulin and ^{99}Tc DTPA in a large population of patients with diverse glomerular disease. The fractional clearance of creatinine relative to inulin was much higher (1.64 ± 0.05). But, on blocking the tubular activity by infusion of Cimetidine, the fractional clearance approached unity (Shemesh et al., 1985). Thus, the overestimation of GFR by creatinine clearance is as a result of its tubular secretion. The fractional secretion of creatinine (the proportion of creatinine in urine as a result of tubular secretion relative to glomerular filtration) was shown to vary inversely with GFR, making it much less reliable in patients with worse kidney function. The authors emphasize the importance of accurate measurement of GFR in patients with glomerular injury; GFR falls less than in proportion to the intrinsic injury to glomerular capillary wall due to a compensatory increase in net ultrafiltration pressure. So, overestimation of GFR will have the effect of significant underestimation of glomerular injury.

1.17 Extra renal excretion of creatinine

There are also reports of extrarenal excretion of creatinine which is significant in patients with poor renal function. In this study radioactive carbon labelled creatinine was administered and its extra- renal excretion was assessed in breath and faeces. They showed that 15.5 to 65.7% of the creatinine formed is metabolised or excreted via extra-renal routes in patients with significant renal dysfunction(Jones and Burnett, 1974).

1.18 Muscle mass and creatinine generation

While endogenous creatinine is formed from muscle metabolism, exogenous creatinine comes from dietary ingestion, primarily from meat. The rate of creatinine generation is dependent on muscle metabolism and diet (Levey, 1990). Creatinine and muscle mass are so closely related that it gave rise to a concept called "creatinine equivalence"; 24 hour urinary creatinine has been used as an index of body muscle mass. Creatinine equivalence (muscle mass in Kg / 24 hour urinary creatinine in grams) has been shown to be a constant rate ranging between 17 and 22 in healthy adults (Heymsfield et al., 1983).

However, serum creatinine has become the most widely used measure of progression of renal disease because of the following reasons(Levey, 1990). Although it is not an accurate measure of GFR, the changes in serum creatinine are a reasonably accurate reflection of changes in GFR. The variability with time of serum creatinine is also lower than GFR. The greatest advantage of serum creatinine is of course the ease with which it can be measured in all clinical laboratories.

1.19 Estimated Glomerular Filtration Rate

A variety of methods have been used to estimate GFR. The Cockcroft-Gault formula, developed for estimation of Creatinine Clearance, not of GFR has been widely used in the past.

The best-validated methods for GFR estimation were derived from the Modification of Diet in Renal Disease (MDRD) Study data. The authors enrolled 1628 patients from the original MDRD study who had undergone GFR measurement by renal clearance of ^{125}I-iothalamate and randomly selected 1070 to the training sample and 558 to the validation sample. Multiple stepwise linear regression analysis showed that seven independent factors were associated with significantly lower GFR; higher serum creatinine, older age, female sex, non-black ethnicity, high blood urea nitrogen, lower serum albumin, and lower urine urea excretion levels. The authors derived two six-variable equations:

1. Equation that uses blood urea nitrogen

$$GFR = 198(Cr^{-0.858} \times age^{-0.167}) \times BUN^{-0.293} \times albumin^{-0.318} \times (1.178 \text{ if black}) \times (0.822 \text{ if female})$$

2. Equation that uses serum albumin

$$GFR = 170(Cr^{-0.999} \times age^{-0.176}) \times BUN^{-0.170} \times UUN^{-0.249} \times (1.178 \text{ if black}) \times (0.822 \text{ if female})$$

The 2 equations were then applied to the validation sample and a six-variable equation containing only demographic and serum variables were chosen to simplify the prediction of GFR (Levey et al., 1999). In the year 2000, Levey et al. published the MDRD4 equation, which is a simplified formula arrived at following a reanalysis of the original 1628 patients. The analysis excluded urine urea, serum albumin, and blood urea. This formula was first recommended for clinical use by 2002 national kidney disease practical guidelines:

$$GFR=186(Cr^{-1.154} \times age^{-0.203}) \times (1.212 \text{ if black}) \times (0.742 \text{ if female})$$

The abbreviated or four-variable equation which uses age, sex, creatinine, and race to estimate GFR is now most commonly used (Levey AS, 2000). In the UK and most parts of the world, laboratories calculate eGFR on all samples sent for creatinine measurement and this forms the basis of (CKD) chronic kidney disease staging.

The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) investigators developed a GFR estimating equation from a large database pooled from 10 studies. The CKD-EPI equation uses the same four variables as the MDRD study equation; serum creatinine level, age, sex, and race. In some studies it has been shown to be more accurate than the MDRD equation in estimating GFR, especially at higher GFR values (Levey and Stevens, 2010). A 6 year follow up study in 2823 patients showed that estimation of GFR using the CKD-EPI equation more appropriately stratifies patients with type 2 diabetes according to the risk of all-cause and cardiovascular mortality compared with the MDRD study equation (Targher et al., 2012). The CKD-EPI equation is discussed in detail in chapter 4 of this thesis.

1.20 Chronic kidney disease (CKD) definition and classification by the American National

Kidney Foundation, Kidney Disease Outcome Quality Initiative (KDOQI):

http://www.kidney.org/professionals/kdoqi/guidelines_ckd/p1_exec.htm

1. Kidney damage for ≥ 3 months, as defined by structural or functional abnormalities of the kidney with or without decrease in glomerular filtration rate (GFR), manifest either by pathological abnormalities or markers of kidney damage; including abnormalities in the composition of blood or urine, or abnormalities in imaging tests, or

2. GFR< 60 ml/min/1.73m^2 for ≥ 3 months, with or without kidney damage.

Table 1.1: CKD classification

CKD Stage	Description	GFR (mL/min/1.73m^2)
1.	Kidney damage with normal or ↑ GFR	≥ 90
2.	Kidney damage with mild ↓ GFR	60-89
3.	Moderate ↓ GFR	(3a) 44-59 (3b) 30-43
4.	Severe ↓ GFR	15-29
5.	Kidney Failure	<15 (or dialysis)

Variability of creatinine and estimated Glomerular Filtration Rate

1.21 Variability in estimated GFR

The various CKD stages imply different prognosis and different degrees of intervention for the patient. Treatment choices are also often determined by the estimated GFR and CKD stage. So, it is vital that GFR estimation is as close to the actual measured GFR as possible. There are many factors that can potentially introduce inaccuracies into GFR estimation: These can be broadly classified into:

1. Inaccuracies resulting from the GFR estimating equation used

2. Pre-analytical and analytical (Joffe et al., 2010) variability

3. Biological (Toffaletti and McDonnell, 2008) variability in creatinine measurement, muscle mass, exercise, and state of hydration.

1.22 Inaccuracies resulting from the GFR estimating equation used

These result from the fact that the commonly used 4-variable MDRD equation was derived from a predominantly non-diabetic population with chronic kidney disease. This equation was developed from a population of 1628 patients with chronic kidney disease whose mean weight was 79.6 ± 16.8 Kg, and only 99 (6%) had diabetes (Levey et al., 1999). The MDRD formula and Cockcroft-Gault introduced significant biases and, in average terms, underestimated GFR. The Cockcroft-Gault formula performed better with increasing weight

and 4-variable MDRD equation performed worse with increasing body weight in patients with newly diagnosed diabetes (Chudleigh et al., 2008).

GFR is traditionally indexed to body surface area (GFR $_{corrected}$ = GFR$_{measured}$ x (1.73/BSA m^2)) and expressed as ml/minute/1.73m^2. Indexing is performed so that renal functions of individuals with varying sizes can be compared and also to allow for a single normal range to be defined. Body Surface Area (BSA) was calculated based on the Du Bois and Du Bois formula (BSA (m^2) = [weight (kg) $^{0.425}$] x [height (cm) $^{0.725}$] x 0.007184) (Du Bois D, 1915).

This reference method by DuBois and DuBois to measure BSA was developed in 1915 by the following ingenious method, but has several limitations. They used a heterogeneous group of 9 participants including a 2 year old child with rickets, a 36 year old man with physical development of an 8 year old and an 18 year old subject with type 1 diabetes with very low body mass index (this was before insulin was discovered as treatment for type-1 diabetes). Participant's bodies were covered with moulds of gummed Manila paper; the moulds were cut open, placed flat on photographic paper and then exposed to sunlight. The unexposed paper was cut and weighed. Knowing the density of the photographic area, the BSA was deduced. They then developed a formula based on weight and height for BSA estimation with this limited and very heterogeneous sample (Du Bois D, 1915, Delanaye et al., 2005).

Indexing is based on the assumption that the average BSA is 1.73m^2, but it is much higher in the obese subjects. Indexing GFR to body surface area of 1.73m^2 in obese patients has been shown to significantly reduce the GFR and some authors have suggested that unindexed GFR is preferable in routine practice and clinical studies for the obese population (Delanaye et al., 2005) (Delanaye et al., 2009) (Peters and Glass, 2010)BSA has been shown to increase

with body weight in several studies (Delanaye et al., 2005). Schmieder investigated the effect of indexing to BSA on renal plasma flow and suggested that similar consideration should be made when adjusting GFR to body size. In this study renal plasma flow was measured as single injection clearance of I^{131} labelled para-aminohippuric acid in 215 adults with BMI ranging between 17.5 to 45.9 Kg/m^2. Age, weight, and BP were the most powerful determinants of renal plasma flow but obesity did not influence it. So the authors concluded that correction of renal plasma flow by BSA was not supported by their data (Schmieder et al., 1995). As the weight increases, although there is no increase in the number of nephrons, the single nephron GFR increases probably due to increased trans-capillary hydraulic pressure. In a study by Chagnac with 12 obese subjects (with BMI>38) and 9 healthy controls, the GFR and renal plasma flow in the obese exceeded controls by 51 and 31% respectively. The increase in renal plasma flow suggests a state of vasodilatation of the afferent arterioles. The authors suggested that abnormal transmission of increased arterial BP to the glomerular capillaries through a dilated afferent arteriole could account for the increased trans capillary pressure (Chagnac et al., 2000). Hence indexing GFR to BSA (which increases with obesity), leads to underestimation of GFR.

A cross-sectional study of 895 adults who had their kidney function measured with ^{51}Cr EDTA showed that GFR indexed to BSA in patients with a BSA <1.60 m^2 overestimated GFR with a bias of 10.08 ml/min (11.46%) and underestimated GFR in those with a BSA >2 m^2 with a bias up to −20.76 ml/min (−23.59%). The authors proposed that BSA is not a good normalization index in patients with extreme body sizes (Redal-Baigorri et al., 2014). The authors recommend that until a better normalization index is found, clinicians should use

absolute GFR to calculate individual drug chemotherapy dosage as well as express individual kidney function.

This issue with inaccuracies due to a normalising index has been shown to be particularly true in children and infants. In a study comparing BSA and vECF (extra cellular fluid volume) as variables against which to index GFR found that vECF was more valid physiologically for indexing GFR than BSA. A measurement of vECF (the ratio of administered radioactivity and the zero time intercept of the terminal exponential) is however more difficult to obtain than BSA (Peters et al., 2000). Several other parameters have been used to index GFR including body weight, lean body mass, total body water and plasma volume.

In the normal sized adult population indexing for BSA does not cause any clinically significant discrepancy between the absolute and indexed GFR (Peters et al., 2000). This is believed to be the reason why indexing for BSA has been a widely accepted as a routine process during GFR measurement.

1.23 Pre-analytical and analytical variability

Pre-analytical variability mainly includes factors introduced during sample collection and handling. These factors if considered and avoided can improve accuracy and reproducibility of test results. A study by Shepherd et al. looked at the effects of delayed centrifugation of blood, which is an important pre-analytical variable. Multiple blood samples were drawn from 5 subjects at the same time point but centrifugation was performed at prespecified times ranging from immediate to over 30 hours after blood sampling. Delayed

centrifugation of the blood sample was shown to introduce significant pre-analytical variability in creatinine values and miss-classification of CKD (Shepherd et al., 2007).

Most laboratories across the world use Isotope Dilution Mass Spectrometry (IDMS) aligned creatinine assay to limit the analytical variability of creatinine measurements.

The alkaline picrate assay or kinetic Jaffe method is used in most UK laboratories for creatinine measurement. Many substances have been shown to interfere with the Jaffe method including glucose and acetoacetate; this is an important consideration as patients with diabetes form a good proportion of the CKD population (Myers et al., 2006, Da Rin G, 1995). Another commonly used methods are the enzymatic creatinine assays which have been reported to have lesser degree of interferences compared to the Jaffe method in some studies (Myers et al., 2006). Isotope dilution mass spectrometry (IDMS) is considered the reference method for establishing the true concentration of creatinine in serum because of its excellent specificity and relative SD <0.3% (Myers et al., 2006). This method is only available in a few highly specialised laboratories worldwide. The manufacturer of assays calibrates creatinine measured by other methods like the kinetic Jaffe and enzymatic methods to IDMS. This helps to reduce assay variability and its effect on eGFR to some extent.

1.24 Biological variability in creatinine measurement and GFR

The main causes for biological variability in creatinine values are thought to be state of hydration of the subject, cooked meat consumption, blood pressure, blood glucose levels and exercise.

The percentage variation in creatinine due to time of the day was found to be very low at 1.4% in a study by Pocock et al in 1989 (S J Pocock, 1989). This study measured creatinine on non-fasting blood and serum samples collected between 9 am and 7 pm from a representative group of 7685 British middle-aged men.

Heavy and prolonged exercise in healthy men have shown to significantly raise creatinine levels (Refsum and Strömme, 1974), this study however includes extreme sport of over 50 miles of skiing. Although it is generally agreed that state of hydration affects creatinine levels, the evidence for this is sparse (Sjöström et al., 1988).

Cooking meat converts creatine in skeletal muscles to creatinine and subsequently leads to a rise in serum creatinine and fall in eGFR, which is considered in detail in the introduction to chapter 6 in this thesis.

Protein intake is also known to cause a true increase in renal plasma flow and GFR. Bosch et al showed significant increase in creatinine clearance with progressive increase in acute protein load in healthy volunteers. The authors described this capacity of kidneys to increase its function in response to a protein load as "renal functional reserve". In this study the healthy volunteers showed the maximum increase in GFR at two and a half hours after the protein load (Bosch et al., 1983). GFR can be made to rise by several mechanisms; the

most commonly used stimuli are high protein meal and intravenous amino acid infusion. Dopamine infusion is also known to increase GFR by a different mechanism. It is generally agreed that animal protein produces the largest increase in GFR. The main mechanism for rise in renal plasma flow and consequent rise in GFR is thought to be renal vasodilatation induced by amino acids. There is also some evidence for a rise in the trans-capillary hydraulic pressure gradient following a meal. Other possible mediators of renal reserve are glucagon, prostaglandin and the renin angiotensin system (Thomas et al., 1994).

1.25 Diabetic nephropathy in Pima Indians

Diabetic nephropathy in Pima Indians is relevant to my thesis as they are a population known to have high prevalence of obesity and diabetic nephropathy. Diabetic nephropathy has been extensively studied in the Pima Indians of the Gila River community in Arizona because of the following characteristics:

1. They have one of the highest incidences of type2 diabetes and up to 21% develop diabetic nephropathy. In one study the Incidence of diabetes was 19 times that in the predominantly white population of Rochester, Minnesota (95% confidence Interval, 16 to 22 times) (Knowler et al., 1978).

2. They have 2 yearly OGTT performed systematically as part of a longitudinal study of diabetes and its complications conducted in this community since 1965; allowing for the onset of type2 diabetes to be documented. It is difficult to know the exact duration of type2 diabetes in less characterised populations due to late diagnosis.

3. They develop diabetes in the 3rd to 5th decade of life, which reduces confounding due to old age; aging can by itself lead to fall in GFR possibly related to age related renovascular disease due to atherosclerosis. Unlike European populations with type 2 diabetes renal involvement occurs relatively early in life in Pima Indians.

Myers et al studied 20 Pima Indians diagnosed with type2 diabetes within 3 years and 28 controls with normal glucose tolerance tests from the same population. They measured Iothalamate and PAH (para-aminohippuric acid) clearance to represent GFR and renal plasma flow respectively. The measured GFR averaged 140±6 ml/min in the diabetic group, which was 15% higher than controls suggesting hyper-filtration. Standard correction for BSA reduces this difference to only 7%. Similarly the absolute renal plasma flow was 12% higher in the diabetes group but only 5% difference remained after correction for BSA. It is notable that in this study the mean BMI was 31.6 ±1.4 in the control group compared to 37.3 ± 1.6 in the diabetes group. This study demonstrated an elevation of absolute GFR and a trend towards enhanced albumin excretion rate fairly soon after diagnosis of type2 diabetes. They also demonstrated increased trans-glomerular passage of dextran showing that there is a barrier size selectivity dysfunction in early type2 diabetes (Myers et al., 1991).

The Pima Indians in addition to having a high incidence of diabetic nephropathy also have a higher rate of progression to develop renal function loss despite having lower blood pressure and lipid levels. They have been found to have a combination of lower number of glomeruli (inherited glomerulopaenia) and glomerulomegaly that results in

greater incremental loss of single-nephron GFR (SNGFR) with each nephron lost to sclerosis. In a group of 34 Pima Indians the authors observed average rate of GFR loss of 13.8mls/min/1.73m^2 and the majority of these patients progressed to end stage renal disease by 10 years of follow up (Lemley, 2003).

Pima Indian studies also give us some insight into the phenomenon of hyper-filtration in early diabetic nephropathy. Nelson et al studied glomerular function in 194 Pima Indians in different stages of development and progression of diabetic nephropathy. GFR and renal plasma flow were measured using Iothalamate and PAH clearance respectively at baseline, 2 years and 4 years. The GFR in the groups with newly diagnosed diabetes, normo and microalbuminuria exceeded that in the group with normal glucose tolerance by 16%, 24% and 26% respectively. GFR was proportionally more elevated than renal plasma flow in the normo or microalbuminuria group. On the other hand in the macro albuminuria group the GFR and plasma renal flow was lower than the other groups. The authors suggested that a progressive loss of intrinsic ultrafiltration capacity is the predominant cause of declining GFR; and in subjects with macro-albuminuria glomerular hyper-perfusion no longer compensates for the reduced ultrafiltration capacity (Nelson et al., 1996).

CHAPTER 2

METHODS

2.1 Recruitment

Study subjects were recruited from the diabetes clinics at Aintree University Hospital and the diabetic nephropathy clinics at St Helens and Knowsley hospital. Healthy volunteers were recruited from amongst the staff and colleagues at Aintree university hospital. All study participants received a patient information leaflet and gave written informed consent before any study related intervention was undertaken. Liverpool Research Ethics Committee and local R&D departments approved all the studies included in this thesis.

2.2 Subjects

Chronic kidney disease was defined as either kidney damage (pathological abnormalities or markers of damage, including abnormalities in blood or urine tests or imaging studies) or eGFR< 60 for > 3 months (KDOQI, 2002).

Body mass index (BMI) was defined as weight (Kg) / height (m) ^{2}. The study participants were divided into two categories based upon their BMI as follows:

BMI <30 kg/m^2 and

BMI ≥30 kg/m^2 (obese category)

Subject numbers and baseline characteristics were different in various studies and have been described separately in each subsequent chapter.

2.3 Sample handling and centrifugation

Venous samples were drawn from an indwelling cannula and immediately centrifuged at 3000rpm for 15 minutes. This was to minimise the effect of delayed centrifugation on measured variables particularly serum creatinine. The supernatant serum was extracted and the rest discarded. One serum aliquot was sent to biochemistry laboratory and the other frozen at -80 ^{0}C for future use.

First void morning urine sample was collected in universal urine containers and subjects brought them in for study visits; an aliquot was frozen at -80 ^{0}C for future use. The remaining sample was sent to biochemistry laboratory for albumin- creatinine ratio (ACR).

2.4 Creatinine assay

Serum creatinine was measured using the kinetic alkaline picrate assay on an Olympus AU640 analyser.

Picric acid in an alkaline medium reacts with creatinine to form an orange coloured complex and the intensity of the colour is proportional to the concentration of creatinine (Delanghe and Speeckaert, 2011)

Figure 2.1 Kinetic alkaline picrate assays

Creatinine **+** Alkaline picrate **=** Orange coloured complex
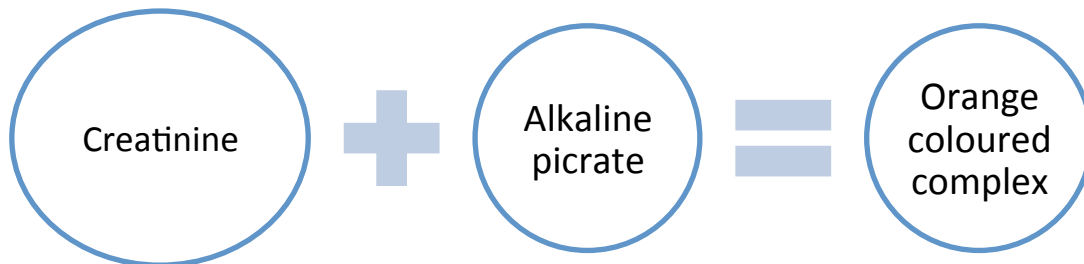
The kinetic part means that the rate of formation of the product between two time points is also considered which reduces assay interference. This is because acetoacetate may interfere early in the reaction and protein may interfere later.

