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TESI DI DOTTORATO

Renal function reserve (RFR) in Living donor renal transplantation (LDRT) and phenotypic characterization of urinary extracellular vesicles (uEVs) pre and post nephrectomy

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To my teachers, parents and family!

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Abstract

Background

In living donor renal transplantation pre operative donors renal function reserve (RFR) may correlate with post nephrectomy residual kidney function in donors. The aim of this study was to evaluate kidney donor (KD) renal function (RF) before nephrectomy and investigate the predictive performance of pre-donation RFR with protein load and to perform phenotypic analysis of uEVs pre and post 7 days of kidney donation.

Methods

Twenty eight living donors were considered for RFR test between February 2019-2021 in the Department of Nephrology, Dialysis and Transplantation, Hospital Maggiore della Carita' di Novara, Italy. RFR was measured with a renal stress test (RST) before nephrectomy through an oral protein loading test (1 g/kg of body weight). For uEVs characterization first urine sample was collected pre nephrectomy. Second urine samples were collected post 7 days of nephrectomy. The expressions of uEVs were characterized by bead-based multiplex analysis by flow cytometry.

Results

A significant increase in GFR pre transplant renal stress test (RST) was observed among donors post protein load (*p=0.0001)*, with corresponding high RFR values; 29.5 (4.2-100) ml/m/1.73m^2. CD133 and CD24 were detected in the majority of the donors after 7 days of nephrectomy.

Conclusion

Pre and post RFR along with pre and post uEVs assessment may represent a useful screening tool for LDRT. Post nephrectomy rise in the levels of CD133 and CD24 may reflect the involvement of progenitor cells providing regenerative renal potential.

Chapter 1: Introduction

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1.1 Kidneys

Kidneys are one of the most vital and complex organs in the body, performing crucial functions like, metabolic waste removal, elimination of foreign compounds, maintaining acid-base balance of the body, maintaining blood pressure, blood and interstitial electrolyte homeostasis, erythropoietin and bone mineral regulation [1].

These functions are carried out by a group of cells that form the structure of glomeruli and tubules of the nephron [2]. Apart from filtering, kidneys also produce certain chemicals and hormones, calcitriol, an active form of vitamin D that maintains calcium levels in the bones and keeps normal chemical balance in the body [3], rennin regulates blood pressure, and erythropoietin regulates the production of red blood cells, the kidneys also regulate several chemicals in the body like, calcium, Phosphorus, Sodium and Potassium levels [4] [5].

Human kidneys accommodate round about 1.0-1.2 million nephrons. A nephron is the primary unit of the kidney. It is consists of different subunits and includes the glomerulus, proximal tubule, loop of Henle, distal tubule and the collecting duct [6]. All subunits commit to the excretory role of the kidney in three steps:

- Glomerular filtration
- Tubular reabosorption and
- Tubular secretion [7].

On an average, the kidneys receive approximately 20% of the cardiac output. This computes around 1 L/minute and 600 ml of plasma. Throughout glomerular filtration, blood plasma is filtered in the glomerulus, a bunch of process capillaries bound by a membrane and specified epithelial cells that allow solute and waste, plus drugs and their metabolites, and water to pass across while ensuring larger substances, like blood cells, and proteins persist in the circulatory system [8].

Protein bound molecules, as well as drugs, are removed by proximal tubular secretion by a well harmonized function of uptake by the tubular cells at the blood-

facing basolateral site and excretion into the tubular lumen. Tubular reabsorption starts as soon as the filtrate gets inside the lumen of the proximal tubule, and involves the reabsorption of organic nutrients, such as hormonal-regulated reabsorption of ions coupled with passive water reabsorption and glucose [9]. Megalin and cubilin receptors at the apical membrane are accountable for endocytosis –mediated re-uptake of filtered low-molecular weight proteins, like β2-microglobulin [10]. As the filtrate moves along the nephron, hydrogen ions, ammonia and some drugs are secreted into the collecting tubule [11].

The circulatory anatomy inside the kidney determines the final urine composition. First glomerular filtration rate (GFR) essentially influences the amount of solute and water that is excreted [12].

1.2 Glomerular filtration

The core function of the glomerulus is to perform as a filtration barrier that allows the passage of water and other solutes and restricts the movement of certain molecules. For example, filtration of water, sodium, urea, and creatinine are essential to proper toxin clearance, volume balance, and electrolyte homeostasis. The glomerular function guarantees that the vital plasma proteins are retained in blood and the filtrate is excreted out as urine. The fenestrated endothelium, glomerular basement membrane (GBM) and highly specified podocytes are the three main glomerular filtration assemblies [13].

An initial separation of ultrafiltrate from plasma takes place across the glomerular capillary wall into Bowman's space for urine production. Starling's forces are the major determinant of ultrafiltrate formation across the capillary wall [14]. These forces are proportional to glomerular permeability and the balance between hydraulic and oncotic pressure gradients. Hence, GFR can be explained by the following equations:

GFR= (capillary porosity X surface area) X (Δ hydraulic pressure ─ Δ oncotic pressure)

GFR= (capillary porosity X surface area) X ([P_{GC} ─ P_{Bs}] ─ S [π_p─π_{bs}])

GFR= (capillary porosity X surface area) X ($P_{GC} - P_{Bs} - \pi_p$)

Where P_{GC} and P_{Bs} are hydraulic pressures in glomerular capillary and Bowman's space, respectively and s is the reflection coefficient of proteins across capillary wall (a measure of permeability) and $\pi_p - \pi_{Bs}$ are the onctic pressure of plasma in glomerular capillaries and Bowman's space respectively. Since π_{Bs} is zero (because the filtrate must be protein free) and the capillary wall is completely permeable, thus making s=1, the last equation,

GFR= (capillary porosity X surface area) X ($P_{GC} - P_{Bs} - \pi_p$), represents the formula for GFR. Normally, hydraulic pressure in the capillaries and Bowman's space persists constant whereas oncotic pressure in plasma rises dramatically with formation of a protein-free ultrafilrate. Hence, at some point across the capillary loop, the total filtration gradient becomes zero and filtration equilibrium occurs [15, 16].

Changes in renal plasma flow (RPF) can change the GFR. RPF plays a vital role in determining GFR in the presence of filtration equilibrium, as it impacts glomerular capillary oncotic pressure. Hence, an increase or fall of GFR is proportional to alterations in RPF [17].

1.2.1 Autoregulation

RPF and GFR are maintained constant by autoregulation of the renal circulation and are depended on two mechanisms:

a. The myogenic response (MR) , and
b. The tubuloglomerular feedback (TGF)

MR is a function of smooth muscle, which provokes vasoconstriction on an increase in arterial pressure, hence, permits autoregulation. On other hand TGF is a more complex process specific to the kidney that allows constriction of the afferent arteriole in response to a rise in sodium chloride concentration in the early distal tubule, which is a function of tubular flow rate [18]. As, autoregulation function decides the amount of pressure variations reaching the glomerulus, peritubular

capillaries, and the medullary circulation, and so, it is vital for filtration, reabsorption, hypertensive renal damage and nutriuresis [19].

1.2.2 Clinical assessment of GFR

For the management of patients with kidney disease measurement of GFR is an important tool. Functioning renal mass can be well determined by measuring total kidney GFR, which is the total of filtration rates of working nephron units. Regular GFR measurement leads to identify kidney disease, drug toxicity, improvement or progression of kidney function, initiation of renal replacement therapies (RRTs) when there is total shut down of renal function. To determine GFR accurately, the substance used a marker must be filtered freely by the glomerulus and should not be secreted, reabsorbed or metabolise by the kidney. GFR can be calculated by the following formula:

$$\text{GFR} = \frac{\text{Urine concentration A X volume}}{\text{Plasma concentration A}}$$

Where A is the substance that suits best as an ideal marker [20]. Inluin is one of the best markers for GFR, because of its feature, inulin clearance precisely reflects GFR. Though it is not used widely because it is administered intravenously and is more expensive [21].

Thus, other less expensive and representative markers is used to measure GFR and kidney function, such as creatinine, urea, Cystatin C, ß-trace protein, radioactive markers [22] and recently urinary extracellular vesicles [23].

A. Creatinine

It is the most common used marker, which is produced from the metabolism of skeletal muscle creatine. It is released into plasma at a stable rate in normal individuals and filtered freely at glomerulus. Unluckily, it enters urine through secretion by organic cation transporter in proximal tubule, overcapitalizing GFR by 10-20%. As kidney function drops or declines the rate of tubular creatinine secretion increases and in such condition creatinine clearance may overestimate true GFR [24].

Creatinine is generally used to monitor progression of kidney disease and function. The normal creatinine clearance (CrCl) ranges from 110-150 ml/m in male and 100-130 ml/m in female. The National kidney Disease Education Programme suggests calculating glomerular filtration rate (GFR) from serum creatinine (SrCr) concentration [25].

CrCl is calculated by the following formula that uses SrCr concentration and a 24-hour urine for Cr concentration and urine volume:

$$CrCl = \frac{UCr \times Volume}{SCr}$$

Where, Cr is creatinine, Cl is clearance, U is urine and SCr is serum creatinine [26].

B. Urea

Urea is the main nitrogenous end product of protein and amino acid catabolism, produced by liver and distributed throughout intracellular and extracellular fluid. In kidneys urea is filtered out of blood by glomerulli and is somewhat being reabsorbed with water [27]. The most specific clinical indicators for the estimation of kidney function are based on the serum urea concentration. It is useful in the differential diagnosis of acute renal failure (ARF) and pre-renal condition where the ratio of blood urea nitrogen to creatinine increases [28].

Urea clearance is a poor predictor of glomerular filtration rate (GFR) because the rate of overproduction depends on many other non-renal factors that include diet and urea cycle enzymes. Increased blood urea nitrogen (BUN) has been seen in association with kidney disease or failure, urinary tract obstruction due to kidney stones, congestive heart failure, dehydration, fever, shock, and gastrointestinal bleeding. High levels BUN can sometimes occur during late pregnancy or result from eating large amounts of protein-rich foods. If BUN is above 100 mg/dL, it indicates severe kidney damage, while decrease in BUN is observed during fluid excess. Low levels are also seen in trauma, surgery, opioids, malnutrition, and anabolic steroid use [29].

BUN is firmly related to kidney function and neurohormonal activation in heart failure (HF). It is filtered through the glomerulus, and urea is reabsorbed in the tubules. Subsequently, plasma BUN isn't just reliant upon GFR, yet, also on tubular function, and closely related to neurohormonal activity such as the renin-angitensin system (RAS) or renin-angiotensin-aldosterone system (RAAS) activity [30]. Urea is principally reabsorbed in the proximal tubules, but also more distally in the collecting ducts under stimulation of V2 receptors of vasopressin [31].

C. Cystatin C

The Cystatin C is a non-glycosolated low molecular weight protein and protease inhibitor. It is used as a biomarker because it is produced by all nucleated cells at a constant amount and filtered freely through glomerulus and fully catabolised in the proximal tubules. GFR determines the concentration of serum Cystatin C making it as an endogenous marker for GFR [32]. Dharindharka *etal* found that Cystatin C was a superior than serum creatinine (SrCr) as a GFR marker [33]. It was found to be an efficient marker for GFR in patients with liver cirrhosis following transplantation [34, 35]. It has been also found to be more helpful in early detection of kidney dysfunction in both type 1 and 2 diabetic patients [36]. Eventually Cystatin C was found to be involved in mild early renal dysfunction in patients with increased risk of cardio vascular events, peripheral arterial disease and heart failure (HF) [37].

Furthermore, the CKD-EPI formula that includes SrCr and Cystatin C was shown to have greater accuracy and precision in finding GFR and hence is better than MDRD and Cockcroft-Gault calculators for GFR [38].

D. ß-trace protein (BTP)

This protein has potential to meet criteria for use as a suitable biomarker of GFR, because it’s filtered at glomerulus and then reabsorbed in proximal tubule or excreted in urine [39]. It has been reported to be a better indicator of reduced GFR upon comparison with serum creatinine (SCr) [40] [41]. Serum BTP has been reported to be elevated in patients with kidney diseases [42].

E. Radioactive markers

In recent years radioactive markers have been used as markers to estimate GFR. Some of the well known radioactive markers are 125 iodine (I)-iothalamate, 99 m Tc-DTPA (diethyl triamine penta acetic acid), 99 mTc mercapto acetyl triglycine and 51 Cr EDTA ethylenediamine terta acid.

Geeta Bajaj et al showed the efficiency of subcutaneous injection of renal 125 iodine (I)-iothalmate clearance is the simple and accurate test for measuring GFR in adults. The same author discovered renal clearance of 125 iodine (I)-iothalamate, simple and effective in healthy children and those with mild and advanced kidney disease [43]. In one of the study compairing Cystatin C and 125 iodine (I)-iothalamate clearance among hypertensive patients showed the mean extraction of Cystatin C was equal to the mean renal extraction of 125 iodine (I)-iothalamate, suggesting tubular excretion of Cystatin C [44]. It was possible to get an accurate determination of 51 Cr EDTA clearance from a single plasma sample in adults by using the mean sojourn time methodology has been shown to accurate for determination of 99 m Tc-DTPA single sample clearance [45]. 51 Cr EDTA-GFR is advisable in patients with systemic lupus erythematosus (SLE) with suspected renal involvement when the serum creatinine concentration and creatinine clearance are normal [46]. Only limitation with this marker is that, it is overestimated in patients with severe oedema [47].

F. Urinary extracellular vesicles (UEVs)

Isolated from various types of body fluids, including urine, Cell-derived extracellular vesicles have achieved an important acceptance as potential diagnostic biomarkers in kidney disease as their cargo consists nucleic acids, proteins, and other cellular components, which likely reflect the physiological and possibly pathophysiological state of cells along the nephron. Research evidence suggests the practicality of utilising EVs as biomarkers for diagnostic, prognostic, and therapeutic purposes in different types of renal disease, such as acute kidney injury (AKI), glomerulonephritis (GN), and living donor renal transplantation (LDRT) [23].

EVs are secreted by most of the cell types of the kidney. Proteomic analysis of uEVs has established that proteins within the kidneys may arise from all segments of nephron, which includes proximal tubules, the thick ascending limb of Henle's Loop, the distal tubule, podocytes, and the collecting duct. Hence, exosomal proteins can be exploited as biomarkers for location-specific diseases [48].

uEVs may reduce the necessity for an invasive renal biopsy in glomerular disease. Specialized epithelial cells like podocytes which forms the glomerular filtration barrier with the glomerular basement membrane, are the main cell-types which are more affected in glomerular disease. Therefore, podocyte-derived EVs can be a possible index of glomerular injury. In a disease with significant podocyte damage known as focal segmental glomerulosclerosis (FSGS), transcriptional factor, which is needed for normal kidney development, i.e Wilm's tumor-1 (WT-1) was examined in urinary exosomes as a possible marker for podocyte damage. Expression of WT-1 was found to be increased in urinary exosomes resulting urinary albumin excretion by one week in a mouse model of collapsing glomerulopathy [40]. In addition, in human subjects with FSGS, exosomal WT-1 levels were remarkably elevated in children with active nephrotic syndrome (NS) caused by FSGS compared with healthy controls or patients with steroid-sensitive nephrotic syndrome (SSNS). Moreover, there was a significant fall in urinary exosomal WT-1 in patients with remission of SSNS following steroid treatment [49].

