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TESI DI DOTTORATO

DEVELOPMENT OF A CLINICAL PROGRAM AIMED TO IMPROVE THE SAFETY OF LIVING DONOR KIDNEY TRANSPLANTATION: THE ROLE OF RENAL FUNCTIONAL RESERVE, RADIOISOTOPE GLOMERULAR FILTRATION RATE AND URINARY BIOMARKERS

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0. Summary

Background and aim. As living kidney donors (LKD) undergo a medically unnecessary procedure, their safety has always been the focus of attention in the transplantation community. After nephrectomy, LKD usually develop an episode of Acute Kidney Injury (AKI) and may have an increased risk of chronic kidney disease (CKD) progression, especially in "medically complex" patients (older age, hypertension, etc.). A reduced Renal Functional Reserve (RFR), defined as the capacity to increase glomerular filtration rate (GFR) in response to certain physiological or pathological stimuli requiring a higher functional demand, plays a key role in functional recovery and progression to CKD. The aim of this study was the dynamic evaluation of RFR, radioisotope measured GFR (mGFR) and urinary injury biomarkers before donation, in the immediate postoperative period (day 7) and 1 year after surgery, in order to investigate their predictive performance and propose a RFR threshold below which donation may be considered not safe.

Patients and methods. 112 LKD were evaluated at different time points (before donation, during hospital stay for donation, 1 year after donation) for serum creatinine (sCr), eGFR, mGFR with 51Cr-EDTA or 99mTc-MAG (both before and after donation), sequential scintigraphy with 99mTc-MAG to determine left vs. right renal function. 61/112 LKD underwent RFR evaluation with oral protein load (IRRIV protocol) before donation and 1 year after. 27/61 LKD were also tested for urinary biomarkers NGAL, Nephrocheck (IGFBP7/TIMP2) and extracellular vesicles expressing the stem cell marker CD133 (CD133+uEVs) detected by flow cytometry (MACSPlex) before donation, 7 days and 1 year after donation.

Results. At study admission, median sCr was 0.69 mg/dL, mGFR 97 mL/min; right kidney split function at scintigraphy was 47.4%. After nephrectomy, renal function worsened (AKI episodes) with median sCr values of 1.19 mg/dL. After 7 days, renal recovery was observed in all KDs. mGFR (99mTc-MAG) was measured at 1 year 63.5 mL/min (IQR 56.5-71) and compared with the split mGFR of the same kidney before donation 46.1 (41-51.4), with a median compensatory increase of 18 mL/min (9.4-23.1), representing 35% (20.4-51.3). Pre-donation median RFR was 27.3 (mL/min, 13.2-34.6). LKD age and basal mGFR represented two independent factors associated with renal function 1 year after nephrectomy at a multivariate linear regression. There was no correlation between pre-donation RFR and post-donation AKI, whereas a moderate linear correlation between RFR and the compensatory mGFR (99mTc-MAG) increase after 1 year was

observed (r=0.58). Moreover, we performed a ROC curve to establish the RFR value with the best predictive value for a mGFR compensatory increase of 10 mL/min 1 year after donation. The RFR threshold value of about 18 ml/min showed the most accurate performance (sensitivity = 90.2%, specificity = 84.2%; AUC 0.88). Of note, after 1 year LKD still maintained a percentage of RFR (9.7 mL/min). Injury biomarkers (NGAL and [TIMP-2]·[IGFBP7]) were all negative 7 days and 1 year after donation; moreover, the percentage of CD133+uEVs was significantly increased 7 days after nephrectomy (24% vs. 4.5%), suggesting a regenerative potential of the remnant kidney. The levels of CD133+uEVs 1 year after donation were comparable to those observed before donation.

Conclusions. The dynamic evaluation of renal function after kidney donation and the use of urinary biomarkers represent the next steps for a safer selection and follow-up of LKD. Radioisotope measurement of GFR is feasible and allows a precise determination of renal function, in particular the compensatory GFR increase after donation. RFR assessment by using a glomerular stress test with protein load showed a good correlation with the compensatory GFR increase after donation and could become a valid tool for LKD screening, in particular in “medically complex” cases due to the presence of multiple comorbidities. Urinary biomarkers of kidney injury are negative in the immediate postoperative period whereas extracellular vesicles showed markers of regeneration (CD133, CD24). In addition, one year after donation, restoration of normal levels of CD133 and the persistent negativity of both NGAL and [TIMP-2]·[IGFBP7] suggested a physiological adaptation of the remnant kidney.

0. Summary [Italian version]

Background. La sicurezza del donatore di rene rappresenta un'assoluta priorità per la comunità trapiantologica. Dopo la nefrectomia a scopo donativo, i donatori sviluppano AKI (Acute Kidney Injury) secondo i criteri delle linee guida KDIGO; studi inoltre dimostrano sul lungo periodo un'incidenza aumentata di insufficienza renale cronica (CKD), soprattutto nella popolazione di donatori "medically complex" con criteri di accettazione sempre più ampliati (età avanzata, ipertensione arteriosa, intolleranza glicidica…). La riserva funzionale renale (RFR) definita come la capacità di aumentare la velocità di filtrazione glomerulare in contesti fisiologici o patologici di aumentata richiesta funzionale, rappresenta un ruolo chiave per la ripresa funzionale post AKI e la progressione AKI to CKD, se ridotta. Scopo di questo lavoro è costruire una valutazione dinamica della funzione renale tramite GFR radioisotopico (mGFR), RFR e biomarker urinari pre e post donazione, sia nell'immediato post operatorio sia ad un anno dalla donazione e verificare se la valutazione della RFR pre donazione abbia un potere predittivo sulla funzione renale post donazione e se ci sia una soglia di RFR di idoneità a nefrectomia a scopo donativo.

Pazienti e metodi. Sono stati arruolati 112 donatori di rene, la cui funzione renale è stata studiata con creatinina sierica (Crs), eGFR, GFR radioisotopico (mGFR con Cr-EDTA or 99mTc-MAG) e concomitante scintigrafia renale per quantificare la ripartizione precisa della funzione renale tra i due emuntori. La funzione renale è stata monitorata nell'immediato post donazione con creatinina sierica (fino a 7 giorni) e 1 anno post donazione con GFR radioisotopico, fino a 5 anni post donazione con creatinina sierica. 61 donatori sono stati sottoposti a misurazione della riserva funzionale renale glomerulare con carico proteico (protocollo IRRIV) pre donazione ed 1 anno post. 27 donatori sono stati testati pre donazione, 7 giorni e un anno post donazione per biomarker urinari: NGAL, Nephrocheck (IGFBP7/TIMP2) e microvescicole urinarie che esprimevano marcatore si staminalità CD133 (CD133+uEVs) tramite citofluorimetria (MACSPlex).

Risultati. Pre donazione, la Crs mediana era 0.69 mg/dL, mGFR radioisotopico 97 mL/min; contributo funzionale del rene 47.4%. Dopo la nefrectomia, la funzione renale è peggiorata (AKI) con una Crs mediana di 1.19 mg/dL (IQR, 1-1.4). 7 giorni dopo, si è assistito ad un parziale recupero funzionale in tutti i donatori. Un anno dopo, è stato misurato GFR radioisotopico (mGFR con 99mTc-MAG) e confrontato con la funzione renale dello stesso rene pre donazione: rispettivamente 63.5 mL/min (56.5-71) vs 46.1 (41-51.4) rispettivamente, con un incremento

funzionale mediano pari a 18 mL/min (9.4-23.1), ossia il 35% (20.4-51.3) in più rispetto alla funzione renale dello stesso rene pre donazione. La RFR mediana pre donazione era pari a 27.3 (mL/min, 13.2-34.6). Una regressione lineare multivariata ha confermato che età e funzione renale basale (mGFR) rappresentano due fattori indipendenti associati alla funzione renale (mGFR) 1 anno post nefrectomia. Non si è dimostrata nessuna correlazione tra RFR ed l'AKI post donazione o recupero funzionale a 7 giorni. E' risultata una moderata correlazione (r=0.58) tra la RFR e l'incremento compensatorio assoluto o relativo del GFR radioisotopico ad 1 anno. Abbiamo inoltre eseguito un'analisi ROC per stabilire il miglior valore predittivo di RFR pre donazione per ottenere un incremento compensatorio di almeno 10 mL/min (misurato con radioisotopo) 1 anno post donazione. Il livello soglia di RFR pre donazione è 18 mL/min, con un'ottima sensibilità (90,2%) e specificità (84,2%), AUC 0,88: proponiamo così un livello soglia per la sicurezza del donatore pari a 20 mL/min. Da sottolineare il dato che ad un anno i donatori mantengono una certa RFR (mediana 9,7 mL/min), considerando la condizione di monorene. I biomarcatori urinari di danno renale (NGAL and [TIMP-2]·[IGFBP7]) sono risultati entrambi negativi a 7 giorni ed 1 anno post donazione mentre le CD133+uEVs sonor risultate significativamente aumentate a 7 giorni dalla donazione (24% vs. 4.5%), indicando un potenziale rigenerativo del monorene in acuto post donazione, per tornare ai livelli pre donazione ad 1 anno.

Conclusioni. La valutazione dinamica della funzione renale pre e post donazione di rene contribusice ad aumentare la sicurezza del donatore, sia nel momento della selezione dello stesso, sia nel follow up post donazione. La misurazione radioisotopica del GFR è uno strumento estremamente preciso e informativo sulla funzione renale pre e post donazione, permettendo una comparazione affidabile e una misurazione accurata dell'incremento compensatorio del GFR nel monorene. La misurazione della RFR glomerulare con carico proteico ha una buona correlazione con l'incremento compensatorio della funzione renale (misurata con radioisotopo) ad 1 anno dalla donazione e potrebbe essere inserito tra i test utili nella selezione del donatore di rene, soprattutto per la popolazione "medically complex". I biomarker urinari di danno renale e la mancanza di correlazione con la RFR delineano un profilo di AKI e CKD diverse nel post nefrectomia (funzionali e non patologiche) mentre le microvescicole urinarie CD133 positive suggeriscono un adattamento fisiologico del monorene post donazione.

1. Introduction

Kidney transplantation (KT) is considered the best available treatment for end-stage renal disease (ESRD) considering the reduction in mortality and the improvement of the quality of life for most nephropathic patients when compared with dialysis. As for the different types of KT, living donor kidney transplantation (LDKT) provides a better patient and allograft's survival when compared with deceased-donor, especially when the living donor transplant is performed before the onset of dialysis, both hemodialysis and peritoneal dialysis [1,2]. During the last decade, its growing development has led to an extension of donor selection criteria, also due to persistent shortage of deceased donors' organs [3].

Living donors undergo a medically unnecessary procedure, and their safety has always been the focus of attention in the transplantation community. The short-term risk is well established with a 0.03% risk of mortality and <1% risk of major morbidity [4,5] but this risk could increase in the current kidney donors with more liberalized selection criteria used in the last years: this procedure must be performed minimizing the impact on the donors' health with all the means at our disposal.

Earlier studies claimed that living kidney donors neither have an increase in all-cause mortality nor an increased risk of ESRD compared to the general population. However, KD are selected from a group of very healthy individuals thoroughly screened for conditions such as arterial hypertension, diabetes, kidney and coronary diseases, so they are not comparable to the general population. Recent studies comparing KD with equally healthy controls indicate an increased risk of kidney failure in living KD, which has led to greater interest in assessing the risk of ESRD in KD [6,7]. The quantification of estimated risk of ESRD associated with donation can be derived from two recent matched-cohort studies [8,9]: KD have a large relative risk (Mjøen: 11.4, Muzaale: 8.0) compared to a close-to-zero baseline risk (Mjøen 0.06%, Muzaale 0.04%) but the absolute risk difference between donors and controls in small. The categories of donors with an increased risk of later de novo ESRD are: first-degree relatives, young age donors, black race, males. In 2016, a meta-analysis published on NEJM by Grams et al. estimated the long-term risk of ESRD according to 10 pre-donation demographic and health characteristics assessed together. Furthermore, they developed an online risk tool to help evaluate and counsel living kidney-donor candidates and improve the acceptance process. They found that the risk of ESRD was highest among donors in

the youngest age group, particularly in black people. The 15-year observed risks after donation in the United States were 3.5 to 5.3 times as high as the projected risks in the absence of donation. [10] However, population and selection criteria for kidney donation in USA are highly different from European and Italian ones. Consequently, these data are not strictly applicable to our kidney donors' population.

As suggested by the recent KDIGO guidelines on evaluation and care of living kidney donors, an important advance is quantification of the combined impact of a donor candidate's pre-donation demographic (e.g., age, sex, and race) and health characteristics at the time of evaluation (e.g., kidney function, blood pressure [BP], body mass index [BMI], etc.) on the risk of serious adverse outcomes after donation [10]. However, robust predictors of long-term risk in kidney donor populations still lack.

Focusing on the evaluation of the pre-donation kidney function, according to KDIGO guidelines (2017), it should be expressed as glomerular filtration rate (GFR) in mL/min/1.73m², estimated from serum creatinine (eGFRcr) for initial assessment and then it should be confirmed using one or more of the following measurements, depending on availability: measured GFR (mGFR) using an exogenous filtration marker, preferably urinary or plasma clearance of inulin, urinary or plasma clearance of iothalamate, urinary or plasma clearance of 51Cr-EDTA, urinary or plasma clearance of iohexol, or urinary clearance of 99mTc-DTP. Moreover, measured creatinine clearance (mCrCl) and estimated GFR from the combination of serum creatinine and cystatin C (eGFRcr-cys) can be used. If parenchymal, vascular or urological abnormalities or asymmetry of kidney size on renal imaging are present, single kidney GFR should be assessed using radionuclides or contrast agents that are excreted by glomerular filtration (e.g., 99mTc-DTPA). In the process of donor selection, GFR of 90 mL/min per 1.73m² or greater should be considered the ideal level of acceptability of kidney function for donation. The decision to approve donor candidates with GFR 60 to 89 mL/min per 1.73m² should be individualized, based on demographic and health profile in relation to the transplant program's acceptable risk threshold while GFR less than 60 mL/min per 1.73m² should not donate. When asymmetry in GFR, parenchymal abnormalities, vascular abnormalities, or urological abnormalities are present but do not preclude donation, the more severely affected kidney should be used for donation. As for counselling, KDIGO suggest that donor candidates be informed that the future risk of developing kidney failure necessitating treatment with dialysis or

transplantation is slightly higher because of donation; however, average absolute risk in the 15 years following donation remains low [11].