2.5 Urine albumin - creatinine ratio

We used albumin: creatinine ratio (ACR) which is a widely accepted surrogate for the albumin excretion rate (Miller and Bruns, 2009), and is expressed in milligrams albumin per millimol creatinine.

2.6 eGFR estimation

We used the four-variable Modification of Diet in Renal Disease Study (MDRD) formula as this was used in most laboratories including in our centre:

$$175 \times [\{(serum\ creatinine\ (\mu mol/l)\ (-intercept)/slope\} \times 0.011312]^{-1.154} \times$$

$$[Age]^{-0.203} \times [0.742\ if\ \ female] \times [1.212\ if\ AfroCarribean]\ (ml/min\ per$$

$$1.73m^2)$$

This is traceable to IDMS-creatinine and adjusted for Olympus methodology used in our laboratory. The intercept and slope for Olympus methodology are 16.4 and 0.955 respectively.

2.7 Isotopic GFR measurement

We used [51]Chromium-EDTA clearance method, which has been validated against the gold standard inulin clearance (Ditzel et al., 1972, Brøchner-Mortensen, 1985). [51]Chromium-EDTA clearance is the most commonly used technique used in UK and also the rest of Europe and has been well validated. It is also the technique recommended by the European Association of Nuclear medicine and the British Nuclear Medicine Society (Piepsz et al., 2005, Fleming et al., 2004). [51]Chromium has a relatively long half-life of 27.7 days, which allows a lot more time between blood sampling and counting of the samples compared to other radionuclides used for GFR measurement (Murray et al., 2013).

Figure 2.2: The 4 stages of GFR measurement using radionuclide-based studies of GFR are illustrated in the diagram below (Murray et al., 2013).

The 4 stages of radionuclide-based GFR studies. Anthony W. Murray et al. J. Nucl. Med. Technol. 2013;41:67-75
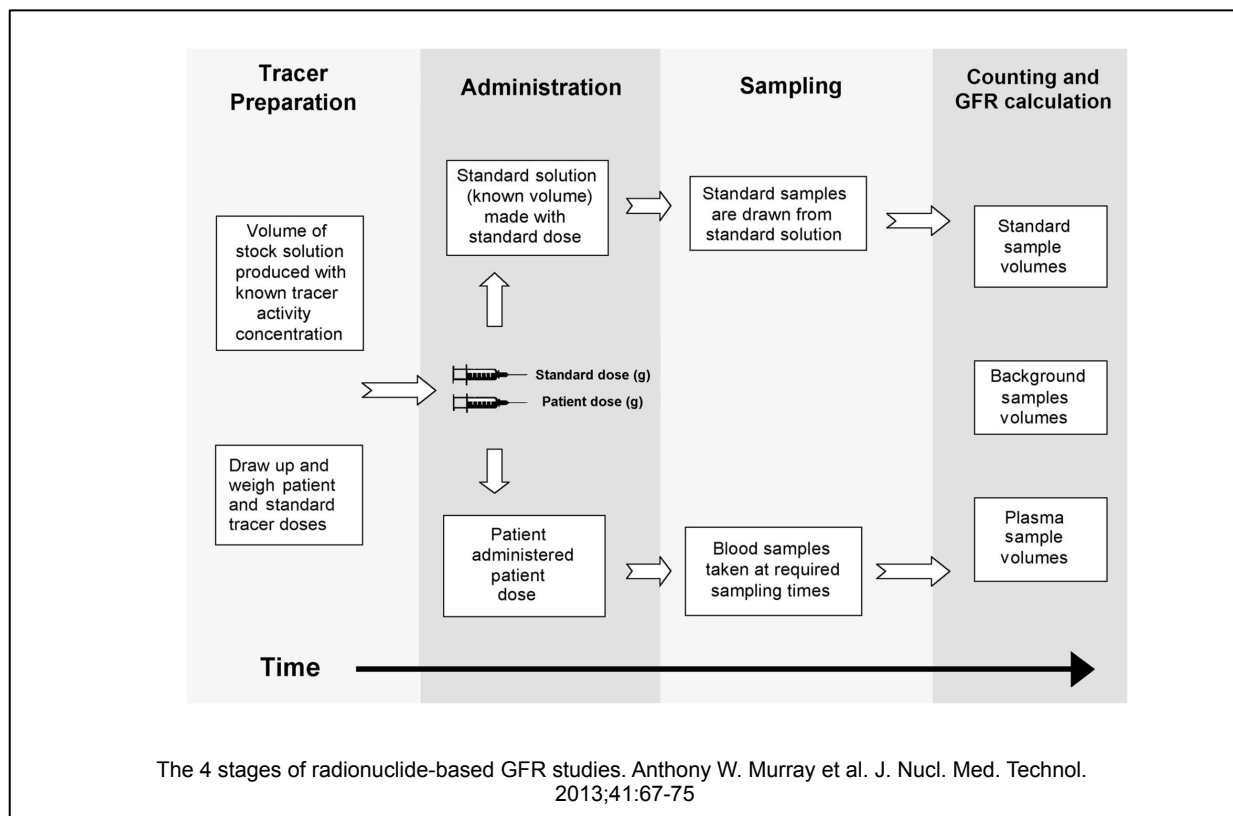
1. Tracer Preparation: A stock solution is prepared as per manufacturer's recommendation and a patient dose and a standard dose are drawn into syringes from it. The quantity of tracer in the 2 doses, the time of measurement, the weight of the patient, and the standard doses are recorded before administration. The standard dose is diluted in a 1litre volumetric flask.

2. Administration: Before administration of the dose the intravenous line is checked by injecting 10ml 0.9% saline to reduce risk of extravasation. A bolus of 3 to 3.5 MBq ^{51}Cr-EDTA is administered to the subjects intravenously at 0 minutes. The weights of the empty syringes from the patient and standard doses are recorded for residual-dose correction purposes.

3. Sampling: A cannula is inserted in the vein in the contralateral arm and 2 blood samples (5 ml of serum sample for creatinine estimation and 4 ml of blood in EDTA tube for glycated haemoglobin) taken from this cannula at 0 hour (baseline). Three further samples are taken at 120, 180 and 240 minutes

4. Counting and GFR calculation: The whole blood is centrifuged at 1000g for 10 minutes to isolate plasma and then taken in 1ml counting vials. The standard sample is also prepared in 1ml vial. Each vial is counted for more than 10000 counts in a gamma counter.

   The slope-intercept GFR was measured from three samples taken at 120, 180 and 240 minutes. The slope intercept method is preferred to full area under the curve method, which is too time consuming for routine use. This GFR is corrected for one-pool approximation using the quadratic Brøchner-Mortensen equation (Fleming et al., 2005) to correct for the relatively small fraction of the AUC missed due to not including the fast exponential in the mesurements. GFR is indexed to body surface area (GFR $_{corrected}$= GFR$_{measured}$ x (1.73/BSA m^2)) and expressed as ml/minute per1.73m^2. Body Surface Area (BSA) was calculated based on the Du Bois and Du Bois formula (BSA (m^2) = [weight (kg)$^{0.425}$] x [height (cm)$^{0.725}$] x 0.007184) (Du Bois D, 1915).

This radioisotope test to assess kidney function is routinely being performed in the Department of nuclear medicine at Royal Liverpool University Hospital (RLUH). Following the test participants were free to leave the hospital after each participant spending approximately 5 hours at RLUH.

2.8 Cooked meals

Our hospital canteen cooked the meals for study participants as per instructions on the packaging. The Aberdeen Angus beef burgers, which contain 23 grams of protein, were grilled from frozen at moderate heat for 25 minutes. The meat free burgers containing 21 grams of protein were grilled at moderate heat for 15 minutes as per cooking instruction. These were served along with 2 buns and vegetable salad, followed by 250 mls of water.

Statistical methods

Statistical analysis was carried out using SPSS (version 17.0 for Windows: SPSS, Chicago, IL) and Microsoft offices excel 2007. The 4-v MDRD eGFR results were compared with isotopic GFR by means of two-tailed paired and unpaired t tests as appropriate. Results are presented as mean ± SD unless otherwise indicated. $P < 0.05$ was taken to indicate statistical significance.

Bland Altman plots were constructed using the SPSS programme. It is a graphical method used to compare two measures. The Bland Altman plot consists of the average of the difference between paired values from each method on the x-axis and the difference of

each pair of readings on the y-axis. It allows us to look for any systematic difference (fixed bias) and also identifies outliers. This statistical method is proposed when a new measure is to be evaluated by comparison with an existing technique. Bland and Altman explain the importance of agreement rather than correlation when comparing two measurement methods (Bland and Altman, 1986). The Bland Altman plot analysis is a simple way to evaluate a bias between the mean differences, and to estimate an agreement interval, within which 95% of the differences of the second method, compared to the first one, fall (Giavarina, 2015). The upper 95% confidence interval was expressed as *(standard deviation X 1.96) + mean* and the lower 95% confidence interval as *mean- (standard deviation X 1.96).* If the differences within mean ± 1.96 SD are clinically insignificant, the two methods may be used interchangeably. The mean paired difference between the two measurements is called the "bias" quantifies how much higher (i.e., positive bias) or lower (i.e., negative bias) values are with the new method compared with the established one.

CHAPTER 3

EFFECT OF BODY MASS INDEX ON THE ESTIMATED GLOMERULAR FILTRATION RATE IN PATIENTS WITH TYPE 2 DIABETES

3.1 INTRODUCTION

Diabetic nephropathy affects 25-40% of diabetic patients and is the leading cause of end-stage renal disease in the United States and Europe (Molitch et al., 2004). The increased cardiovascular disease risk in diabetes is known to multiply in the presence of diabetic nephropathy (Valmadrid et al., 2000) (M B Mattock, 1992) (Buckley et al., 2009).It is important to be able to screen and accurately stage patients with diabetes and chronic kidney disease to enable timely intervention to delay progression as recommended by national and international guidelines (KDOQI, 2007, RCP, 2006).

The US National Kidney Foundation recommends estimating eGFR in subjects at risk of kidney disease such as patients with diabetes. Patients with CKD are classified primarily on the basis of the eGFR into different stages of severity. This has been endorsed in the UK. Furthermore, in patients with CKD, it is recommended that kidney function be followed regularly every 6 or 12 months in stable patients, depending on the stage of CKD.

Renal Association and the Department of Health, UK has recommended using the MDRD equation for estimating eGFR, as this equation was found to be superior to the previous estimates. The MDRD equation (Levey et al., 1999) takes into account the fact that the relationship between serum creatinine and GFR differs between Caucasians and African-Americans. Africans have a proportionally higher muscle mass compared to Asians and Caucasians, so for the same GFR the creatinine may be higher.

This equation was derived from a population with impaired renal function and only 6% of patients had type2 diabetes mellitus (Levey et al., 1999), this may explain the limitation of

the equation in predicting renal function in healthy subjects and in patients with diabetes mellitus.

Historically, proteinuria (and microalbuminuria) have been used to diagnose and assess progression of diabetic kidney disease, but there is increasing recognition of the high prevalence of non-proteinuric diabetic kidney disease (Pugliese G, 2010) and of the importance of Glomerular filtration rate (GFR) as a predictor of cardiovascular events, independent of proteinuria status (Florenz H, 2010). Moreover, treatment choices for concomitant conditions are also often influenced by estimates of renal function. GFR is a key indicator of kidney function, but measuring it with inulin clearance or isotopic methods are time consuming, expensive and clinically inapplicable. Hence kidney function is now commonly assessed and monitored using creatinine based estimated GFR (eGFR). When compared to the renal clearance of ^{125}I-Iothalamate, the equation developed from the Modification of Diet in Renal Disease (MDRD) study was shown to provide a more accurate estimate of GFR than measured creatinine clearance or other commonly used equations(Levey et al., 1999). Currently, the 4-variable MDRD formula, based on serum creatinine, age, sex, and race is the most widely employed (Carter et al., 2006). Most biochemistry laboratories now routinely report estimated GFR on samples sent for measurement of renal function.

A study by Chudleigh et.al, measured GFR in 293 subjects with newly diagnosed type 2 diabetes by [51]Chromium-EDTA clearance method and compared it with estimated GFR using Cockcroft-Gault equation and the 4-variable MDRD equation. The MDRD formula showed significant underestimation of GFR in subjects with newly diagnosed type2 diabetes without proteinuria and this bias increased with increasing body weight (Chudleigh et al., 2008). We

speculated that this bias continues into later stages of diabetic chronic kidney disease. This will have significant implications for management of patients with Type 2 diabetes, over 85% of whom are overweight or obese, where treatment choices are influenced by GFR. For example, metformin, a drug with proven cardiovascular benefits to overweight Type 2 diabetic patients(UKPDS, 1998) may be denied to them due to GFR underestimation.

The aim of the present study was to estimate the bias between isotopic glomerular filtration rate (GFR) and eGFR in patients with Type 2 diabetes and relate it to their body mass indices (BMI). We used non-corrected GFR measured using ^{51}Cr EDTA method and calculated 4v-MDRD eGFR using IDMS calibrated creatinine which has been proposed as a robust methodology to highlight the bias between GFR and eGFR (Delanaye et al., 2006).

Indexing GFR to body surface area in obese patients has been shown to introduce underestimation of the GFR. Because the number of nephrons does not increase with increasing body fat, increasing obesity must result in an increase in the single-nephron GFR. This has been discussed previously in the general introduction in the context of Pima Indian studies. Absolute GFR reflects this phenomenon whereas correcting GFR for BSA obscures it. Nyengaard and Bendsten estimated glomerular number in autopsy specimen using a method called unbiased stereological method and found the average glomerular number to be 617000. Glomerular number showed a negative correlation to age of the subject and positive correlation to kidney weight. BSA correlated positively to kidney weight and total glomerular volume but not to number of glomeruli (Nyengaard and Bendtsen, 1992). Another autopsy study on 35 neonates who died within 2 weeks of birth showed a negative correlation between glomerular number and size with low birth weight.

It has been proposed that being born with decreased number of nephrons (Nephron under-endowment) may increase risk from hypertension and the rate of progression of renal disease (Manalich et al., 2000).

Previous studies have recommended that uncorrected GFR must be preferred in routine practice and clinical studies for the obese population (Delanaye et al., 2005) (Delanaye et al., 2009). We report both uncorrected GFR and GFR indexed to BSA and also examine the difference between them in the obese and non-obese categories.

3.2 RESEARCH DESIGN AND METHODS

Study population

The study population comprised of 111 Caucasian subjects, 55 females and 56 males with type 2 diabetes recruited from diabetes clinics at Aintree University Hospital. They had a median age of 66, median HbA1c of 8.1% and median serum creatinine of 98 µmol/l. The eGFR (4v-MDRD) ranged between 18.5-to 124.8 mls/min/1.73 m^2.

The local research ethics committee approved the study and all participants gave written informed consent.

Serum Creatinine assay and eGFR calculation

Serum creatinine was measured using the kinetic alkaline picrate assay on an Olympus AU640 analyser.

The modified MDRD equation traceable to IDMS-creatinine and adjusted for Olympus methodology (ml/min per 1.73m^2):

175 x [((serum creatinine -16.14)/0.955) x 0.011312]$^{-1.154}$ x [age]$^{-0.203}$ x [0.742 if female] x [1.212 if AfroCarribean] was used to calculate eGFR (Reynolds and Twomey, 2007).

GFR measurement

We used ^{51}Chromium-EDTA clearance method, which is discussed in the method section.

Statistical analysis

The 4-v MDRD eGFR results were compared with isotopic GFR by means of two-tailed paired and unpaired *t* tests as appropriate. Results are presented as mean ± SD unless otherwise indicated. *P* < 0.05 was taken to indicate statistical significance. A Bland Altman plot was constructed using the SPSS programme to plot the difference and mean of the indexed GFR and eGFR estimated by 4 variable MDRD.

3.3 RESULTS

Table 3.1: Demographics and Bias between eGFR and measured GFR from all subjects

N	111
Male/female	56/55
Age (years)	65.7±10.7 (66.0[58.0-74.0])
Weight (Kg)	90.74±22.4 (87.9[73.9-102.8])
BMI (Kg/m^2)	33.0±7.3 (32.3 [27.7-37.5])
BSA (m^2)	1.97±0.25 (1.95[1.7-2.1])
HbA1c (%)	8.3±1.6 (8.1 [7.1-9.4])
Creatinine (µmol/L)	107.6±31.8 (98.0 [87-126])
Uncorrected GFR	85.4±38.8 (76.6 [56.7-108.0])
Indexed GFR	75.1±32.1 (68.8[51-91.5])
eGFR	66.7±24.2 (65.6[47.9-84.6])
Bias 1 (95% CI)	-18.70(-26.9 to -10.4) p=0.000
Bias 2	-8.45 (-15.7 to -1.1) p=0.024

Demographics and Bias between eGFR and measured GFR
Data presented as means ±SD (median [interquartile range]). Bias1 is the mean difference between eGFR and uncorrected measured GFR. Bias2 is the mean difference between eGFR and indexed GFR.

Table 3.2: Demographics and Bias between eGFR and measured GFR from non-obese subjects (BMI<30)

N	37
Male/female	23/14
Age (years)	69.3±11.2 (72[63-77])
Weight (Kg)	71.9±11.5 (71.0[63.1-80.3])
BMI (Kg/m^2)	25.3±2.8 (25.9 [23.2-27.9])
BSA (m^2)	1.81±0.17 (1.82 [1.6-1.9])
HbA1c (%)	8.7±1.7 (8.3 [7.4-9.5])
Creatinine (μmol/L)	103±23.3 (99.0 [87.5-113.0])
Uncorrected GFR	74.6±24.7 (71.4 [54.9-86.9])
Indexed GFR	71.4±24.1 (65.9 [51.4-83.3])
eGFR	68.6±23.0 (69.4 [52.9-87.2])
Bias 1 (95% CI)	-6.04 (-17.6 to- 5.5) p=0.299
Bias 2	-2.86 (-14.1 to 8.3) p=0.609

Demographics and Bias between eGFR and measured GFR
Data presented as means ±SD (median [interquartile range]). Bias1 is the mean difference between eGFR and uncorrected measured GFR. Bias2 is the mean difference between eGFR and indexed GFR.

Table 3.3: Demographics and Bias between eGFR and measured GFR from obese subjects

N	74
Male/female	33/41
Age (years)	63.9±10.0 (64.0 [57.0-73.0])
Weight (Kg)	100±20.6 (97.2 [86.1-112.7])
BMI (Kg/m^2)	36.8±5.6 (35.1 [32.3-40.3])
BSA (m^2)	2.05±0.24 (2.02 [1.89-2.21])
HbA1c (%)	8.1±1.5 (8.0 [6.8-9.3])
Creatinine (µmol/L)	109.6±35.2 (97.5 [84.5-128.0])
Uncorrected GFR	90.8±43.4 (88.4 [58.0-110.8])
Indexed GFR	77.0±35.4 (71.1 [49.4-97.0])
eGFR	65.7±24.9 (62.1 [46.2-84.9])
Bias 1 (95% CI)	-25.02 (-35.9 to -14.1) p=0.000
Bias 2	-11.25 (-20.7 to -1.7) p=0.021

Data presented as means ±SD (median [interquartile range]). Bias1 is the mean difference between eGFR and uncorrected measured GFR. Bias2 is the mean difference between eGFR and indexed GFR.

The 4-v MDRD formula significantly underestimates GFR in the obese patients with Type 2 diabetes as shown in Fig.3.1 (a). A significant bias persisted into CKD stage 3 in the obese (BMI>30) subjects as shown in fig.3.1 (b).
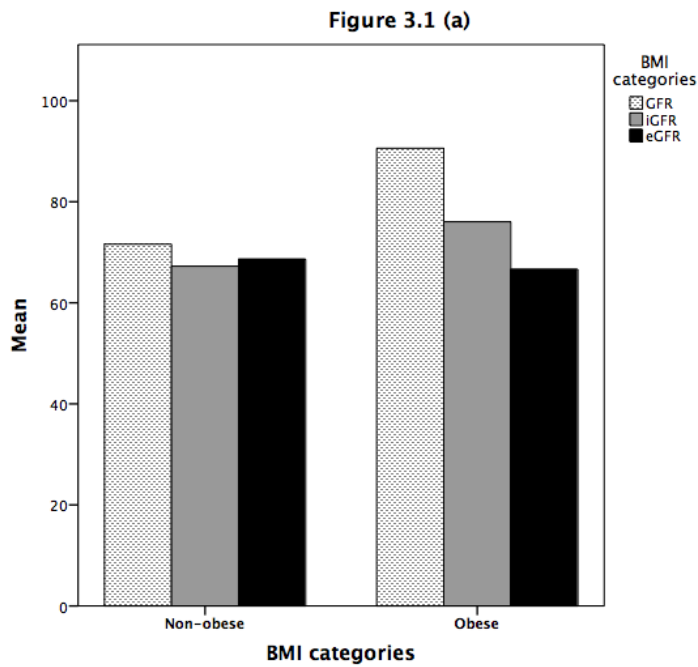
Figure 3.1 (a)

Figure 3.1:

(a) Showing the bias in the obese and non-obese categories. The figure also shows significant reduction in measured GFR when it is indexed to BSA in the obese population.