WT-1 expression in uEVs was also found to be higer in patients with diabetes, where, podocyte injury is an initial characteristic. Kalani *et al*. pointed WT-1 in urinary exosomes of diabetic patients, which correlated with declining renal function, highlighting exosomal WT-1 as a biomarker of podocyte injury in diabetic nephropathy (DN) [50].

EVs may play an important role in cilia biology and biomarkers for polycystic kidney disease (PKD), because cystin and ADP ribosylation-factor-like-6 are abnormally expressed in urinary EVs of patients with PKD [51].

1.2.3 Renal function markers and laboratory testing

Renal dysfunction occurs in variety of diseases and conditions. Acute kidney injury (AKI) occurs due to trauma to the kidney like an accident or medical procedure including Intensive Care Unit (ICU) acute renal failure (ARF) [52]. Chronic kidney disease (CKD) results from another disease like hypertension (HTN), diabetes mellitus (DM) or from an inherited syndrome (Table 1.1) [53]. Early detection of any kind of renal dysfunction plays an important role in preventing further deterioration of renal function.

A. Diagnosis

Indications for testing:

- Assess for any early renal abnormalities.
- Any risk for CKD

B. Laboratory testing

- Serum creatinine, BUN, and eGFR: use for initial diagnosis for AKI or CKD.
- Urine protein/albumin: initial test for assessing kidney function.

Acute kidney injury (AKI)	**Chronic kidney disease (CKD)**
Trauma	Hypertension (HTN)
Sepsis	Diabetes Mellitus (DM)
Blood loss	Polycystic kidney disease (PKD)
Hypotension	Vascular disease
Contrast induced	Nephrotoxic drugs • Nonsteroidal anti-inflammatory drugs (NSAIDs) • Radio contrast.

Table 1.1: Differential diagnosis of renal injury.

C. Screening

eGFR and albumin/protein in patients with HTN, DM, cardiovascular disease (CVD) [54] [55].

D. Pathophysiology

- Tubular proteinuria results when glomerular function is normal but the proximal tubules have diminished absorbing capacity [56].
- Established biomarkers of chronic tubular dysfunction – acute and chronic
  - Glomerular filtration rate (GFR)/BUN/ serum creatinine – provide estimates of renal function
    - BUN/creatinine – biomarkers of protein metabolism
    - eGFR is best measure – accounts for age, BMI, and sex
    - Useful in both acute and chronic renal failure
  - Albumin (urine)
    - Normally very little excreted by the kidney
    - Albuminuria – 30-300 mg albumin/24 hours or 30 mg/g creatinine
    - Sensitive marker of glomerular disease in patients with diabetes, chronic kidney disease
    - Limited ability to predict disease progression
  - Cystatin-C (serum and urine)
    - Cysteine protease inhibitor is a marker of GFR
    - Not influenced by changes in muscle mass – may make it a better marker than creatinine
    - Urine test measures proximal tubular injury
    - Affected by steroid use and thyroid dysfunction
  - Beta-2-microglobulins (urine)
    - Filtered freely in the glomerulus and nearly completely reabsorbed – normally <1% appears in urine
    - Occur during the course of advanced diabetic nephropathy
    - May be useful as a marker of progressing idiopathic membranous nephropathy

- Alpha-1-microglobulins (α_1-MG) (urine)

- Evaluates primarily proximal tubular region (may also be assessed by urinary retinol binding protein)
- Occur during the course of nephritis or advanced diabetic nephropathy
- Occur after heavy metal exposure or treatment with nephrotoxic medications.
- Occur in urinary tract infections (UTIs), where elevated α_1-MG concentrations signal renal involvement
- May be a promising candidate as a biomarker of acute renal failure (ARF).

- Alpha-2-macroglobulin (α_2-MG) (serum)
  - One of a family of protease inhibitors that includes alpha-1-antitrypsin
  - α_2-MG is a protease inhibitor capable of irreversibly binding, and therefore inhibiting, a wide variety of proteases, including plasmin, pepsin, trypsin, chymotrypsin and cathepsin-D
  - α_2-MG molecule tends to remain intravascular due to its large size; levels increase during renal disease where smaller proteins are leaked into the urine
  - α_2-MG is synthesized in the liver

- May also be increased in the following
  - Estrogen stimulation due to pregnancy, contraceptives
  - Nephrotic syndrome – retained by damaged glomerular membranes because of its large size
  - Diabetes mellitus with renal disease
  - Hepatorenal syndrome
  - Interruption of blood/brain barrier; presence of α_2-MG in cerebrospinal fluid (CSF) [57] [58].

1.2.4 GFR estimates

Different formulas have been used worldwide using SrCr concentration and other clinical and laboratory data to calculate more accurately estimate GFR (eGFR),

which includes the Cockcroft-Gault formula, Modification of Diet in Renal Disease (MDRD) formula and CKD-EPI creatinine equation. These formulas use serum creatinine concentration, age, gender, race, and body size, and are considered to be better estimates of GFR than serum creatinine concentration alone [59].

1.2.4.1 Cockcroft-Gault formula

The equation given below estimates creatinine clearance [60]:

$$\frac{[140\text{-age (years)}] \text{ X weight (kg)}}{72\text{X serum creatinine (mg/dL)}} \text{ X } 0.85 \text{ for females.}$$

Although it gives an adequate estimate of GFR, the MDRD equations are more accurate.

1.2.4.2 Modification of Diet in Renal Disease (MDRD) formula:

MDRD equation 7 is the most preferred formula but it needs serum blood urea nitrogen (BUN) and albumin concentrations. The MDRD formula is as follows:

$$170 \text{ X } [\text{SCr (mg/dL)}]^{-0.99} \text{ X } [\text{age (years)}]^{-0.176} \text{ X } [0.762 \text{ if female}] \text{ X } [1.18 \text{ if African American}] \text{ X } [\text{albumin (g/dL)}]^{+0.318}$$

An abbreviated form of the MDRD equation that doesn't require serum BUN or albumin concentration was also developed and is follows [61]:

$$186 \text{ X } [\text{ SCr (mg/dL)}]^{-1.154} \text{ X } [\text{age (years)}]^{-0.203} \text{ X } [0.742 \text{ if female}] \text{ X } [1.21 \text{ if African American}].$$

This abbreviated form is reasonably accurate. The MDRD equation has been tested and developed in over more than 500 patients with a range of kidney diseases and ethnicities (Table 1.2) [62].

1.2.4.3 CKD-EPI equation:

Another equation for estimation of GFR is CKD-EPI creatinine equation (chronic kidney disease epidemiology collaborations).CKD-EPI equation is developed in 2009 by a research group established by National Institute of Diabetes, Digestive and Kidney Disease [63].

The CKD-EPI is a single expressed equation as follows:

$eGFR=141X \min(SCr/K,1)^{\alpha} X \max(SCr/K,1)^{-1.209} X 0.993^{age} X 1.018$[if female] X 1.159 [if African American]

Where, K=0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males. Tables 1.3 show the CKD-EPI equations to be implemented in clinical laboratories [64].

Marker	Study year	Characteristics	GFR measurements	Equation	References
Creatinine	MDRD 1999	N=1628, CKD mean mGFR=40	Urinary clearance of iothalamate	6-variable MDRD Study equation and 4-variable MDRD Study equation	6-variable: Levey et al[63] (*Annals*, 1999); 4-variable: Levey et al[65] (*JASN* abstract, 2000)
	MDRD 2006	Same as above	Same as above	6-variable MDRD Study equation	6-variable: Levey et al[63] (*Annals*, 1999); 4-variable: Levey et al[65] (*JASN* abstract, 2000)

				and 4-variable MDRD Study equation	

Table 1.2: GFR estimating equations developed by MDRD study group.

Race	Sex	SCr (mg/dL)	Equation
African American	Female	≤ 0.7	$GFR = 166 \times (Scr/0.7)^{-0.329} \times (0.993)^{Age}$
		> 0.7	$GFR = 166 \times (Scr/0.7)^{-1.209} \times (0.993)^{Age}$

	Male	≤0.9	$GFR = 163 \times (Scr/0.9)^{-0.411} \times (0.993)^{Age}$
		> 0.9	$GFR = 163 \times (Scr/0.9)^{-1.209} \times (0.993)^{Age}$
White or Others	Female	≤ 0.7	$GFR = 144 \times (Scr/0.7)^{-0.329} \times (0.993)^{Age}$
		> 0.7	$GFR = 144 \times (Scr/0.7)^{-1.209} \times (0.993)^{Age}$

	Male	≤0.9	$GFR = 141 \times (Scr/0.9)^{-0.411} \times (0.993)^{Age}$
		> 0.9	$GFR = 141 \times (Scr/0.9)^{-1.209} \times (0.993)^{Age}$

Table 1.3 CKD-EPI for estimating GFR.

1.3 Renal replacement therapy (RRT)

Renal replacement therapy (RRT) restores the normal blood filtering function of the kidneys and is used when kidneys stop functioning which is known as renal or kidney failure, which includes acute kidney injury (AKI) and chronic kidney disease (CKD). RRT comprises of various ways of blood filtration such as dialysis (hemodialysis or peritoneal dialysis), hemofiltration and hemodiafiltration [66].

Hemodialysis (HD), hemofiltration (HF), and hemodiafiltration (HDF) can be continuous or intermittent and can use an arteriovenous route or a venovenous route [67].

It also includes renal transplantation which is an ultimate type of replacement where old kidney is replaced by donor kidney (living kidney or deceased kidney) [68].

1.3.1 Renal transplantation

Dr. Joseph Murray in 1954 conducted first successfully kidney transplantation. Since then there have been vital evolution in the field of transplantation and immunology, allowing an extensive selection of acceptable donors and recipients [69]. Kidney transplantation is done to improve and prolong the lives of patients with end-stage renal disease (ESRD). Open and laparoscopic surgery is the two methods for both procurement and transplantation [70]. It is often the best treatment available for ESRD [71]. Patients with ESRD have better long-term survival than those who stay on dialysis. Moreover those who undergo transplantation have better quality of life and graft estimated survival of over 10 years among those who stay on dialysis [72].

The prevalence of ESRD is rising worldwide very rapidly. The most common cause of ESRD is hypertension (HTN) and diabetes. Other causes of ESRD/CKD are glomerular disease, tubulointerstital disease [73]. Patients with chronic kidney disease (CKD) stage 4 and glomerular filtration rate (GFR) less than 30 mL/min/1.73 m, must be educated about kidney failure and RRTs, including transplantation [74].

A. Contradictions for kidney transplantation

Inability to tolerate surgery due to severe cardiac or pulmonary diseases, active malignancy, active infection, active drug abuse, and uncontrolled psychiatric diseases are absolute contradictions for kidney transplantation [71]. Relative contraindications varies and may be different depending on the institution and geographic region: morbid obesity with a recommended body mass index (BMI) less than 40 kg/m, history of noncompliance with medication regimen or dialysis schedule , frailty, psychiatric problems, and limited life expectancy (defined as less than the anticipated waiting time for a kidney) [75].

B. Preparations for kidney transplantation

I. Recipient selection

Most End Stage Renal Disease (ESRD) patients have multiple co-morbidities and complications due to kidney disease. And therefore they are screened for their ability to tolerate surgery and subsequent immunosupression which goes hand to hand with

transplant surgery [71]. Summary of evaluation of co-morbid conditions in transplant recipients are described below:

- Cardiovascular disease: one of the leading causes of death post kidney transplantation is cardiovascular disease [76]. Therefore ESRD patients need to be carefully evaluated for cardiovascular disease. Non-invasive procedures can be implied for testing those with high risk or symptoms. For example, a dobutamine stress echocardiogram has been proved to have superior accuracy for predicting preoperative cardiac events [77]. Therefore, patients should undergo cardiac revascularization before kidney transplantation if tested positive.
- Cerebrovascular disease: A patient should be evaluated for carotid artery disease with history of a cerebrovascular accident, including transient ischemic injury [78]. Also any history of polycystic kidney disease (PKD) warrants a magnetic resonance angiogram for screening aneurysms [79]. Duplex Ultrasound (duplex USG) and computed tomography angiography (CTA) are advisable in case of abnormalities in peripheral pulse examination, suggestive of peripheral vascular disease (PVD) and vascular surgery to be considered before kidney transplantation [80].
- Gastrointestinal disease: Any transplant recipient with personal or family history of colon cancer or above the age of 50 years should have screening colonoscopy as per USPSTF recommendations [81]. Any recipient with history or active chronic liver disease (CLD) or viral hepatitis must consult hepatologist for possible consideration of Liver-Kidney transplant.
- Haematological disorder: Transplant recipients with a history of thrombosis should be evaluated for hypercoagulable disorder which may require treatment with anticoagulants [82]. Those with diathesis, full panel of coagulation should be done.
- Infections: Active infection is an absolute contradiction to a kidney transplant. It is recommended to send a serology panel to test for viral infections, tuberculosis, etc. Vaccination should also be up to date.

- Malignancy: Depending on the type of cancers, most transplant centre requires 2-5 years of cancer free period. It is required to minimise the post transplant recurrence or metastasis potentiated by immunosuppressive therapy [83].
- Pulmonary disease: Pulmonary function test and an echocardiogram to be performed to rule out possibilities of pulmonary hypertension in patients who have been on dialysis for long time, those with heart dysfunction, known case of chronic obstructive pulmonary disease (COPD), extensive use of tobacco, history of sleep apnea [84]. Those with pulmonary HTN, preoperative treatments with vasodilators are recommended before transplantation, along with pulmonary clearance.
- Frailty: Short physical performance with an objective score is performed to check the recipients' fitness for transplant. This frailty criteria domain includes self-reported exhaustion, weakness, slow walking speed, low physical activity and unintentional weight loss. These scales are especially useful in the elderly demographic, in particular with recipients above the 60 years of age [85].