The assessment of living donor's kidney function should be as more complete, precise and accurate as possible, both before donation to ideally predict with reliability the outcome, and after donation during the follow-up period. Donor safety, in particular lifelong sufficient renal function, is the key requirement in LKD organ transplantation.

Final acceptance of a living donor is still highly dependent on GFR. There are well known limitations of relying on creatinine as the only glomerular filtration marker, which can lead to inaccurate GFR estimates in certain populations due to the influence of non-GFR determinants of serum creatinine (e.g., protein intake, muscle mass, physical activity, age, gender). To this end, the CKD-EPI combined creatinine-cystatin C equation (eGFRcr-cys) was developed in 2012 and demonstrated superior accuracy to equations relying on creatinine or cystatin C alone (eGFRcr or eGFRcys). New filtration markers, such as Beta2-microglobulin (B2M) and beta-trace-protein (BTP) have been proposed as candidates for improving both GFR estimation and risk prediction [12].

After nephrectomy, as a result of the decrease of the nephron mass, kidney donors (KD) develop a partial loss of renal function, classically defined as Acute Kidney Injury (AKI) according to KDIGO guideline for Acute Kidney Injury criteria [13]. However, "real" clinical AKI is an abnormality of kidney structure or function occurring abruptly, either resolving or progressing to chronic kidney disease (CKD). The risk for AKI is increased by exposure to factors or presence of factors that increase susceptibility. Thus, the risk for AKI is a composite mosaic of factors encompassing a delicate balance between susceptibility and level of exposure (stressor) [14].

The recovery of renal function with a rapid increase in glomerular filtration rate following AKI is mainly ascribed to the concept of renal functional reserve (RFR), defined as is defined as the capacity of the kidney to increase glomerular filtration rate in response to certain physiological or pathological stimuli requiring a higher functional demand including, for example, high protein intake, pregnancy, acute kidney injury (AKI) episodes, sepsis, unilateral nephrectomy, chronic kidney disease (CKD), and congestive heart failure [15].

During an anatomical or functional nephron loss, RFR allows for increase GFR in the residual nephrons, trying to maintain the whole-organ GFR. In case of intact RFR, if the insult of an AKI episode creates a damage in less than 50% of the nephrons, the syndrome may remain subclinical (subclinical AKI), reducing or even completely deleting RFR without clinical evidence, even if the exposure is remarkable. All insults may stay subclinical leading to significant damage of nephrons without however affecting baseline GFR. If RFR is lost in subsequent insults, the patient will display an increase in susceptibility and may develop AKI, evident by a rise in serum creatinine, even if the exposure is of only limited severity. Finally, the recovery would be incomplete or absent (non-recovery) leading to the development of CKD. The loss of functioning nephrons affecting RFR and eGFR has been invoked as an underlying mechanism for acute kidney disease and AKI-to-CKD transition [14, 16, 17]. FIGURE 1.

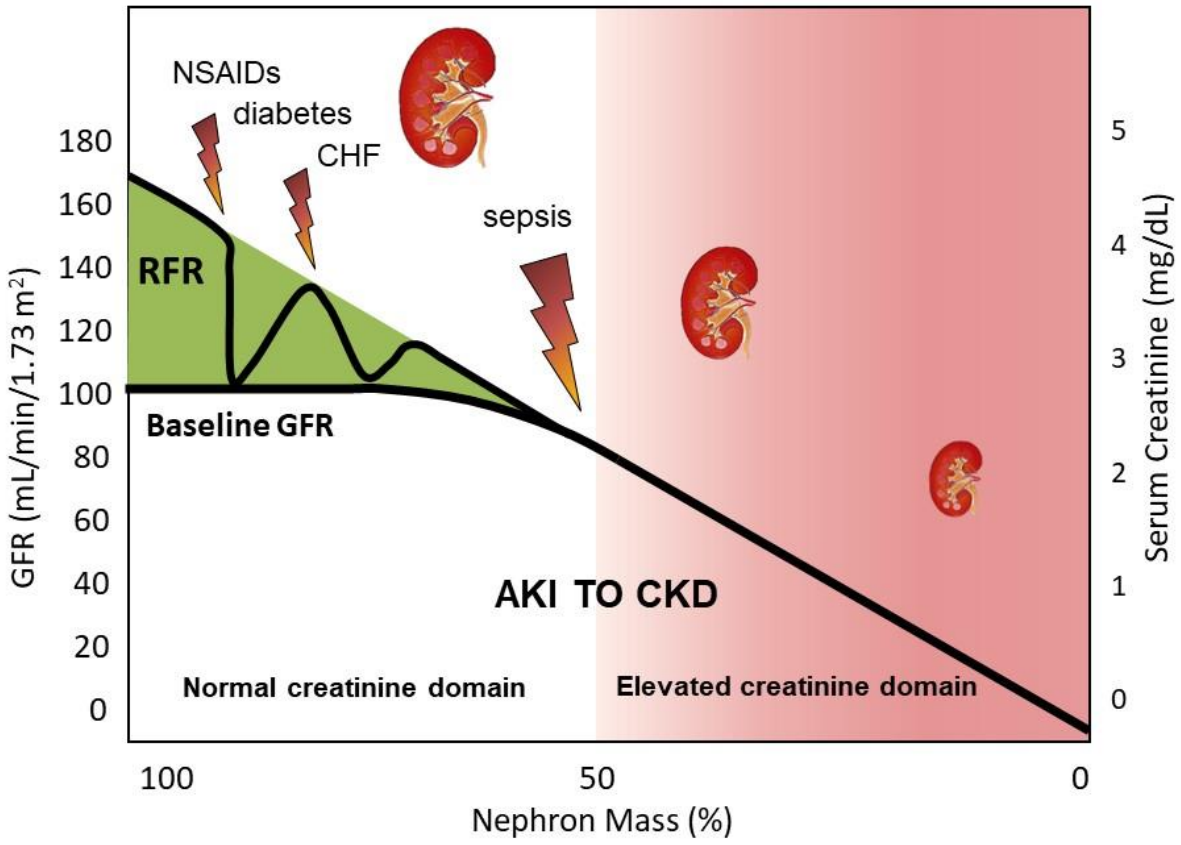


FIGURE 1. When RFR is intact, AKI episodes may remain subclinical, not affecting baseline GFR. When RFR is consumed, AKI to CKD transition will develop.

Currently, no standardized definition of renal recovery from AKI exists and the clinical standard diagnostic tools for AKI detection and AKI recovery include monitoring of serum creatinine and urine output, both of which are markers of renal function but not kidney injury. Over the last few years, several new AKI biomarkers have been discovered and validated to improve early detection, differential diagnosis, and stratification of patients into risk groups for progressive renal failure, need for renal replacement therapy (RRT), or death. Biomarkers of AKI have been developed to identify tubular injury; they are measurable in urine or plasma of patients with AKI, including neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1), interleukin 18 (IL-18), liver-type fatty acid-binding protein (L-FABP), tissue inhibitor of metalloproteinase 2 (TIMP-2), insulin-like growth factor-binding protein 7 (IGFBP7), calprotectin, urine angiotensinogen (AGT), and urine microRNAs. After AKI occurs, biomarker levels remain elevated for a certain period of time. None of the reported biomarkers is entirely specific for AKI [18].

FIGURE 2

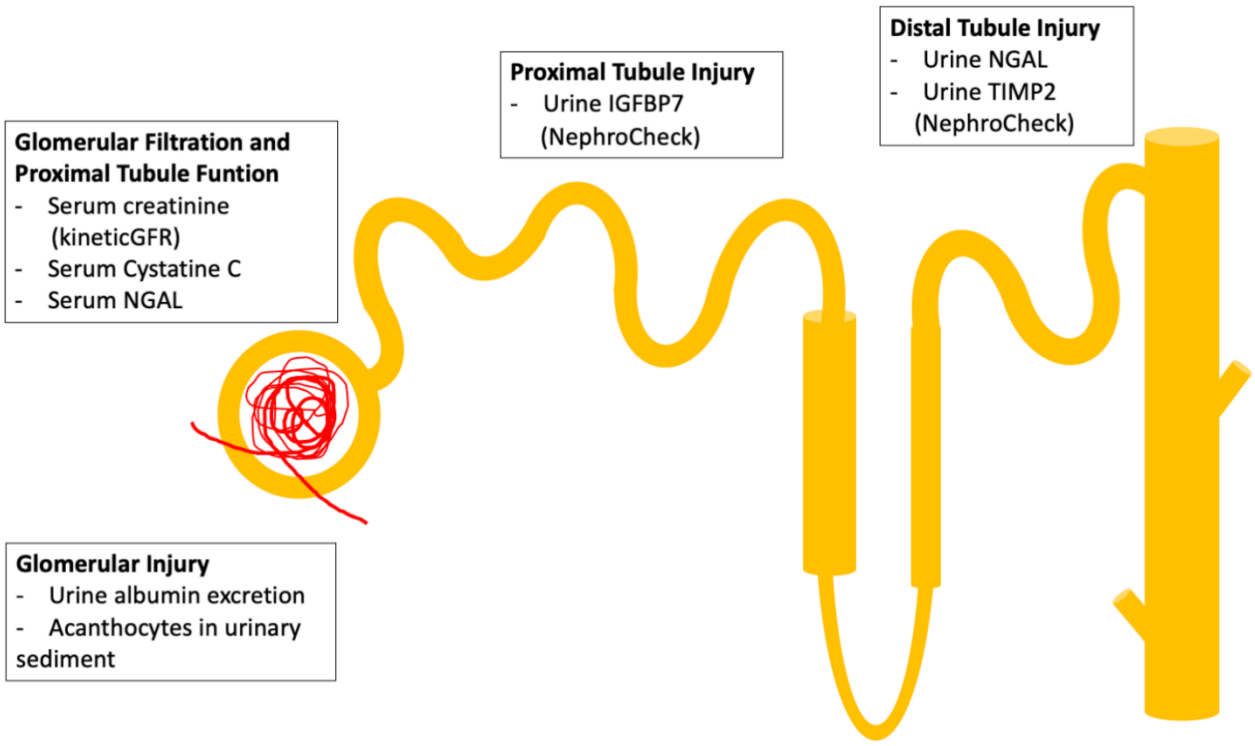


FIGURE 2. Biomarkers of AKI.

Extracellular vesicles (EVs) could represent useful potential biomarkers of AKI and renal recovery. EVs is a general term which includes membrane structures of different size released by cells after fusion of endosomes with the plasma membrane (exosomes), shed from plasma membrane (macrovesicles), or released during apoptosis (apoptotic bodies). EVs are subsequently taken up

by neighboring or distant target cells (paracrine or endocrine effect) and mediate a wide range of physiological and pathological processes, including renal diseases, maladaptive repair after AKI and tubular regeneration after acute tubular necrosis. Their bioactive cargo includes costimulatory/inhibitory molecules, cytokines, growth factors and functional miRNAs that modulate expression of cell target genes. [19] FIGURE 3

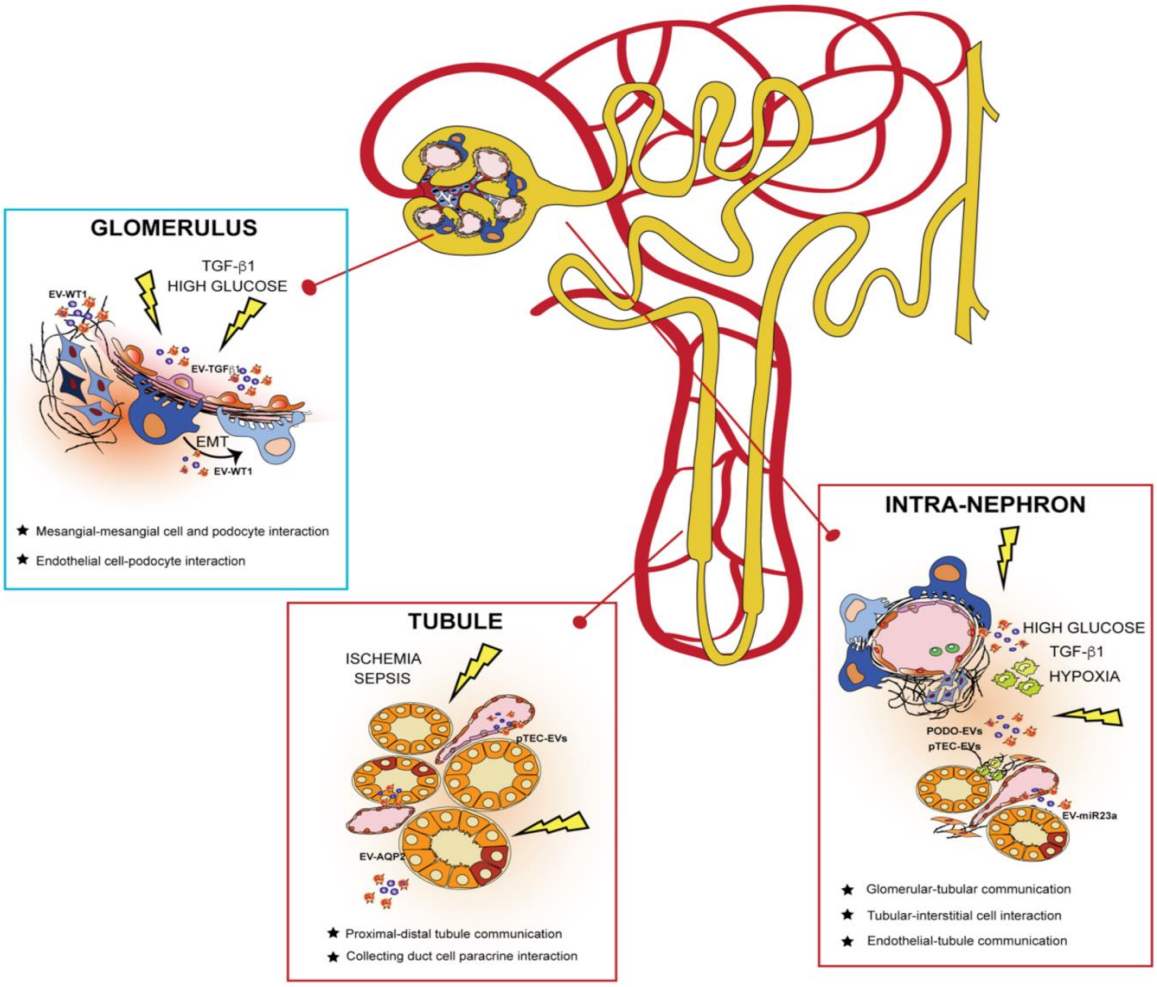


FIGURE 3. Role of extracellular vesicles in the kidney

Focusing on biomarkers we used in this study:

- Neutrophil Gelatinase-Associated Lipocalin (NGAL) is a secretory protein of activated neutrophils with a single polypeptide and a molecular weight of 25 kDa. NGAL is a critical regulator of Iron homeostasis and a significant component of the innate immune system required for fighting against bacterial infections. The Iron-containing NGAL, after

interaction with the cell surface receptors, is internalized and then releases iron inside the cell, stimulating iron-dependent genes. In parallel, non-iron bound NGAL, interacting with cell surface receptors, chelates and shuttle the intracellular iron out of the cell. Initially, NGAL was localized inside the granules of the neutrophils during maturation in the bone marrow. Its expression was then found in multiple human tissues, including the kidney, heart, liver, and lung. The systemic NGAL is filtered by glomerulus, while the proximal tubule reabsorbs it. The kidney production of NGAL is located in the thick ascending limb of the loop of Henle and in the intercalated cells of the collecting duct. It is one of the highly upregulated genes in the injured kidney: the regenerating tubular epithelial cells express higher levels of NGAL following AKI. While the plasma and urinary NGAL concentrations are very low and often undetectable, the plasma and urinary NGAL are elevated following AKI and CKD in humans. Interestingly, high NGAL levels often occur before the variation of traditional kidney function markers such as serum creatinine. [18]

- Tissue inhibitor of metalloproteinase 2 (TIMP-2) and insulin-like growth factor-binding protein 7 (IGFBP7) [Nephrocheck®]: the so-called cell cycle arrest biomarkers. These proteins trigger cell-cycle arrest during the initial stage of cellular damage, causing the cell-cycle to stop in the G1 phase: IGFBP-7 regulates p53 and p21, while TIMP-2 triggers p27 expression, inhibiting the cyclin-dependent protein kinase complexes and therefore resulting in cell cycle arrest in G1 phase of cell cycle. blocking the impact of cyclin-dependent protein kinase complexes gives cells a chance to repair DNA damage and restore functionality. Al that process takes place in the earliest stages of cellular stress and might support cellular energy stability, stop additional DNA damage, and limit cell proliferation: this is a protective mechanism designed to avoid the cells to divide when DNA has been damaged. In addition, if tubular cells become arrested at the G1 phase for prolonged periods, senescence and fibrosis will develop: cell cycle arrest may also provide a mechanistic connection between AKI and CKD.

- CD133-positive urinary Extracellular Vesicles (uEVs) and CD133: in the kidney, EVs are potent intercellular messengers released by all urinary system and involved in cell cross talk. Moreover, urine is a reservoir of EVs coming from the systemic circulation after crossing the glomerular filtration barrier or originating directly in the kidney. Nowadays,

the scientific community has a great interest in using uEVs as diagnostic and prognostic biomarkers, although they represent only 3% of the whole urinary proteome. Renal progenitor cells exhibit the stem/progenitor cell marker CD133, which is also highly expressed in healthy people' uEVs. The CD133 molecule represents a marker for a population of tubular cells intercalated between other epithelial cells possessing the capacity to survive after damage and to proliferate in case of cell damage. CD133 induces proliferation of tubular cells through activation of the Wnt/β-catenin pathway. Considering that, CD133 can be used a marker of renal regenerative capability. [19]

According to Brenner's hyperfiltration theory, a major loss of nephron mass induces hyperfiltration, which leads to progressive nephrosclerosis and kidney failure when driven by glomerular hypertension. After nephrectomy, characterized by a loss of about 50% of nephrons, an adaptive increase in the GFR of the remnant kidney occurs due to hemodynamic and structural changes of its glomeruli. In this process, every single nephron uses its reserve capacity to a certain degree, so it may be expected that the remaining RFR will be lower after donation.

Since the 1980s, several methods have been tested to quantify the RFR [20,21]; no author could actually describe a single method or a single dose of the stress needed to quantify RFR in an easy and accurate way that which could be used in clinical practice, also be due to fewer trials done for RFR evaluation. Furthermore, the kidney presents a functional reserve capacity both at glomerular and tubular level. In presence of appropriate stimuli, a subject with intact nephron mass can increase his GFR and tubular secretion. The difference between maximal capacity and baseline function represents the RFR. The ability to test this reserve may represent an excellent diagnostic possibility to reveal subclinical disease or silent loss of nephron mass. In normal subjects, baseline GFR (bGFR) tends to have some changes during the day depending on physiological requirements. Glomerular RFR (RGR-G) is defined as the capacity to increase GFR in response to a stimulus such as an acute oral protein load (weight-adjusted doses of protein of 1-2 g/kg are equally effective in increasing, intravenous amino acid infusion or dopamine infusion [23-25]. bGFR (unstressed), a surrogate of kidney function, averages in healthy subjects from 110 to 130 ml/min/1.73 m2 in females and males, respectively, and changes with age, sex and body size, with considerable variation among individuals depending on diet and other situations. Normal subjects display a

significant capacity to increase GFR under physiological stimuli (e.g., pregnancy, solitary kidney) or pathological states (e.g., diabetes and hypertension). The increase in GFR in subjects with intact RFR-G varies between 20 and 70 ml/min/1.73 m² during glomerular stress test [21, 27-29]. RFR-G is lower in the elderly and in the initial CKD stages although sCr is still normal [28].

Different theories have been proposed to explain the increase in GFR during a glomerular stress test. Glomerular hyperfiltration may occur, leading to an increase in the filtration fraction (FF) [31]. In 1993, Woods suggested that an overall increase in renal blood flow is the main mechanism, rather than a temporary variation in FF [32]. This theory seems to be supported by the observation of the decrease in renal vascular resistance in response to protein load with afferent arteriolar vasodilatation [33]. Another theory is that the GFR increases by recruiting the "dormant cortical nephrons," which are not working during resting conditions but potentially available under stress [34]. According to this theory, solitary kidneys – with fewer nephrons (and thus a limited RFR) – may have an increased susceptibility to develop AKI.

Up until now, probably the most important study showing its potential clinical was performed by Husain-Syed and colleagues. They demonstrated that among elective cardiac surgical patients with normal resting glomerular filtration rates, preoperative RFR was highly predictive of AKI. A reduced RFR appears to be a novel risk factor for AKI, and measurement of RFR preoperatively can identify patients who are likely to benefit from preventive measures or to select for use of biomarkers for early detection [20].

Theoretically, the evaluation of RFR in potential living kidney donors could provide more information about the quality of the kidneys, which usually don't undergo biopsy and consequently about the suitability of LDKT both in the donor's point of view (predictor of compensatory GFR post donation) and in the recipient's, whose kidney function would be affected by transplant-related factors such as immunosuppressive drugs, infections, rejection, vascular and urological problems, recurrent disease, etc.). Moreover, it could possibly increase the number of donors that could safely be accepted for living kidney donation, providing a better pre-donation risk-stratification.

To the best of our knowledge, there are only a few studies about RFR in kidney donors and its real value in kidney donation, before and after that. It is well known that the loss of renal mass from unilateral nephrectomy in living kidney donors is associated with compensatory changes in the remaining kidney such as hypertrophy [33] and increase of GFR comparing to the immediate postoperative time since GFR after nephrectomy is 60-75% of its pre-donation value, and not 50% as theoretically should be [34-36]. However, the predictive value of the pre-donation RFR for GFR in the immediate postoperative period and in the medium and long term and the compensatory GFR increase post-donation are not clarified yet. Finally, in most of the studies the GFR increase is calculated on eGFR or mGFR (eg. with 125-Iothalamate), assuming that the split renal function is 50% so the results are not highly precise.

In 2017, Spinelli et al. published a study evaluating the safety and feasibility of preoperative evaluation of RFR with protein load (1 g/kg of body weight [37]) in routine clinical practice during living kidney transplantation. They performed the test in KD before donation and after transplantation (2-4 years after, median time 3 years) in both donors and recipients and compared basal and stress renal functions before and after transplantation. Seven pairs of living kidney donors and recipients were enrolled. RFR was defined as the difference between the maximum value of creatinine clearance after protein load (stress glomerular filtration rate, sGFR) and baseline creatinine clearance (basal GFR, bGFR). Results: before transplantation, a significant difference between sGFR and bGFR ($p = 0.04$) was observed in donors, with an RFR = 30.6 (11.9–41.5) mL/min/1.73 m2. After kidney transplantation, sGFR was similar to bGFR for both donors and recipients ($p=0.13$), with a limited RFR 7.9 [6.70 – 19.25] and 14.90 [–6.67 to 25.53] mL/min/1.73m², respectively). The sum of the donor's and recipient's post-transplant sGFR was similar to the pre-transplant donor's sGFR ($p=0.73$). They concluded that renal stress test with protein load is a safe, feasible, and an inexpensive tool that is able to quantify RFR. In living kidney transplantation, it can be used in clinical practice to measure the original global filtration capacity of the donor's kidneys (sGFR) and to quantify the susceptibility of donors and recipients in developing postoperative kidney dysfunction. This study revealed that the RFR preoperatively measured in living kidney donors was not preserved after the transplantation: it may be explained by part of the reserve capacity being utilized by the remaining (solitary or transplanted) kidney, through glomerular hyperfiltration, to maintain a normal organ function under resting conditions. RFR is not only based on functional but also on anatomical mechanisms. Accordingly, the division

of total nephronic mass between donors and recipients resulted also in the division of RFR while the global renal capacity, expressed as the maximum GFR during stress conditions, is preserved and distributed between donors and recipients [40].

Then, in 2018, Van Londen et al. showed the results of prospective cohort study evaluating the mGFR as the urinary clearance of 125I-iothalamate and RFR (as the increase of mGFR in response to low-dose dopamine), 4 months before, and 3 months and 5 years after donation in 937 kidney donors and the predictive performance of pre-donation RFRdopa on post-donation GFR. Before donation, mean GFR was 114±22 mL/min, which increased to 149 124±24 mL/min after stimulation with dopamine (1.5 μg/kg/min), resulting in a pre-donation RFRdopa of 9±10 mL/min. In response to dopamine, most donors had an increase of effective renal plasma flow, a decrease in renal vascular resistance and filtration fraction. 11% of donors had a negative RFRdopa. Three months after donation mean single kidney GFR was 72±15, rising to 75±15 mL/min after dopamine, resulting in a post-donation RFRdopa of 3±6 mL/min. The compensatory GFR increase after donation (expressed increase of GFR above 50% of pre-donation GFR) was 15±9 mL/min. Pre-donation RFRdopa was not associated with pre-donation GFR, but was positively associated with GFR 3 months after donation. In the subgroup of donors with 5-year follow-up data, RFRdopa was not associated with GFR at 5 years post-donation. The authors concluded that RFRdopa is a predictor of short-term GFR after living kidney donation, but not of long-term kidney function so not a useful tool for donor screening. They gave the explanation that the compensatory GFR increase in the first period after donation is mostly due to early hemodynamic changes and this matches the mechanism of action of the dopamine response, as dopamine induces renal vasodilatation and hence a GFR increase (so the correlation between RFRdopa and GFR 3 months after donation). After 5-15 years, gradual GFR increase in most donors is mechanistically different from the short-term hemodynamic response; they hypothesize that it reflects more structural changes in single remaining kidney, like benign increase of glomerular size, tubule-interstitial hypertrophy and changes in recruitment of renal arteries. They reported also that this gradual GFR increase is dependent on donor age [41].

Recently, the first systematic review of renal functional reserve in adult living kidney donors has been published by Figurek, Luyckx and Mueller. They performed a systematic literature review on RFR articles published from 1956 to 2019, including in their analysis only studies which reported

RFR in living donors both before and/or after kidney donation. A total of 3250 studies were identified but only 23 studies were included in final analysis. In total, RFR measurements were performed for 1547 donors, while both pre- and post-donation data were available for 1425 donors. Several methods had been used in these studies for GFR and RFR measurements with changes in GFR, effective renal plasma flow (ERPF), RFR, and filtration fraction (FF) such as creatinine clearance, inulin clearance, radiolabeled iothalamate, diethylenetriamine pentaacetic acid or para-amino-hippurate for ERPF. Moreover, different stimuli had been used to induce RFR, including oral protein loading, either containing a fixed amount of protein (60–150 g) or adjusted according to donor body weight (1–1.2 g/kg), i.v. dopamine, amino acids (AAs), or dopamine with AAs. Finally, the intervals between kidney donation and post-donation RFR measurements were variable across the selected studies, ranging from 1 month to 22 years. RFR is reported as the percent increase in baseline GFR on stimulation of kidney function. Analysis of RFR before and after living kidney donation suggests that RFR is reduced after kidney donation, and the reduction is relatively greater among older, overweight, and possibly hypertensive donors. The Authors concluded that due to the heterogeneity of methods used and of the timing of measurements, the overall small donor numbers, the predominantly cross-sectional analyses and the lack of long-term data limit comparison of results, strong conclusions regarding the value of RFR in living kidney donors' selection were not permitted. [42]

Although RFR testing has been studies for decades, it has not entered yet into everyday clinical practice, in part because it is quite cumbersome in part because the true clinical impact has not been rigorously determined. The best method to induce and measure RFR, its normal value in different clinical contexts, as well as its predictive value, especially in more marginal donors, remains still unknown. Consequently, the RFR threshold below which donation may not be safe has never been established. On the other hand, an in-depth functional assessment of living donors' kidney function is an absolute need to enhance safety of living donation.