(b) Shows the bias in chronic kidney disease stages 1-4 in the obese subjects with type2 diabetes.

Figure 3.1 (b)

Figure 2. Correlation between Bias and BMI

Pearsons corelation=0.535
p=0.000
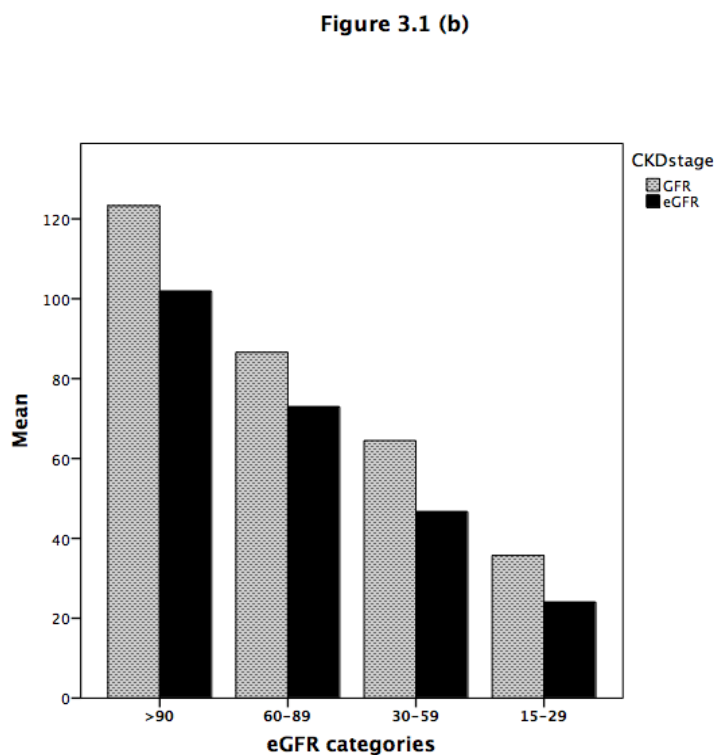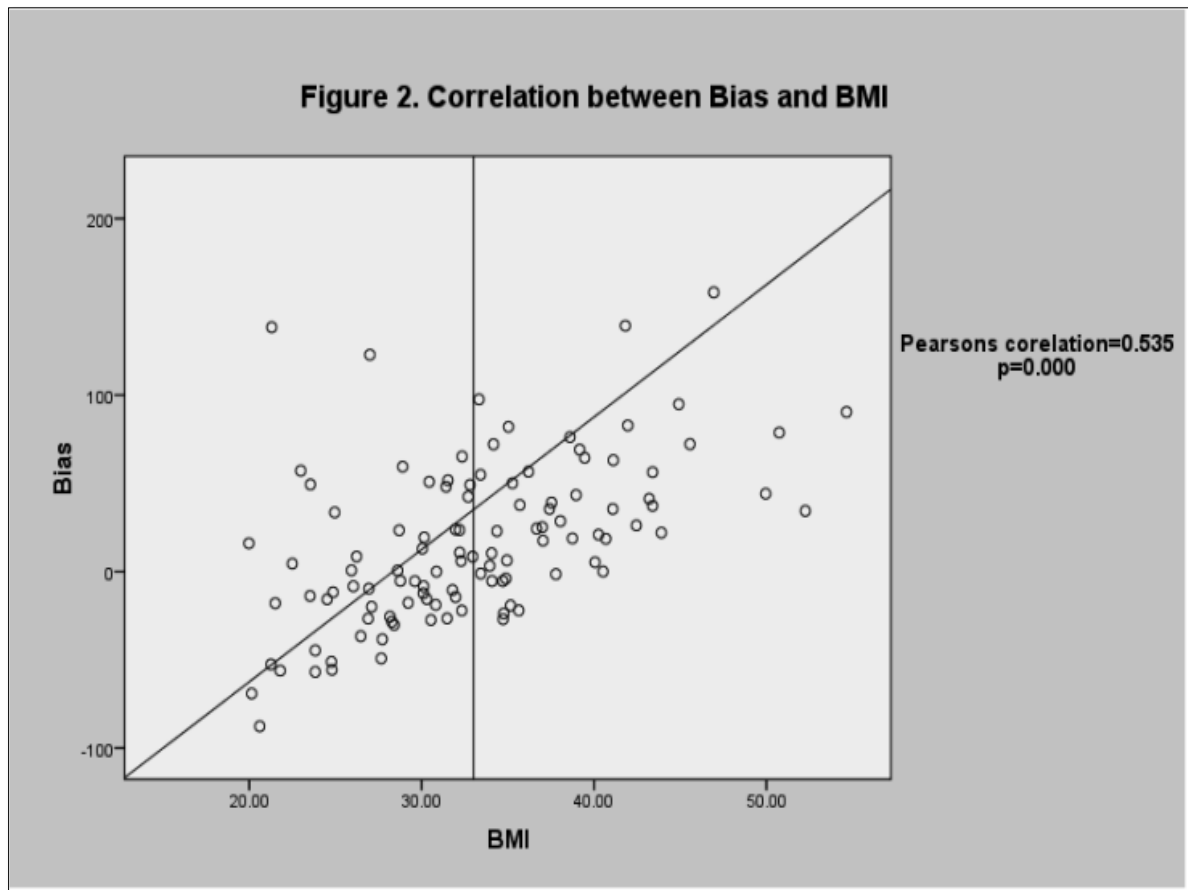
Figure 3.2: Graph above shows a significant positive correlation between the difference between indexed GFR and eGFR and BMI across the whole study population

It was also noted that the mean body surface area increased with increasing body mass indices and was 2.05±0.24 m^2 in the obese subjects. The mean difference between the uncorrected GFR and the corrected GFR (indexed GFR) was 13.02±53.2 (p=0.039) in the obese subjects. This discrepancy between the measured GFR before and after indexing to a BSA of 1.73 m^2 can also be visualised from fig1 (a).
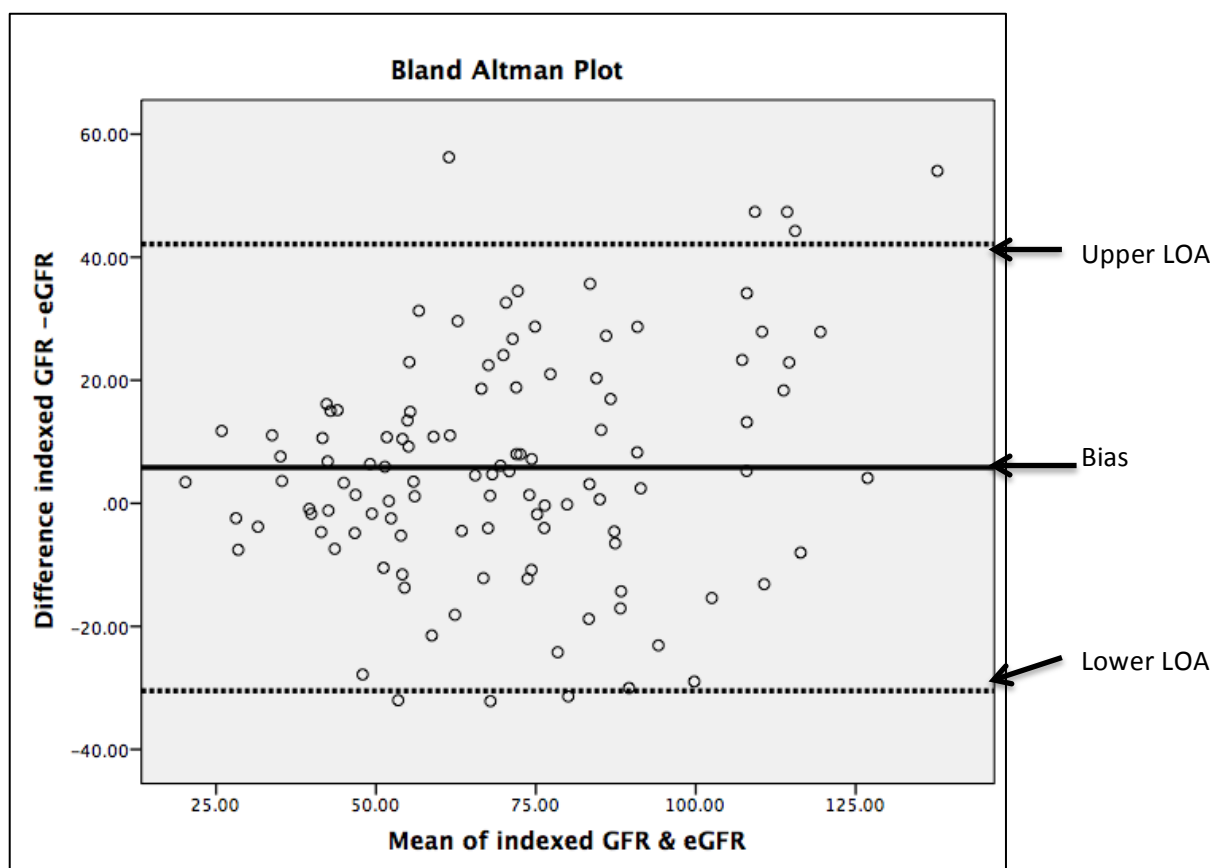
Figure 3.3: Bland Altman plot of the difference between indexed GFR and 4-v MDRD eGFR and the mean of the two measurements from the whole study population. The continuous line represents the mean of the difference between the 2 measure and the dotted lines represent the upper and lower 95% confidence intervals.

3.4 DISCUSSION

Our study shows that the 4-v MDRD formula underestimates GFR in overweight and obese patients with Type 2 diabetes. The bias between estimated and measured GFR in the obese type 2 diabetic subjects persists across the range of CKD stages. This may have Implications for management of obese patients with Type 2 diabetes, where treatment decisions in relation to management of hyperglycaemia, hypertension and other concomitant conditions are often determined by estimation of GFR.

The bias between measured and estimated GFR translates to a misclassification of study subjects in the obese category into higher CKD groups. 40% of subjects in CKD stage 2 were misclassified into stage 3 and 54% from stage 3A to stage 3B.

One of the limitations of the study is that GFR measurement using the slope intercept method may not be accurate in patients with GFR less than 30 mls/min/1.73m^2. The tracer is substantially equilibrated by 2 hours in the normal subject, but complete equilibration could take much longer. But the fraction not equilibrated by 2 hours is relatively very small and is insignificant (Chantler and Barratt, 1972). However, in the presence of renal failure defined as GFR less than 15 mls/min/1.73m^2, the apparent clearance of the small fraction becomes more important. Some author recommends plasma sampling for 6 to 8 hours in these circumstances (Maisey et al., 1969). This might have contributed partially to the very high bias that we saw in our cohort of patients with CKD stage 4.

Rule et al. studied MDRD equation in CKD with healthy kidney donors all of whom had an Iothalamate clearance GFR measurement. These analyses indicate that although the abbreviated MDRD equation was reasonably accurate in patients with chronic kidney

disease, it significantly underestimated GFR in healthy persons. The authors proposed that this was probably due to the exclusion of healthy persons from the study participants used to develop this equation (Rule et al., 2004). The study by Chudleigh *et al.* also with subjects with newly diagnosed diabetes and no known kidney disease showed significant bias between MDRD eGFR and measured GFR. Our study complements these results by examining the bias between measured and estimated GFR in subjects with CKD stages 1 to 4, which include the particularly important category with eGFR between 30 and 60 where treatment decisions are often determined by an eGFR cut-off value. Also unlike in the early CKD stages, a percentage bias in the more advanced CKD stages will have significant clinical implications.

The Cockcroft-Gault formula (140-age) X body weight/plasma creatinine X 72 X (0.85 if female) was established in 1976 to predict creatinine clearance (Cockcroft and Gault, 1976). As body weight is factored in the numerator in this formula, it overestimates clearance in the obese subjects. In a cross sectional study using data from 271 potential kidney donors who underwent GFR measurement using [125]I Iothalamate clearance method, Cockcroft-Gault formula had significantly higher bias than MDRD or CKD-EPI formulae in patients with the highest BMI. MDRD provided 97% accuracy compared to the measured GFR while Cockcroft-Gault formula only provided 57% in the obese subjects (Michels et al., 2010). Another study comparing Cockcroft-Gault and MDRD equations with GFR measured by ^{99}Tc DTPA showed that Cockcroft-Gault underestimates GFR in lean people (-13 mls/min/1.73m^2) and overestimates GFR in the overweight (+10 mls/min/1.73m^2) (Verhave et al., 2005). These observations suggest that Cockcroft-Gault formula may not be suitable for use in people with high BMI.

Most national and international guidelines advise close monitoring of patients on metformin if eGFR is less than 45ml/min/1.73m^2. More than half of our obese subjects with eGFR >45 were misclassified into the lower category by the 4v-MDRD equation. This can lead to earlier than necessary initiation of insulin in these patients with possible adverse effects like weight gain and hypoglycaemia. Obesity is associated with significant reduction in insulin sensitivity (Jallut et al., 1990). The dose of insulin required is hence often very high in the obese patients which lead to further cost escalation. As we move away from a glucocentric approach to managing basic pathophysiology in type 2 diabetes (Defronzo, 2009), the overweight and obese patients may be denied important therapeutic options because of an underestimated GFR.

This study also highlights the problem with indexing measured GFR as it is practiced now in the obese population. Indexing is based on the assumption that the average BSA is 1.73m^2 but our study shows it to be much higher in the obese subjects. As GFR is indexed by multiplying it by a factor of 1.73/BSA, the higher the BSA the lower will be the indexed GFR. This practice of indexing does not cause major difference in the normal weight and the slightly overweight populations, but it leads to significant underestimation of the GFR in the obese subjects. Our obese study subjects had an average BSA of 1.9m^2. Part of the underestimation of GFR in obesity by the MDRD equation is likely to be due to the fact that it was derived from indexed GFR. Our data shows worse bias when MDRD eGFR is compared with unidexed GFR.

In conclusion, the 4-variable MDRD equation significantly underestimates GFR in obese patients with type2 diabetes. There is a need for better equations for estimating GFR in the obese subjects which are validated against the gold standard isotopic GFR in this population.

Until such an equation becomes available, clinicians should consider this bias when making treatment decisions in obese patients with type2 diabetes.

CHAPTER 4

**THE PERFORMANCE OF CHRONIC KIDNEY DISEASE
EPIDEMIOLOGY COLLABORATION (CKD-EPI) FORMULA
COMPARED TO THE 4-VARIABLE MDRD EQUATION IN EGFR
IN TYPE 2 DIABETIC CHRONIC KIDNEY DISEASE**

4.1 Introduction

The Chronic Kidney Disease Epidemiology collaboration formula was developed to improve estimation of GFR from serum creatinine and other readily available clinical parameters especially when GFR is >60 mL/min per $1.73m^2$. There are several studies showing MDRD equation to have significant bias in patients with higher GFR.

Poggio et al. compared MDRD and CG formulae against measured ^{125}I-iothalamate GFR in 828 patients with chronic kidney disease and 457 potential kidney donors with normal GFR. In the kidney donor group, MDRD equation significantly underestimated the measured GFR when compared with the CG formula, with a bias of −9.0 versus 1.9 ml/min per 1.73 m2, respectively (P < 0.01). In this study neither the MDRD equation nor the CG formula adequately represented the relationship between measured GFR and serum creatinine in kidney donors. The authors concluded that this is probably due to the narrower range of renal function in the donors than the CKD group and also differences in serum creatinine between two donor patients are likely to predominantly reflect measurement error and non-renal biologic factors (Poggio et al., 2005).

Ibrahim et al. sowed similar bias in a study comparing the performance of Modification of Diet in Renal Disease (MDRD) and the Cockcroft-Gault formulae against GFR measured by Iothalamate clearance in 1286 individuals from the Diabetes Control and Complications Trial (DCCT). The DCCT participants had normal serum creatinine and were also younger compared to MDRD study population, which led to biased and highly variable estimates of GFR when these formulae were applied to the DCCT subjects. In the range of GFR observed in the DCCT population and analyzed for this validation effort (60 to 300 ml/min per 1.73

m^2), the MDRD and the CG formulas underestimated renal function at levels of GFR that were <120 ml/min per 1.73 m2 and so individuals with levels of renal function between 60 and 120 ml/min per 1.73 m2 are more likely to have a measured GFR that is higher than that predicted by the equations (Ibrahim et al., 2005).

Levey et al. developed a new equation to estimate GFR called the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation (Levey et al., 2009). They intended to respond to the limitations of MDRD equation in estimating GFR in the normal renal function range. Their aim was to develop a new equation that worked as well as the MDRD equation at GFR less than 60-ml/min/1.73 m2 and more accurately at higher GFR. Data were obtained from clinical studies and clinical populations who had their GFR measured using an exogenous filtration marker. They had 8254 participants for development (5504 participants) and internal validation (2750 participants) and 3896 participants, for external validation. They used linear regression analyses to relate measured GFR and available clinical characteristics. The independent variables included were serum creatinine, age, race, and gender in all models as in the original MDRD study equation derivation. Some models also included diabetes [yes/no], previous organ transplantation [yes/no], and weight. Performance of these models were compared with each other and the MDRD equation, and based on performance and ease of application, they selected a single model as the best equation for general use called the CKD-EPI equation. Although other factors such as weight, diabetes, and transplant were considered as potential variables during its derivation, the final equation that was proposed had the same four variables as the MDRD equation (Levey and Stevens, 2010).

The CKD-EPI equation is expressed as:

GFR = 141 X min (Scr/κ, 1)^{α} X max(Scr/κ,1)$^{-1.209}$ X 0.993^{Age} X 1.018 [if female] X 1.159 [if black]

Where Scr is serum creatinine (mg/dL), κ is 0.7 for females and 0.9 for males, α is –0.329 for females and –0.411 for males, min indicates the minimum of Scr/κ or 1, and max indicates the maximum of Scr/κ or 1.
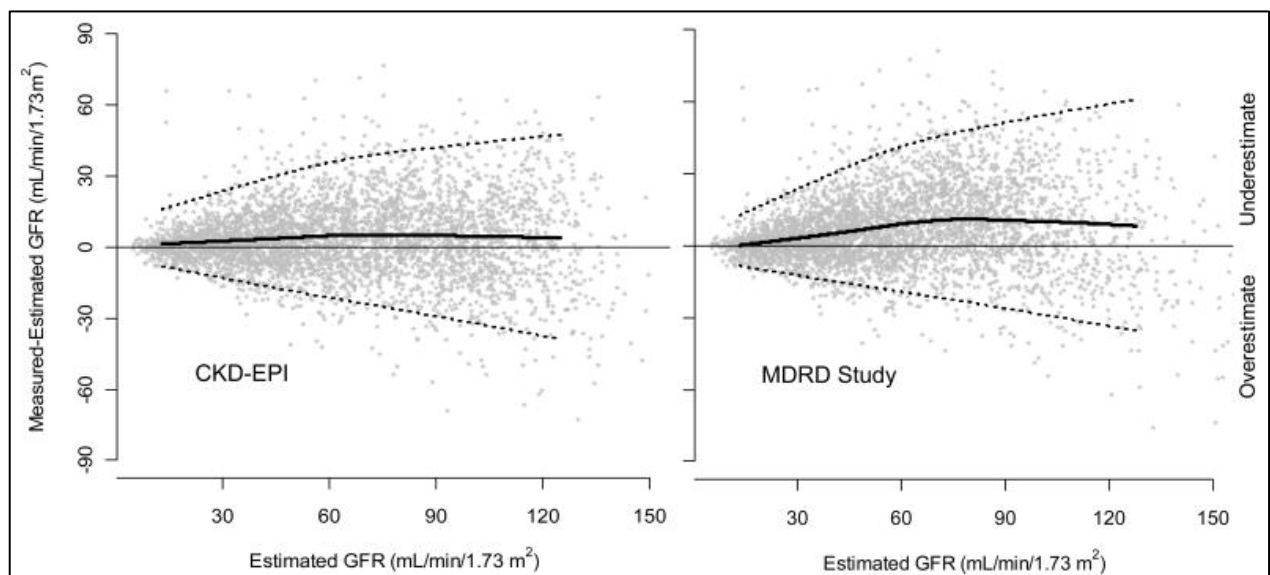

Figure 4.1: (reproduced with permission) shows improvement in the bias between estimated and measured GFR in the >60 ml/min/1.73m^2 group of subjects in the first panel (CKD-EPI) compared to the second panel (MDRD)

The CKD-EPI equation has been evaluated in a large metaanalysis by the CKD prognosis consortium. To be included in the consortium a study had to have over 1000 participants, have information at baseline of eGFR and urine albumin levels and a minimum of 50 events for any of the outcomes of interest (all cause/CV mortality and ESRD). The metaanalysis of data from 1.1 million adults from 25 general population cohorts, 7 high risk cohorts and 13 CKD cohorts showed that CKD-EPI equation classified fewer individuals as CKD and more accurately categorised the risk of mortality and ESRD than MDRD equation. The mean eGFR was higher with CKD-EPI than MDRD equation in the general population but comparable in the CKD cohort. Also the participants reclassified upwards had lower risk of mortality and ESRD and the authors concluded that CKD-EPI is a better predictor of risk factors. As the equation has the same variables as the MDRD equation, there is no additional laboratory costs involved its implementation. They also suggest that estimated GFR reporting by CKD-EPI equation could contribute to more efficient and targeted prevention and management of CKD-related outcomes (Matsushita et al., 2012).