II. Donor Selection

There are two types of donors:

a. Deceased donors
b. Living donors and

- **Deceased donors:** They are of two types, Brain dead donors (BDD) and those who donate after cardiac death (DCD). BDDs are those who fulfil the criteria of brain death testing. DCD are those who do not fulfil the criteria of brain death, but are those who are unlikely to experience meaningful neurologic recovery [86]. In case of DCD procurement cannot be initiated until heart has stopped beating and an independent physician pronounces the patients following terminal extubation. United Network for Organ Sharing (UNOS) stratified guidelines for deceased donors for organ quality. Thus, deceased donors need to meet standard

criteria (SCD) or otherwise, it will fall into umbrella for extended criteria donation (ECD). ECD kidneys are linked with shortened graft survival secondary to donor risk factors: over 60 years of age, or those between 50-59 years with a history of HTN, creatinine concentration above 1.5 mg/dL, or cerebrovascular cause of death [87].

In 2004 SCD and ECD classifications were replaced by the kidney donor profile index (KDPI), a more objective graft quality measure. It is derived from the kidney donor risk index (KDRI), the percentage of donors in a reference population defined by the Organ procurement and transplantation network (OPTN). Factors used on determination of KDPI are, donor age, height, weight, HCV status and DCD status [88]. Multiple studies has proved that kidney transplantation based on high-KDPI kidneys is still linked low morbidity rate and improvement in life expectancy and is a cost-saving treatment plan when compared with patients who carried on with maintenance dialysis [89].

- **Living kidney donor (LKD):** Living donor renal transplantation (LDRT) offers the best possibilities of graft and recipient survival, even in paired kidney exchange, which involves transport of the organ before implantation [90]. Eligibility criteria for LKDs are 18-70 years of age, no active infection, no active malignancy, and adequate renal function (~ GFR > 80). BMI more than 40 kg/m, diabetes, active malignancy, human immunodeficiency virus (HIV) positivity, GFR less than 70 mL/min/1.72m, HTN requiring more than medication, albuminuria, horseshoe kidneys, and psychiatric disorders are main contraindications to LKDs [71].

C. Recent developments in living kidney donations

Living kidney transplantation is the best available treatment for patients with ESRD for various reasons including:

i. Better long term graft survival
ii. No need to wait for transplant on waiting list from deceased kidney transplant list.
iii. Lower risk for graft rejection and delayed graft function (DGF) [91].

The living donor programme was expanded by new modes of living donation and extended by the living donor pool [91]. A well developed paired kidney donation is the fundamental element for creating a donor pool [92]. Paired kidney donation helps donation for non-compatible donor and recipient that would need desensitization [93]. Other possible approach to increase donor pool is ABO-incompatible transplantation [94].

It has been already described over the last decade about the long-term risk of kidney donation. Living donors believed to have a higher risk of developing ESRD, especially in obese donors and also for African Americans with an apolipoprotein L1 (APOL1) high-risk genotype. African Americans with high risk APOL1 was found to have an almost three times more accelerated fall in eGFR [95]. Hence researchers need to focus on unaddressed concerns of living related renal transplantation (LDRT) and with respect to living donors.

D. Procedure of kidney transplantation (Technique):

Kidney transplant surgery always consists of two surgeries, the donor and the recipient. Procedure of the surgery can be performed in a minimally invasive fashion or via open surgery for the living donor. Implantation of the kidney is done in an open fashion, where the organ is placed heterotopically in the pelvis, anastomosing the vessels to the external iliac vessels and the ureter to the bladder. The iliac vessels are preferentially exposed retroperitoneally as the peritoneum is retracted medially. Intra-peritoneal placement is also acceptable.

In the open surgical technique for living donor procurement, a subcostal incision is made, and the retroperitoneal space is exposed. The ureter is followed down to the iliac vessels and ultimately divided there before extraction. The kidney is isolated on its vascular pedicle, and once the recipient team is ready, the renal artery and vein are transected, and the organ is delivered to the back-bench. The tributary stumps are then ligated or oversewn. Any residual perinephric fat is pruned as the kidney is prepared for implantation [71]. Figure 1.4 describes the process of kidney transplantation diagrammatically.

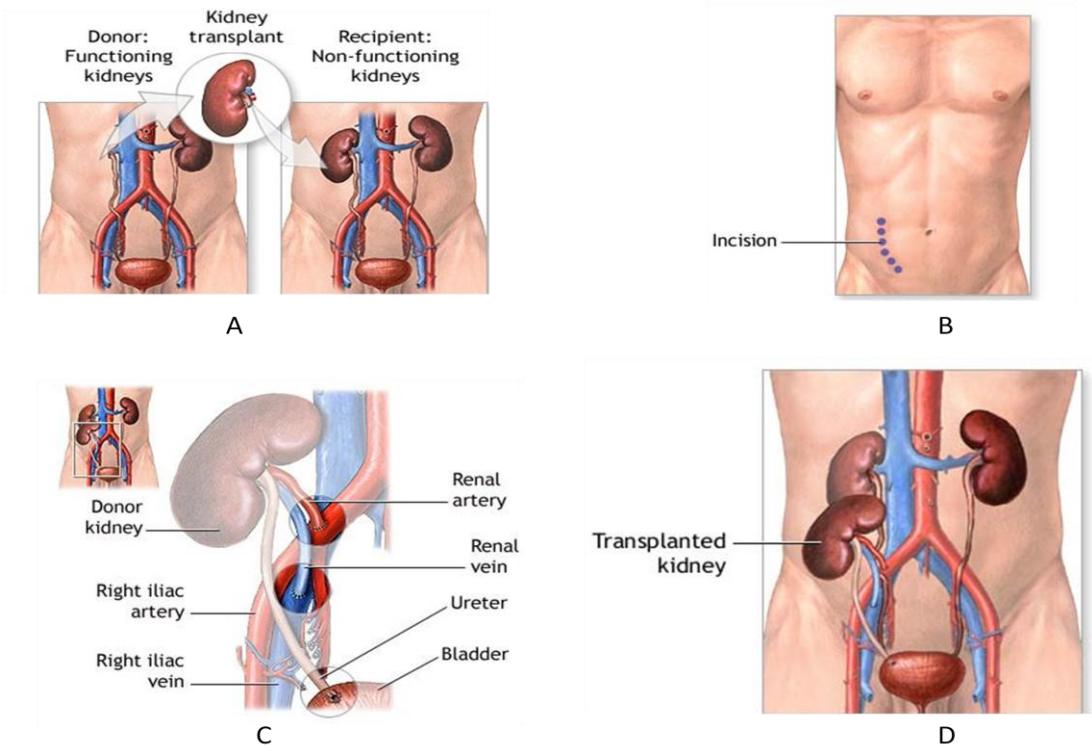

Figure 1.3:

A. Donor kidney obtained from living donor.
B. Under the general anaesthesia, an incision or cut is made in the lower right quadrant of the abdomen. The donor kidney is implanted into the lower pelvis of the recipient.
C. The new kidney is sutured into place. The vessels of the new kidney are connected to the vessels leading to the right leg (the iliac vessels) and the ureter is sutured to the bladder.
D. Transplanted kidney.

Images adapted from Kidney transplant series—Incision: Medline plus Medical Encyclopaedia.

1.3.2 KDIGO guidelines for the evaluation and care of living kidney donors (LKDs) to clinical practice

In 2017 Kidney Disease: Improving Global Outcomes (KDIGO), a global non-profit organisation dedicated for developing, improving and implementing evidence based clinical practice guidelines in kidney disease published the first “Clinical Practice Guidelines on the Evaluation and Care of Living Kidney Donors (LKDs)”, which

includes donors evaluation and care before, during and after donation in organised 19 chapters [96]. Table 1.4 briefs the items for the evaluation, care and follow-up of the LKDs described in the chapters 1-19 of the KDIGO guidelines.

Chapter	Topic	Checklist Item
1	Evaluation goals, decision-making framework, roles and responsibilities	• Provide the donor candidate individualized estimates of short- and long-term risks
		• Evaluate medical risks with respect to predetermined transplant program acceptance threshold
2	Informed consent	• Obtain consent from the donor candidate for evaluation and donation
3	Compatibility testing, incompatible transplantation, paired donation	• Determine ABO blood type and human leukocyte antigen compatibility
		• Inform incompatible donors about exchange programs and incompatible living donor transplantation options
4	Preoperative evaluation and management	• Conduct a preoperative assessment as per local guidelines to minimize risk
5	Predonation kidney function	• Estimate GFR using serum creatinine–based estimating equations and confirm with one or more of the following according to availability: measured GFR using an exogenous filtration marker, measured creatinine clearance, eGFR from the combination of serum creatinine and cystatin C, or repeat eGFR with serum creatinine
6	Pre-donation albuminuria	• Assess albuminuria using albumin-to-creatinine ratio in an untimed urine specimen

		and confirm albuminuria with albumin excretion rate in a timed urine specimen or by repeating albumin-to-creatinine ratio if albumin excretion rate cannot be obtained
7	Pre-donation hematuria	• Perform testing to identify cause of microscopic hematuria that is not reversible
8	Kidney stones	• Assess history and kidney imaging for nephrolithiasis
9	Hyperuricemia, gout, and mineral and bone disease	• Assess history of gout
10	Predonation BP	• Measure BP prior to donation on at least two occasions
11	Predonation metabolic and lifestyle factors	• Assess metabolic and lifestyle risk for CKD and/or cardiovascular disease by obtaining the following prior to donation:
		• Body mass index measurement
		• History of diabetes mellitus and gestational diabetes and family history of diabetes
		• Fasting blood glucose and/or glycated hemoglobin (hemoglobin A_{1c})
		• Fasting lipid profile, including total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides
		• Present and past use of tobacco products
12	Preventing infection transmission	• Screen for the following infections before donation:
		✓ HIV
		✓ Hepatitis B virus
		✓ Hepatitis C virus

		✓ Cytomegalovirus
		✓ Epstein–Barr virus
		✓ *Treponema pallidum* (syphilis)
		✓ Urinary tract infection
		✓ Other potential infections on the basis of geography and environmental exposures
13	Cancer screening	• Perform cancer screening as per local guidelines
14	Evaluation of genetic kidney disease	• Assess family history of kidney disease
15	Pregnancy	• Confirm a negative quantitative human chorionic gonadotropin pregnancy test immediately before donation in women of childbearing potential
16	Psychosocial evaluation	• Perform face-to-face psychosocial evaluation, education, and planning session with one or more trained, experienced health professionals
17	Acceptable surgical approaches for donor nephrectomy	• Select optimal surgical technique by an experienced surgeon
18	Ethical, legal, and policy considerations	• Respect donor autonomy during all phases of evaluation and donation
19	Postdonation follow-up care	• Perform annual postdonation follow-up care that includes the following:
		• BP measurement
		• Body mass index measurement

		• Serum creatinine measurement with GFR estimation
		• Albuminuria measurement
		• Review and promotion of healthy lifestyle practices, including exercise, diet, and abstinence from tobacco
		• Review and support of psychologic health and well-being

Table 1.4: Checklists for the evaluation, care and follow-up of the LKDs [97].

KDIGO guidelines also discusses about assessment of pre-donation GFR as kidney function and the use of GFR as risk estimation (Chapter 5) (Figure 1.4) [97].

1.3.3 Measurements and procedures post donation

The following measures and procedures should be done at least once or twice post donation:

i. Blood pressure (BP), Body Mass Index (BMI), Serum Creatinine (SCr), eGFR, albuminuria, healthy life style including physical activities, etc.

ii. Donors should be monitored for CKD and those who meet the criteria for CKD must be managed according to 2012 KDIGO CKD guidelines [98].

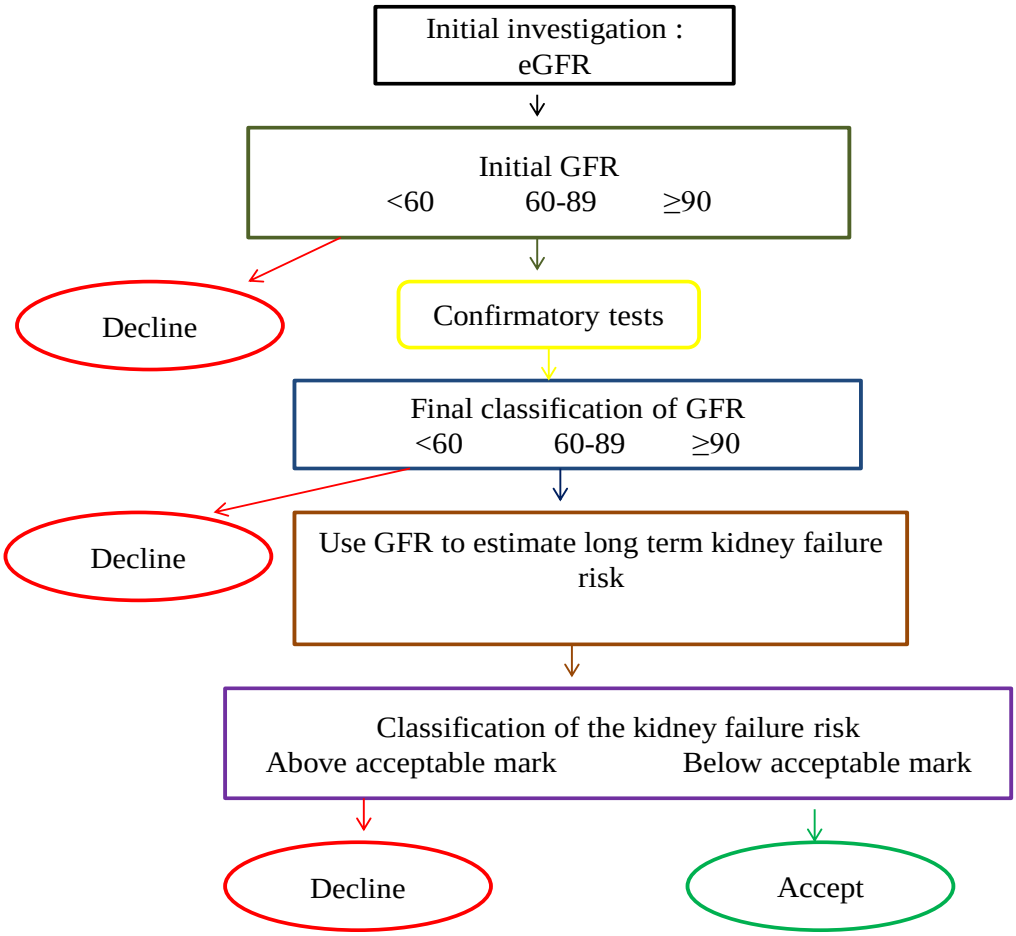

Figure 1.4: Stepwise approach to estimate GFR in kidney donor selection.

1.4 Renal function reserve (RFR) in living kidney donors (LKDs)

Kidney transplantation grants a quality and survival of life compared with dialysis and is more cost-effective over the long-term [99]. Living kidney donation gives a vital source of organs given the shortage of deceased kidneys.