2. Aim of the study

The aim of the present prospective cohort study was to precisely characterize KD renal function before nephrectomy, in the immediate postoperative period and every year after donation (minimum 1, maximum 5) together with dynamic tests (RFR) and urinary biomarkers.

The renal function of all the donors before donation has been assessed with serum creatinine and eGFR CKD-EPI, creatinine clearance, measured GFR (mGFR) with 51Cr-EDTA (with Brochner-Mortensen's correction) and a concomitant sequential functional scintigraphy with 99mTc-MAG to determine split renal function. Since May 2019, due to the interruption of the production of 51Cr-EDTA, plasma clearance of 99mTc-DTPA (with Brochner-Mortensen's correction) has been used for GFR measurement. In the immediate postoperative period, serum creatinine was used to monitor the renal function. One year after the donation, both serum creatinine and mGFR with 51Cr-EDTA or 99mTc-MAG have been performed in order to obtain a precise determination of GFR of the remnant kidney and be able to compare it with its own GFR before donation. During follow-up period (minimum 1 year, maximum 5 years), serum creatinine and eGFR were assessed annually.

Starting from June 2016, KDs underwent a kidney glomerular stress test to assess their RFR with protein load before donation, as a tool to complement the assessment of their dynamic kidney function. The same test has been repeated one year after donation to evaluate whether or not the solitary kidney preserved its RFR.

In this study, we investigated also the predictive performance of pre-donation RFR with protein load on post-donation (sCr), seven days after donation (sCr) and 1 year after donation (mGFR with 51Cr-EDTA or 99mTc-MAG) and the relative compensatory GFR increase of the remnant kidney. Moreover, we proposed a threshold of RFR below which donation may not be safe.

In a subgroup of patients, samples of plasma and urine collected during KST with protein load (before donation and 1 year after) and on post-operative day 7 were analyzed for [TIMP-2]·[IGFBP7] (NephroCheck®) and NGAL to identify the presence of a putative injury of the tubular compartment before donation, during glomerular stress test, 7 days and 1 year after nephrectomy. Finally, additional samples of urine before and 7 days after donation were analyzed

for urinary extracellular vesicles (uEVs) to characterize them and their potential role in the solitary kidney immediately after donation and 1 year after.

3. Patients and Methods

From 2003 to April 2023, 168 living donor kidney transplants were performed in the Kidney Transplantation Unit of Maggiore della Carità University Hospital in Novara (Italy), with a rapid growth during the last years: 126 from January 2014.

All donors were older than 18 years old, normotensive or had controlled hypertension (maximum of two antihypertensive drugs), no diabetes or abnormal glucose tolerance tests, no cardiovascular events, no kidney diseases, with normal kidney function expressed as mGFR (51Cr-EDTA or 99mTc-DTPA) >70 mL/min, normal proteinuria (<150 mg/24h) and albuminuria (<30 mg/24h).

This study was performed in accordance with the principles of the Declaration of Helsinki. All donors were informed of the objectives of the study and gave consent for the use of their data and samples.

3.1 Measurement of Renal Functional Reserve (RFR)

From June 2016, KD underwent a kidney stress test (glomerular) with protein load to assess their RFR two weeks before and 1 year after donation, respectively [IRRIV protocol, International Renal Research Institute of Vicenza, Prof. Ronco].

All the donors were on a standard diet. 8 hours of fasting was required to assess the kidney glomerular stress test after an adequate oral hydration (8 mL/kg body weight) in 30 minutes and voiding of the bladder. After that, the urine volume was replaced with equal volume of water by mouth. Two measurements of 1-hour Creatinine Clearance (CrCl) were obtained in resting conditions and the mean value of them was considered the baseline. Then, an oral protein load (cooked read meat, 1.2 g of proteins/kg of body weight of the patient [14]) was given and eaten in 30 minutes. 1-hour CrCl was assessed in the following four hours after protein load. The difference between the higher CrCl obtained after protein load and the baseline CrCl defined RFR.

Urinary creatinine (uCr) and serum creatinine (sCr) was measured by enzymatic method with an automated analyzer (Siemens ADVIA 1800®, Siemens Healthcare Diagnostics Inc., Japan/Canada).

CrCl was calculated and corrected for 1.73m² of body surface area (BSA using Dubois method) as follow: CrCl = uCr (mg/dL)/sCr (mg/dL)* urinary volume (mL)/time (minute)* 1.73/BSA (m²)

3.2 Definition of Acute Kidney Injury (AKI)

AKI was defined according to the KDIGO consensus criteria [13] as the increase of sCr by ≥0.3 mg/dL (≥26.5 µmol/L) or increase of sCr to ≥1.5 times baseline, or urine volume <0.5 mL/kg/h ×6-12 h stage 1. Stage 2: SCr 2.0-2.9 times baseline, or urine volume <0.5 mL/kg/h for ≥12 h. Stage 3: sCr 3 times baseline or increase sCr to ≥4 mg/dL (≥353.6 µmol/L) or initiation of renal replacement therapy, or anuria for ≥12 h. Baseline sCr was defined as the lowest value among the following: the sCr value that was measured at a time closest to nephrectomy within the prior 1 year or the minimum sCr value within 3 months before admission.

The percentage of worsening in sCr was calculated as [(zenith of sCr – baseline sCr)/baseline sCr]*100.

The recovery of renal function at discharge was calculated as {[(sCr at discharge – baseline sCr)/baseline sCr]*100} - {[(peak of sCr – baseline sCr)/baseline sCr]*100}.

3.3 Measurement of compensatory GFR increase

All the KD in Our Centre underwent the radioisotope measurement of GFR with chromium-51 labeled ethylenediamine tetraacetic acid (51Cr-EDTA) and a concomitant sequential functional scintigraphy with 99mTechnetium labeled mercaptoacetyltriglycine (99mTc-MAG). Since May 2019, 99mTc-MAG was used both for measurement of GFR both for the sequential functional scintigraphy.

From 2015, we also prospectively measured GFR (51Cr-EDTA or 99mTc-DTPA) one year after donation.

All these tests were performed at the Nuclear Medicine Unit of Maggiore della Carità University Hospital.

All the patients were adequately hydrated prior and during the study and had a light breakfast.

They avoided excessive intake of drinks containing caffeine and high protein meals the day before the exam. None of the donors was using drugs that could modify renal blood flow and/or GFR (Angiotensin Converting Enzyme Inhibitors, Angiotensin Receptor Blockers, diuretics, nonsteroidal anti-inflammatory drugs, antibiotics).

51Cr-EDTA, the tracer recommended by the European Association of Nuclear Medicine (EANM) and British Nuclear Medicine Society (BNMS), is considered a standard radiopharmaceutical for GFR measurement in Europe. The administered dose is calculated according to the patient's weight, equal to 37KBq/Kg. In order to obtain the maximum precision, the method of double weighing of the syringe, before and after injection, has been adopted, to know the net weight of the injected 51Cr-EDTA. After intravenous injection, EDTA is eliminated from plasma exclusively by glomerular filtration without tubular excretion or reabsorption. Given time "0" the time of the tracer injection, three blood samples are obtained after 120, 180 and 240 minutes from the contralateral arm to the infusion site, to reduce the risk of contamination. The blood samples are then centrifuged to obtain plasma samples that are placed in a scintillation well. Then, the residual radioactivity expressed in counts per minute (CPM) is calculated for each sample. Radioactivity in the 3 samples is reduced over time and is an expression of the filtration capacity of the kidney. Just for example, greater is the loss of radioactivity between the 3 samples, better is the glomerular filtration. GFR is calculated from the area under the plasma clearance curve (AUC) obtained by a mono-exponential fit of the 3 blood sample count using the so called "slope intercept" method. It should be recalled that in reality the plasmatic disappearance curve of the tracer follows a bi-exponential pattern of which the first "fast" exponent shows rapidly falling kinetics of radioactivity due to the redistribution of the tracer in bodily fluid. After mixing, a second "slow" exponent, starting from the 2th hour of the injection, reflects the filtration capacity of the kidney. The use of a mono-exponential fit just of the second exponent, not considering the first exponent, leads to an overestimation of glomerular filtration rate, especially for high GFR value (e.g. hyperfiltration states). Brochner-Mortensen's formula is used to correct the overestimation due to the absent of the early compartment of AUC. The GFR value obtained is corrected for the body surface area. [43]

A concomitant sequential functional scintigraphy with 99mTc-MAG is performed to determine split renal function. MAG is actively excreted by renal tubules without glomerular filtration or tubular reabsorption. A dynamic acquisition is performed with patient in a supine position and

gamma camera positioned under the bed, immediately after the intravenous administration of 74-111MBq of the tracer. The images obtained allow a good evaluation of its distribution in the kidney cortex after extraction from the bloodstream (so called parenchymal phase occurring usually within the first 5 minutes of acquisition) and the evaluation of the subsequent excretory phase. The “split renal function” is calculated during parenchymal phase and represents the contribution in percentage of each kidney respect to the total parenchymal function; to calculate split renal function 2 Region of Interest (ROIS) are drawn around the shape of the kidneys. The amount of radioactivity expressed in CPM within the 2 ROIS by the arithmetic calculation: CPM right kidney/(CPM right kidney + CPM left kidney) represents the percentage contribution of each kidney. The tracer is than excreted via the ureters into the urinary bladder.

In late 2018, the production of 51Cr-EDTA, was halted. Consequently, 99mTc-DTPA has been validated and used for GFR measurement, since no clinically relevant differences were found between the plasma clearance of 99mTc-DTPA and that of 51Cr-EDTA. [44]

The pre-donation single kidney GFR is calculated as: mGFR (mL/min) * the percentage (%) of the function, determined by scintigraphy, of the kidney that will remain solitary in the donor after donation.

The compensatory GFR increase post-donation is defined as 1 year post-donation mGFR minus pre-donation single kidney mGFR.

3.4 Assessment of Tubular Injury

Samples of urines of a subgroup of donors were taken at the following time points: during KST before and 1 year after donation (at the beginning of the test as basal urine and 4 hours after the protein load), and on post-operative day 7 in basal conditions. Urine samples were tested for [TIMP-2]·[IGFBP7] (NephroCheck®) and NGAL to investigate the presence of tubular injury during glomerular stress test, some days after nephrectomy and 1 year after. Samples were centrifuged and the supernatant frozen at -80°C until laboratory use.

In the postoperative period, we decided to collect and analyze samples of urine taken in 7^{th} day after surgery to avoid the potential interference due to the intraoperative handling of the donated kidney.

The two biomarkers TIMP-2 and IGFBP7 were measured converting fluorescent signals from each of the two immunoassays by Astute 140 meter® (Astute Medical, San Diego, CA, USA). All values for ([TIMP-2] x [IGFBP7])/1000)-NC- are reported in units of [(ng/ml)^2/1000]. NC score: ([TIMP-2] x [IGFBP7])/1000)>0.3 [(ng/ml)^2/1000] was considered positive.

The assay used to measure urinary NGAL was chemiluminescent microparticle immunoassay (CMIA) by Architect® (Abbott Laboratories, Abbott Park, IL, USA). A value of NGAL ≥100 ng/mL was considered as positive.

Both measurements were performed in collaboration with IRRIV (International Renal Research Institute), Vicenza, Italy, research partner of the present study (Prof. C. Ronco).

3.5 Urinary extra-cellular vesicles characterization

Urinary extra-cellular vesicles (uEVs) were characterized by bead-based multiplex analysis by flow cytometry (MASCSPlex Exosome Kit, human, Miltenyi Biotec). All urinary samples were centrifuged at 3000g for 15 minutes and filtered through 0.22µM filter. One hundred and twenty microliters of each urinary sample were loaded onto a 1.5 mL tube and 0.5 µL of protease inhibitor (Sigma) were added. After that, 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 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
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

These measurements were performed in collaboration with the Department of Medical Sciences and Molecular Biotechnology Center, University of Torino, Torino, Italy, research partner of the present study (Dr. S. Bruno, Prof. G. Camussi).

3.6 Statistical analysis

Statistical analyses were performed by STATA v.17.0. Normality distribution was assessed preliminarily by Kolmogorov–Smirnov and Shapiro–Wilk tests. Quantitative variables were expressed by median, interquartile range (IQR) and min-max range, while categorical variables were expressed by absolute and relative frequencies. Differences between independent groups were estimated by non-parametric Mann–Whitney U-test. Association between categorical variables was evaluated by Chi-Squared test or Fisher's Exact test if needed. Correlation between continuous variables was evaluated by non-parametric Spearman correlation. A p-value <0.05 will be considered significant for all statistical tests (two-tailed).