Camargo et al. analyzed the performances of Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) and of Modification of Diet in Renal Disease (MDRD) study equations to estimate glomerular filtration rate (GFR) in 56 patients with Type2 diabetes mellitus with GFRs >60 ml/min, and 55 healthy volunteers. Glomerular filtration rate was measured by the ^{51}Cr-EDTA single-injection method (^{51}Cr-GFR). In diabetic individuals, ^{51}Cr-GFR was 106 ± 27ml/min/1.73 m^2, CKD-EPI-estimated GFR 82 ± 18 ml/min/1.73 m^2 and MDRD-estimated GFR 80 ± 21 ml/min/1.73 m^2 (P < 0.001). In healthy volunteers, the corresponding values were 98 ± 20, 89 ± 13 and 84 ± 14 ml/min/1.73 m^2 (P < 0.001). The

accuracy of CKD-EPI was higher in healthy volunteers than in diabetic patients (90 vs. 66%, respectively, P < 0.001). They concluded that CKD-EPI equation performed as poorly as the MDRD equation in individuals with Type 2 diabetes (Camargo et al., 2011).

The aim of our study was to examine the bias between measured and estimated GFR (using CKD-Epi and MDRD equations) in the obese and non-obese groups in our Database.

4.2 Methods and Results

We investigated the bias between 4 variable MDRD eGFR and measured GFR in 111 patients with type 2 diabetes who had chronic kidney disease stages 1 to 4 (S Nair, 2011). We measured GFR using our reference method of ^{51}Cr-EDTA plasma clearance method and indexed it to a BSA of 1.73m^2. Serum creatinine was measured using the kinetic alkaline picrate assay on an Olympus AU640 analyser. The modified MDRD equation traceable to IDMS-creatinine and adjusted for Olympus methodology was used for estimating GFR and expressed as ml/min per 1.73m^2. We calculated GFR using the CKD-EPI equation (Levey and Stevens) and also its bias when compared to GFR and GFR indexed to a BSA of 1.73m^2 as shown in the table 1.

The study population comprised of 111 Caucasian subjects, 55 females and 56 males with type 2 diabetes recruited from diabetes clinics at Aintree University Hospital. They had a median age of 66, median HbA1c of 8.1% and median serum creatinine of 98µmol/l. The eGFR (4v-MDRD) ranged between 18.5 to 124.8 ml/min/1.73 m^2.

Table 4.1: MDRD vs CKD Epi equation

	4 v MDRD equation	CKD Epi equation
eGFR	67.2 ± 27.3	51.13 ± 17.7
eGFR– GFR	-16.9 (-25.0, -8.4) p<0.001	-34.2 (-41.7, -26.7) p=0.024
eGFR– Indexed GFR	-5.8 (-9.3, -2.3) p=0.01	-24.04 (-30, -17) p<0.001

4.3 Discussions

One of the criticisms of the CKD-EPI equation is that high-risk patients with clinical CKD, characterized by an average measured GFR <60 ml/min per 1.73 m^2 constituted 71% (n = 5858) of the development population. This is despite the fact that the equation was expected to improve estimation of higher GFR. The external validation was performed using a sample size of 3896, 72% (n = 2810) of whom came from high-risk populations and the rest had GFR>60 ml/min per 1.73 m^2. This may be the reason the CKD-EPI equation performed better in the external validation sample than MDRD equation that was derived using only a high-risk population. However the authors recognized that a single equation is unlikely to work equally well in all populations (Rule, 2010).

As shown by Camargo et al, analysis of our study cohort also shows significantly worsened bias when eGFR was calculated using the CKD-Epi compared to the 4 variable MDRD formulas in patients with type2 diabetes and chronic kidney disease.

The bias is potentially multifactorial; in the original MDRD study (Levey et al., 1999) from which the equation was derived the mean body weight was 79.6 ± 16.8 kg and hence not typical of a type 2 diabetic patient population, the other factor is the inaccuracies introduced by indexing glomerular filtration rate to body surface of $1.73m^2$. The mean body surface area in our obese category was much higher at 2.05±0.24. We would also like to point out that the study population in our study had a much higher BMI at 36.8±5.6 and a wider range of eGFR 66.6±25.0, which may account for the differences observed by Camargo *et al. (Camargo et al., 2011).*

Our study, suggests this may be due to high rates of obesity in type2 diabetes. Our study population is also different to the CKD-EPI population in some important baseline characteristics. The patients with diabetes only accounted for 30% of the CKD-EPI population; they had a mean BMI of 28 and BSA of $1.93m^2$. In comparison all our patients had diabetes, the mean BMI was 33 and mean BSA was 1.97 m^2. The mean GFR in the CKD-EPI population was 68 mls/min/1.73 m^2 compared to our study population which had a mean GFR of 85.4 mls/min/1.73 m^2. It is possible that having more subjects in the higher GFR range (>60 mls/min/1, 73 m^2) in our study makes the CKD-EPI equation less reliable.

Although CKD-EPI was proposed to be more generalizable than MDRD in various clinical settings, it has the same 4 variables as the MDRD equation. It was also developed from a population of which >70% were high risk (defined by GFR<60 mls/min/1.73 m^2). Some authors believe that CKD-EPI performed better than MDRD in the validation cohort purely because MDRD was derived exclusively from a high-risk population (Rule, 2010).

The authors of CKD-EPI equation did suggest that a single equation is unlikely to work for all populations. Our study population of patients with type2 diabetes is likely to be different from the general population in terms of their BMI, BSA and GFR.

CHAPTER 5

DERIVATION OF OBESITY CORRECTION EQUATION FOR THE

4-VARIABLE MDRD EQUATION

5.1 INTRODUCTION

The aim of the present study was to measure the effect of obesity on estimate GFR and to derive a 'correction factor' for the 4-variable MDRD equation to adjust for the equation's underestimation of true (isotopic) GFR in obese subjects with Type 2 diabetes. We studied diabetic subjects with CKD stages 1 to 4 (eGFR 90 to 30 mls/min per 1.73 m2), where treatment decisions may be influenced by eGFR and formulated linear non-linear regression equations to correct the bias between estimated and measured GFR in our study population. Correction coefficients for the MDRD equation for Chinese and Japanese ethnicity have been reported previously (Ma et al., 2006, Matsuo et al., 2009), but there are no established correction factors for the obese.

 Saracino et al. proposed a correction factor for the overestimation of creatinine clearance by Cockcroft Gault formula in obesity. They simultaneously determined creatinine clearance using 24-hour urine collection (Cr-cl) and the Cockcroft Gault clearance (CG-cl) in 52 subjects with a body mass index (BMI) > 25. The percentage difference between the 2 clearances (delta %) was correlated with BMI for each patient using simple linear regression analysis and derived the following CG-cl correction formula for obese subjects: Corrected CG-cl = CG-cl (1.25 - 0.012 BMI). This was validated in 20 subjects with BMI>25 and shown to correlate well with 24-hour urine collection creatinine clearance (Saracino et al., 2004)

We also used our correction equations in an external dataset to establish their external validity.

5.2 RESEARCH DESIGN AND METHODS

5.2.1 Study population

The study population comprised 55 females and 56 males with type2 diabetes. They had a mean age of 65.7±10.7, median HbA1c of 8.1% and median serum creatinine of 98 µmol/l. The mean eGFR (4v-MDRD) was 67.29 ± 24.32.

An external dataset with 169 obese subjects with newly diagnosed type 2 diabetes from a study conducted in Wales (Chudleigh et al., 2008) with similar methodology served as the "validation set". The study subjects had a mean age of 55.2, mean BMI of 31.52, HbA1c of 7.79 and Creatinine of 80. The mean eGFR was 114.86 ± 22.35.

Serum Creatinine assay and eGFR calculation

Serum creatinine was measured using the kinetic alkaline picrate assay on an Olympus AU640 analyser.

The modified MDRD equation traceable to IDMS-creatinine and adjusted for Olympus methodology (ml/min per $1.73m^2$):

175 x [((serum creatinine -16.14)/0.955) x 0.011312]$^{-1.154}$ x   [age]$^{-0.203}$ x [0.742 if female] x [1.212 if AfroCarribean] was used to calculate eGFR (Reynolds and Twomey, 2007).

This equation has a power law dependence on serum creatinine and age (i.e., eGFR varies as a power of creatinine and age. Relatively small changes to creatinine and age could have

disproportionately high effect on eGFR), together with ratio correction factors for gender and ethnicity

The external dataset serum creatinine was measured using the OCD dry-slide system on the Vitros 750 X RC and 950analyser (HP12 4DP: Johnson & Johnson, High Wycombe, UK). The correction factor for creatinine for the OCD dry-ice method is (Creatinine – 7.71)/0.988. So the $eGFR_{175}$ MDRD equation used was:

175 x [{(serum creatinine – 7.71)/0.988} x 0.011312]$^{-1.154}$ x [age]$^{-0.203}$ x [0.742 if female] x [1.212 if AfroCarribean]

GFR measurement

We used [51]Chromium-EDTA clearance method to measure GFR, which is discussed in detail in the methods section of this thesis. Measure GFR indexed to BSA is referred to as iGFR in the subsequent regression analyses.

GFR measurement in the external dataset was also using the [51]Chromium-EDTA clearance method and corrected for BSA.

5.2.2 Statistical analysis

Statistical analysis was carried out using SPSS (version 16.0 for Windows: SPSS, Chicago, IL) and Microsoft Office Excel 2007. Differences in bias with various equations were compared

using Student's *t* test for paired samples. Pearson's correlation coefficients were calculated to estimate the linear correlations between measured GFR and eGFR corrected using the various equations.

Bland Altman plots were also constructed using SPSS software by plotting the mean of the two measurements on the x-axis and the paired difference between them on the y-axis.

5.3 RESULTS

The 4-v MDRD formula underestimates GFR in overweight /obese patients with Type 2 diabetes. This bias is greatest in the obese and persists after correction for body surface area.

5.3.1 Correction of the bias using linear non-linear equations

Given the form of the MDRD formula, it is mathematically appropriate to fit the measured GFR against eGFR and the corrective factor BMI, using an additive linear model and also to fit the relation of measured to estimated GFR with additive or power law models. Applying this approach led us to derive the following 3 possible correction equations from our data. The three proposed predictive equations along with their Pearson's correlation coefficients and also Bland Altman plots compared to indexed GFR are presented.

1. Multiple linear regression analysis with iGFR as the outcome variable and eGFR and BMI as the independent variables produced this correction equation to predict GFR

Predicted (p) GFR-1 = 0.88*(BMI)+0.88*(eGFR)-15.6

Pearsons correlation with measured GFR = 0.797
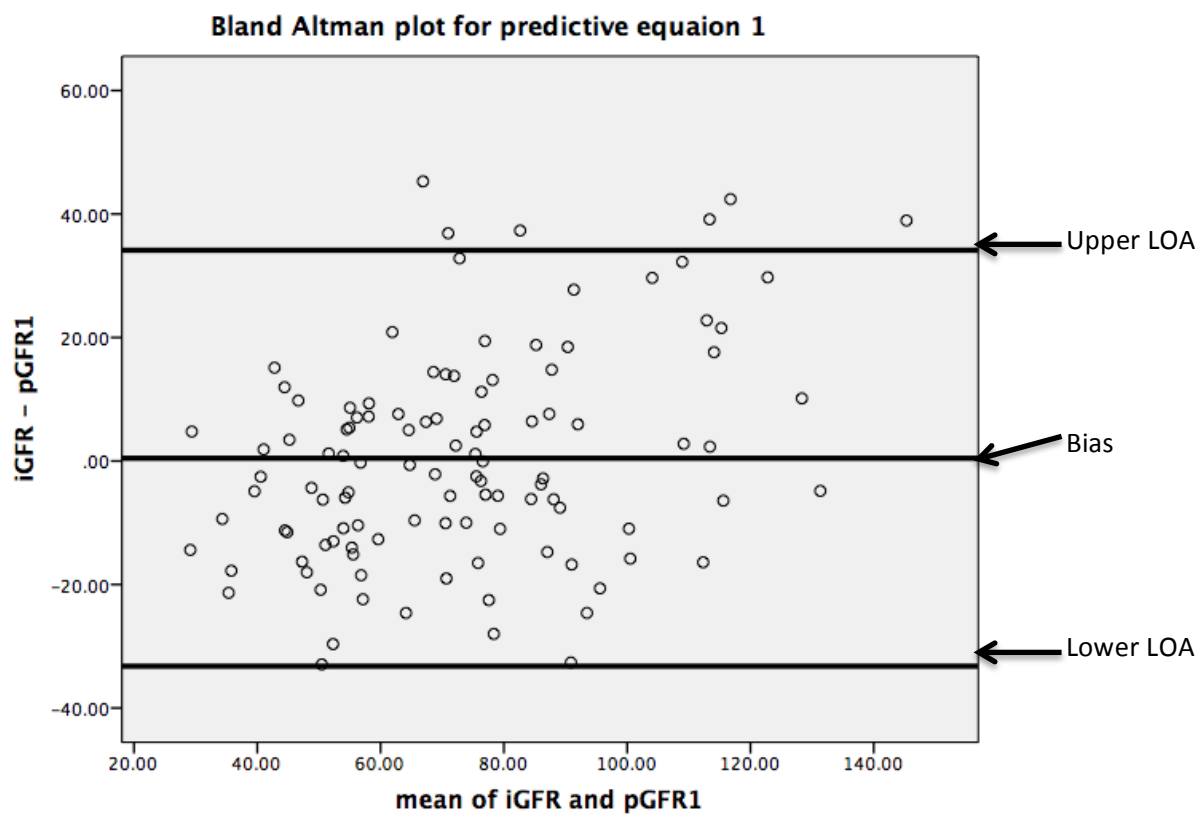
Fig 5.1: Bland Altman plot between the difference and mean between indexed GFR and the predictive equation 1. The bold line is the mean and dotted lines are the 95% confidence intervals representing upper and lower limits of agreement (LOA).

2. This model was obtained by fitting the log (iGFR) using log (BMI) and log (eGFR) as independent covariates: Log (pred_GFR) = log (BMI) x 0.322 + log (eGFR) x 0.812 - 0.264, leading to temp= $bmi^{0.32}$ x $egfr^{0.81}$ and consequently to

pGFR-2 = 0.808*$bmi^{0.32}$ x $egfr^{.812}$ -0.599

**Pearsons correlation with measured GFR = 0.808**
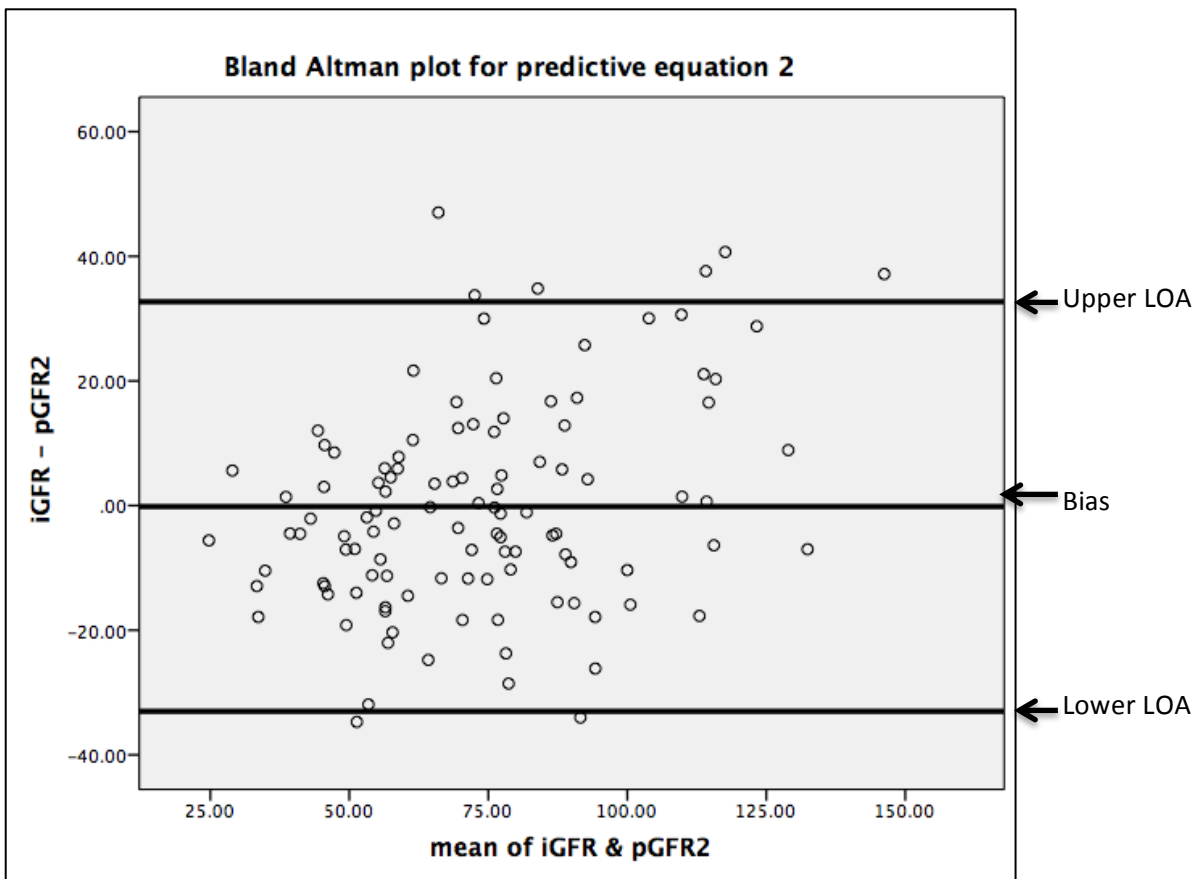
Figure 5.2: Bland Altman plot between the difference and mean between indexed GFR and the predictive equation 2. The bold line is the mean and dotted lines are the 95% confidence intervals representing upper and lower limits of agreement (LOA).

3. A ratio model obtained by modelling iGFR/eGFR as a linear function of BMI and slightly approximating the parameters: 0.0062->0.005 and 0.8889->1. This reduces the correlation coefficient with rGFR by only ½ of 1 %, from 0.7983 to 0.7943 and provides a much simpler equation:

pGFR-3 / eGFR = 0.010 * BMI +0.792

**Pearsons correlation with measured GFR = 0.804**
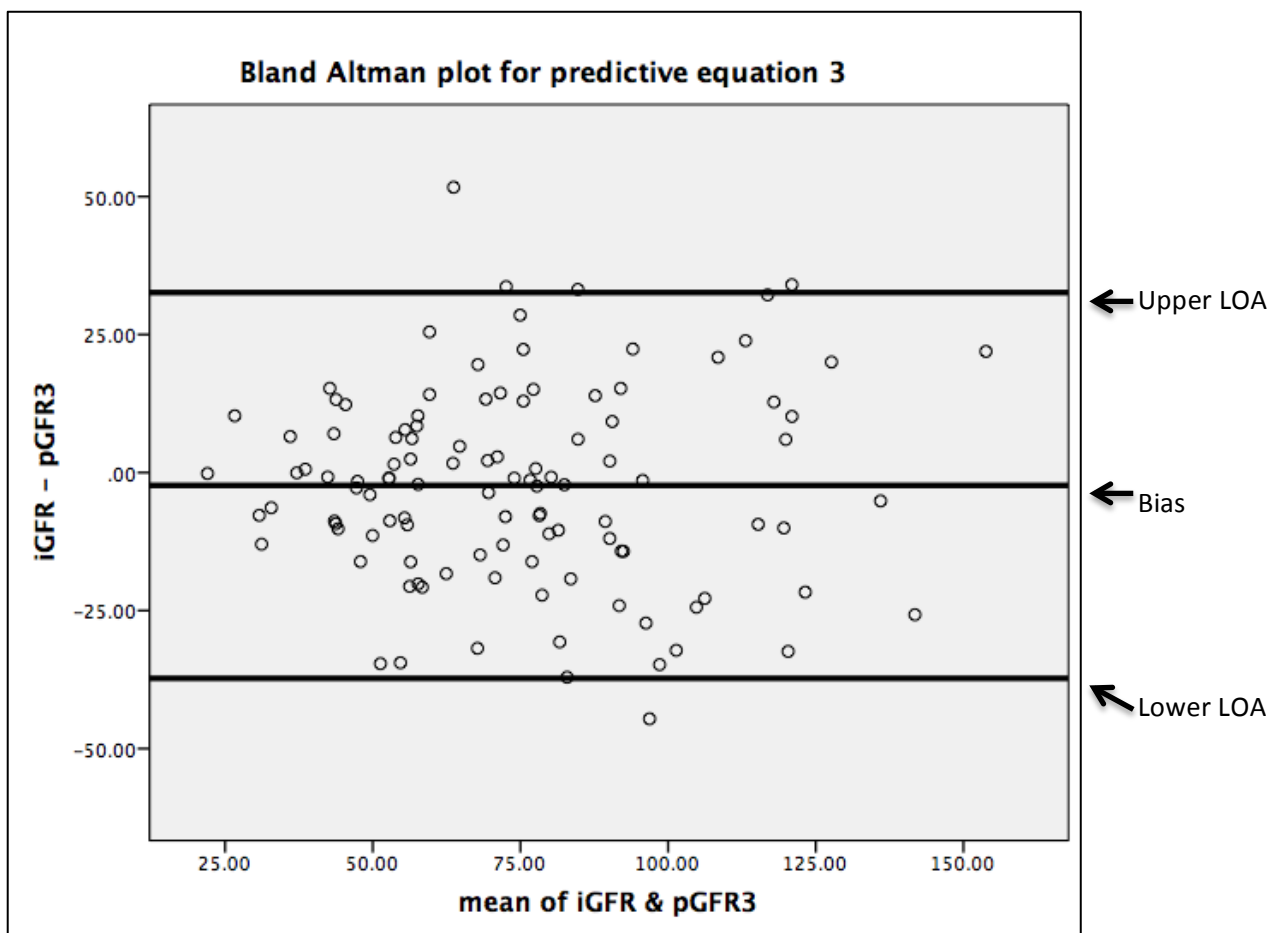
Figure 5.3: Bland Altman plot between the difference and mean between indexed GFR and the predictive equation 3. The bold line is the mean and dotted lines are the 95% confidence intervals representing upper and lower limits of agreement (LOA).