Studies on short and long-term outcomes on living kidney donors (LKDs), has led to the inclusion of more border-line donors like, older age, controlled hypertension (HTN), BMI up to 35 and low grade proteinurea [100]. The final call of the transplant nephrologist in living kidney selection is still depends on eGFR, which is, however, not an ideal method to determine

actual renal function providing the known factors affecting its accuracy, for example, age, sex, physical activity, BMI, protein intake, etc [101]. Recent studies have shown poorer long –term outcomes in certain donor populations, such as those in African American origin, obese donors [102][103][104].
Generally all living donors are well screened pre donation; routine clinical findings (blood pressure, urine routine, renal function, BMI) have not been robust predictors to long- term risk in such donor population [104]. Final call for kidney acceptance however still highly relays on GFR, which may be influenced by multiple non-renal factors (age, sex, physical activity, protein intake, etc), which when estimated leads to underestimated measured GFR in LKDs [105] [106].

After nephrectomy, the single kidney increases its function approximately by 35% [104] [107] [108]. The ability of a kidney to increase its GFR on higher functional demand is termed as renal function reserve (RFR) [109]. Studies done on 11 of 12 nephrectomies in rats showed that hyperfiltration due to glomerular hypertension resulted in progressive renal failure [110] [111]. How accurate these data are in the case of a 50% loss of nephron mass in healthy kidney donors is not clear given the general long-term safety of living kidney donations. Although, this may be more applicable to borderline kidney donors.

As serum creatinine (SrCr) gives an estimate of how accurate kidney is working, but in the absence any measurement or estimate GFR, it is difficult to predict what is happening to the kidney after clinical insults, such as infections, exposure to nephrotoxic drugs and autoimmune complex depositions. The serum creatinine (SrCr) concentration can’t sensitively detect alterations in kidney function [112] [113] [114]. In a person who is at risk to develop kidney insult, current serum creatinine (SrCr) concentrations, especially near normal, can’t predict who will develop kidney injury. The baseline GFR may not reflect the full functional and anatomical features, including reserve in the kidney [112] [115] [116] [117].

Since human kidneys don't always work at full capacity, thus the baseline GFR doesn't reflect full function of the kidney. Similarly, serum creatinine (SrCr) is not a sensitive measure for kidney injury or function. In healthy individuals the GFR physiologically increases in response to certain stress or stimuli, such as protein loading. Multiple studies have proved that an assortment of protein load can increase GFR, some studies have shown up to 62-81% increase in GFR [112] [118] [119].

Renal function reserve (RFR) is defined as the difference between the maximal GFR and the baseline GFR, which is generally calculated after an oral protein or intravenous (IV) amino acid loading [112] [119] [120] [121]. The absence of a normal RFR can be helpful to identify patients who are more susceptible to kidney injury [115].

The RFR plays a sensitive method to detect sub-clinical kidney injury, and hence can be helpful to identify patients susceptible to kidney damage, as it provides more information about whole kidney function and remaining reserved function [115] [117] [120].

RFR or renal stress test is useful in determining and predicting the risk of developing kidney damage as it uncovers the loss of renal function mass when there is no evidence of clinical kidney damage [118].

1.4.1Physiology of RFR

Oral protein intake or intravenous (IV) amino acid infusion increases GFR. This effect happens both in normal and impaired kidneys. An increase in GFR usually occurs in the first hour after protein loading, and the maximum effect occurs around 2-2.5 hours [112] [113] [116] [122]. GFR can be increased by an increase in renal plasma flow (RPF) or filtration fraction (FF).

GFR=FF X RPF

The increase in GFR after protein intake is due to increased RPF without a change in FF [115]. Three main mechanisms involved in an increased GFR [121]:

1. **Metabolic mechanisms**

   Metabolic mechanisms involve amino acid stimulation of other metabolic processes in the kidney that include tubular sodium reabsorption and thus increased oxygen consumption by the kidney [119] [121].

2. **Humoral factors**

   There are number of humoral factors involved in RFR. Endothelium derived relaxing factors, such as nitric oxide, prostaglandins and the rennin-angiotensin –aldosterone system (RAAS) can alter renal hemodynamics. Nitric oxide causes vasodilation and an increase in GFR.

   Multiple studies also suggested that increased glucagon and amino acid levels increase renal plasma flow [113] [116] [118].

3. **Intrinsic renal mechanisms**

   Intrinsic mechanisms are usually involved in tubuloglomerular feedback; although these need an intact function of both tubules and glomeruli. After a protein load, there is an increase in tubular amino acid absorption. There is an increase in NaCl transport due to co-transportaion of amino acids in the proximal tubule with sodium. This results in decrease in sodium delivery to the distal tubule and macula densa which provides feedback to release prostaglandin and nitric oxide. All these events lead renal vasodilatation. GFR doesn’t increase after protein loading in patients with proximal tubule dysfunction (Fanconi syndrome) or with macula densa dysfunction. This implies that prostaglandins and nitric oxide have vital roles in regulating GFR [119] [121].

Renal function reserve decreases in advanced chronic kidney disease (CKD). RFR was reported to fall in normal population from 23.4% to 6.7% in CKD stage IV [114] [123].

Donor nephrectomy is one of the best examples to understand how RFR responds to an acute loss of renal function. There is a 20-40% increase in GFR of a single kidney within days after nephrectomy. Increased renal blood flow and hyperfiltration are responsible behind this increase in GFR [124].

RFR can be used in identifying susceptible donors and recipients for post-operative kidney injury in living donor renal transplants (LDRTs) [125].

RFR can be easily utilised in clinical practice. It can be helpful in identifying patients who are susceptible to kidney injury and can guide nephrologists for adverse kidney outcomes in patients with low RFR. Though more studies on RFR is needed and might be easily introduced into clinical studies and trials.

Chapter 2: Aim of the project

Chapter: 2

2.1 Aim of the project

Post neprectomy , living kidney donors (LKDs) develop a partial loss of kidney function, defined as acute kidney injury (AKI) as per KDIGO guidelines. The recovery following AKI is due to renal function reserve (RFR), which is described as the capacity of the kidney to increase glomerular filtration rate (GFR). Recent studies comparing living kidney donors (LKDs) with healthy controls show an increased risk of end stage renal failure (ESRD) in LKDs. This leads to have a greater interest in assessing the risk of LKDs. There are only few studies on RFR in kidney donors and correlation with renal function (RF) outcomes and identifying pre and post nephrectomy urinary extracellular vesicles (uEVs) biomarekers.

Hence the aim of the project was:

1. To analyse kidney donor (KD) renal function (RF) pre nephrectomy and to investigate the predictive performance of pre-donation RFR with protein load.
2. To analyse pre and post nephrectomy urinary extracellular vesicles (uEVs) and to investigate the predictive biomarkers in KDs post 7 days of nephrectomy.

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Chapter3: Materials and methods

Chapter: 3

Materials and methods

3.1 Study design

3.1.1 Subjects

Living donor kidney transplantation in adult patients (defined as > 18 years of age) was performed in between February 2019 -2020 in the Department of Nephrology, Dialysis and Transplantation, Hospital Maggiore della Carita' di Novara, Italy.
In this study only living donors were enrolled for kidney/renal stress test before nephrectomy. It was a case controlled and single-centre study.
This study was performed according to the principles of Declaration of Helsinki [126]. The study was approved by the Institution Review Board (IRB) and ethics committee. All the patients were informed about the objectives of the study. An informed consent was taken from all the patients who underwent renal stress test.

Inclusion Criteria:

i. Living kidney donors
ii. Informed consent
iii. Both genders
iv. Donors above than 18 years old.

Exclusion Criteria

i. HIV positive donors
ii. Pregnancy
iii. Acute systemic infections
iv. Cirrhosis
v. Active neoplasm
vi. Haematological malignancy
vii. Active autoimmune disease
viii. Intestinal malabosprption

ix. Chronic bowel disease

x. Pancreatic insufficiency

xi. Abnormal blood chemistry. Total cholesterol ≥ 300 mg/dL, Triglycerides ≥ 400 mg/dL, W.B.C count ≤ 300/ µl, platelets ≤ 75 X 10^3 / µl, abnormal SrCr ≥ 1.2 mg/dL.

xii. Denial of informed consent

3.2 Sample size

From February 2019-2021, 28 prospective living kidney donors (LKDs) were enrolled for kidney stress test or renal stress test (RST) with protein load to assess their renal function reserve (RFR) two weeks before donation, respectively.

3.3 Patients and methods

Kidney donor evaluation in the Department of Nephrology, Dialysis and Transplantation, Hospital Maggiore della Carita' di Novara, Italy:

Twenty eight enrolled kidney donors were evaluated before donation with,

A. SrCr
B. eGFR (CKD-EPI)
C. Complete urine analysis (routine and microscopic)
D. Cr clearance
E. 24-hours urine protein (including albuminuria)
F. Abdominal angio CT scan
G. Radioisotopic GFR (rGFR)
H. Renal Stress test (RST) or Renal Function Reserve (RFR) with protein load.

All the donors were on a standard diet. They avoided excessive intake of caffeine and high protein diets the day before the RST. None of the donors were using any drugs or medications that could modify renal blood flow and/or GFR (Angiotensin Converting Enzyme Inhibitors (ACE), Angiotensin Receptor Blockers (ARBs), diuretics, nonsteroidal anti-inflammatory drugs (NSAIDs) and antibiotics).

Eight hours of fasting or starvation was required to asses' kidney glomerular stress test or renal stress test (RST) after an adequate oral hydration (8 ml/kg body weight) Time 0 (T0) in 30 minutes and voiding of the bladder.
After that, the urine volume was replaced with equal volume of water by mouth (1 hour T1). Two measurements of 1-hour creatinine clearance (CrCl) were obtained in resting conditions and the mean value of them was considered the baseline (basal) (2 hours and 3 hours post T0). Then, an oral protein load (cooked red meat or protein shake) of 1.2 g of proteins /kg weight of the patient was given and eaten in 30 minutes. One hour CrCl was assessed in the following four hours (three determinations 1.5, 2.5 and 3.5 hours) after protein load.
The difference between the higher CrCl obtained after protein load and the baseline (basal) CrCl defined RFR. Blood samples for SrCr were collected at 30 minutes and 90 minutes after T0.

3.4 Measurements

Urinary creatinine (uCr) and serum creatinine (SrCr) was measured by enzymatic method with and automated analyser (Siemens ADVIA 1800, Simens Healthcare Diagnostics Inc, Japan/Canada).
CrCl was calculated and corrected for $1.73m^2$ of body surface area (BSA) using Dubois method as follows:

CrCl = uCr (mg/dL) / SrCr (mg/dL) X urinary volume (ml/time in minute) 1.73/BSA (m^2).

Two values of uCr and SrCr were obtained before the protein load and the mean value of CrCl obtained was considered as the baseline or basal GFR (bGFR). The maximum CrCl obtained after the protein load was considered as the stress GFR (sGFR). Glomerular RFR was defined as the difference between sGFR and bGFR.

RFR=CrCl Max post protein load-Mean basal CrCl.

3.5 Protein load

Cooked red meat, 1.2 g of proteins/kg of body weight of the patients or protein shakes " Fresubin Protein Powder" 1.2 g of proteins /kg of body weight of the patients were administered for kidney stress test.

3.6 Urine collection

First urine sample was collected pre-nephrectomy or donation. Second urine samples were collected post 7 days of nephrectomy or donation.

3.7 Urinary extra cellular vesicles (uEVs) characterisation

Urinary extra-cellular vesicles (uEVs) were characterized by bead-based multiplex analysis by flow cytometry (MASCSPlex Exosome Kit, human, Miltenyi Biotec [127] [128]. All urinary samples were centrifuged at 3000g for 15 minutes and filtered through 0.22µM filter. One hundred and twenty micro liters of each urinary sample were loaded onto a 1.5 mL tube and 0.5 µL of protease inhibitor (Sigma) were added. After, 15 µL of MACSPlex Exosome Capture Beads (containing 39 different antibody-coated bead subsets) were added to each tube and samples were incubated over night at room temperature using an orbital shaker. To wash the beads, 1 mL of MACSPLEX buffer (MPB) was added to each tube and washed at 3000 g for 5 minutes. For counterstaining of EVs bound by capture beads with detection antibodies, 5 µL of each APC-conjugated anti-CD9, anti-CD63, and anti-CD81 detection antibodies were added to each tube and then incubated on an orbital shaker for 1 hours at room temperature, protected from light. In this study, we mostly used a mixture of all three antibodies (pan tetraspanin) in order to cover most EVs being present in the samples. To wash the beads, 1 mL of MPB was added to each tube and washed at 3000 g for 5 minutes. This was followed by another washing step with 1 mL of MPB, incubation on an orbital shaker protected from light for 15 min at room temperature and then washed at 3000 g for 5 minutes. After washing, 1 mL of the supernatant was carefully aspirated, leaving about 150 µL in the tubes, ready to be acquired.

Flow cytometric analysis was performed, with a Cytoflex (Beckman Coulter, Brea CA, USA). Approximately 5000–8000 single bead events have been recorded per sample. Median fluorescence intensity (MFI) for all 39 capture bead subsets were

background corrected by subtracting respective MFI values from matched media controls that were treated exactly like EV-containing samples (buffer/medium + capture beads + antibodies). All bead populations can be identified and gated based on their respective fluorescence intensity according to manufacturer instructions.