4. Results

4.1 Pre-donation characteristics

We included in this study 112 living kidney donors who donated in Our Transplant Center from 1st January 2014 to 31st March 2022. Main demographic and clinical characteristics of the patients investigated are shown in Table 1. Median (IQR; min-max) age at the time of donation was 53 years (47-61; 30-76), 73 (65%) were females. Before donation, median sCr was 0.69 mg/dL (0.6-0.84; 0.46-1.18), median eGFR CKD-EPI 101 mL/min/1.73m² (92.5-106; 68-122), median CrCl 114 mL/min/1.73m² (96.3-133.3; 62-171.1), median mGFR with 51Cr-EDTA or 99mTc-DTPA 97 mL/min (88-108; 72-137). The split function, evaluated by a concomitant scintigraphy using 99mTc-MAG, was as follows: the median percentage of renal function of right kidney was 47.4% (46-49.7; 43-56) and left 52.6% (50.3-54; 44-57), respectively. In our Center all left nephrectomies were performed (except in one single case in which right nephrectomy was performed), therefore, we calculate the pre-donation single kidney mGFR of the right kidney (or left kidney in that single case), 46.1 mL/min (41-51.4; 32.9-65.1), in order to be able to compare it with the post-donation GFR of the solitary remnant kidney. TABLE 1

Variables	**Statistics**
Sex (male)	39 (35%)
Age at donation (years)	53 (47-61; 30-76),
Height (cm)	165 (159-174; 147-190)
Weight (Kg)	70 (60-82; 43-113)
BMI (Kg/m²)	25 (23-27.6; 15.2-32.3)
BSA (m²)	1.75 (1.63-1.92; 1.34-2.38)
Hypertension	28 (25%)
Dyslipidemia	40 (36%)
Active smoker	16 (14%)
Baseline sCr (mg/dL)	0.69 (0.6-0.84; 0.46-1.18)
Baseline eGFR (CKD-EPI) (mL/min/1.73m²)	101 (92.5-106; 68-122)
Baseline CrCl (mL/min/1.73m²)	114 (96.3-133.3; 62-171.1)
Baseline mGFR (51Cr-EDTA or 99mTc-DTPA)	97 (88-108; 72-137)
Spilt function of right kidney (%)	47.4 (46-49.7; 43-56)
mGFR right kidney pre donation	46.1 (41-51.4; 32.9-65.1)

TABLE 1. Main characteristics of the population of the study. Data are described as median (IQR; min-max) or as relative and absolute frequencies, respectively.

4.2 Post donation renal function

4.2.1 Within 7 days after donation

All 112 patients were diagnosed with AKI within 48-72 hours after surgery and staged according to positive creatinine criteria of KDIGO guidelines [12]; none exhibited positive urine output criteria. Most AKI cases were classified as stage 1, 8% (9/112) AKI stage 2.

Zenith creatinine levels were observed in 93% of patients within 48 hours following nephrectomy.

Median serum creatinine levels at zenith and at 7th day after surgery were significantly higher than at baseline (Friedman test, $p<0.0005$). In particular, median (IQR) serum creatinine levels at zenith and at 7th day after surgery were respectively 1.19 mg/dL (1.03-1.43, 0.74-2.32) ($p<0.0005$ vs baseline, Bonferroni's correction) and 1.04 mg/dL (0.89-1.26, 0.63-2.2) ($p<0.0005$ vs baseline, Bonferroni's correction). The partial recovery of renal function, as estimated by a significantly lower creatinine levels than zenith, was observed in all donors within 7 days and before hospital discharge ($p<0.0005$ vs zenith, Bonferroni's correction). Serum creatinine variations within 7 days after donation are shown in FIGURE 4.

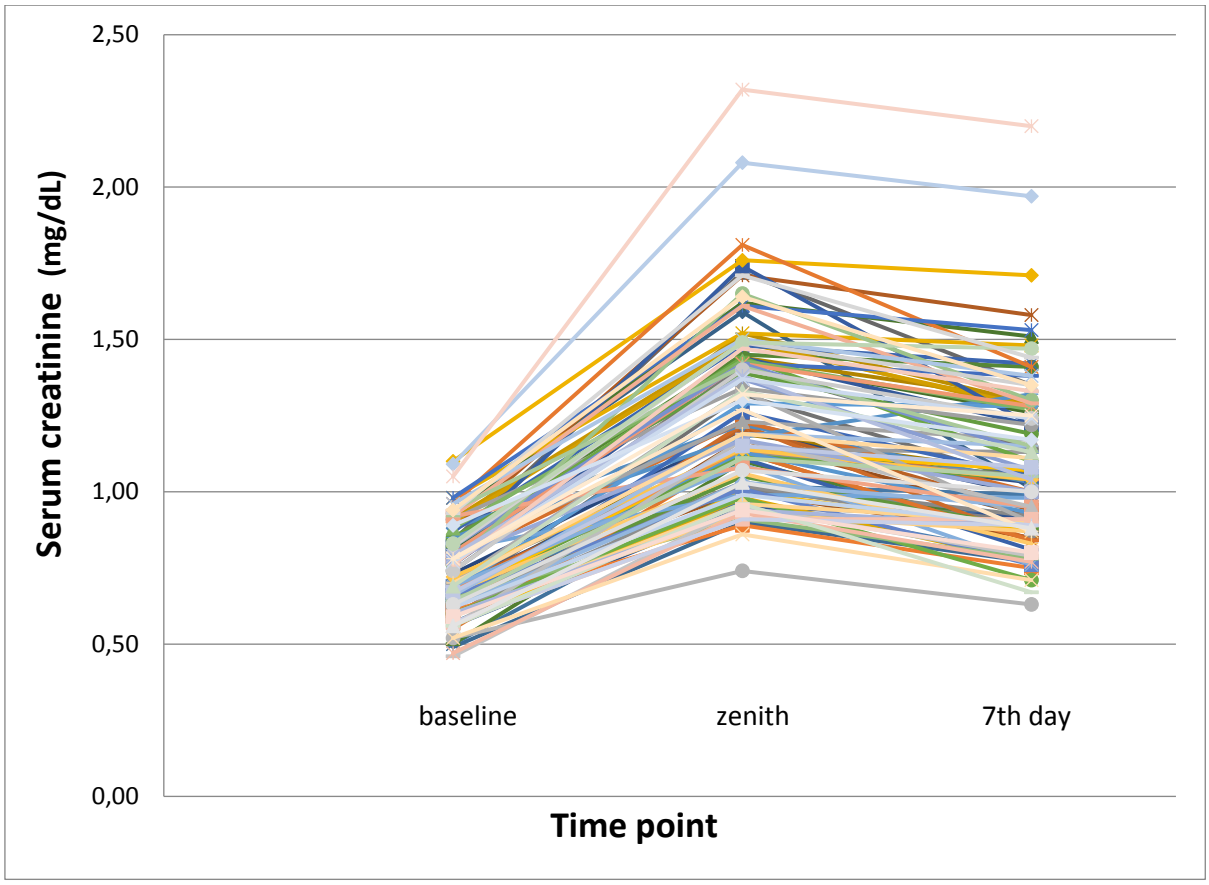


FIGURE 4. Variations in serum creatinine among the 112 patients investigated from baseline to 7th day after surgery.

4.2.2 One year after donation

92 donors underwent mGFR determination with 51Cr-EDTA or 99mTc-DTPA 1 year after donation. The median mGFR (51Cr-EDTA or 99mTc-DTPA) of the solitary kidney was 63.5 mL/min (56.5-71; 36-95) vs pre donation mGFR of the same kidney 46.1 ml/min (41-51.4; 32.9-65.1). The median compensatory GFR increase after donation (expressed as 1 year post donation mGFR minus pre-donation single kidney mGFR) was 18 mL/min (9.4-23.1; 1-45.6), with median percentage increase of 37% (20.4-51.3; 0-110) vs the pre-donation right kidney mGFR ($p<0.0005$, non parametric Wilcoxon test for paired samples). None of the donors displayed a GFR decrease of the solitary kidney left after donation. Most of donors (72/83, 87%) increased the GFR of the single kidney more than 5 mL/min (or more than 10%) vs the pre-donation GFR of the same kidney. A visual representation of the GFR increase of the single kidney at 1 year is shown in FIGURE 5.

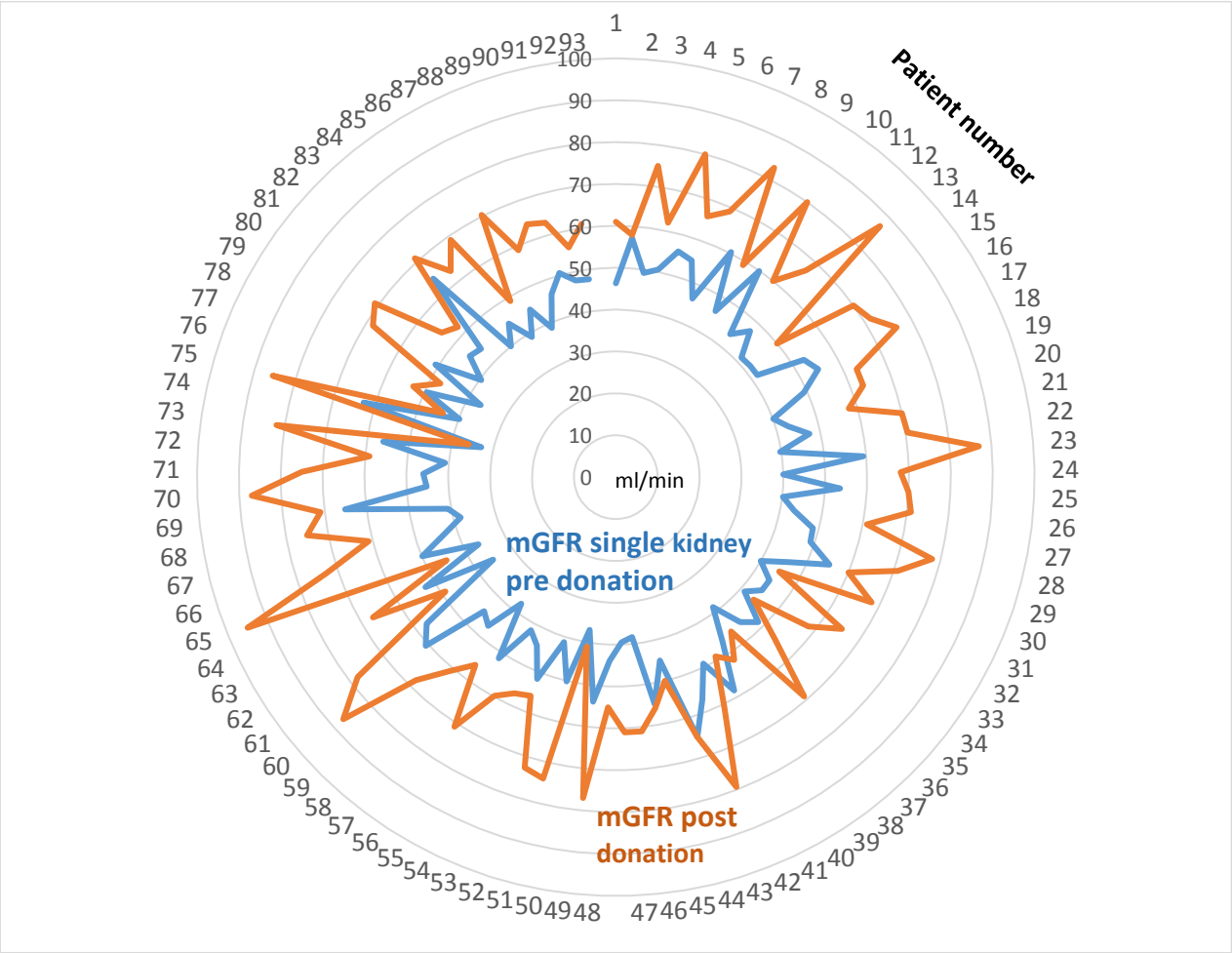


FIGURE 5. Radar chart for the GFR increase of the single kidney at 1 year in the patients underwent nephrectomy. In blue and in orange are shown the mGFR levels at the first scintigraphy + mGFR (before nephrectomy) and after 1 year after, respectively.

The value of mGFR after 1 year was associated with age and mGFR of the same kidney before donation (p=0.02 and 0.01 respectively) and not associated with sex (p=0.464), BMI (p=0.715), hypertension (p=0.250), dyslipidemia (p=0.361) and smoking (p=0.659). TABLE 2

Variable	Regression coefficient	Standard error	t	p-value	IC 95%
mGFR remnant kidney	0,506	0,191	2,65	**0,010**	0,123 – 0,890
Donor Age	-0,467	0,146	-3,20	**0,002**	-0,758 – -0,175
BMI	0,132	0,360	0,37	0,715	-0,588 – 0,852
Sex (M vs F)	2,157	2,923	0,74	0,464	-3,694 – 8,009
Hypertension	3,580	3.084	1,16	0,250	-2,592 – 9,753
Dyslipidemia	-2,459	2,670	-0,92	0,361	-7.803 – 2.885
Smoking	-1,209	2.724	-0,44	0,659	-6.661 – 4.243

TABLE 2. Multivariate regression analysis of predictors of mGFR 1 year after donation

Moreover, we considered the median value of mGFR at 1 year as a threshold to identify donors with adequate long-term renal function after donation (mGFR > 65 mL/min) and studied predictors of that endpoint with a multivariate logistic regression analysis. Age at donation and radioisotope GFR of the single kidney (that will be the remnant one) were independently associated with mGFR > 65 ml/min at 1 year after donation. Every increase in mGFR unit (1 mL/min) determines a 19% increase in the likelihood of an adequate 1 year mGFR. In contrast, every decrease in age unit (1 year) determines a 12% increase in in the likelihood of an adequate 1 year mGFR. TABLE 3

Variable	OR	Standard error	z	p-value	IC 95%
RFR (per unit)	1,035	0,034	1,06	0,290	0,971 – 1,104
mGFR remnant kidney (per unit)	1,196	0,098	2,18	**0,029**	1,018 – 1,404
Donor age	0,886	0,058	-1,85	**0,065**	0,778 – 1,007
BMI per unit	0,955	0,163	-0,27	0,788	0,684 – 1,334
Sex (M vs F)	0,834	0,862	-0,18	0,861	0,110 – 6,321

TABLE 3. Multivariate logistic regression analysis of predictors of mGFR > 65 mL/min 1 year after donation

4.3 Characterization of Renal Functional Reserve (RFR)

From June 2016, 61 donors underwent the kidney stress test (glomerular) with a protein load to assess their RFR (pre-donation), as described in the Method section. Median pre-donation RFR was 27.3 mL/min/1.73m² (13.2-34.6; 0-64.4).