Figure 5.4: shows the reduction in bias by 3 predictive equations compared to the 4v-MDRD equation. The CKD-EPI formula performs worse than the 4v-MDRD equation in our dataset.
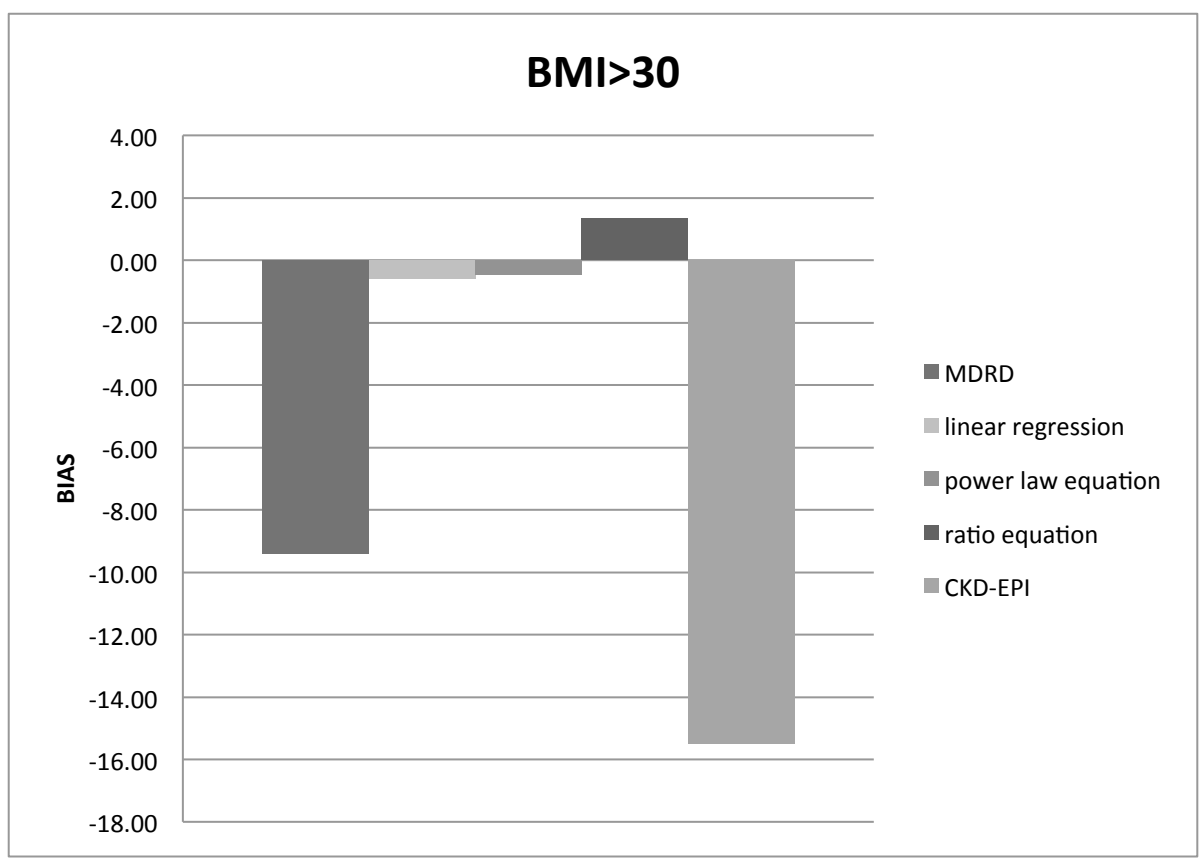
5.3.2 Validation in the external dataset

We validated the linear and 2 non-linear equations in the external dataset, all of which showed significant reduction in the bias in the obese subjects as shown in figure 3. The predictive equations reduced bias by 17.5%, 22.5% and 59.1% respectively compared to the 4v-MDRD equation.

Figure 5.5: Graph showing the performance of correction equations in an external dataset
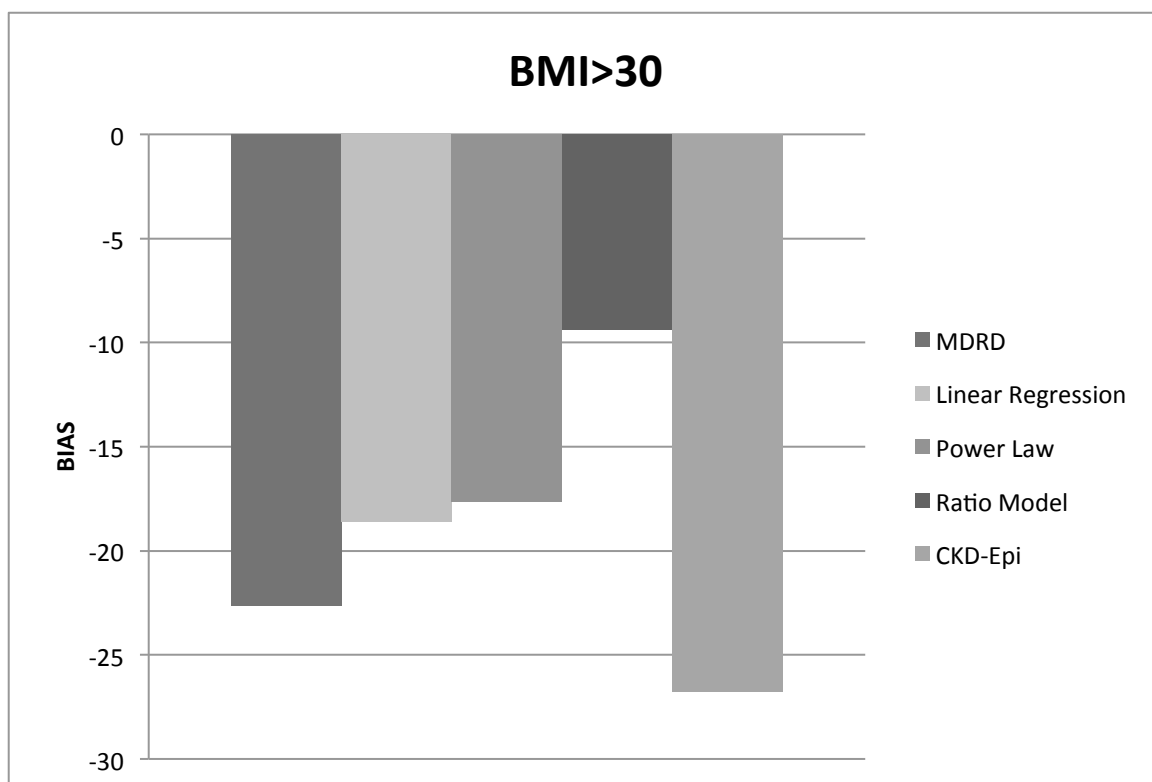
5.4 DISCUSSION AND CONCLUSIONS

In this study we derived equations employing regression analysis from our dataset of obese patients with type 2 diabetes and various stages of CKD to correct underestimation of GFR by the 4v-MDRD equation in this population. Our study as in chapter 3, confirms observations made by others that the 4-v MDRD formula underestimates GFR in overweight and obese patients with Type2 diabetes. The bias between estimated and measured GFR is greatest in the obese and persists after correction for body surface area. This may have Implications for management of obese patients with Type 2 diabetes, where treatment decisions in relation to management of hyperglycaemia, hypertension and other concomitant conditions are often determined by estimation of GFR.

The linear and non-linear equations derived from our study reduce the bias between measured and estimated GFR significantly in the external dataset; this improvement being more pronounced in the obese subjects and is best achieved by the ratio model equation. This equation is the only predictor to score consistently well across all three ranges of GFR studied, including good positive predictive value in CKD stage 3 in the obese. This simple corrective factor, which we have externally validated, can be used when making management decisions in the obese with type2 diabetes based on eGFR. This is particularly relevant when contemplating starting or withdrawing drugs such as metformin or the newer GLP-1 based agents in the overweight / obese patients with type 2 diabetes. This study highlights the significant problem of underestimation of GFR in the obese with type2 diabetes and proposes a possible solution using a mathematical correction factor.

CHAPTER 6

EFFECT OF A COOKED MEAT MEAL ON CREATININE AND EGFR IN

DIABETES RELATED CHRONIC KIDNEY DISEASE

6.1 INTRODUCTION

As discussed in previous sections, it is important to be able to screen and accurately stage patients with chronic kidney disease (CKD) and intervene as recommended by national and international guidelines (KDOQI, 2007, RCP, 2006).

Variations in serum creatinine could lead to misclassification of patients to a particular CKD stage with clinical implications to the patient and cost implications to services. Some of the factors known to be responsible for biological variability in Creatinine values are: ingestion of cooked meat, fluid status, diurnal variation and delay in centrifugation of blood samples (Diskin, 2007). Most blood samples in the original modification of diet in renal disease (MDRD) study were drawn after overnight fasting (Preiss et al., 2007). This is however overlooked in most clinical situations, and laboratories calculate eGFR routinely from all blood samples sent for renal function tests.

The intra individual biological variation in creatinine measurement is significantly higher in people with CKD (CV= 5.3%) than in healthy people (CV=2.7%) (Holzel, 1987).

Creatine is stored in muscle as phosphocreatine and is an important source of energy. Creatine turns into creatinine in muscle by removal of water and the formation of a ring structure (Figure 6.1).

Figure 6.1: Schematic showing conversion of Creatine to creatinine
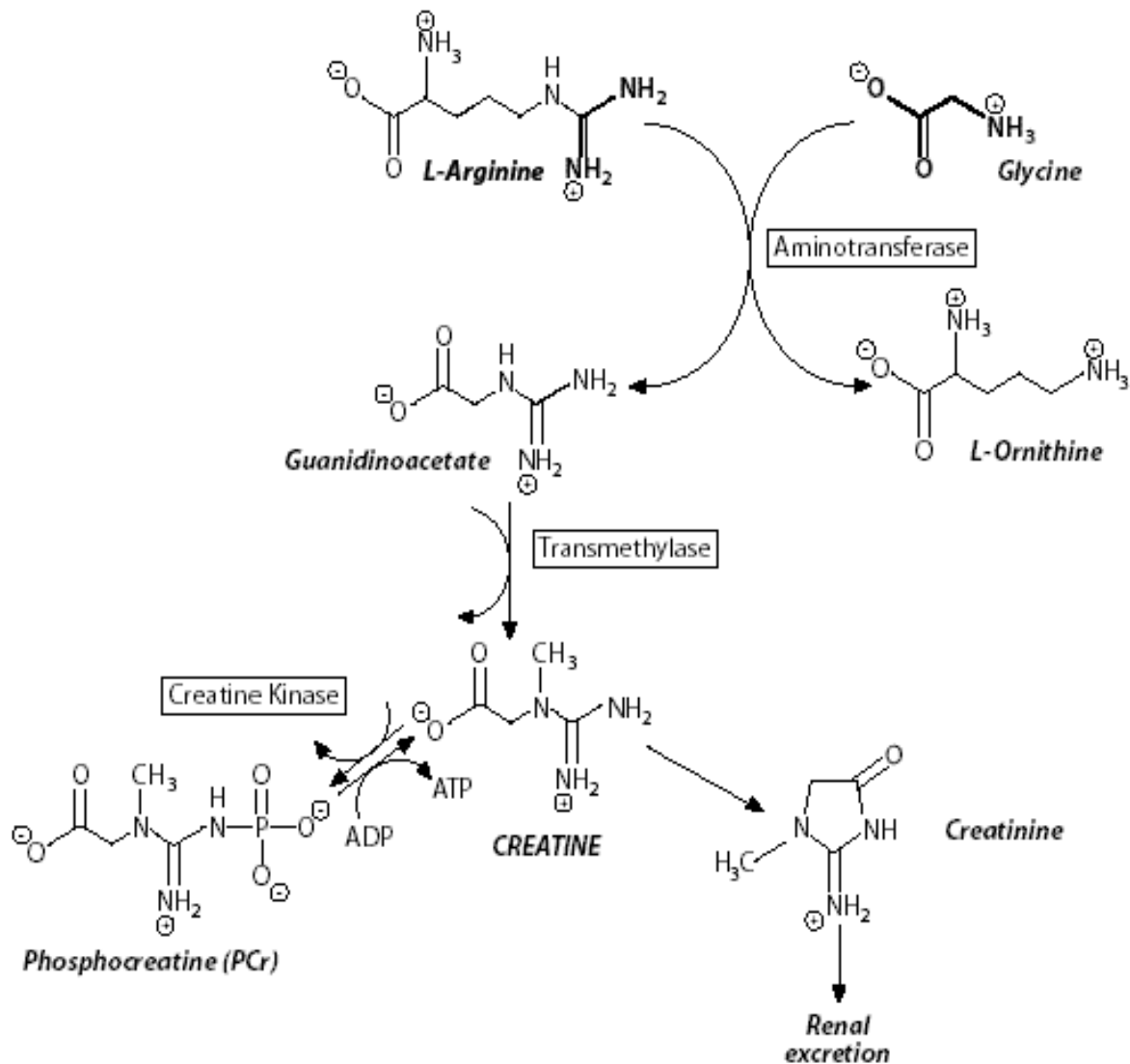
Cooking meat converts creatine in skeletal muscles to creatinine. Studies looking at the effect of cooking on acid-extractable chemical constituents of beef have shown significant increase in creatinine and decrease in eGFR levels(Robert, 2006) (Purchas, 2004). Ingestion of meals containing cooked meat caused a marked postprandial increase in serum

creatinine concentration, whereas ingestion of raw meat had no effect on serum creatinine in normal human subjects (Jacobsen et al., 1979). A large cooked meat meal (225gm) was shown to cause an average increase of 52% in creatinine compared to the mean value on a control meal in 6 healthy individuals (Mayersohn et al., 1983).The maximum concentration of serum creatinine was achieved 2 hours after the cooked meat meal.

A study looking at the effect of acute protein load with a 0.75 gm. per Kg body weight of cooked meat in renal transplant and healthy controls showed 30% increase in serum creatinine levels, with the rise in absolute terms being higher in the transplant group. A significant rise in creatinine was observed in the transplant group at 3 and 8 hours after meal but only at 3 hours in the control group. The authors suggest that the "functional reserve" to deal with a protein load is reduced when renal function is impaired leading to this difference between the transplant and control groups (Sterner, 1991). Hence the effect of cooked meat on serum creatinine level is likely to be higher in subjects with more advanced CKD stages. Moreover, patients with chronic kidney disease have been shown to be unable to increase their urinary creatinine excretion in response to a protein load compared to normal individuals (Herrera and Rodríguez-Iturbe, 1998). Hence the effect of cooked meat on serum creatinine level is likely to be higher in subjects with more advanced CKD stages. Patients with chronic kidney disease have been shown to be unable to increase their urinary creatinine excretion in response to a protein load compared to normal individuals (Herrera and Rodríguez-Iturbe, 1998).

A more recently reported study by Preiss *et al.* found a median serum creatinine concentration increase from 80.5 micromoles/L pre-prandial to 101.0 micromoles/L 1-2 h postprandially, and 99.0 micromoles/L 3-4 h postprandially. Median eGFR decreased from

84.0 mL/min/1.73 m2 pre-prandial to 59.5 mL/min/1.73 m2 1-2 h postprandially (P<0.0001), and 64.0 mL/min/1.73 m2 3-4 h postprandially (P<0.0001) following intake of a normal helping of cooked meat (Preiss et al., 2007). This study was conducted on 17 healthy volunteers and 15 randomly selected patients from a care of the elderly day unit, who had chronic kidney disease stages 3 or less. The study showed that the rise in creatinine measured by three different methods (kinetic Jaffe, IDMS and enzymatic methods) was similar after a cooked meat meal.

None of the previously published studies have looked into the effect of cooked meat on creatinine in a well defined patient population with different stages of diabetes mellitus related CKD. The effect of a cooked meat meal on creatinine levels more than 4 hours postprandially is also uncertain. In the above study the difference between 2 hrs & 4 hrs post prandial values of creatinine were small(Preiss et al., 2007). This raises questions regarding the timescale of changes in serum creatinine levels, because patients attending a morning clinic may well have consumed a large cooked meat meal the previous evening.

The aim of the present study is to estimate the biological variation in creatinine levels caused by a standardised cooked meat meal in subjects with Diabetes mellitus who have various stages of chronic kidney disease, compared to healthy volunteers.

Hypotheses

1. A standardised cooked meat meal will cause a significant rise in serum creatinine in diabetic patients with various stages of CKD compared to normal volunteers

2. Taking a blood sample after overnight fasting will eliminate the confounding effect of a cooked meat meal in the evening, on creatinine levels in this population

6.2 RESEARCH DESIGN AND METHODS

This was a prospective, experimental, controlled study which involved a study group consisting of patients with diabetes in different stages of CKD and a control group with healthy volunteers. Each group consisted of 16 participants based on sample size calculations described in the statistical section below. The study group consisted of 4 subgroups: CKD stages 1 & 2, stage 3A, stage 3B and stage 4. We included CKD stages 1 & 2 into one group, as the expected effect of cooked meat meal based on previous studies may not be very clinically relevant. We did not include CKD stage 5 as they are under close monitoring by the nephrologists and being prepared for renal replacement therapy.

Figure 6.2: Control and study groups

Control	• Healthy volunteers
CKD 1& 2	• eGFR >60 + Microalbuminuria
CKD 3A	• eGFR 45 -59
CKD3B	• eGFR 30-44
CKD4	• eGFR 15-29

The study was reviewed and approved by the Liverpool Research Ethics Committee and all study participants gave written informed consent.

6.2.1 Study subjects and Sampling

Study participants were recruited from the diabetic nephropathy and diabetes clinics at Whiston and Aintree Hospitals. Healthy volunteers were drawn from staff and the general public recruited via advertisements. As our sampling strategy, we employed quota sampling, selecting consecutively to achieve equal numbers within the study groups to allow progressional analyses through the stages.

Inclusion criteria

- Type 1 or 2 Diabetes mellitus,
- CKD stages 1 to 4 and
- Age >18 years

Exclusion criteria

- Patients known to have non-diabetic renal disease
- Anyone who cannot eat meat and
- Patients on renal replacement therapy

6.2.2 Intervention

The intervention constituted of a standardised meal containing cooked meat in a quantity considered a normal helping. Although there is no evidence in the literature for renal damage from an acute protein load, we kept the protein load within the recommended daily allowance for CKD patients. The effect of dietary protein restriction in patients with chronic kidney disease remains controversial and research in this area has produced conflicting results (Hartley, 2001). A Cochrane review published in 2007 based on 12 studies with type1 and type2 diabetes for at least 4 months, restricted protein intake appeared to slow progression of diabetic kidney disease, but not by very much on average. The authors concluded that although reducing protein intake appears to slightly slow progression to renal failure but this was not statistically significant. The actual protein intake in the intervention groups ranged from 0.7 to 1.1 g/kg/day (Robertson LM, 2007) The European Renal Care association (2002) recommend that the pre-dialysis patient be advised to achieve a protein intake of 0.6-1.0g/kg body IBW/day. We provided patients with one meat and one non meat meal each providing approximately 54g protein, which comprises no more than 75% of each patient's daily protein allowance (1.0g protein/kg IBW per day).

On the first study visit participants attended after an overnight fasting and a pre-prandial and 3 post-prandial blood samples 1, 2, and 4 hours after the meat meal were obtained. The evening before the second visit they were advised to have the standardised meat meal and attend following overnight fasting. A fasting and 3 post-prandial blood samples 1, 2, and 4 hours after the standard meat free meal were obtained. Participants also provided a fasting and 4 hour post-prandial urine sample on both study visits.