EV-surface marker description		**Role**
CD1c	APC cells surface glycoprotein	Antigen-presenting protein
CD2	T and NK cell surface antigen	Mediator of adhesion between T-cells and other cell types
CD3	T cells surface glycoprotein	Mediator of signal transduction
CD4	T cells transmembrane glycoprotein	Co-receptor for MHC class II molecule
CD8	T cells transmembrane glycoprotein	Co-receptor for MHC class I molecule
CD9	Tetraspanin super-family – EV-surface protein	Regulator of cell adhesion
CD11c	Integrin alpha-X	Receptor for fibrinogen
CD14	Monocyte differentiation antigen	Co-receptor for bacterial lipopolysaccharide
CD19	B-lymphocyte antigen	Co-receptor for the B-cell antigen receptor complex (BCR)
CD20	B-lymphocyte antigen	Regulation of cellular calcium influx necessary for the development, differentiation, and activation of B cells
CD24	Signal Transducer	Modulator of B-cell activation responses
CD25	Interleukin-2 receptor	Marker for immune cell activation

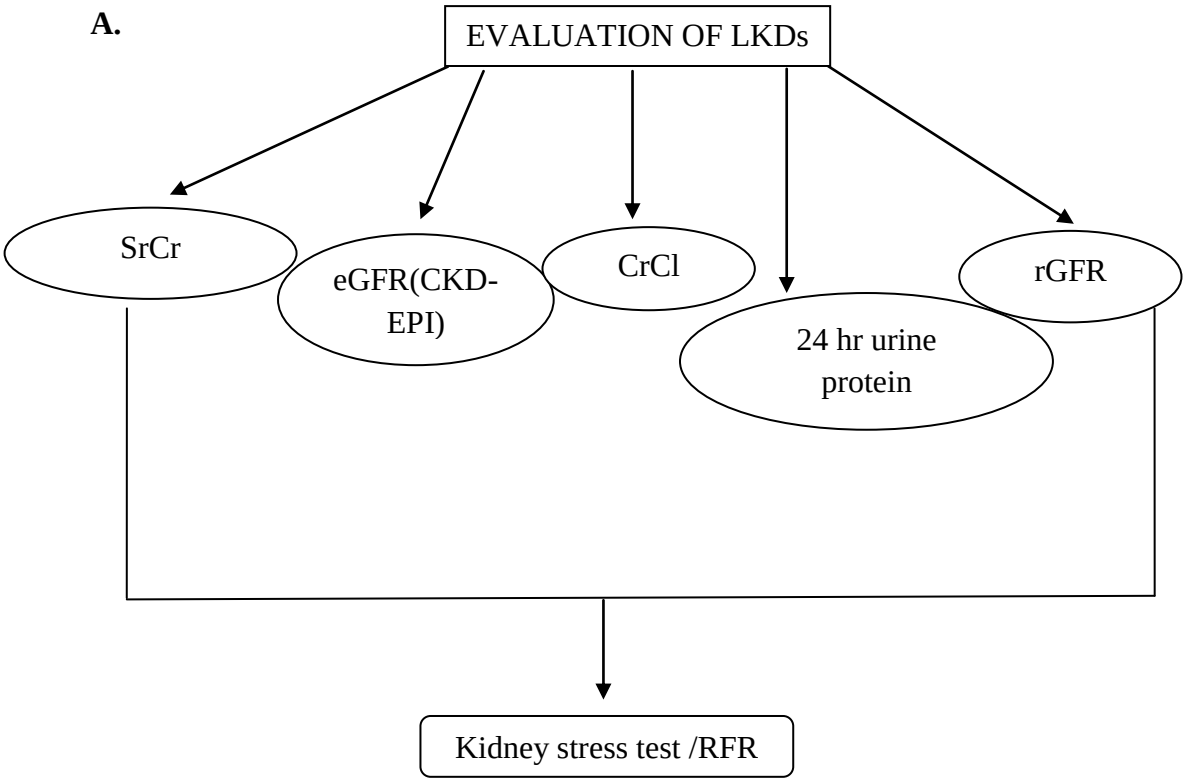
	subunit alpha	
CD29	Integrin beta-1	Extracellular matrix component
CD31	Platelet endothelial cell adhesion molecule	Regulator of leukocyte trans endothelial migration (TEM)
CD40	Costimulatory surface molecule	Co-stimulator of T and B cells
CD41b	Integrin alpha-IIb	Receptor for fibronectin, fibrinogen, plasminogen, prothrombin, thrombospondin and vitronectin
CD42a	Platelet glycoprotein 9	Mediator of platelet adhesion to blood vessels
CD44	Cell-surface receptor	Regulator of activation, recirculation and homing of T cells
CD45	Receptor-type tyrosine-protein phosphatase C	Positive regulator of T-cell coactivation
CD49e	Integrin alpha-5	Receptor for fibronectin and fibrinogen
CD56	Neural Cell Adhesion Molecule 1	Cell adhesion molecule involved in neuron-neuron adhesion, neurite fasciculation, outgrowth of neurites
CD62P	P-selectin	Mediator of interaction between activated endothelial cells or platelets with leukocytes
CD63	Tetraspanin super-family – EV-surface protein	Modulator of signal transduction
CD69	Early activation antigen	Signal transmitting receptor in lymphocytes, natural killer cells, and platelets

CD81	Tetraspanin super-family – EV-surface protein	Modulator of signal transduction
CD86	T-lymphocyte activation antigen	Co-stimulator of T cells proliferation and interleukin-2 production
CD105	Endoglin	Vascular endothelium glycoprotein that regulates angiogenesis
CD133/1	Prominin-1	Regulator of cell differentiation, proliferation and apoptosis
CD142	Tissue factor	Coagulation regulator
CD146	Melanoma Cell Adhesion Molecule	Cell adhesion molecule
CD209	C-type lectin receptor	Pathogen-recognition receptor
CD326	Epithelial cell adhesion molecule	Cell adhesion regulator
HLA-1	Major Histocompatibility Complex class I	Immune response regulator
HLA-DR	Major Histocompatibility Complex class II	Immune response regulator
MCSP	Melanoma-associated Chondroitin Sulfate Proteoglycan	Regulator of cell proliferation and migration
ROR1	Neurotrophic Tyrosine Kinase, receptor-related 1	Neurite growth modulation in central nervous system
SSEA-4	Stage-Specific Embryonic Antigen-4	Marker of bone-marrow derived very small embryonic-like stem cells

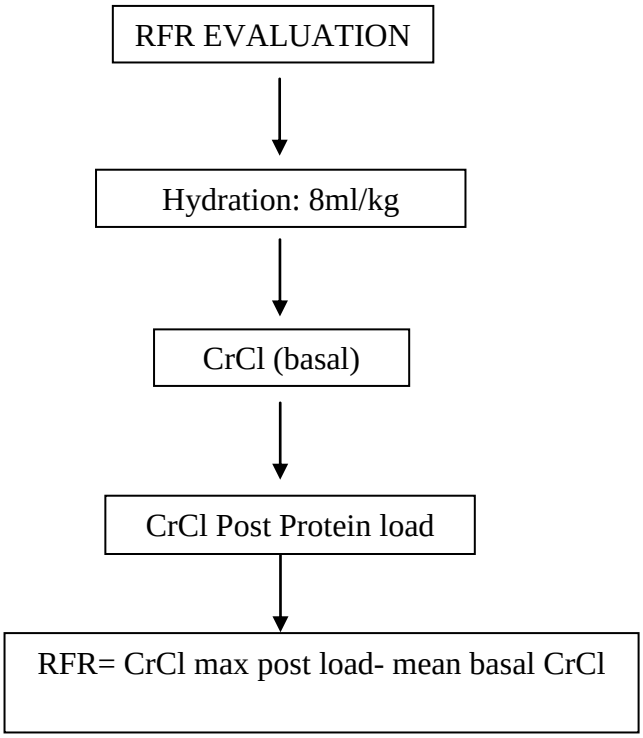
Table3.1 : List of 37 EV surface antigens

3.8 Study flow chart

A.


EVALUATION OF LKDs
SrCr
eGFR(CKD-EPI)
CrCl
24 hr urine protein
rGFR
Kidney stress test /RFR

B.


RFR EVALUATION
Hydration: 8ml/kg
CrCl (basal)
CrCl Post Protein load
RFR= CrCl max post load- mean basal CrCl

3.9 Statistical analysis

Data analysis was performed by usingGraphPad Prism 6.0. Results are expressed as mean ± standard deviation (SD) or standard error of the mean (SEM) where indicated. Statistical analyses were performed by employing: student's t test, one way analyses of variance (ANOVA) or two-way ANOVA with a multi comparison test where appropriate. A p value of <0.05 was considered statistically significant.

Chapter 4 : Results

Chapter: 4

Results

4.1 RFR test

Twenty eight kidney transplantation from living donors were performed between February 2019-2021 in the Department of Nephrology, Dialysis and Transplantation, Hospital Maggiore della Carita' di Novara, Italy. Out of these one was excluded from the RFR test because of non evaluable data. The enrolment flow chart of pre-transplant RFR test, mean baseline SrCr, mean baseline eGFR and mean RFRare shown in figure 4.1.

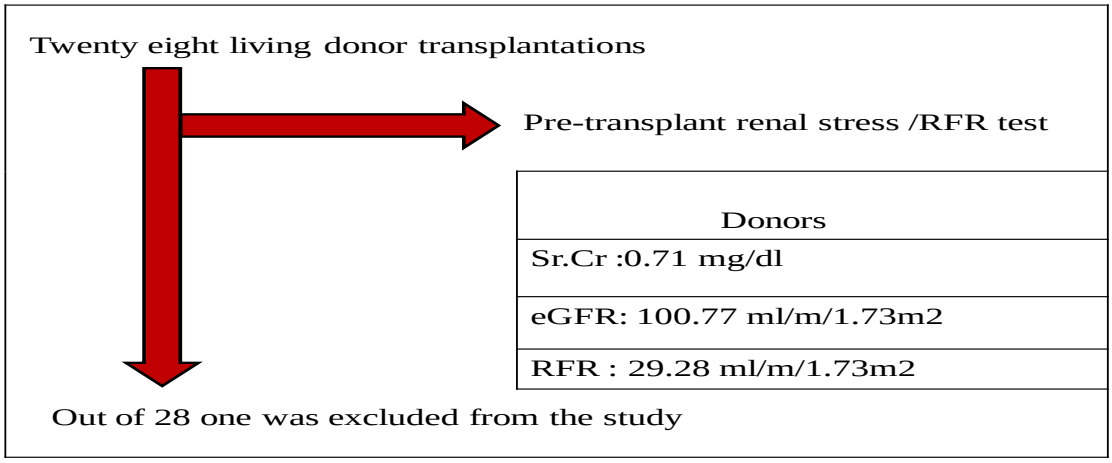


Figure4.1: Enrolment flowchart. Twenty eight living donor kidney transplantation was performed between 2019-2021 in the Department of Nephrology, Dialysis and Transplantation, Hospital Maggiore della Carita' di Novara, Italy.

Clinical characteristics of the living donor pre nephrectomy and renal function reserve are summarized in table 4.1 respectively.

Living donor parameters	Before Nephrectomy
Age	Mean=52.75 Years Median=53.5 (34-69) Years
Female, N=18	64.28%
Male, N=10	35.71%
BMI, N=28	Mean=22.82 kg/m^2 Median=22.7 (15.2-28.5) kg/m^2
Base line SrCr	Mean=0.71 mg/dl Median=0.67 (0.49-1.11) mg/dl Mean±SEM =0.71 ±0.02 mg/dl
Base line CrCl	Mean=129.83ml/m Median= 129.615(70.41-165.05) ml/m Mean±SEM = 129.83 ±4.86 ml/m
Base line eGFR	Mean=100.77 $ml/m/1.73m^2$ Median=102.0 (70-124) $ml/m/1.73m^2$ Mean±SEM = 100.77 ±2.61 $ml/m/1.73m^2$
sGFR	Mean= 158.44 $ml/m/1.73m^2$ Median=160.7 (92.40-264.3) $ml/m/1.73m^2$ Mean±SEM =158.44 ±6.66 $ml/m/1.73m^2$
RFR	Mean=29.28 $ml/m/1.73m^2$ Median= 29.5 (4.2-100) $ml/m/1.73m^2$ Mean±SEM = 29.2856 ±4.046 $ml/m/1.73m^2$

Table 4.1: Demographics of living kidney donors

4.2 Clinical characteristics and pre-nephrectomy RFR/Renal stress test (RST) in donors

The median age of donors pre-nephrectomy was 53.5 (34-69years), with 64.28% of them being female and 35.71% male (Figure 4.2).

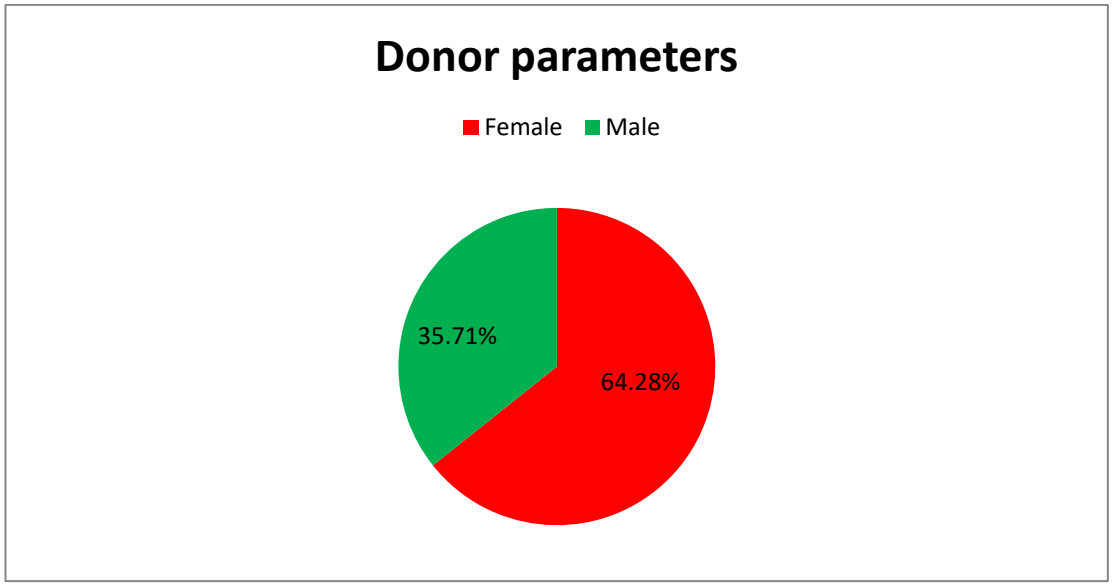


Figure 4.2: Graph representing distribution of female and male donors.

The median baseline eGFR was 102.0 (70-124) ml/m/1.73m^2,and sGFR was 160.7 (92.40-264.3) ml/m/1.73m^2 and were statistically significant (p= < 0.0001) figure 4.3. Basal creatinine clearance (CrCl) and stress GFR (sGFR) were significantly different (figure 4.4 and 4.5).

Median RFR was 29.5 (4.2-100) ml/m/1.73m^2. The median base line SrCr was 0.67 (0.49-1.11) mg/dl, body mass index (BMI) was 22.7 (15.2-28.5) kg/m^2 , baseline CrCl was 129.615(70.41-165.05) ml/m. Demographics of living kidney donors are summarised in table 4.1.