5 donors underwent mGFR with 51Cr-EDTA during the kidney stress test: the infusion of radionuclide had been done immediately before the protein load with three blood samples obtained after 120, 180 and 240 minutes: no differences between mGFR under glomerular stress and basal mGFR were observed (data not shown), probably because mGFR is calculated as an AUC while RFR is a peak of GFR in a short time.

61 kidney donors (61/112, 55% of the entire cohort) had complete data about their kidney function before donation (with pre-donation RFR), in the postoperative period (within 7 days after

nephrectomy) and 1 year after donation (serum creatinine, eGFR, radioisotope GFR). This subgroup was comparable with all other 51 subjects: in particular, subgroups did not differ for sex (p=0.761), age (p=0.404), BMI (p=0.683), BSA (p=0.810) and mGFR (p=0.164).

Among these 61 donors, median (IQR; min-max) age at the time of donation was 57.5 years (51-64; 33-72), 38 (62%) were females. Before donation, median sCr was 0.68 mg/dL (0.6-0.83; 0.49-1.18), median eGFR CKD-EPI 103 mL/min/1.73m² (93-106; 63-124), median CrCl 115 mL/min/1.73m² (98-138; 74.6-171.1), median mGFR with 51Cr-EDTA 96 mL/min (86-105; 72-137). The split function, evaluated by a concomitant scintigraphy using 99mTc-MAG, was as follows: the median percentage of renal function of right kidney was 47.5% (45.3-51.5; 43-56) and left 52.5% (48.5-54.7; 44-57), respectively (Table 2). Finally, we calculated the pre-donation single kidney mGFR (that one of the future remnant kidney): its median value was 44.6 mL/min (40-50.6; 32.9-65.1), in order to be able to compare it with the post-donation GFR of the solitary kidney after donation. TABLE 4

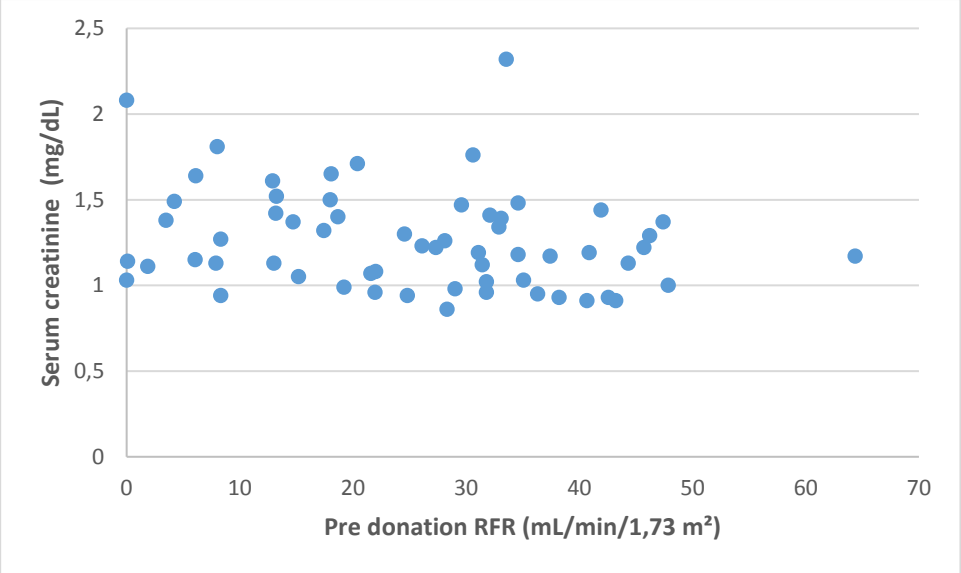
Variables	**Statistics**
Sex (male)	23 (28%)
Age at donation (years)	57.5 (51-64; 33-72)
Height (cm)	167 (160-174; 147-187)
Weight (Kg)	71 (62-79; 42-94)
BMI (Kg/m²)	25 (23.2-27.3; 15.2-31.2)
BSA (m²)	1.79 (1.64-1.92; 1.4-2.2)
Hypertension	14 (23%)
Dyslipidemia	18 (30%)
Active smoker	9 (15%)
Baseline sCr (mg/dL)	0.68 (0.6-0.83; 0.49-1.18)
Baseline eGFR (CKD-EPI) (mL/min/1.73m²)	103 (93-106; 63-124)
Baseline CrCl (mL/min/1.73m²)	115 (98-138; 74.6-171.1)
Baseline mGFR (radioisotope)	96 (86-105; 72-137)
Spilt function of right kidney (%)	47.5% (45.25-51.5; 43-56)
mGFR right kidney pre donation (radioisotope) (ml/min)	44.6 (40-50.6; 32.9-65.1)

TABLE 4. Main characteristics of the sample of 61 donors who underwent RFR study.

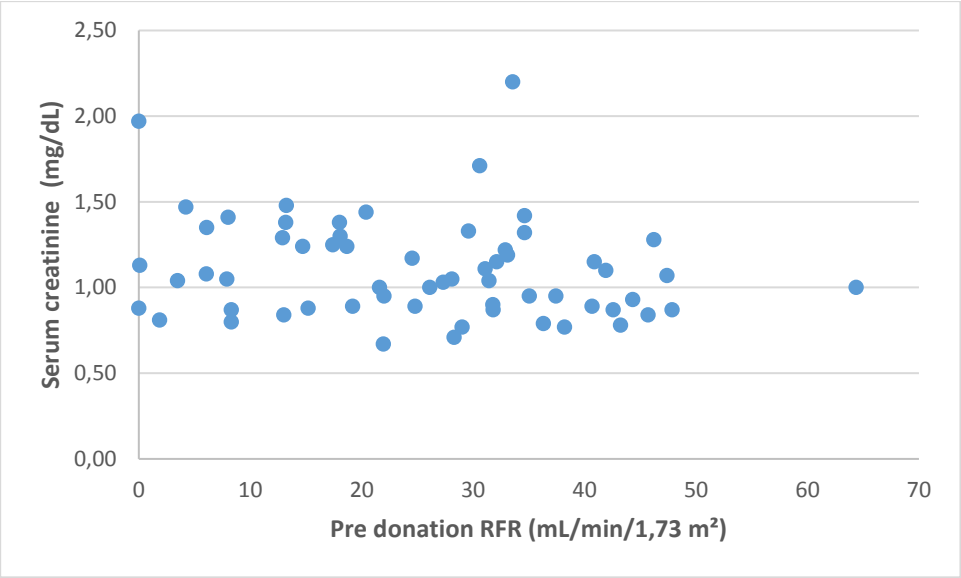
We did not find a correlation between RFR and age (r -0.11), mGFR before donation (r 0.12), serum creatinine (r -0.11), eGFR (r 0.1) or other clinical characteristics sex, BMI, hypertension, dyslipidemia, active smoking.

4.3.1 RFR and renal function within 7 days after donation

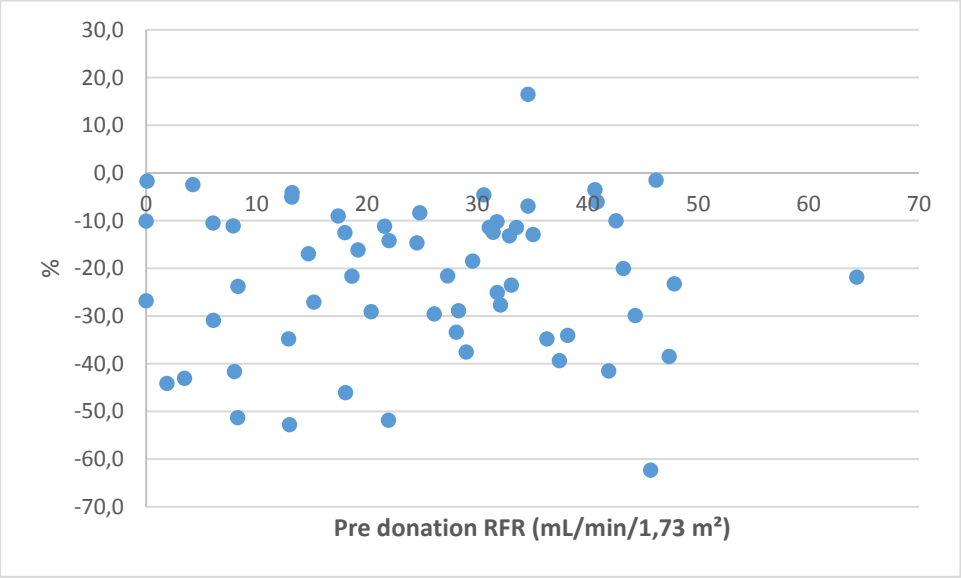
No association between pre-donation RFR and worsening of sCr ("AKI") after nephrectomy was observed. Indeed, pre-donation RFR was not associated either with increase (absolute or in percentage) of sCr at zenith compared to baseline (p=0.562), or increase (absolute or in percentage) of sCr 7 days after compared to baseline (p=0.752), or the relative (%) lowering of serum creatinine 7 days after donation compared to zenith (p=0.585). GRAPH 1, 2, 3.



GRAPH 1. Correlation between RFR and sCr at zenith



GRAPH 2. Correlation between RFR and sCr 7 days after donation



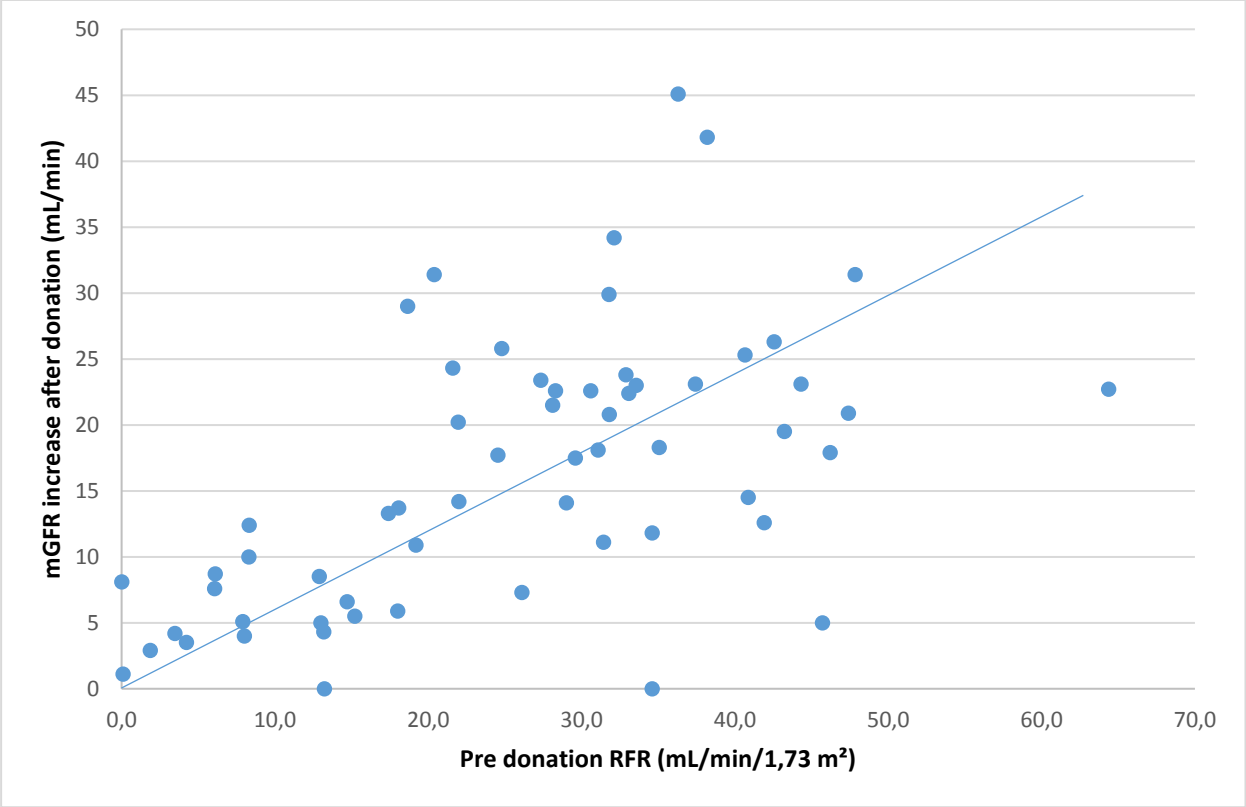
GRAPH 3. Correlation between RFR and relative lowering of sCr