Figure 6.3: Study visits and procedures

Visit 1
- Consent
- History & Physical examination
- Fasting blood sample
- Cooked meat brunch
- Blood samples 1,2, & 4hrs postprandially
- participant allowed home

Visit 2
- Cooked meat meal the previous evening
- Fasting blood sample
- Cooked meat brunch
- Blood samples 1,2, & 4hrs postprandially
- participant allowed home
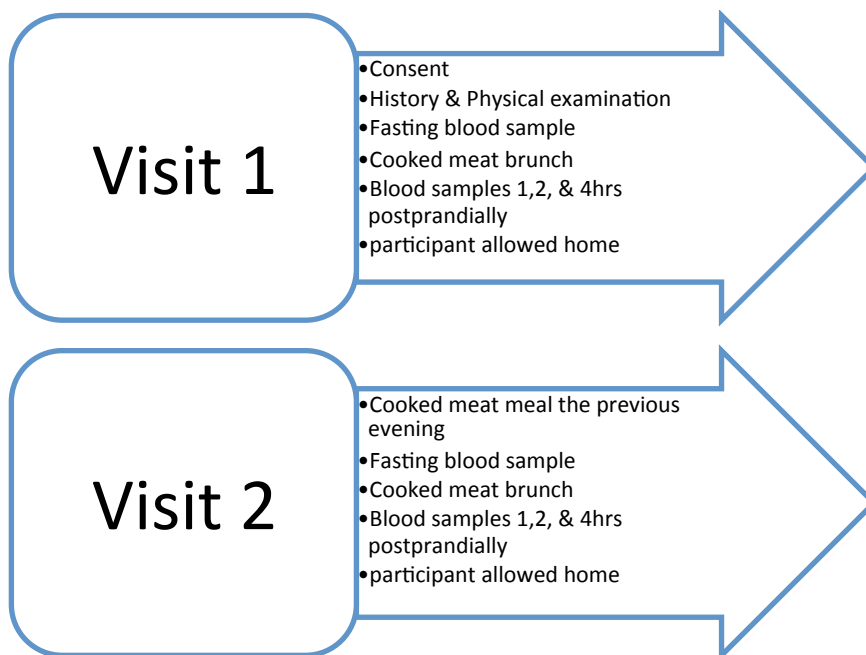
Measured variables

The principle variable in the study was serum creatinine, which was measured using the kinetic alkaline picrate assay on an Olympus AU640 analyser. The modified MDRD equation traceable to isotope dilution mass spectrometry (IDMS)-creatinine and adjusted for Olympus methodology (ml/min per 1.73m^2):

$$175 \times [((serum\ creatinine\ -16.14)/0.955) \times 0.011312]^{-1.154} \times [age]^{-0.203} \times [0.742\ if\ female] \times [1.212\ if\ AfroCaribbean]$$ *was* used to calculate eGFR (Reynolds and Twomey, 2007).

The other measures are urea, sodium, potassium, bicarbonate, phosphate, and urine for albumin-creatinine ratio.

6.2.3 Statistical considerations:

We regarded a clinically significant (effect size) change in creatinine as greater than 10 µmol/L, as by Preiss et al. (David Preiss: personal communication). By this is interpreted that a minimum clinically significant change in creatinine is a change of 10 micromoles/L.

Individual differences between baseline pre-prandial and each of the 1hour, 2hour and 4hour post-prandial measurements were computed. Differences for subjects with CKD were compared to differences for healthy volunteers using un-paired Mann-Whitney tests as the data are non-normal.

The study by Preiss et al. listed as an appendix individual serum creatinine data for 32 subjects before a cooked meat meal and then 1-2 hours and 3-4 hours afterwards. Using the Kolmogorov-Smirnov test each of these distributions could be regarded as coming from a normal distribution with standard deviations of 12.5 (Jaffe) and 14.2 (IDSM) for differences between baseline and 1-2 hours and of 10.4 (Jaffe) and 12.8 (IDSM) for differences between baseline and 3-4 hours. These measures of scatter are about the same as the minimum clinically significant difference.

To detect a mean shift of 10 micromoles/L (roughly equivalent to one standard deviation) between post minus pre-prandial values with a power of 80%, p=0.05 in a paired t-test requires 10 participants in each group and 13 participants for a power of 90%. However, for non-normal distributions, to use Wilcoxon sign rank test number of participants required for power of at least 90% is 15. Statistical analysis was carried out using SPSS (version 17.0 for Windows: SPSS, Chicago, IL) and Graph pad prism 5 for Windows. The shifts in creatinine and eGFR values in all groups were compared using Wilcoxon signed rank test as most data

were non-normally distributed. Results are presented as median and interquartile ranges and $p < 0.05$ was taken to indicate statistical significance.

6.3 RESULTS

We obtained results from 80 participants following the standard cooked meat and non-meat meals. 66 of the participants were males and all were Caucasians. The mean age was 62.2 ±15.1. The mean HbA1c among the 64 participants with diabetic chronic kidney disease was 62.7± 16.1 mmol/mol.

Figures below demonstrate the fasting and post-prandial creatinine and eGFR values in the participants in all 5 groups. Post-prandial values are 1 hour, 2 hours and 4 hours following the meat and non-meat meals.

Significant differences were found in serum creatinine and eGFR values following the standard meat meal in healthy volunteers and the participants with chronic kidney disease, but there were no significant differences following a non-meat meal.

Figure 6.4: fasting and post-prandial creatinine and eGFR healthy volunteers
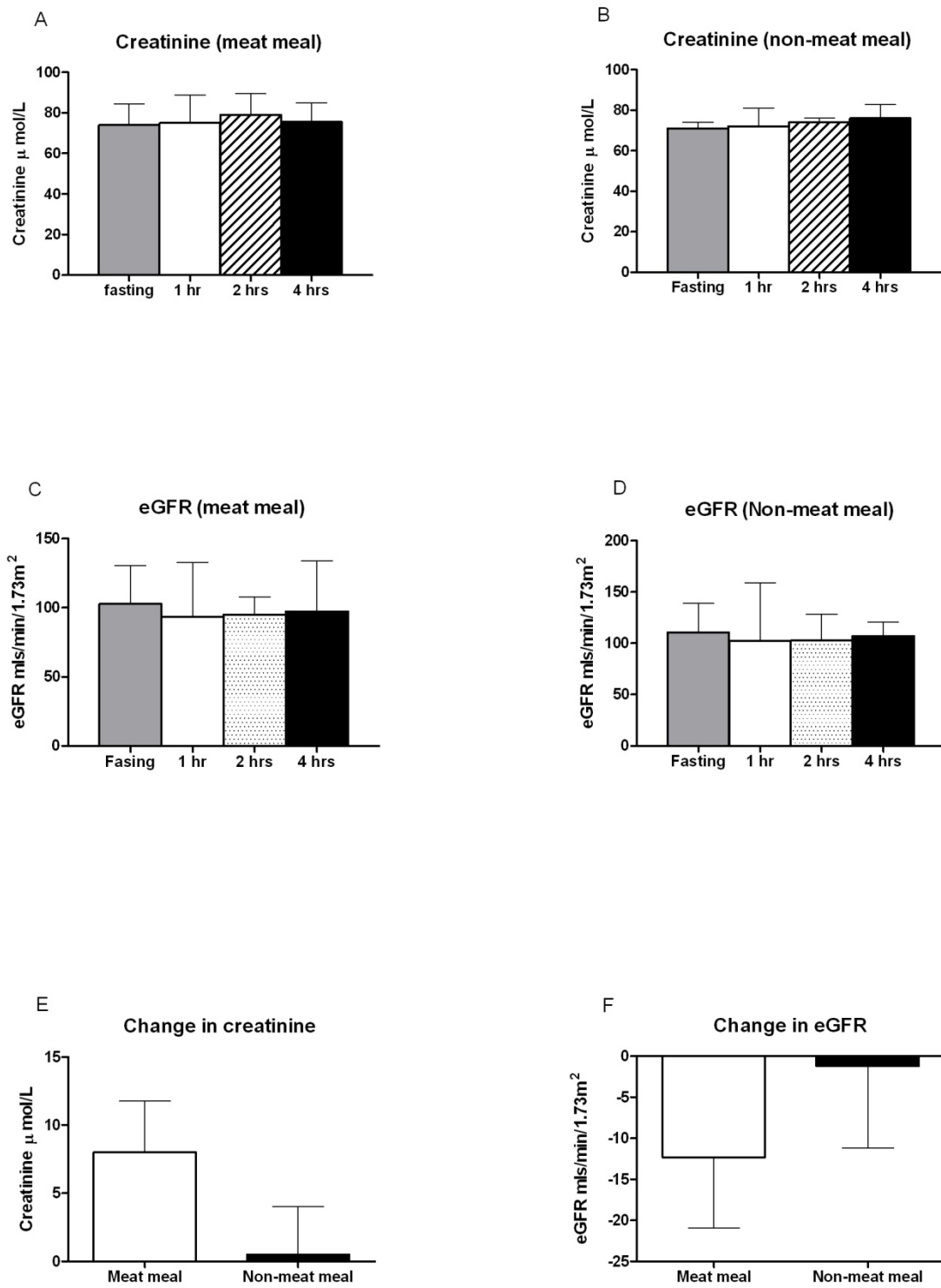
Figure 6.5: fasting and post-prandial creatinine and eGFR in CKD 1& 2
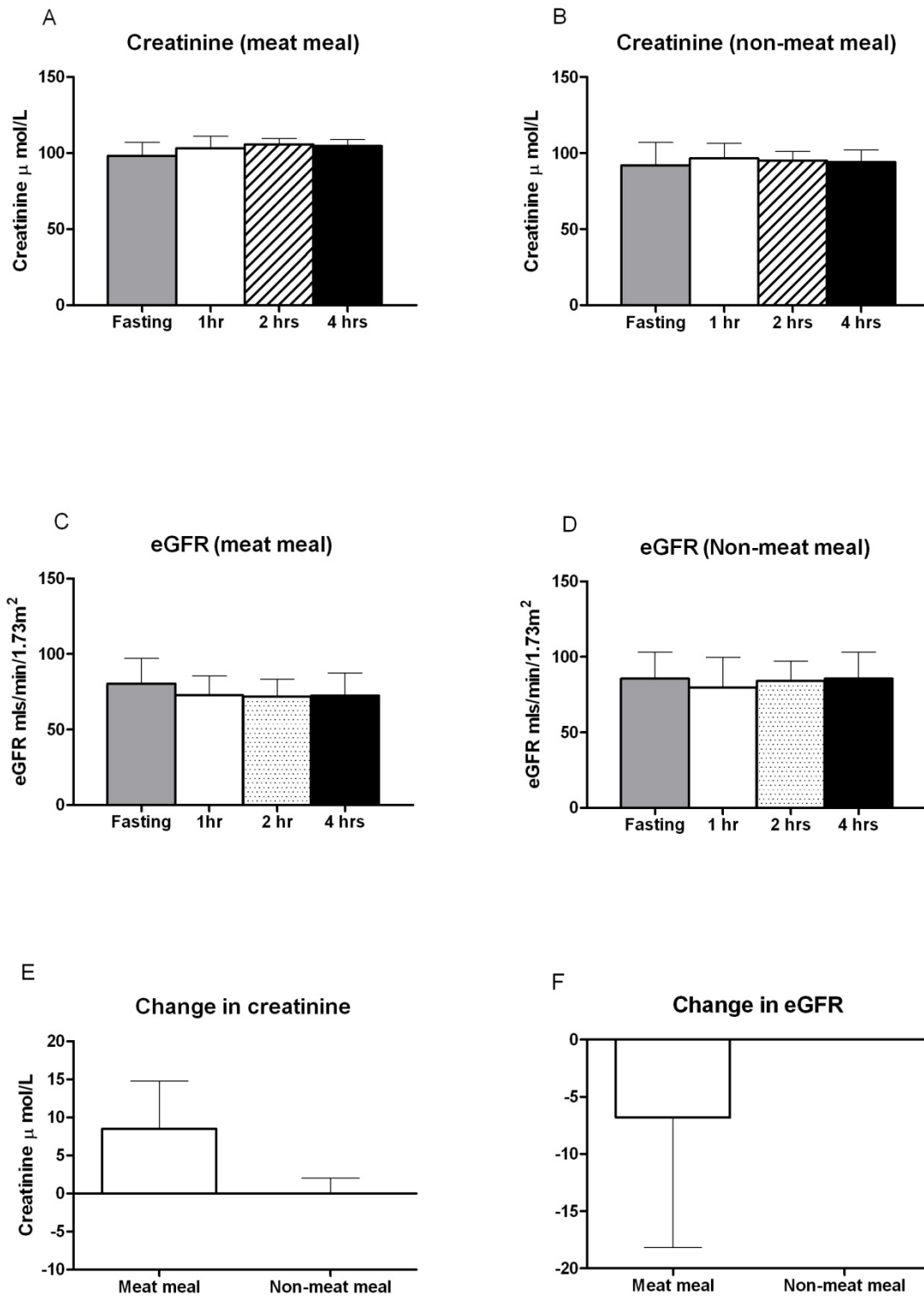
Figure 6.6: fasting and post-prandial creatinine and eGFR in CKD3a

A

Creatinine (meat meal)

B

Creatinine (non-meat meal)

C

eGFR (meat meal)

D

eGFR (Non-meat meal)

E

Change in creatinine

F

Change in eGFR
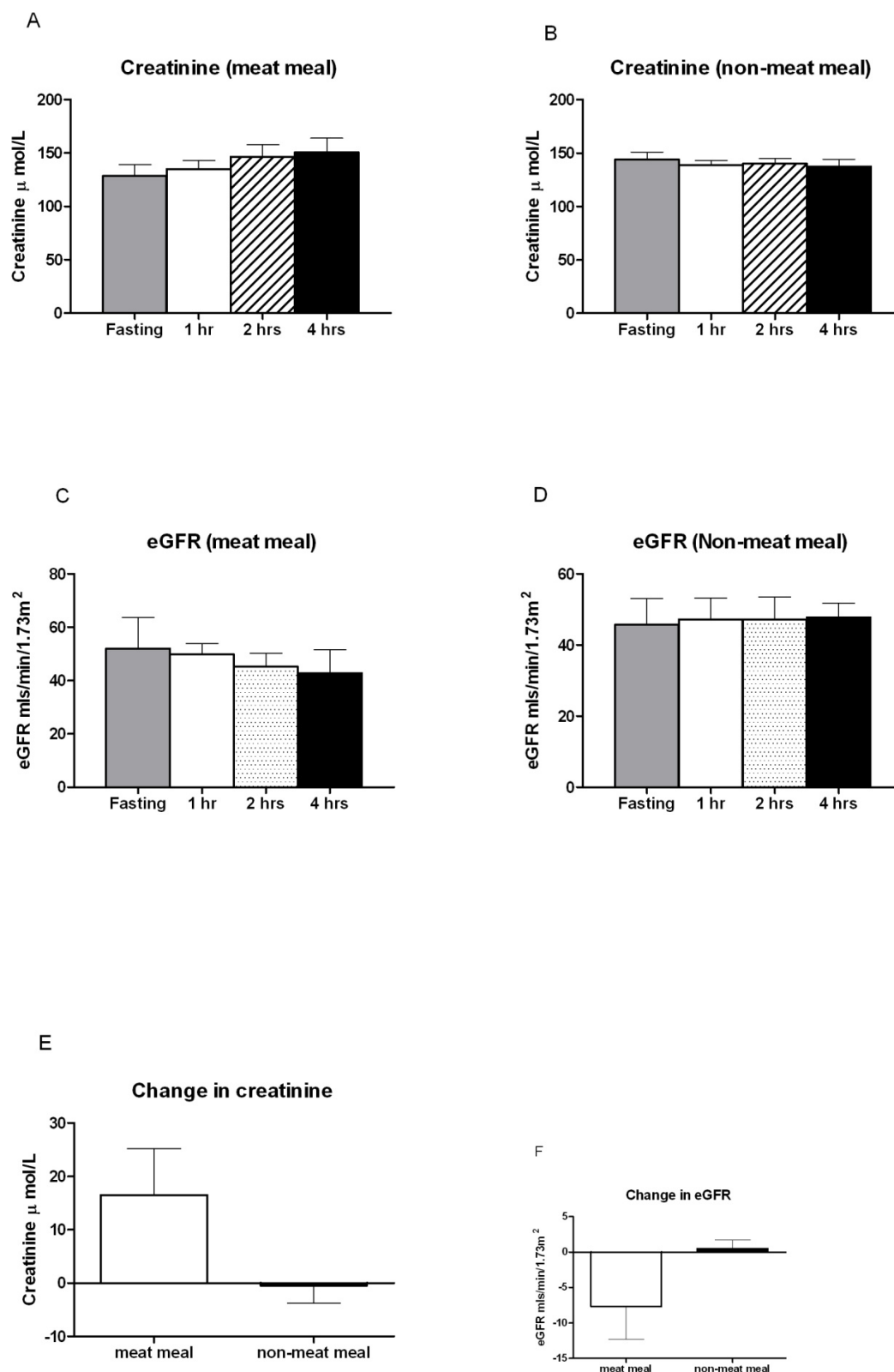
Figure 6.7: fasting and post-prandial creatinine and eGFR in CKD3b
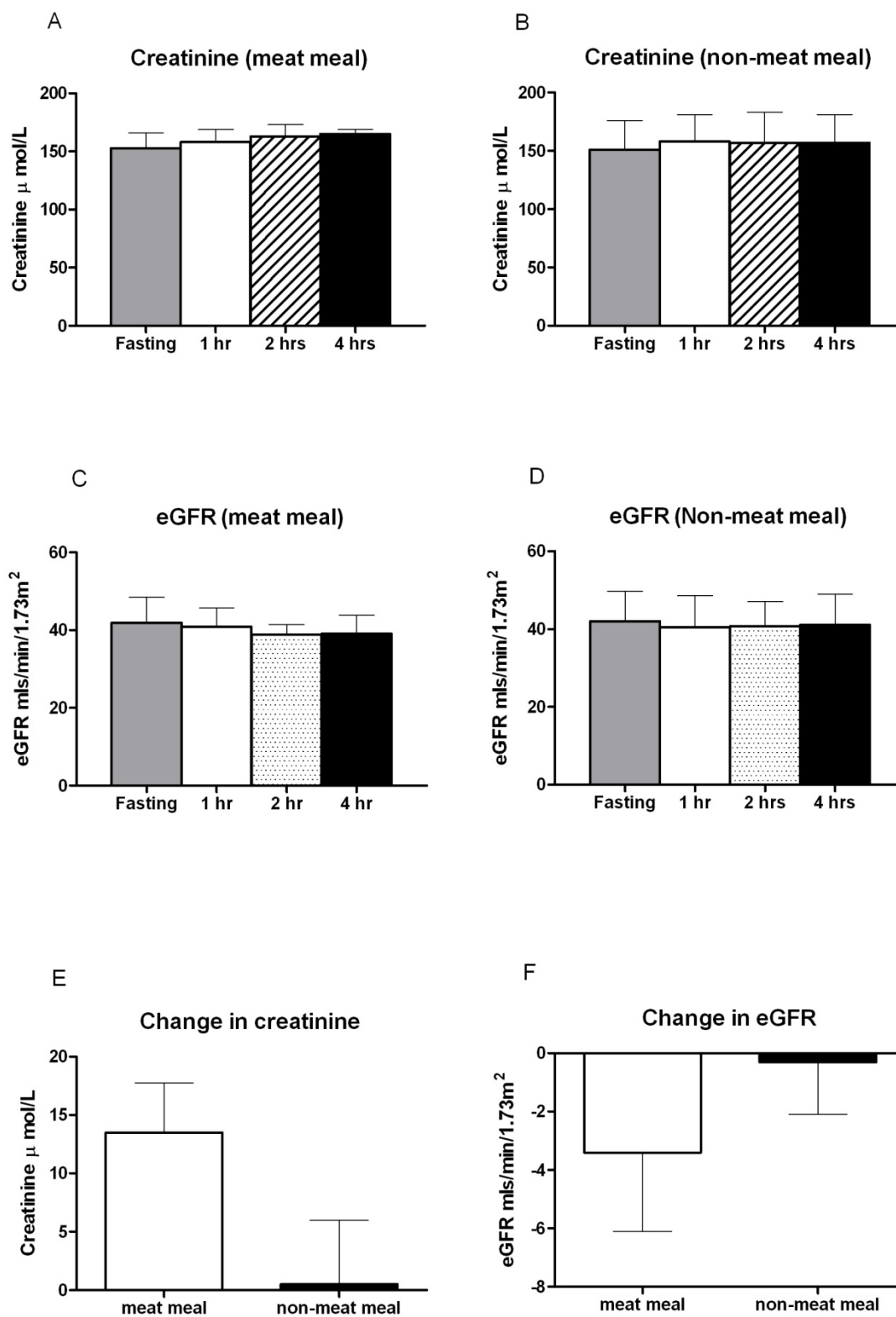
**Figure 6.8: fasting and post-prandial creatinine and eGFR in CKD4**
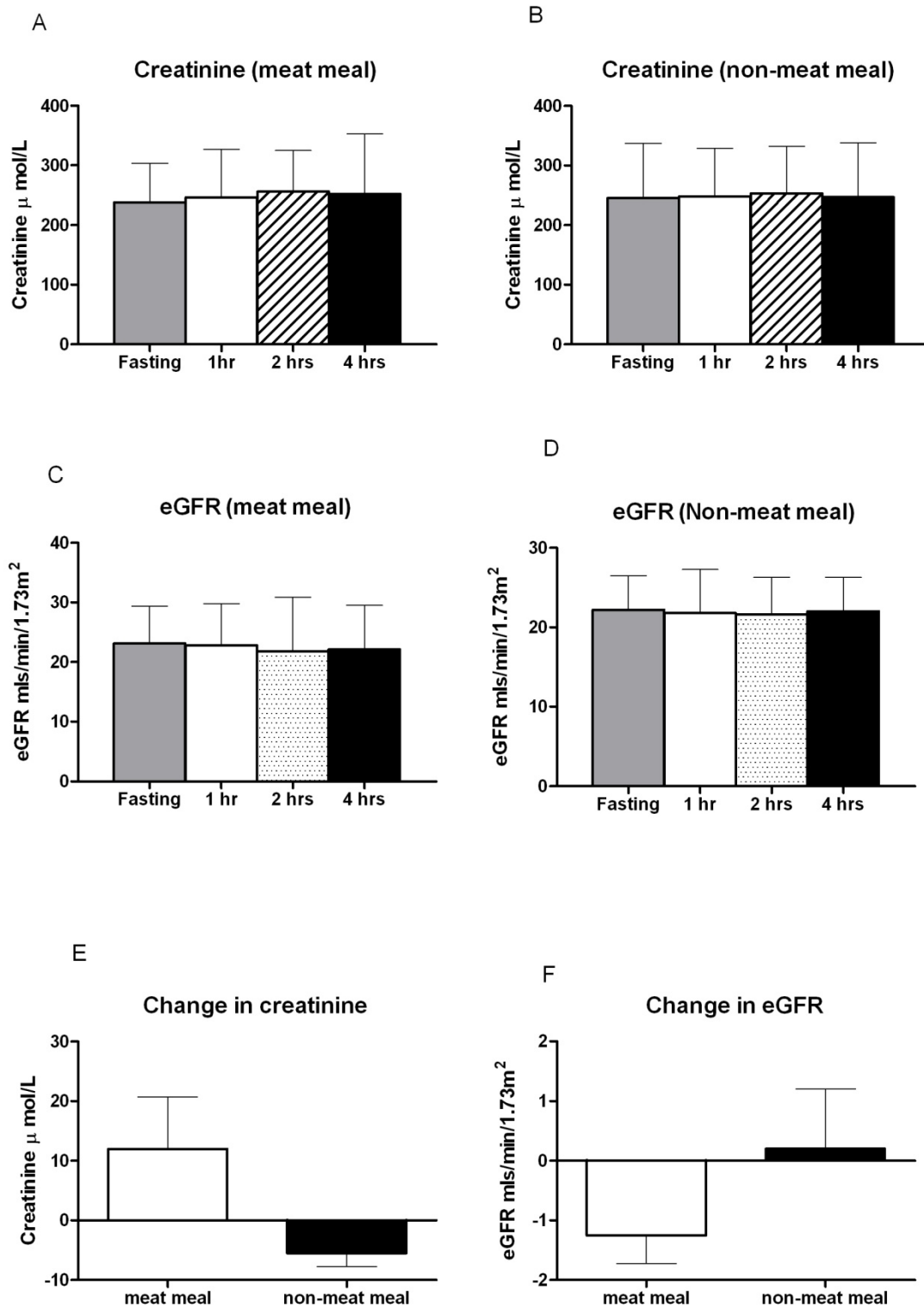
Figure 6.9: demonstrates the shift in serum creatinine and eGFR following the meat and non-meat meals. In healthy volunteers and CKD stage 1 & 2, the maximum rise in creatinine was noted at 2 hrs, while in CKD 3a and 3b the maximum rise was seen at 4 hours post-prandially. In CKD 4, the 4 hour creatinine was only 0.5 µmol/L lower than the 2 hour value (table 1).
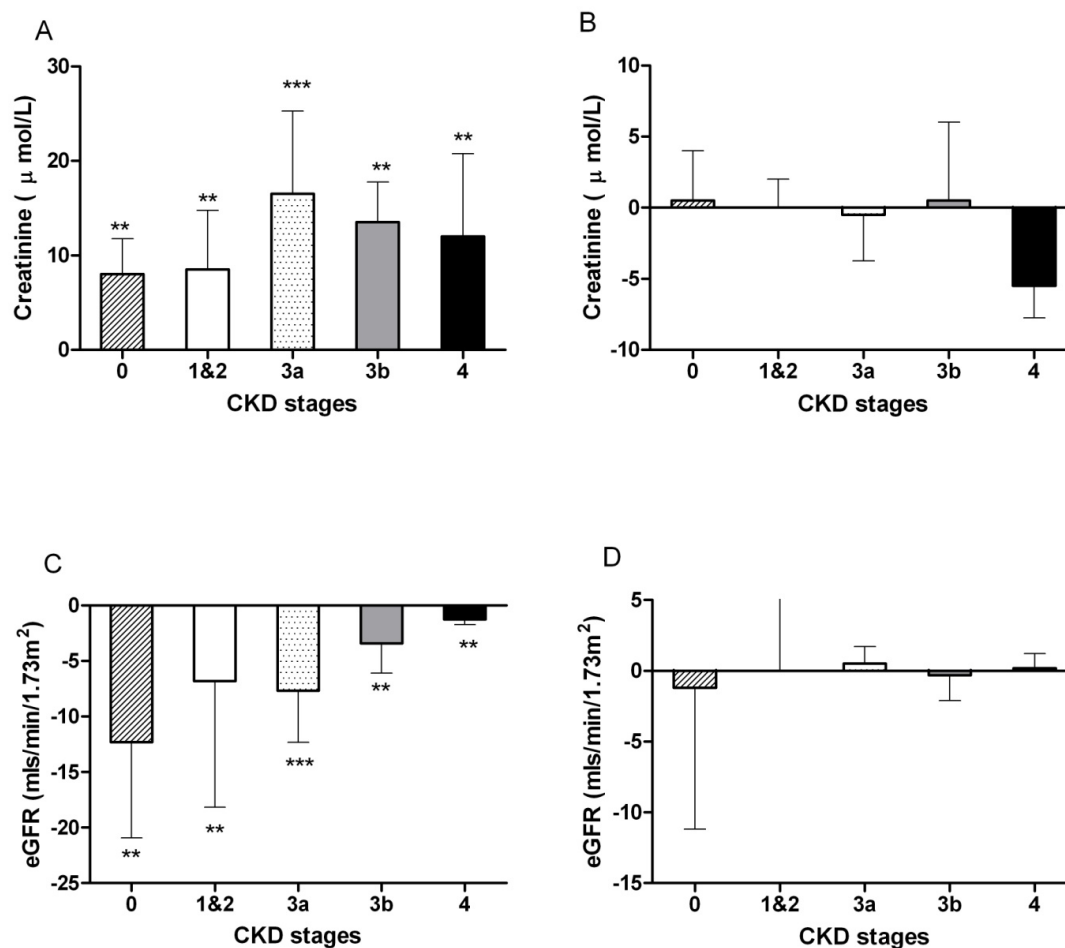
Panel A & B shows median and interquartile ranges of shift in serum creatinine 2 hours after a meat and non-meat respectively. Panel C & D shows median and interquartile ranges of shift in eGFR following meat and non-meat respectively. (** $p < 0.01$ and *** $p < 0.001$ compare baseline and 2 hour values)