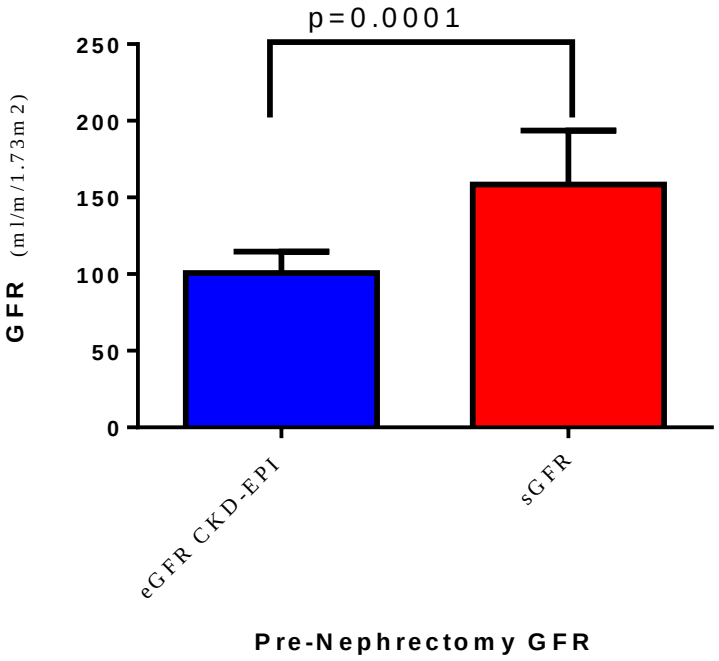


Figure 4.3 : Graph representing baseline eGFR and sGFR post protein load among donors. Data in the graph are expressed in mean and standard deviation. Paired t-test (two-tailed), number of pairs 26 between eGFR and sGFR are significantly different (p= < 0.0001). Mean±SEM of eGFR was 100.77 ±2.61 ml/m/1.73m^2 (N=28) and Mean±SEM of sGFR was 158.44 ±6.66 ml/m/1.73m^2(N=27) and are significantly correlated with correlation coefficient (r)= -0.05998.

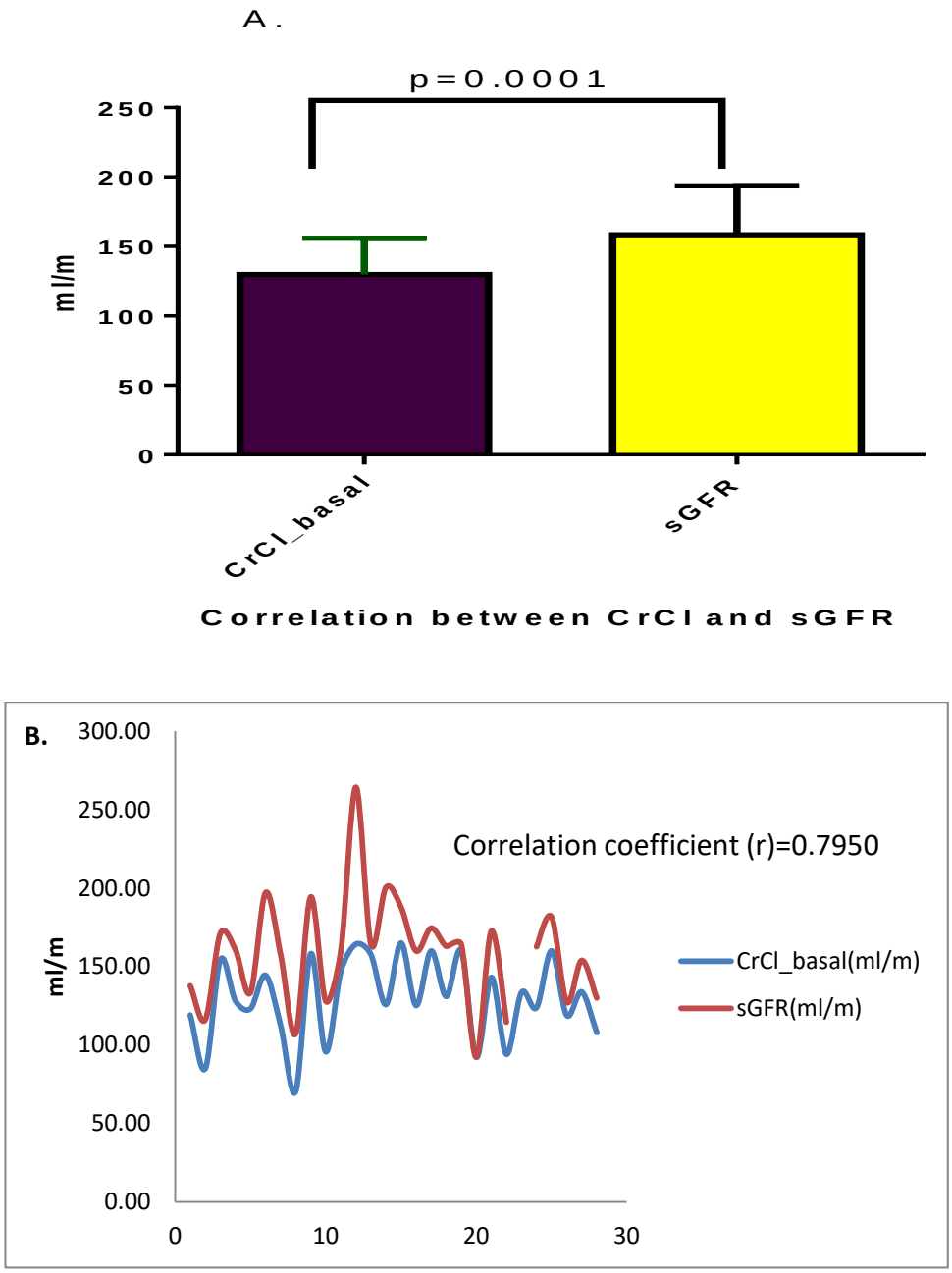


Figure 4.4: (A) Graph representing correlation between basal creatinine clearance (CrCl) and stress GFR (sGFR) expressed in ml/m. Data expressed in mean and standard deviation in the graph. Basal CrCl and sGFR was significantly different (p=0.0001).

(B) Correlation coefficient,r was 0.7590 and basal CrCl and sGFR was not significantly correlated to each other.

Post donor nephrectomy, all living donors had poor renal function, the median SrCr was 1.17 (1-1.5) mg/dl and renal recovery was observed after 7 days and the median SrCr was 0.66 (0.49-0.98) mg/dl (Figure 4.5). Median mGFR with 51Cr-EDTA of the right kidney post-nephrectomy was 100 (86-153) ml/m/1.73m^2 (Table 4.2).

	Post-nephrectomy(01 day)	Post-nephrectomy (7th day)
SrCr	Mean= 1.17mg/dl Median= 1.06 (1-1.5) mg/dl Mean±SEM =1.17±0.19 mg/dl	Mean=0.70 mg/dl Median= 0.66 (0.49-0.98) mg/dl Mean±SEM =0.70 ±0.025 mg/dl
GFR		Mean=100.70 ml/m/1.73m^2 Median= 100 (86-153) ml/m/1.73m^2 Mean±SEM = 100.70 ±3.80 ml/m/1.73m^2

Table4.2: Renal function of living donors post nephrectomy where SrCr is expressed in mg/dl and GFR in ml/m/1.73m^2.

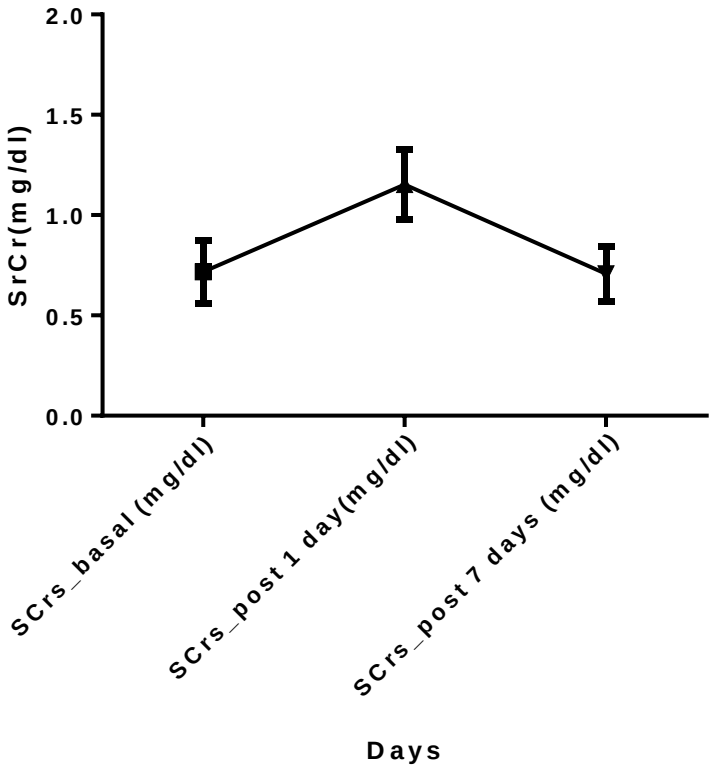


Figure 4.5 : Graphical representation of kidney function test (SrCr) pre and post nephrectomy. X-axis represents SrCr (mg/dl) and Y-axis represents number of days (pre and post nephrectomy). Serum creatinine Pre, post 1 day and 7 day nephrectomy was statistically significant (p= < 0.0001).

Serum Creatinine pre and post 7 days of nephrectomy was not statistically significant (p=>0.9999) and indicating renal recovery post donation (figure 4.6).

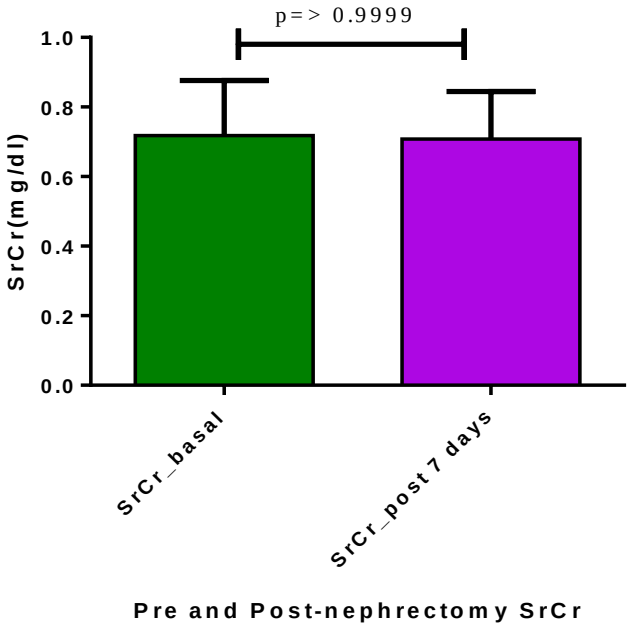


Figure 4.6: Graph representing data in mean and standard deviation of serum creatinine pre and post 7 days of nephrectomy expressed in mg/dl. Paired t-test between pre and post nephrectomy SrCr was not significantly different (p=0.9999). Correlation between basal SrCr and SrCr post nephrectomy was significantly correlated to each other (correlation coefficient, r= -0.09633, p value (one tailed) = 0.3163.

The median eGFR before nephrectomy was 102.0 (70-124) ml/m/1.73m^2 and median GFR post nephrectomy was 100 (86-153) ml/m/1.73m^2. Pre and post GFR was not significantly different (p=0.9254) (figure 4.7 and figure 4.8).

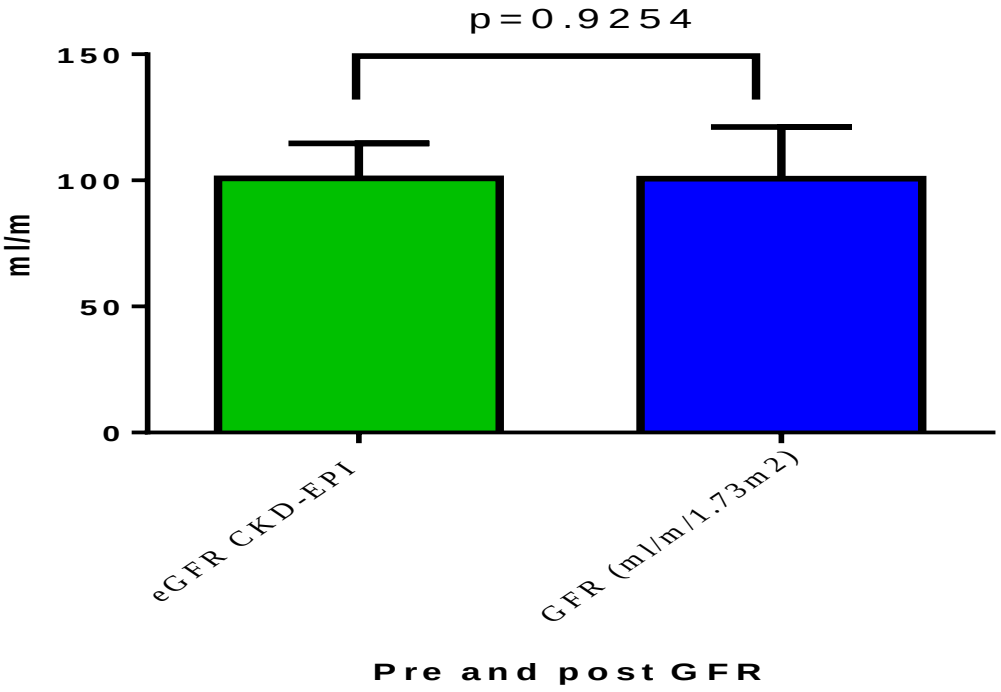


Figure 4.7 : Pre and post GFR expressed in ml/m/1.73m^2 was not significantly different (p=0.9254).

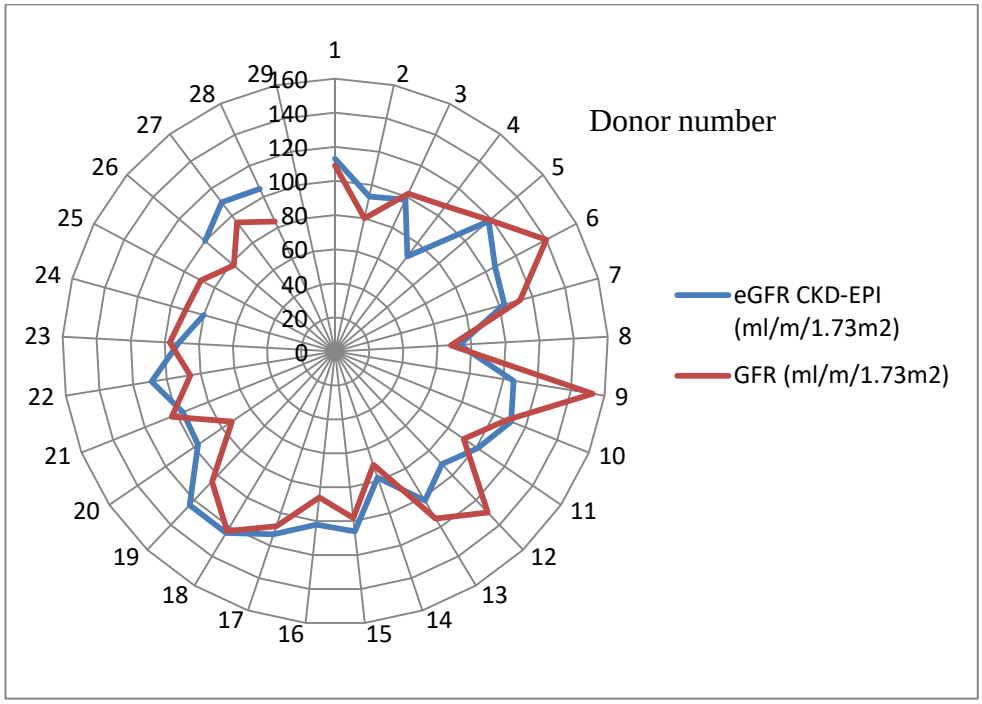


Figure 4.8 : Radar chart for the GFR in donors pre and post nephrectomy. In blue eGFR of donors before nephrectomy and in red GFR of donors post nephrectomy.