7 days after donation compared to zenith

4.3.2 RFR and renal function 1 year after donation

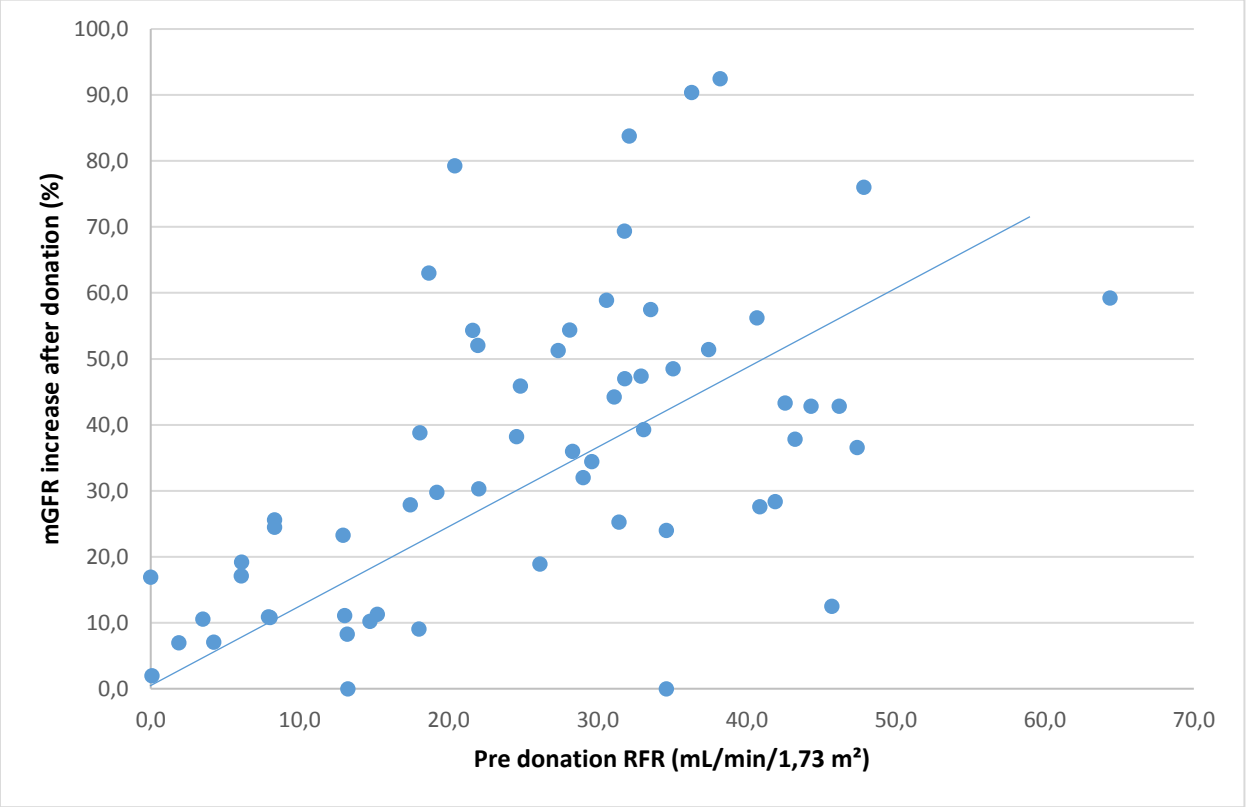
As reported in Methods paragraph, mGFR was determined with 51Cr-EDTA or 99mTc-DTPA: its increase is derived by the difference between the mGFR 1 year after donation and mGFR of the same kidney before donation. We performed a statistical analysis to verify whether there is a correlation between RFR and compensatory mGFR increase 1 year after donation. The results are reported below.

Pre-donation RFR was correlated with the compensatory mGFR increase at 1 year after donation with a moderate relationship (r=0.58). [53] GRAPH 4



GRAPH 4. Correlation between pre-donation RFR and post-donation mGFR absolute compensatory increase.

The correlation coefficient r between RFR pre donation and the percentage compensatory mGFR increase at 1 year after donation was 0.56. GRAPH 5



GRAPH 5. Correlation between pre-donation RFR and post-donation percentage mGFR compensatory increase.

Consequently, we performed statistical analysis in order to establish a RFR threshold for donation, depending on mGFR compensatory increase 1 year after donation. FIGURE 6

-RFR threshold: 10 mL/min/1.73 m²

Group 0 (RFR < 10 mL/min/1.73 m²) [12 KD]: median mGFR increase 4.6 mL/min (3.3-8.4; 1.1-12.4)

Group 1 (RFR ≥ 10 ml/min/1.73 m²) [49 KD]: median mGFR increase 19.5 mL/min (11.8-23.4; 0-45.1)

-RFR threshold: 15 mL/min/1.73 m²

Group 0 (RFR < 15 mL/min/1.73 m²) [17 KD]: median mGFR increase 5 mL/min (3.5-8.1; 0-12.4)

Group 1 (RFR ≥ 15 ml/min/1.73 m²) [44 KD]: median mGFR increase 20.8 mL/min (13.9-24.1; 0-45.1)

-RFR threshold: 20 mL/min/1.73 m²

Group 0 (RFR < 20 mL/min/1.73 m²) [23 KD]: median mGFR increase 5.9 mL/min (4-10; 0-29)

Group 1 (RFR ≥ 20 ml/min/1.73 m²) [38 KD]: median mGFR increase 21.9 mL/min (17.5-24.3; 0-45.1)

-RFR threshold: 25 mL/min/1.73 m²

Group 0 (RFR < 25 mL/min/1.73 m²) [29 KD]: median mGFR increase 8.1 mL/min (4.3-13.7; 0-31.4)

Group 1 (RFR ≥ 25 ml/min/1.73 m²) [32 KD]: median mGFR increase 21.9 mL/min (16-23.6; 0-45.1)

-RFR threshold: 30 mL/min/1.73 m²

Group 0 (RFR < 30 mL/min/1.73 m²) [35 KD]: median mGFR increase 8.7 mL/min (5-17.7; 0-31.4)

Group 1 (RFR ≥ 30 ml/min/1.73 m²) [26 KD]: median mGFR increase 22.5 mL/min (17.9-25.3; 0-45.1)

-RFR threshold: 35 mL/min/1.73 m²

Group 0 (RFR < 35 mL/min/1.73 m²) [46 KD]: median mGFR increase 12.1 mL/min (5.5-22.4; 0-34.2)

Group 1 (RFR ≥ 35 ml/min/1.73 m²) [15 KD]: median mGFR increase 22.7 mL/min (17.9-26.3; 0-45.1)

-RFR threshold: 40 mL/min/1.73 m²

Group 0 (RFR < 40 mL/min/1.73 m²) [50 KD]: median mGFR increase 13.5 mL/min (5.9-22.6; 0-45.1)

Group 1 (RFR ≥ 40 ml/min/1.73 m²) [11 KD]: median mGFR increase 20.9 mL/min (14.5-25.3; 5-34.1)

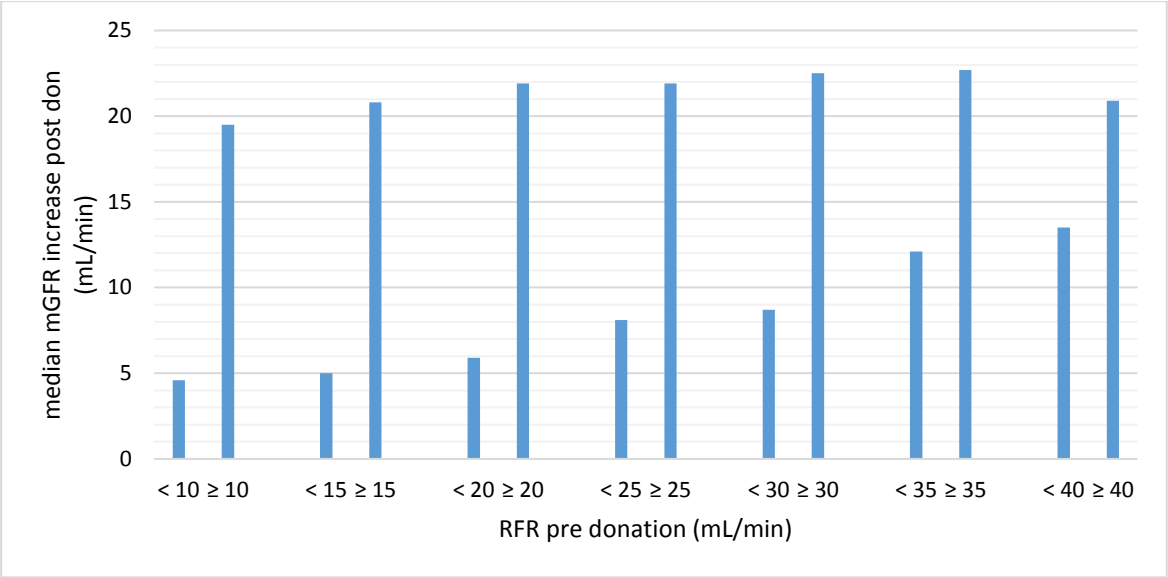
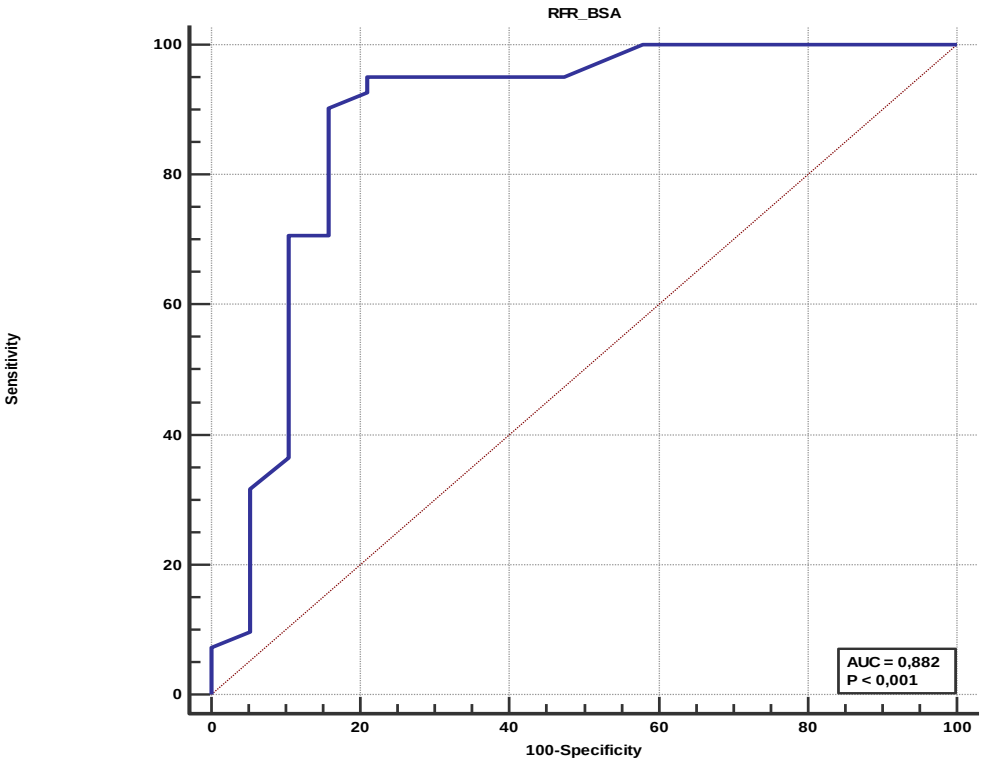


FIGURE 6. Different RFR thresholds pre donation and outcomes in compensatory GFR increase post donation (expressed as median mGFR increase 1 year after donation).

Considering that the increase in GFR in healthy subjects with intact RFR-G varies between 20 and 70 ml/min/1.73 m² during glomerular stress test after a protein load [21, 39, 40], we observed the different outcomes in kidney function 1 year after donation, expressed as the median radioisotope mGFR increase, at different RFR threshold points.

We observed that RFR group < 10 ml/min/1.73 m² is not different from the group < 15 ml/min/1.73 m² and < 20 ml/min/1.73 m² ($p < 0.05$) so we proposed a RFR threshold for a safe donation at 20 ml/min/1.73 m².

Moreover, we performed a ROC analysis to establish the RFR value with the best predictive value for an mGFR compensatory increase of 10 mL/min 1 year after donation. The RFR threshold value above 18 ml/min showed the most accurate performance to identify this subset of patients (sensitivity = 90.2%, specificity = 84.2%; AUC 0.88). FIGURE 6



Criterion	Sensitivity	95% CI	Specificity	95% CI
≥0	100,00	91,4 - 100,0	0,00	0,0 - 17,6
>6	100,00	91,4 - 100,0	42,11	20,3 - 66,5
>8	95,12	83,5 - 99,4	52,63	28,9 - 75,6
>15	95,12	83,5 - 99,4	78,95	54,4 - 93,9
>17	92,68	80,1 - 98,5	78,95	54,4 - 93,9
>18	**90,24**	**76,9 - 97,3**	**84,21**	**60,4 - 96,6**
>25	70,73	54,5 - 83,9	84,21	60,4 - 96,6
>26	70,73	54,5 - 83,9	89,47	66,9 - 98,7
>34	36,59	22,1 - 53,1	89,47	66,9 - 98,7
>35	31,71	18,1 - 48,1	94,74	74,0 - 99,9
>44	9,76	2,7 - 23,1	94,74	74,0 - 99,9
>46	7,32	1,5 - 19,9	100,00	82,4 - 100,0
>64	0,00	0,0 - 8,6	100,00	82,4 - 100,0

Area under the ROC curve (AUC)	**0,882**
Standard Error [a]	0,0594
95% Confidence interval [b]	0,773 to 0,951
z statistic	6,432
Significance level P (Area=0.5)	<0,0001

FIGURE 6. ROC curve showing best sensibility and specificity profile with RFR > 18 mL/min

1 year after nephrectomy, 24 out of 61 donors underwent the kidney stress test (glomerular) with protein load to assess their RFR at this time point (post-donation RFR). Median 1 year post-donation RFR was 9.7 mL/min/1.73m² (6.4-16.9; 1.5-21.9) significantly different from the RFR before donation (median value of the same donors before donation: 29 mL/min/1.73m², IQR 19.8-33, min-max 1.9-45.7), $p<0.05$.

4.3.2 RFR and renal function 2-5 years after donation

The pre donation RFR was not correlated with renal function estimated with eGFR (CKD-EPI) during 5 year-follow up period after donation (1st year r 0.23, 2nd year 0.15, 3rd year -0.08, 4th year 0.16, 5th year 0.28).