Effect of cooked meat meal on serum creatinine after overnight fasting:

The median difference between fasting creatinine 12 hours following meat and non-meat meal was not statistically significant as illustrated in figure 6.10

Figure 6.10: Effect of cooked meat meal on serum creatinine after overnight fasting
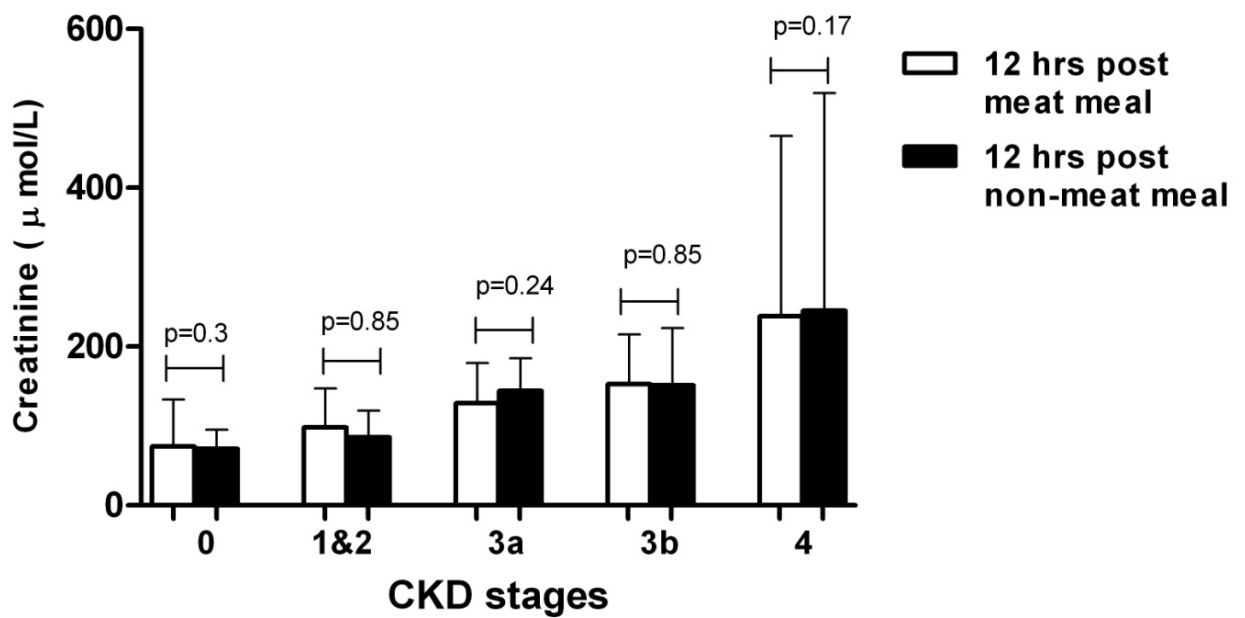
Median and inter quartile ranges of creatinine 12 hours following meat and non-meat meals in healthy volunteers and the 4 groups of participants with chronic kidney disease. No significant differences were noted in any group with all p values>0.05.

Table 6.1: Changes in creatinine following meat and non-meat meals

	Creatinine Fasting	Creatinine 1 hr pp	Creatinine 2 hrs pp	Creatinine 4 hrs pp
Meat Meal				
Healthy Volunteers	74 (68.2 - 84.25)	75 (73.2 - 89.5) P=0.23	79 (73.5 - 89.5) P=0.002	75.5 (69.5 - 84.5) P=0.047
CKD 1 & 2	98 (84 – 107)	102 (88 -110.5) P=0.051	105.5 (93.5 – 109) P=0.001	104.5 (91 -108.7) P=0.002
CKD 3a	128.5 (113 – 139)	135 (127 – 143) P=0.001	146.5 (132 – 158) P<0.001	150.5 (131 - 163) P=0.001
CKD 3b	152.5 (133 – 166)	158 (142 – 169) P=0.014	165 (155 – 173) P=0.001	165 (147 – 169) P=0.009
CKD 4	238 (191 – 303)	246.5 (191 -327) P=0.038	256 (193 -325) P=0.007	255.5 (194 – 334) P=0.038
Non-meat meal				
Volunteers	71 (66.5 - 73.5)	71.5 (64.2 - 79.2) P=0.24	73.5 (65.7 - 75.7) P=0.39	72.5 (57.5 - 81.5) P=0.75
CKD 1 & 2	89 (76 – 106.5)	94.5 (80.2 – 104) P=0.53	93 (83 – 100.2) P=0.80	92 (81 – 101.7) P=0.13
CKD 3a	143 (128 – 150)	135.5 (129 – 143) P=0.47	139 (126 – 145) P=0.88	135 (120 – 143) P=0.71
CKD 3b	150.5 (131 -172)	153 (131 – 178) P=0.91	152 (129 – 180) P=0.95	152 (126 – 179) P=1.0
CKD 4	242 (202 – 322)	245 (197 – 314) P=0.02	247 (198 -316) P=0.059	243.5 (196 – 331) P=0.50

Data are median and inter-quartile ranges. Creatinine is expressed as micromoles/L and p values compare fasting and post prandial results

Table 6.2: Changes in 4-variable MDRD eGFR following meat and non-meat meals

	eGFR Fasting	eGFR 1 hr pp	eGFR 2 hrs	eGFR 4 hrs
Meat Meal				
Volunteers	102.8 (85.3 – 130.4)	93.5 (76.9 – 132.9) P=0.125	95 (76.1 – 107.9) P=0.002	97.3 (77.6 – 133.9) P=0.116
CKD 1 & 2	80.4 (68.6 – 97.2)	71.8 (65.2 – 85.4) P=0.003	71.6 (67.5 – 83.3) P=0.002	72.5 (66.6 – 87.6) P=0.002
CKD 3a	52 (47.1 – 63.6)	49.8 (45.4 – 53.8) P=0.001	45.2 (40.5 – 50.2) P<0.001	42.8 (38.6 – 51.5) P=0.001
CKD 3b	41.9 (38.2 – 48.5)	40.8 (36.9 -45.7) P=0.025	38.6 (36.4 – 41.4) P=0.001	37.7 (35.5 – 43.8) P=0.017
CKD 4	23.1 (17.8 – 29.4	22.8 (15.8 – 29.8) P=0.034	21.8 (15.6 – 30.8) P=0.017	21.5 (14.5 -27.5) P=0.038
Non-meat meal				
Volunteers	109.4 (86.9 – 138)	100.7 (88.1 -158) P=0.19	102.1 (93.8 – 127) P=0.6	102.6 (88.9 – 117) P=0.51
CKD 1 & 2	83 (64 – 102.9)	78.2 (59.9 – 97.8) P=0.85	81.6 (67.4 – 96.7) P=0.92	83.5 (65.9 – 102) P=0.13
CKD 3a	45.4 (42.1 – 52.6)	47 (42.6 – 53.2) 0.73	46.7 (42.9 – 53.1) P=0.97	47.5 (37.4 – 51.4) P=0.41
CKD 3b	41.9 (33.5 – 49.2)	40.1 (33 – 48.5) P=0.83	40.5 (33.2 – 47) P=0.97	40.9 (33.7 – 48.5) P=0.86
CKD 4	21 (12 – 26.4)	21.6 (12.4 – 27) P=0.03	21.3 (12 – 26.2) P=0.14	21.7 (12 – 26.2) P=0.75

Data are median and inter-quartile ranges. eGFR as mls/min/1.73m^2 and p values compare fasting and post prandial results.

6.4 DISCUSSION

Our study demonstrates significant increase in serum creatinine levels following a cooked meat meal in healthy volunteers and participants with diabetic chronic kidney disease stages 1 to 4. This is likely to be due the effect of absorbed creatinine present in the cooked meat. This result in a drop in estimated GFR in all the groups studied. The relative drop in eGFR secondary to rise in creatinine is proportionately less in patients with more advanced CKD stages, but the percentage drop remains significant in all groups. A proportion of patients with CKD could be misclassified to more severe stages depending on their baseline eGFR. Six out of the sixteen patients in CKD 3a were misclassified into CKD 3b due to the effect of cooked meat consumption. This could have significant effect on management of these patients; particularly as the threshold for commencing and withdrawing certain medications are defined by eGFR.

There is some literature suggesting that protein load and intravenous amino acid infusion increases glomerular filtration rate. In a creatinine clearance study conducted in 9 healthy vegetarians after an oral protein load of 100 grams of vegetarian protein 5 subjects showed a significant increase of the creatinine clearance after the protein load of 66.0% (mean). The remaining 4 however did not increase their creatinine clearance following acute protein challenge (Hirschberg et al., 1985). Bosch et al showed an increase in glomerular filtration rate two and a half hours after protein load to a maximal glomerular filtration rate of 171.0 +/- 7.7 ml per minute in healthy volunteers. The authors conclude that the capacity of the kidney to increase its level of function with protein intake suggests a renal function reserve (Bosch et al., 1983). The mechanism by which renal plasma flow and GFR increases following

protein load or amino acid infusion remains unclear. There are several proposed mechanisms such as hormonal response to increase amino acids (insulin, glucagon, and growth hormone levels) and amino acid metabolism by the kidney generating nitric oxide and endothelium derived relaxing factor may increase renal plasma flow (Tolins and Raij, 1991). These studies suggest that there is a true increase in GFR following acute protein load, however our study did not measure creatinine clearance or glomerular filtration rate and was based on creatinine measurement and estimation of GFR using the MDRD equation. Moreover patients with chronic kidney disease have lower renal reserve than healthy volunteers and this increase in GFR may not be seen in our cohort. The blunting of the effect of cooked meat protein due to transient rise in GFR may therefore not be very prominent in CKD patients.

The published studies referenced in the introduction have shown significant effect of cooked meat consumption on serum creatinine irrespective of the methodology. Preiss et al 2007, measured serum creatinine using the kinetic Jaffe method, enzymatic, isotope-dilution mass spectrometry [IDMS]). Jacobson et al used the Jaffe reaction as well. Mayersohn et al used high-pressure liquid chromatography to measure serum creatinine. Sterner et al used a standard Jaffé method and also with a specific HPLC technique. All these studies have shown significant increase in serum creatinine following a cooked meat meal.

We also demonstrate that the peak rise in creatinine following a cooked meat meal is delayed in more advanced CKD stages of 3a, 3b and 4, perhaps due to slower clearance from the blood. After overnight fast, however the effect of cooked meat on serum creatinine does not remain statistically or clinically significant. This argues for the opinion that GFR should be estimated using fasting rather than random serum samples particularly for

patient with CKD. Although this effect has been shown as early as 1983, it was thought to be caused only by excessive amount of cooked meat consumption and not very relevant in clinical situations. Our study and the study by Preiss et al. (Preiss et al., 2007) demonstrate that significant rise in serum creatinine can occur with what is considered a normal helping of cooked meat.

CHAPTER 7

EFFECT OF A COOKED MEAT MEAL ON ALBUMIN-CREATININE RATIO IN DIABETES RELATED CHRONIC KIDNEY DISEASE

7.1 INTRODUCTION

Urinary protein analyses have been known to help with diagnosing several renal disorders (Butler and Flynn, 1958). Urinary low molecular weight proteins such as β2-microglobulin, retinol binding protein and lysozyme are sensitive markers of impaired renal tubular function while excretion of albumin and IgG are sensitive markers of glomerular damage (Arai et al., 1991).

Urine dipstick commonly used to screen for proteinuria can only detect urinary protein from 150-300 mg/L, and hence is suitable only to detect gross proteinuria. Dipstick positive proteinuria usually denotes urinary protein excretion >0.5 grams/day (Abuelo, 1983). Approximately 50% of urinary protein is constituted of Tamm-Horsfall proteins, which are shed by the terminal part of the renal tubules. In health, relatively small amounts of albumin (<30 mg/24 h) are lost in the urine and also a large increase in albuminuria can occur without raising the total protein significantly (Lamb et al., 2009).

Peterson et al studied the differential protein excretion in patients with glomerular and tubular disorders; 15 patients with glomerular disorders such as glomerulonephritis, nephrotic syndrome, and Alport syndrome and 15 patients with tubular disorders such as renal tubular acidosis, chronic Cadmium poisoning and Wilson's disease. They showed that patients with glomerular disorders had increased urinary excretion of albumin and normal or only somewhat increased excretion of β2-microglobulin, but patients with tubular disorders had increased excretion of β2-microglobulin with normal or only slightly increased albumin excretion (Peterson et al., 1969). Although albumin and IgG are known to be good markers of glomerular damage, albumin is more consistently excreted during the natural

course of diabetic kidney disease. The selectivity of proteinuria has been proposed to show a triphasic change during the pathogenesis of diabetic nephropathy. In the first phase, proteinuria is non-selective with IgG clearance equal to or exceeding albumin clearance. As microalbuminuria develops, there is preferential excretion of albumin compared with IgG but in later stages of nephropathy, there is a return to non-selective proteinuria (Jerums et al., 1989). This selectivity at the microalbuminuria stage is due to a loss of anionic heparan sulphate proteoglycan from basement membrane, which in turn leads to a loss of the fixed negative charge of the glomerular basement membrane. In the later stages as the pore size increases the charge selectivity becomes less important (Peter H. Winocour, 1998).

Some authors evaluated the use of protein creatinine ratio instead of 24-hour urine protein estimation. It was shown that in the presence of stable renal function, a protein/creatinine ratio of more than 3.5 (mg/mmol) can be taken to represent "nephrotic-range" proteinuria, and a ratio of less than 0.2 is within normal limits (Ginsberg et al., 1983).

The first radioimmunoassay to detect albumin in low concentration in the urine was proposed in 1963 by Keen and Chlouverakis and the term "microalbuminuria" was used (Keen and Chlouverakis, 1963). Urinary albumin excretion is used to define the extent of glomerular damage in diabetes related chronic kidney disease.

The proteinuria normal ranges have been derived from studies that used 24-hour urine collection or overnight urine collection; however this is burdensome for the patient on a clinical basis. Most national and international guidelines now recommend the use of protein: creatinine ratio (PCR) or albumin: creatinine ratio (ACR). This ratio is suggested to reduce variability due to urine concentration at various timed during the day. Creatinine

was considered the natural denominator as it is excreted at a constant rate in an individual and it is easily measured. The ACR is a ratio of albumin in μg to creatinine in mg and is used as an index of urinary albumin excretion (UAE). The ACR in μg/mg can be converted to SI unit of mg/mmol by dividing by 8.84 (Warram et al., 1996).