Correlation between pre nephrectomy RFR and post nephrectomy GFR was positively significant (correlation coefficient, r=0.2245) (figure 4.9).

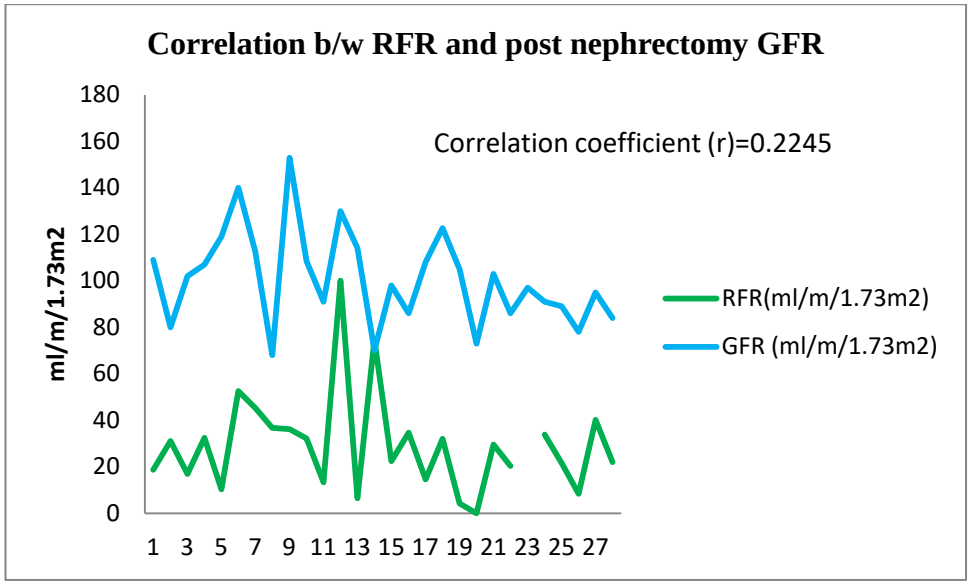


Figure 4.9: Graph representing correlation between pre nephrectomy RFR and post nephrectomy GFR, expressed in ml/m/1.73^2. RFR and post nephrectomy GFR was significantly different (p= <0.0001). Correlation coefficient, r was 0.2245, p-value (one tailed) was 0.1301, which shows RFR and GFR are significantly correlated.

4.3 Urinary extracellular vesicles (uEVs) characterization

Urinary samples from all prospective donors (from 2019-2021) (pre Protein load/nephrectomy and post 7 days of nephrectomy) were subjected to EV characterization using MACSPlex exosome kit, human, Miltenyi Biotec (12, 13). Each uEV markers median intensity (MFI) was normalised to the mean MFI by subtracting the median intensity of control buffer obtained from the signal intensities of the respective beads for specific markers.

Each EV markers MFI was normalised to mean MFI for specific EV markers (CD9, CD63 and CD81) obtaining normalised MFI (nMFI). All analyses were based on nMFI values.

Seven days post nephrectomy, donors showed an increased number of uEVs and mean MFI of the exosomal markers (CD9/CD63/CD81) was significantly higher in donors post nephrectomy (p=0.0002) compared to donors pre-nephrectomy

(p=0.0300) (Figure 4.10). In particular, 22 out of 27 analysed donors showed an increase in the expression of exosomal marker CD63 and 14 patients showed an increase in the expression of exosomal marker CD9. The other exosomal marker CD81 (expressed in 06 patients) did not increase in the same manner (Figure 4.11).

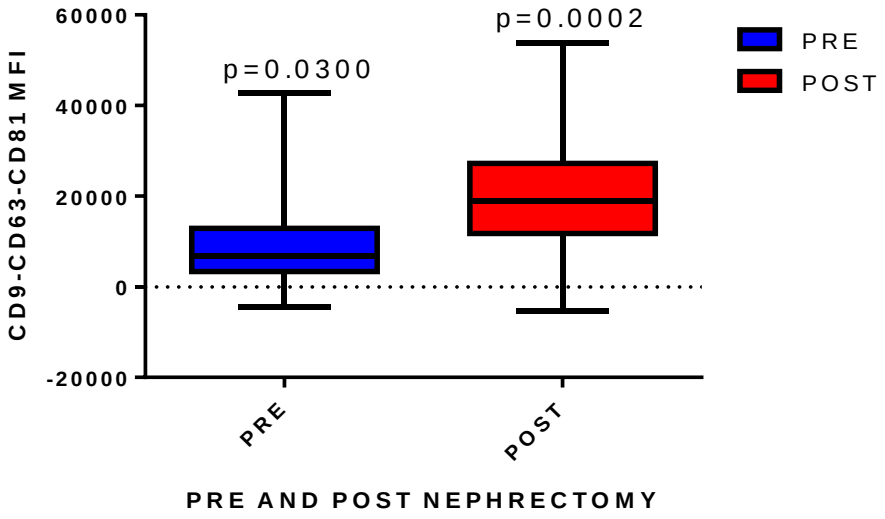


Figure 4.10: Graph representing mean median fluorescence intensity (MFI) for CD9, CD63 and CD81 at MACSPlex exosome analysis/characterisation pre and post nephrectomy.

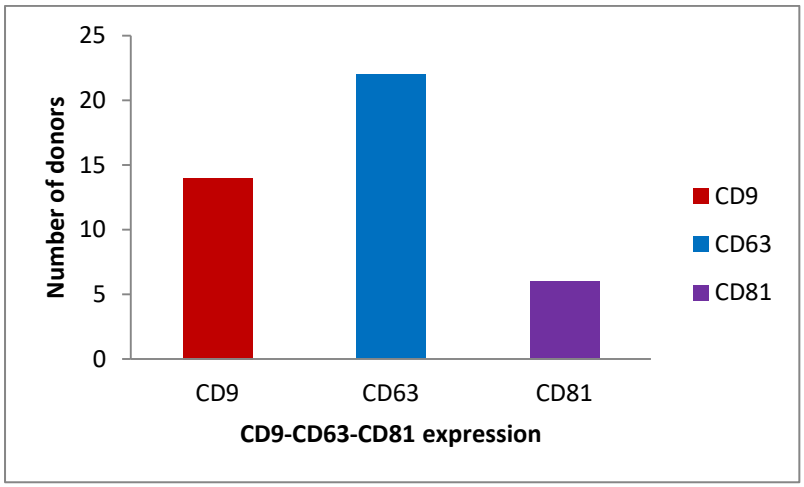


Figure 4.11: Graph representing post nephrectomy uEVs expression of exosomal markers (CD9, CD63 and CD81), 81.4 % of the donors showed an increase

expression of CD63, 51.85% expression of CD9 and 22.2% expression of CD81 respectively.

4.3.1. Phenotypic analysis of uEVs pre and post nephrectomy

Among the 37 EV surface markers, 34 were expressed in donors' pre nephrectomy (Figure 4.12, Table 4.3). In particular, we found the expression of:

- cell adhesion markers**:** CD41b by 11/27(40.74%), CD44 and CD29 by 09/27(33.33%), CD42 and CD326 by 08/27 (29.62%), Melanoma-associated chondrotin sulphate proteoglycan (MCSP) and CD49e by 07/27 (25.92%).
- immune and inflammatory markers**:** CD2 by 11/27(40.74%), CD20 by 10/27 (37.03%), CD56 by 09/27(33.33%), CD62p, CD11c and CD3 by 08/27(29.62%), CD105 and CD142 by 07/27 (25.92%), CD14, CD40 and CD8 by 06/27(22.22%), CD25 by 05/27(18.51%), CD209 and CD69 by 04/27(14.81%) and CD86 by 03/27(11.11%).
- endothelial cell markers**:** CD146 by 03/27(11.11%) and CD31 by 09/27(33.33%).
- T-cell related (CD4) by 09/27(33.33%), B-cell related (CD19) by 08/27(29.62%), leukocyt**e** (CD45) by 11/27(40.74%) and antigen presenting cells (CD1c) by 08/27(29.62%).
- the molecules of major histocompatibilty complex: HLA-1 by 10/27(37.03%) and HLA-DR by 16/27 (59.25%).
- the renal stem cell markers**:**CD133 by 20/27(74.07%) and CD24 by 18/27(66.66%).
- the stage specific embryonic antigen-4 (SSEA-4) by14/27(51.85%).
- the diagnostic tumor cell marker (ROR-1) by 08/27 (29.62%).

HLA-1 and SSEA-4. All of these markers had higher nMFI than the levels found after nephrectomy.

These markers were expressed at different levels. The most expressed markers were the cell adhesion molecules (CD41b, CD29, CD44, CD326), the immune and inflammatory cell markers (CD20, CD56, CD2, CD11c and CD105), HLA-DR,

HLA-1 and SSEA-4. All of these markers had higher nMFI than the levels found after nephrectomy.

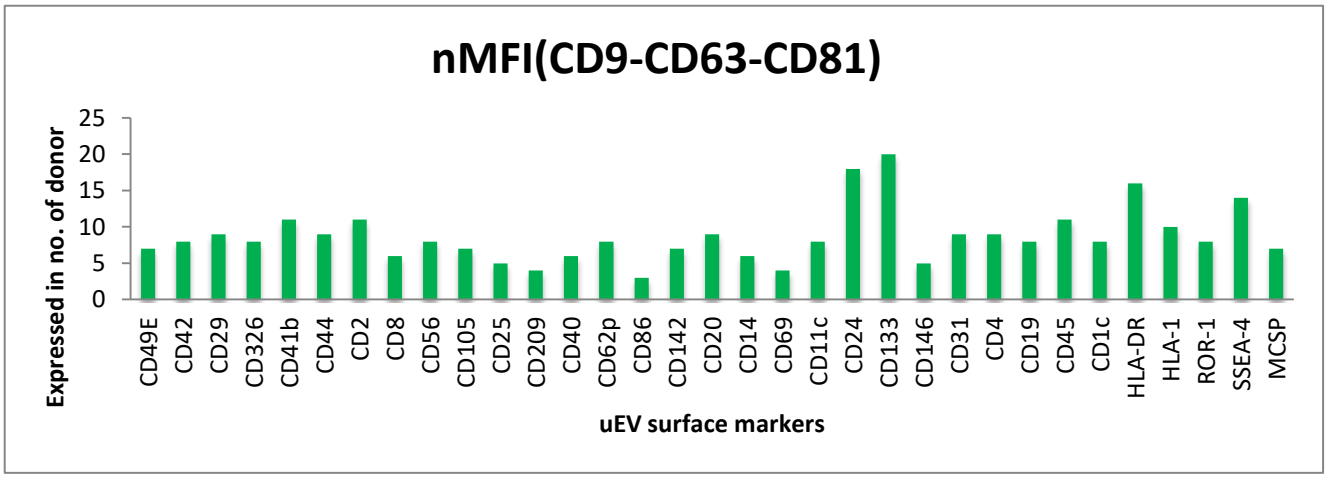


Figure 4.12: Graph representing uEVs surface Ag expression in number of donors' pre nephrectomy (on y-axis) and CD markers (on x-axis).

Seven days after the nephrectomy 29 markers were expressed (Figure 4.13, Table 4.3):

In particular:

- cell adhesion markers: CD326 was expressed by 3/27(11.1%), CD29, CD49e and CD42 by 02/27(7.4%), CD41b and CD44 by 1/27(3.7%) donors;
- T-cell, B-cell and leukocyte markers: CD4 by 02/27(7.4%), CD19 by 01/27(3.7%), and CD45 by 04/27(14.8%)
- antigen presenting cells marker (CD1c) was expressed by 04/27(14.8%);
- immune and inflammatory cell markers: CD40, CD62p and CD14 were expressed by 07/27 (25.92%), CD11c and CD20 by 06/27 (22.2%), CD3, CD142, and CD2 by 05/27(18.51%), CD209 by 04/27(14.81%), CD8 and CD69 by 03/27(11.11%), CD105, CD25 and CD86 by 02/27 (7.40%) and CD56 by 01/27(3.70%).
- HLA-1 and HLA-DR respectively by 09/27(33.33%) and 13/27(48.14%).

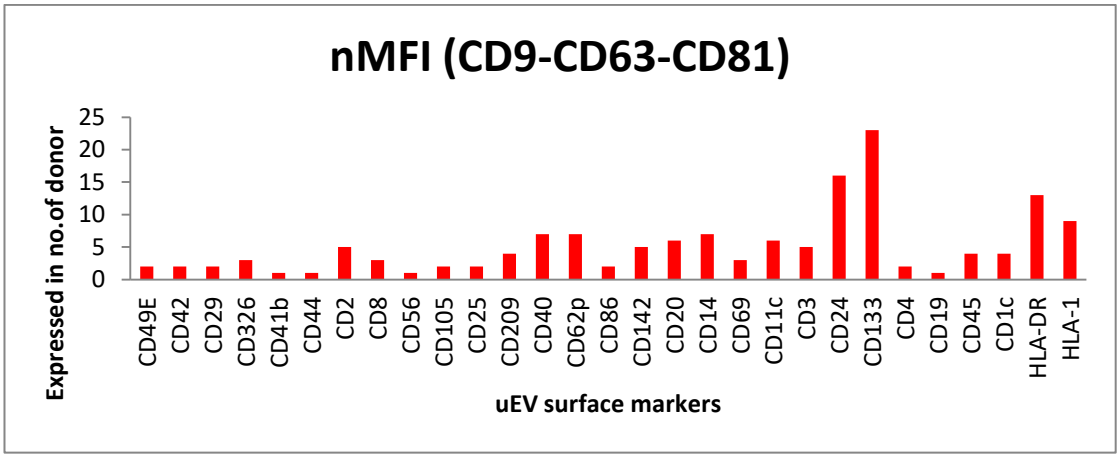


Figure 4.13: Graph representing uEVs surface Ag expression in number of donors post nephrectomy (on y-axis) and CD markers (on x-axis).

Twenty five common EV markers were expressed in donors pre and post nephrectomy:

- fifteen immune and inflammatory cells markers such as CD2, CD8, CD56, CD105, CD25, CD209, CD40, CD62p, CD86, CD142, CD20, CD14, CD69, CD11c and CD3;
- six molecules involved in cell adhesion: CD49e, CD42, CD29, CD326, CD41b and CD44;
- the renal stem cell marker (CD133 and CD24)
- the molecules of major histocompatibility complex (HLA1 and HLA-DR).

Interestingly, the expression of renal stem cell marker CD133 along with CD24 was found to be increased in terms of nMFI 7 days after nephrectomy in 23 and 16 donors respectively (Figure 4.14).

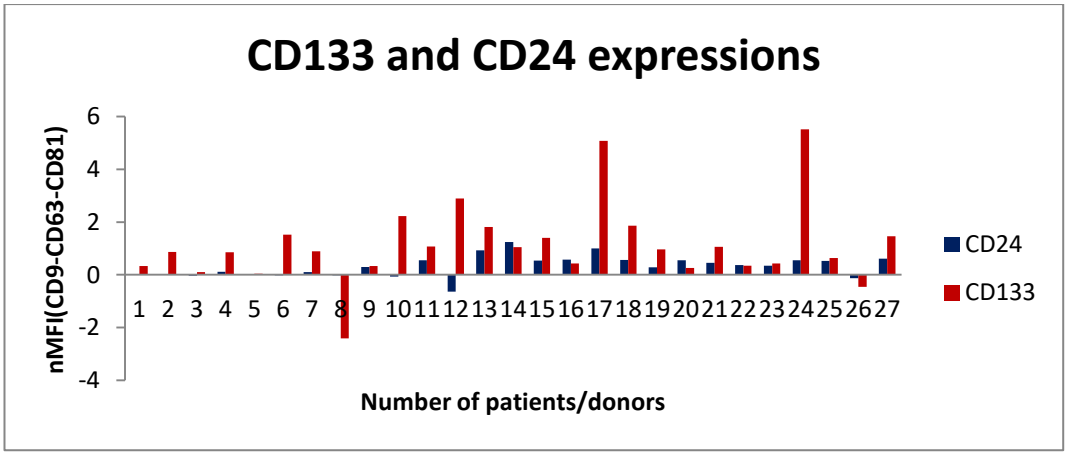


Figure 4.14: Graph representing donor distributions expressing renal stem cell marker CD133 and CD24 in respect to pre nephrectomy and increased nMFI**.**

Further, we classified donors into 2 subgroups: ***Group 1*** includes donors who had renal stem cell markers expression 7 days post nephrectomy and ***Group 2*** includes donors without expression of renal stem cell markers 7 days post nephrectomy. In particular, seven days post nephrectomy three donors did not show any expression for renal stem cell markers (figure 4.14).

Sixteen markers were different between *Group 1* and *2. Group 2* didn't show the expression of: T-cell (CD4) and B-cell (CD19) related markers, cell adhesion markers (CD42, CD44 and CD29), immune and inflammatory markers (CD3, CD8, CD56, CD105, CD25, CD40, CD20, CD11c, and CD69) and the molecules of major histocompatibilty complex (HLA1 and HLA-DR).

Eight common markers were expressed between these 2 groups: CD2, CD49E, CD326, CD62p, CD14, and CD142, CD45 and CD1c.

EV-surface marker description	**Role**	**Pre nephrectomy (N=27)**	**post nephrectomy (N=27)**
CELL ADHESION MARKERS			
1. CD41b	Hematopoiesis	11(40.74%)	-
2. CD29	Extracellular matrix component	09(33.33%)	02(7.4%)
3. CD44	Regulator of activation, recirculation and homing of T cells	09(33.33%)	01(3.7%)
4. CD326	Cell adhesion regulator	08(29.62%)	03(11.1%)
5. CD 42	Mediator of platelet adhesion to blood	08 (29.62%)	02(7.4%)

	vessels		
6. CD49E	Receptor for fibronectin and fibrinogen	07(25.92%)	02(7.4%)
7. MCSP	Regulator of cell proliferation and migration	07(25.92%)	-
IMMUNE AND INFLAMMATORY MARKERS			
8. CD2	Mediator of adhesion between T-cells and other cell types	11 (40.74%)	05(18.51%)
9. CD20	Regulation of cellular calcium influx necessary for the development, differentiation, and activation of B cells	10(37.03%)	06(22.2%)
10. CD56	Cell adhesion molecule involved in neuron-neuron adhesion, neurite fasciculation, outgrowth of neuritis	09(33.33%)	01(3.70%)
11. CD3	Mediator of signal transduction	08(29.62%)	05(18.51%)
12. CD62p	Mediator of	08(29.62%)	07(25.92%)

	interaction between activated endothelial cells or platelets with leukocytes		
13. CD11c	Receptor for fibrinogen	08(29.62%)	06(22.22%)
14. CD105	Vascular endothelium glycoprotein that regulates angiogenesis	07(25.92%)	02(7.40%)
15. CD142	Coagulation regulator	07(25.92%)	05(18.51%)
16. CD14	Co-receptor for bacterial lipopolysaccharide	06(22.22%)	07(25.92%)
17. CD40	Co-stimulator of T and B cells	06(22.22%)	07(25.92%)
18. CD8	Co-receptor for MHC class I molecule	06(22.22%)	03(11.11%)
19. CD25	Marker for immune cell activation	05(18.51%)	02(7.40%)
20. CD209	Pathogen-recognition receptor	04(14.81%)	04(14.81%)
21. CD69	Immune cell activation	04(14.81%)	03(11.11%)
22. CD86	Co-stimulator of T	03(11.11%)	02(7.40%)

	cells proliferation and interleukin-2 production		
ENDOTHELIAL CELL MARKERS			
23. CD146	Melanoma Cell Adhesion Molecule		-
24. CD31	Regulator of leukocyte trans endothelial migration (TEM)		-
T CELL, B CELL, LEUKOCYTES AND AgPCs			
25. CD19	Co-receptor for the B-cell antigen receptor complex (BCR)	08(29.62%)	01(3.7%)
26. CD4	Co-receptor for MHC class II molecule	09(33.3%)	02(7.4%)
27. CD45	Positive regulator of T-cell co-activation	11(40.74%)	04(14.8%)
28. CD1c	Antigen-presenting protein	08(29.62%)	04(14.8%)
MHC-I / MHC-II			
29. HLA-1	Immune response regulator	10 (37.03%)	09(33.33%)
30. HLA-DR	Immune response regulator	16(59.25%)	13(48.14%)
RENAL STEM CELL MARKERS			

31. CD 133	Marker of renal progenitor cell	20 (74.07%)	23(85.18%)
32. CD 24	Marker of renal progenitor cell	18 (66.6%)	16(59.25%)
STAGE SPECIFIC EMBRYONIC ANTIGEN-4			
33. (SSEA-4)	Marker for undifferentiated, pluripotent human embryonic stem cells	14(51.85%)	-
DIAGNOSTIC TUMOR CELL MARKERS			
34. ROR1	Neurite growth modulation in central nervous system	08(29.62%)	-

Table 4.3: Description and role for each EV-surface markers pre and post nephrectomy.

Chapter 5: Discussions

Chapter: 5

Discussions

Kidney/renal transplantation (RTx) is considered the best available treatment for end stage renal disease (ESRD). Only a limited population of patients may have access to it, due the scarcity of donors. Therefore, factors that could affect renal transplant outcomes must be carefully evaluated.

An important asset for patients with ESRD is the "living donor kidney transplantation" (LDRT) which has numerous advantages, both in terms of waiting time and renal outcome, compared to transplantation from a deceased donor. The LDRT procedure must be carried out to minimise the effect on donors' health. Notably, an acceptable residual renal function is normally required in donor subjects to justify living kidney donation strategy. Although, past studies has shown that nephrectomy might not affect short-term or long-term outcomes, development of chronic kidney disease (CKD) and eventually requirement of renal replacement therapy (RRT) have been reported among living kidney donors [129] [130]. Moreover, recent studies have shown the effect of renal mass in living donor outcomes [131]. Therefore, accurate and responsible donor screening strategies, and careful post donation care and follow-up must be performed. Previous studies have particularly compared the renal functional reserve (RFR) of donors and recipients post donation with RFR of healthy volunteer or RFR of solitary kidney [132] [133]. Only some studies have shown RFR in donors' pre donation [134] [135].

In recent years, urinary extracellular vesicles (uEVs) have gained interest for their role as non-invasive biomarkers for kidney related pathologies [136]. Like in several other body fluids, urine is a rich source of EVs originating directly from the cells coating the urinary lumen, consisting of differentiated tubular cells, progenitor cells and infiltrating inflammatory cells. Certain markers of glomerular and tubular damage like NGAL [137], WT-1[138, 139] and ATF3 [140], including renal regeneration marker, such as CD133 [140] [141] [142] [143], have been associated with uEVs. Several studies have shown the expression of CD133 in urine was reduced in acute and chronic glomerular damage [144] and was associated with slow

graft function and recovery [143]. Thus, uEVs represent an important source of information for diagnostic purposes [137] [138] [139] [140] [141]. uEVs being markers of damage or diagnosis, also plays a vital role in providing information on physiopathological state of the kidney and the intrinsic mechanisms of its homeostasis and repair.

Recent studies have indicated that kidney consists majority of cells involved in the continuous renewal and regeneration of epithelia as well as in repair mechanisms after renal injury or damage [145]. A cell population with CD133 expression and progenitor characteristics has been identified in human kidney [146], and its number was found to be increased in the cortex after acute kidney injury (AKI) [147]. Stem cell derived microvesicles are known to act in a paracrine fashion to support the neighbouring cells [148]. The scattered $CD133^+$ progenitor cells along the nephron may release $CD133^+$ EVs with a functional effect along the renal tubules and were found to be positive for proximal tubule and glomerular markers suggesting their origin from the upper part of the nephron [143].

Vesicles from injured cells may favour fibrosis and disease progression whereas those with regenerative potential may promote cell survival. Therefore, uEVs have become a reliable source of non-invasive biomarkers for detecting any change in physiopathology of their parental cell and are also bio activator in kidney diseases as uEVs are shown to be rich in innate immunity proteins including antimicrobial proteins, peptides, bacterial and viral receptors [149].

To the best of our knowledge, this is the first study that investigated the predictive performance of pre-donation RFR with protein load and that characterised uEVs Pre and Post nephrectomy, as predictive renal function markers. The major findings of this study consist in phenotypical profiling of uEVs subpopulations, obtained from urine of donors' pre and 7 days post nephrectomy. We systematically evaluated differentially expressed uEVs antigens.

A significant increase in GFR pre transplant renal stress test (RST) was observed among donors post protein load, with corresponding high RFR values. Correlation

between basal creatinine clearance (CrCl) and stress GFR (sGFR) post protein load was statistically significant.

All living donors had poor renal function after nephrectomy and renal recovery was observed after 7 days. Reduction of 20-40 % in bGFR after nephrectomy has been already reported in literature [150]. The difference between SrCr pre and 7 days post donation was not significant as also for GFR pre and post nephrectomy. Moreover, correlation between RFR and post nepherctomy GFR was statistically significant. This may be due to the utilisation of preserved capacity post nephrectomy by the remaining kidney through glomerular hyperfiltartion, to maintain normal organ function in resting conditions. Personal behaviour and physical characteristics all define bGFR in resting conditions, and hence the amount of RFR used post nephrectomy [151].

Seven days post donation, donors showed an increase number of uEVs. Among the 37 surface markers, 34 were expressed pre nephrectomy at different levels. The most expressed markers were the cell adhesion molecules (CD41b, CD29, CD44, CD326), the immune and inflammatory cell markers (CD20, CD56, CD2, CD11c and CD105), HLA-DR, HLA-1 and SSEA-4. All of these markers had higher nMFI than the levels found after nephrectomy.
Seven days after nephrectomy 29 markers were expressed by the donors in comparison to 33 markers expressed pre nephrectomy, with 25 common markers in between them. The analysis of surface markers of uEVs in post nephrectomy donors showed a high expression of renal stem cell markers CD133 and CD24.

As majority of EVs in urine are released by cells of the nephron and can come up with information on kidney function. Among uEVs markers differentially expressed in donors 7 days after nephrectomy, CD133 and CD24 are of interest because they are markers of renal progenitor cells [152] [153] [154].

Studies have indicated that, cells with progenitor characteristics expressing CD133 cell marker are present in different segments of the human nephron, locally present in proximal tubules, the Bowman capsule of glomeruli and in inner medullary papilla region that includes S3 limb segment and Henle's loop [155] [156]. CD133 cell

population expressed renal embryonic and stem-related transcription factors that were able to differentiate into mature renal epithelial cells [155]. Studies show that levels of urinary $CD133^+$ EV are reduced in patients with end-stage renal disease (ESRD), possibly indicating that these vesicles are only released by functioning renal tissue [143]. The levels of CD133 uEVs was found to be significantly decreased in pediatric patients with acute glomerulonephritis (AGN) and in diabetic patients with albuminuria [144]. However $CD133^+$ levels was restored after subsequent recovery in AGN patients, suggesting that the level of $CD133^+$ uEVs may act as a marker of normal renal physiology and can provide information on regeneration of cells within tubules [157] and in patients with proteinurea (glomerularnephritis) [147]. Moreover, $CD133^+$ cells increased in number after AKI proved that CD133 may represent a marker of renal function and their role in renal repair and damage [143] [154].

Post nephrectomy rise in the levels of CD133 and CD24 detected in the majority of the donors, may reflect the involvement of progenitor cells in renal homeostasis providing regenerative renal potential.

Chapter 6: Conclusions

Chapter: 6

Conclusions

The results of this study suggest that the RFR via oral protein load is a safe, feasible, easy and an inexpensive tool and could be used before transplant to establish the global filtration capacity of the donor kidneys as sGFR. The assessment of the RFR must also be suggested in the clinical follow-up of donors post nephrectomy to provide the possibilities of the evaluation of the single kidney function and to check donors' susceptibility in developing kidney injury before clinical evidences.

Urine is a rich reservoir of extracellular vesicles (EVs) directly originating from the urinary lumen, including differentiated tubular cells, progenitor cells and infiltrating inflammatory cells. Several markers of glomerular and tubular damage as well as of renal regeneration such as CD133 have been identified as an incredible source of information for diagnosis purposes.

Pre and post RFR along with pre and post uEVs assessment may represent a useful screening tool for LDRT, providing us more information about the quality of the kidneys without any surgical intervention (like biopsy) and possibly increase the number of living kidney donors.

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