Some authors have suggested that correction for albuminuria and proteinuria by creatinine is flawed; particularly when we use age, gender, and race for correcting creatinine derived GFR. Although creatinine is excreted at a constant rate in an individual it is dependent on muscle mass. Muscle mass variation will have proportional effect on urinary creatinine as it does on serum creatinine. It is therefore likely that the higher prevalence of microalbuminuria reported in the elderly is due to lower muscle mass and consequently lower urinary excretion of creatinine (Ellam, 2011).

A first void morning urine sample has been shown to correlate better with 24-hour urinary albumin excretion compared to random spot urine as shown in the figure below. In this study 241 participants provided three 24-h urine collections, a first morning void, and spot urine sample, and a regression analysis was performed to establish correlation (Witte et al., 2009). Urinary excretion shows a circadian rhythm with lower excretion rates overnight and also prolonged standing and exercise can increase albuminuria (Ellam, 2011). The mechanism of orthostatic proteinuria is unclear but venous pooling leading to decreased renal plasma flow and activation of Angiotensin II has been proposed. This in turn increases efferent arteriolar resistance and increased filtration of protein (Devarajan, 1993). This could also explain increase in albumin excretion following vigorous exercise (Poortmans and Jeanloz, 1968).

Scatterplots showing the relationship between 24-h UAE and other albuminuria measures.
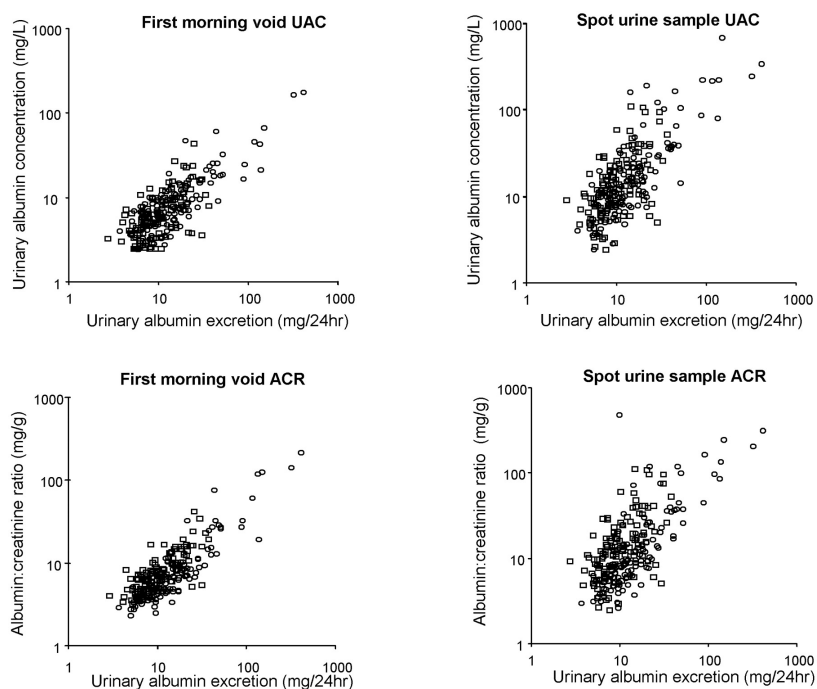
Elsbeth C. Witte et al. JASN 2009;20:436-443

Figure 7.1: The top panel shows correlation between 24-hour UAE versus first morning void and spot urine sample UAC. The bottom panel shows 24-hour UAE versus first morning void and spot urine sample ACR, respectively. •, Male; □, female.

Microalbuminuria was shown to be a predictor of progression to diabetic nephropathy (Mogensen, 1987). It has been shown to be a strong predictor of all cause and cardiovascular mortality and therefore proposed to be an ideal target for early primary prevention using CV-protective therapy regimens (de Zeeuw et al., 2006).

Studies comparing the intra-individual variability of urinary protein estimation and albumin creatinine ratio have not shown any significant difference, which suggested that spot UACR can replace timed urine collection for albumin excretion rate (Cohen et al., 1987b, Claudi T, 2001). In a study by Hutchison et al, timed overnight urine collection from 261 patients attending a diabetes clinic with albumin excretion rate >30 μg /min was compared with spot albumin to creatinine ratio and albumin concentration. They found a linear correlation between the three. In the first morning specimen either an albumin concentration >17 mg/L or ACR >3 mg/mmol predicted an overnight AER >30 μg /min with a 96.8% sensitivity and 90%specificity (Hutchison et al., 1988)

Urine ACR is also used to guide anti-hypertensive and renin angiotensin aldosterone system (RAAS) blockade therapies in patients with diabetes related chronic kidney disease. Improvement in urine ACR is considered to be a surrogate measure of improvement in the pathophysiology of diabetes related kidney disease.

Factors that have been shown to introduce variability in to urinary albumin excretion are physical activity, dietary protein intake, glycaemic control, fever and UTI(Mogensen et al., 1995). Reduced dietary protein has been shown to decrease albumin excretion in a 3 week randomised crossover trial; when randomised to normal protein diet (median 92 (range 55-117) g/day) and a low protein diet (47 (38-57) g/day), the median overnight albumin

excretion rate fell from 23.0 (15.0-170.1) micrograms/min during the normal diet to 15.4 (4.1-97.8) micrograms/min during the low protein diet (Cohen et al., 1987a). Long term dietary protein restriction has also been shown to reduce albuminuria in patients with type 1 diabetes and microalbuminuria in a 2 year randomised study (Dullaart RP, 1993).

A study involving 10 subjects with newly diagnosed type 1 diabetes looking at micro vascular permeability showed significant increase in albumin excretion during poor metabolic regulation, indicating a functional microangiopathy (Parving HH, 1976). Exercise induced albuminuria occurs at lower levels of exercise in patients with type 1 diabetes compared to normal subjects and it improves significantly on insulin treatment (Vittinghus and Mogensen, 1982). Exercise has been proposed as a provocative test for albuminuria in early renal disease in diabetes; exercise increases albuminuria in normal controls, normoalbuminuric and albuminuric subjects with diabetes (Feldt-Rasmussen B, 1985). Urinary tract infection has been shown to be associated with proteinuria and albuminuria which can be ameliorated by antibiotic treatment (Carter et al., 2006).

The immediate effect of a cooked meat meal on UACR in diabetic patients with and without microalbuminuria is unclear from the literature. Although it is advised that UACR be tested on first voided morning urine sample, in clinical practice this is often overlooked.

The aim of our study was to determine the effect of a cooked meat meal on UACR in diabetic patients with diabetes related chronic kidney disease.

7.2 RESEARCH DESIGN AND METHODS

This study was conducted using the data from a prospective, experimental, study that involved 16 participants each with CKD stages 1 & 2, 3A, 3B and 4 and 16 healthy volunteers (total n=80). The research design and method is described in detail in the previous chapter in this thesis.

The participants were provided meat and non-meat meals each providing approximately 54g protein (The meat meal consisted of 2 Aberdeen Angus quarter pounder beef burgers, and the non-meat meal comprised of 2 vegetarian burgers). Each meal included 250 mls of water to drink.

On the first study visit participants attended after an overnight fast and a pre-prandial and 4-hour post-meat meal urine samples were obtained. On the second visit, a fasting urine sample and a second sample 4 hours following the non-meat meal were obtained.

The median changes in urine ACR from fasting to 4-hour post-prandial urine samples were estimated in healthy volunteers and all the 4 study groups.

7.3 RESULTS

Changes in ACR from baseline to 4 hours were -0.08, -0.31, -3.0, -12.1, and -19.1 in the control, CKD 1&2, CKD 3a, CKD 3b, and CKD 4 stages respectively.

The decrements in ACR levels were significant in CKD 3b & 4 stages with p values of 0.046 and 0.028 respectively.

Figure 7.2 Changes in ACR in control and study groups

	Control	CKD 1&2	CKD3A	CKD3B	CKD4
■ ACR Changes	-0.08	-0.31	-3	-12.1	-19.1

Figure 7.3 (a)
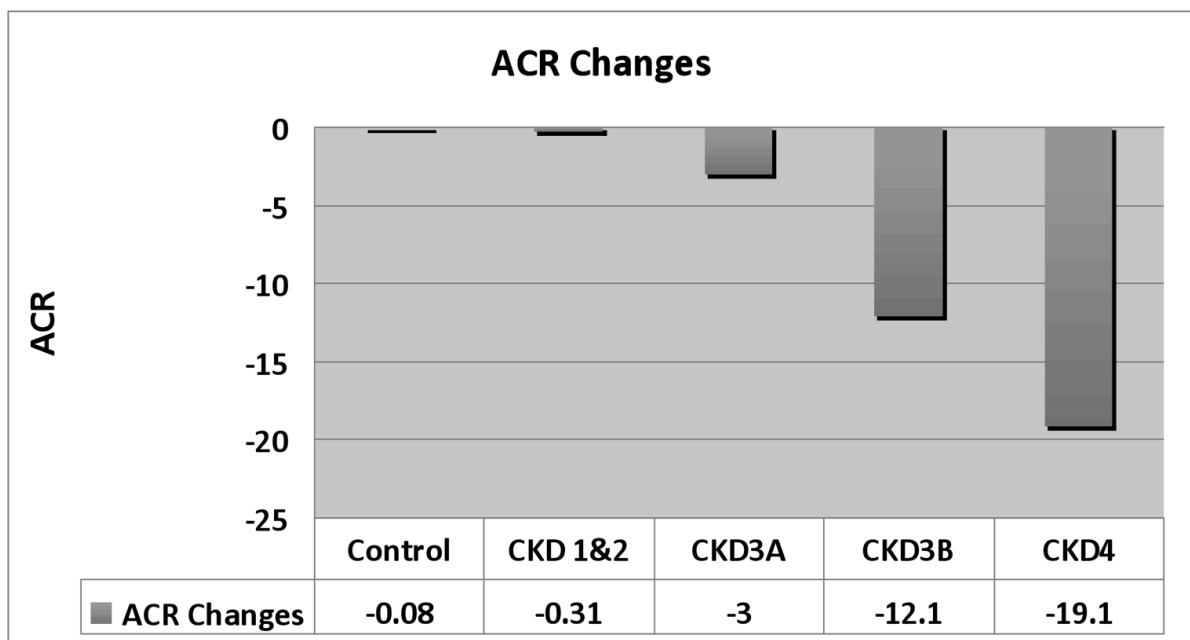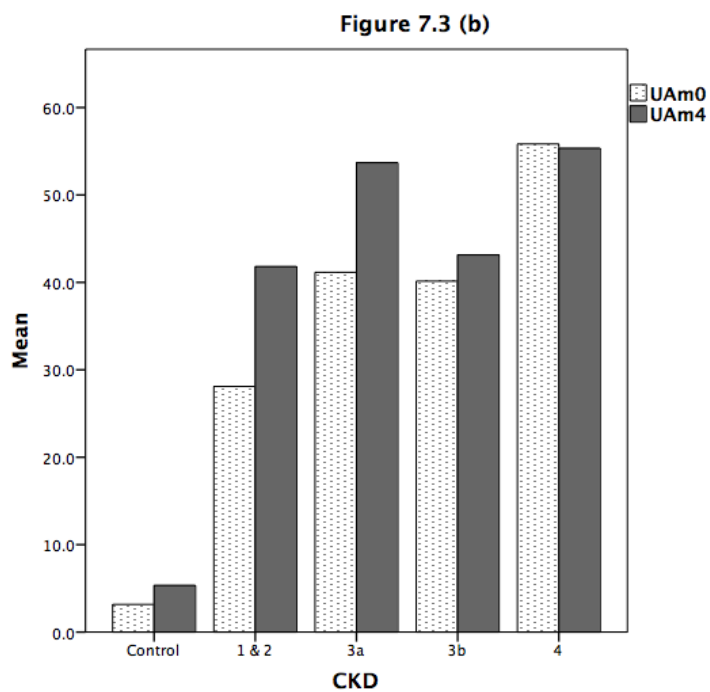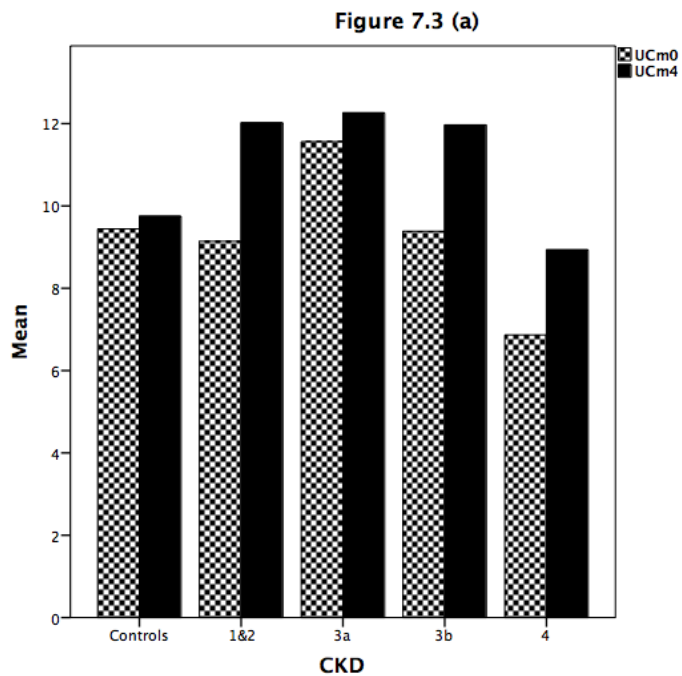
Figure 7.3 (b)

Figure 7.3 (a): UCmo & UCm4 represent the urinary creatinine prior to and 4 hours after the meat meal

Figure 7.3 (b): UAmo & UAm4 represent the urinary albumin prior to and 4 hours after the meat meal

7.4 DISCUSSIONS AND SUMMARY

We demonstrate a fall in ACR after cooked meat meal in patients with diabetes associated chronic kidney disease. A significant rise in serum creatinine following a meat protein meal has been demonstrated previously (Mayersohn et al., 1983) (Preiss et al., 2007) (Nair et al., 2014). In the study by Mayersohn et al cooked meat meal resulted in an average of 180mg in excess compared to that excreted on a control day. Our study showed a subsequent increase in urinary creatinine following a meat protein meal as shown in Fig 7.3 (a). This rise in urinary creatinine excretion following a meat meal can reduce the ACR as creatinine forms the denominator in this ratio.

Warram et al have previously demonstrated variability in ACR based on gender. In this study with 1613 patients with type1 diabetes and 218 healthy volunteers the range of ACR was 25 to 355 µg/mg in females and 17 to 250 µg/mg for males. These gender specific ranges correspond to AER of 30 to 300 µg/min. In the non-diabetic healthy volunteers cohort ACRs were below 16 µg/mg and the 95th centile in females was 25 and 17 for men. The gender difference is explained by higher muscle mass in males and higher serum and urinary creatinine levels with consequent reduction in the ACR (Warram et al., 1996).

An infusion of amino acid or an acute protein load is known to increase creatinine clearance (Bosch et al., 1983, Hirschberg et al., 1985). Our study demonstrates the effect of this increased excretion of creatinine on albumin creatinine ratio. Most of these studies looking at the acute effect of protein load show higher creatinine clearance in healthy volunteers than patients with chronic kidney disease. This should mean that the rise in urinary creatinine following cooked meat meal should be higher in healthy volunteers than patients

with more severe CKD. However, our study shows that the fall in ACR increases with worsening stages of chronic kidney disease.

Cooking converts creatine in meat into creatinine, which is absorbed from the gut. This has been shown to increase serum creatinine by other authors and also in the previous study in the thesis. This increased serum creatinine could raise urinary creatinine excretion, thereby reducing the albumin-creatinine ratio

Spot urine ACR makes clinical assessment for proteinuria immensely more easy compared to timed/overnight urine collection. But clinicians should be aware of the variability of urinary creatinine and the consequent effect on ACR. Cooked meat consumption is a major factor leading to variation in ACR values, which should be considered when interpreting results.

Chapter 8

STRENGTHS, LIMITATIONS AND FUTURE DIRECTIONS

8.1 Strengths and weaknesses of studies

The main strengths of these studies are that they attempt to answer important clinically relevant issues; limitations of estimating GFR in the obese subjects and the reliability of serum creatinine and eGFR following a meat meal. The studies involve real world routine diabetes clinic patients. The main limitation is the relatively small number of participants, which is primarily because of the intense nature of the interventions.

Effect of obesity on eGFR in type 2 diabetes and derivation of obesity correction equations:

This study had a well-characterised study population of patients with diabetes and different stages of chronic kidney disease with a wide range of eGFR from 18.5 to 124.8 mls/min/1.73m^2. The reference method for GFR measurement was ^{51}Cr-EDTA method, which has some limitations as mentioned in the introduction. The results may not be directly comparable to many studies particularly from the United States as the commonly used reference methods there are ^{99}Tc-DTPA and ^{125}I Iothalamate. ^{51}Cr-EDTA however has definite advantages like stability and possibility of using stored samples and also is the technique routinely used in our reference laboratory and across Europe in general. The MDRD study used equation was derived using renal clearance of ^{125}I Iothalamate and this could have contributed to some of the bias we observed.

Another limitation of our study is that in the calculation of GFR possible sex differences are not addressed and also there is an imbalance between males and females in BMI <30. The

MDRD equation includes a factor to correct for gender. Although the study was not powered to investigate gender differences, we have analysed our data by gender categories; in the obese subjects, there was no significant difference in bias across gender but overall, females had higher bias. There is also an imbalance in the number of obese and non-obese subjects in this study. We employed a purposive or quota sampling from a hospital diabetes clinic population to achieve the required numbers in the groups and as expected, the type2 diabetes clinic population was skewed towards obesity.

The suggested prediction equations needs patients BMI to be known which can make automatic result generation challenging. However, we consider it possible that the laboratory can apply such a correction if requested for an overweight or obese patient when eGFR is an important determinant in the choice of antidiabetic medication. These correction equations for the existing 4-variable MDRD equation need validation in larger external datasets to attain better generalisability. One of the main challenges we faced is in accessing data from studies that have used ^{51}Cr-EDTA method for external validation.

Effect of cooked meat protein on creatinine, eGFR and ACR

The main strengths of this study is that our study population had diabetes and well defined CKD stages while previous studies were conducted in healthy volunteers or general day units (Mayersohn et al., 1983, Preiss et al., 2007). This would make the study results more relevant for patients with diabetic nephropathy. The intervention itself was more standardised in that patients received same amount of protein load as they received commercially packed and quantified meals. Like any other study of dietary interventions,

there will be variability in absorption of cooked meal protein in various individuals, which could affect the rise in serum creatinine. However our study draws comparison between rise in serum creatinine in the same individual following meat and non-meat meal. Some previous studies have shown much more pronounced rise in serum creatinine of nearly 100%, but the amount of meat eaten by the healthy volunteers was much higher (Jacobsen et al., 1979) while we administered a meat portion which would a normal helping.

There are some confounders that we were unable to control due to the nature of the study. As the participants were only with us for a maximum of 4 hours in a day the food intake during the rest of the day was not standardised although they were advised to have non-meat meals for the rest of the day. The amount of exercise and state of hydration are other potential confounders in this study. Conducting these experiments in a residential manner, which would significantly increase inconvenience to participants, could control these confounders to a better extent but we feel it could have made recruitment of relatively high numbers of participant more challenging.

8.2 Future directions

It is evident from our studies presented in this thesis and also other studies referenced here that estimation of glomerular filtration has several variability and limitations. This is particularly true when estimating GFR in the obese and morbidly obese subjects who form an increasingly larger proportion of our patient population. This group of patients may be seriously disadvantaged because of underestimation of GFR and being denied treatment options for type2 diabetes and potentially for other medical conditions.

We need more studies with larger number of obese subjects to be able to derive new equations to estimate their glomerular filtration rate. The current trend towards developing well-resourced obesity and bariatric assessment clinics in the NHS and world over should help develop more robust datasets.

Indexing of GFR to BSA is clearly an area that needs more research, as it is a major factor in the significant bias between measured and estimated GFR. This is particularly true for patients with body sizes very different than average. It is possible to take away the effect of indexing on eGFR by multiplying it by BSA and dividing by 1.73; converting eGFR units from mls/min/1.73m^2 to mls/min. A study looking at comparison between measured GFR (un-indexed) and eGFR in mls/min would be interesting to see if the bias can be avoided. Another interesting study would be to compare GFR indexing with BSA calculated from ideal body weight rather than the real body weight.

For the clinicians it is important to recognise the underestimation of GFR in obesity when using the MDRD or CKD-Epi equations. If a major clinical decision is based on the renal function we should consider trends in serum creatinine or other indicators like 24-hour urinary creatinine clearance or Cystatin C derived eGFR to have more information to make a better judgement. Also meat protein meal induced rise in serum creatinine and drop in urinary albumin creatinine ratio should be considered and a fasting sample requested before making major clinical decisions based on these measurements.

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APPENDICES

(Published papers from this work)