4.4 Biomarkers

In 27 kidney donors, samples urine collected during KST with protein load (before donation and 1 year after) and on post-operative day 7 were analyzed for NGAL and [TIMP-2]·[IGFBP7] (NephroCheck®) to identify the presence of a putative injury of the tubular compartment during glomerular stress test, 7 days and 1 year after nephrectomy. Moreover, additional samples of urine before donation, 7 days after and 1 year after were analyzed for urinary extracellular vesicles (uEVs) in the same donors. These samples were subjected to EV characterization using MACSPlex exosome kit, human, Miltenyi Biotec. 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 normalized to mean MFI for specific EV markers (CD9, CD63 and CD81) obtaining normalized MFI (nMFI). All analyses were based on nMFI values. These 27 kidney donors (27/61, 44% of the cohort who underwent RFR test) had complete data about their kidney function before donation (with pre-donation RFR), in the postoperative period (within 7 days after nephrectomy) and 1 year after donation (serum creatinine, eGFR, radioisotope GFR). This subgroup were comparable with all other 34 subjects: in particular, subgroups did not differ for sex (p=0.321), age (p=0.412), BMI (p=0.532), BSA (p=0.785), mGFR (p=0.473), serum creatinine at zenith (p=0.326), serum creatinine 7 days after donation (p=0.422), serum creatinine 1 year after donation (p=0.553), eGFR 1 year after donation (p=0.654), radioisotope mGFR 1 year after donation (p=0.228).

4.4.1 Biomarkers within 7 days after donation

NGAL and [TIMP-2]·[IGFBP7] (NephroCheck®) were negative in all kidney donors.

Considering uEVs, donors showed an increased number of uEVs. Mean MFI of the exosomal markers (CD9/CD63/CD81) was significantly higher in donors 7 days after nephrectomy (p=0.0002) compared to donors before nephrectomy (p=0.03). FIGURE 7

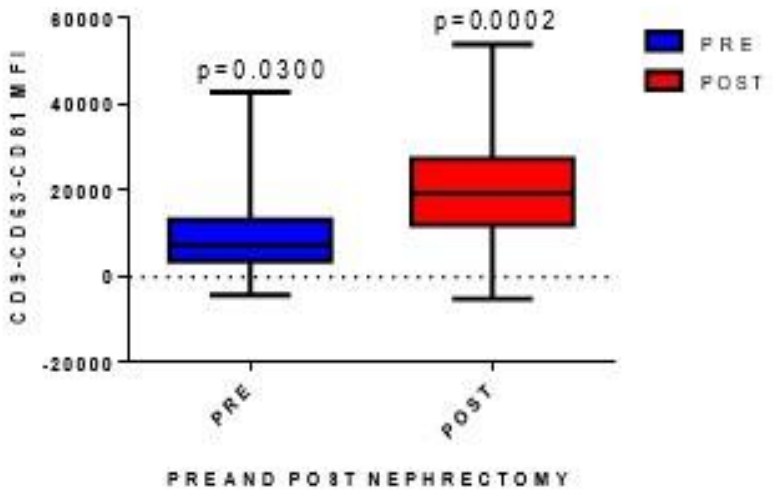


FIGURE 7. Number of uEVs before (blue) and after donation (red).

Characterizing the uEVS, we observed twenty-five common EV markers that were expressed in donors 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 8

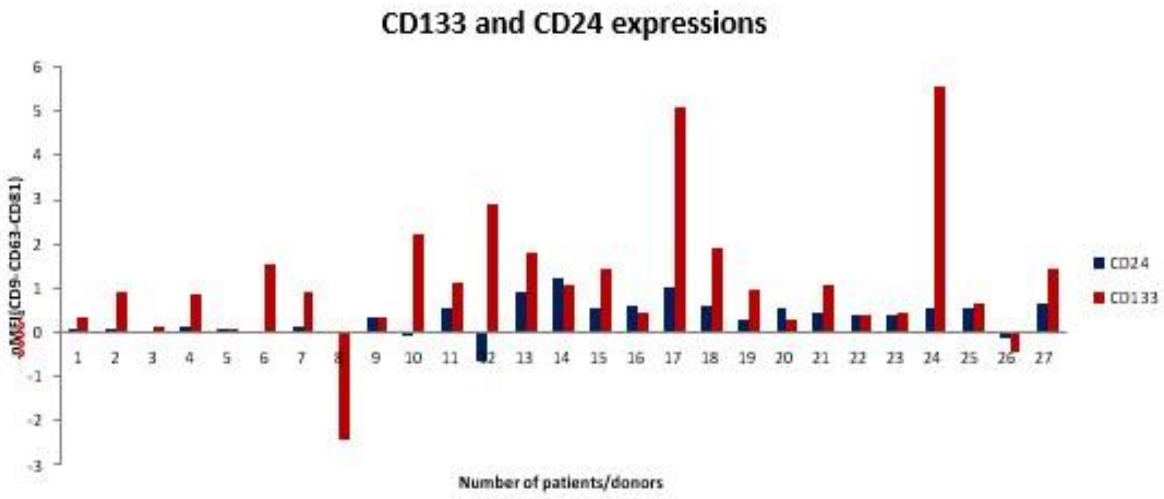


FIGURE 8. CD133+ uEVs showed an increase from 4.5% at baseline up to 24% on the 7th postoperative day.

4.4.2 Biomarkers 1 year after donation

The same donors were tested for NGAL, [TIMP-2]·[IGFBP7] (NephroCheck®) and CD133+ Extracellular Vesicles 1 year after donation.

NGAL and Nephrocheck® remains negative in all patients, while the levels of 133+ uEVs proved to be comparable to those present before the donation, returning to a value of 2,5% (from 24% observed on day 7 after donation). FIGURE 9

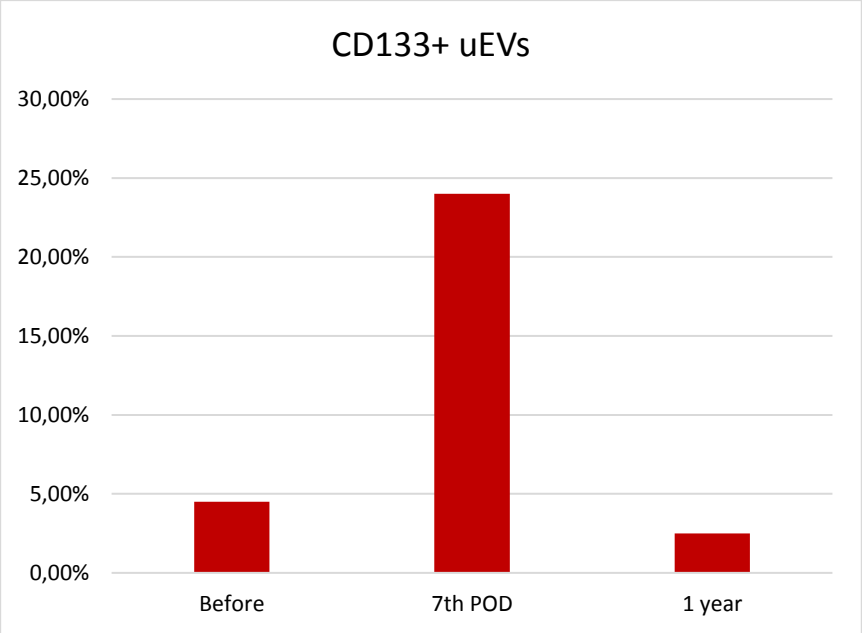


FIGURE 9. CD133+ uEVs before donation, 7 days after and 1 year after donation.

5. Discussion

In the present study, we observed that:

- Radioisotope measurement of GFR is a feasible and precise tool for determination of renal function in kidney donors, in particular the compensatory GFR increase after donation.
- RFR pre donation assessment by using a glomerular stress test with protein load showed a good correlation with the compensatory GFR increase after donation and could become a valid tool for LKD screening, in particular in “medically complex” cases
- Urinary biomarkers of kidney injury are negative in the immediate postoperative period whereas extracellular vesicles showed markers of regeneration (CD133, CD24). One year after donation, restoration of normal levels of CD133 and the persistent negativity of both NGAL and [TIMP-2]·[IGFBP7] suggested a physiological adaptation of the remnant kidney.

Living donor kidney transplantation is considered the best available treatment for end-stage renal disease providing a better patient and allograft’s survival when compared with deceased-donor, reducing mortality and improving the quality of life when compared with dialysis. During the last years, its growing development has led to an extension of donor selection criteria: the minimization of the impact on the donors’ health represents an important clinical need.

According to the recent literature in this field, living kidney donors could have an increased risk of end-stage renal disease, cardiovascular diseases and overall mortality, probably due to the reduction of the nephron mass after nephrectomy, especially if the basal nephron number is reduced, as demonstrated by Schachtner et al. [9, 10, 45].

In the last years, the number of living donor kidney transplant has been significantly increased, the donor selection criteria have been extended and “new” kidney donors, with advanced age and co-morbidities, have been used in the clinical practice. For this reason, the assessment of living donors’ health status and, particularly, kidney function should be as more complete as possible: new dynamic tools and biomarkers discovered by the application of the OMICs technologies are needed to, ideally, predict with reliability the outcomes after donation.

To the best of our knowledge, this is the first study measuring with precision the real compensatory GFR after donation, using 51Cr-EDTA or 99mTc-MAG, which are considered the gold standard tests for GFR measurement, completed with a concomitant sequential functional scintigraphy with 99mTc-MAG to determine split renal function. Indeed, most of the studies reported a value of compensatory GFR calculated based on eGFR or mGFR (e.g. with 125-Iothalamate), assuming that the split renal function is 50%.

As shown in the Results section, none of the donors included in this study displayed a GFR decrease of the remnant kidney after donation, whereas most of the donors increased the mGFR of the single kidney with a clinical significance, with a median value of 18 mL/min, from a pre-donation single kidney median GFR 46.1 mL/min to 63.5 mL/min 1 year after donation (+ 37%, median), independently from sex and co-morbidities (e.g. elevated BMI and hypertension) and with a slight association with age, as expected.

Kidney donors' renal function at 1 year after donation was significantly associated with mGFR of the same kidney before donation and donor age, as expected. Considering a threshold of 65 mL/min as an adequate kidney function (corresponding to the median value of our population and similar to the lower threshold of CKD stage II that is 60 mL/min), we studied the potential predictors of this endpoint with a multivariate logistic regression analysis. We found that age at donation and radioisotope GFR of the single and future remnant kidney were independently associated with mGFR > 65 ml/min 1 year after donation. Each increase in mGFR unit (1 mL/min) determines a 19% rise in the likelihood of an adequate 1 year mGFR. In contrast, every decrease in age unit (1 year) determines a 12% increase in in the likelihood of an adequate 1 year mGFR. All these elements should be considered during the evaluation of a potential kidney donor candidate.

Furthermore, these data showed that if a meticulous donor screening procedure is performed, despite the increasing "medical complexity" of the actual donor cohort, the impact of donation on donor's health could be minimized. The radioisotope GFR assessment is feasible and allows a precise determination of the renal function before as well as after nephrectomy, thus allowing to perform also a careful post-donation follow-up. Based on these findings, we propose to use radioisotope GFR measurement at least once 1 year post donation in order to obtain a renal

function assessment as more precise as possible and not replaceable with eGFR or creatinine clearance.

The Renal Functional Reserve (RFR) is defined as the capacity of the kidney of increasing glomerular filtration rate in response to certain physiological or pathological stimuli including pregnancy, AKI episodes and, obviously, nephrectomy. Since the GFR after donation is 60-75% of its pre-donation value, and not about 50% as expected, the RFR assessment could theoretically be used to predict the renal adaptation of the remnant kidney after donation.

In the literature, there is only one study, published a few months ago by a Dutch Group, investigating the predictive performance of pre-donation RFR, assessed with dopamine infusion, on post-donation GFR measured with 125I-iothalamate. This study showed that pre-donation RFRdopa is a predictor of short term GFR (3 months after donation) but not of long-term kidney function (5 years after), since the compensatory GFR increase in the first period after donation is mostly due to early hemodynamic (comparable to mechanism of action of the dopamine response): the conclusion of the study were that RFR assessment with dopamine is not a useful tool for donor screening [41].

In our Center we usually perform a glomerular stress test with protein load to assess donors' RFR, as described in more details in the Methods section, using creatinine clearance to measure GFR during the test. In this study we tried to find a potential correlation between pre donation RFR and kidney function in the immediate postoperative period (within 7 days) and 1 year after donation.

In our cohort study, we cannot find a correlation between pre-donation RFR and the peak of sCr ("AKI") as well as the partial recovery observed 7th day after nephrectomy, probably because in the immediate postoperative period there are many factors that can interfere with the clinical evidence of RFR (e.g. intravenous infusions, postoperative nausea, mobilization time, etc.).

On this basis, our results are not completely comparable to the Dutch study for the following aspects: donors' pre-donation RFR was assessed with dopamine infusion using GFR measured with 125I-iothalamate, whereas we used a glomerular stress test with a protein load using creatinine

clearance. Furthermore, they analyzed if there was a correlation between pre-donation RFR and mGFR 3 months and 5 years after donation, whereas we used serum creatinine and mGFR with 51Cr-EDTA or 99mTc-MAG to assess renal function in the immediate postoperative period and 1 year after, respectively.

Another interesting aspect of the present study is that the worsening of sCr after nephrectomy could be identified as an AKI episode according to the KDIGO criteria. However, from a physiopathological point of view, the observed increase of sCr seems to be dependent on a physiological adaptation of the remnant kidney and it is surely different from a “real” AKI episode in which an abnormality of kidney structure or function occurs abruptly in pathological situations. Classification of AKI includes pre-renal AKI, acute post-renal obstructive nephropathy and intrinsic acute kidney diseases: kidney donation cannot belong to any of them. Unilateral nephrectomy causes an abrupt reduction of the nephron mass. In the following hours, some different mechanisms of compensatory adaptation occur: functional changes of renal plasma flow, glomerular filtration rate and exertion of electrolytes (e.g. clearances and segmental tubular Na+) occur to restore a sufficient kidney function in a short time period, followed by a compensatory hypertrophy of the remaining kidney. A disproportional increase of functional over structural changes may be responsible of hyperfiltration, a condition that could lead to renal damage and progression toward chronic kidney disease (CKD) [46]. The physiopathology of end stage renal disease (ESRD) in kidney donors do not belong to AKI-to-CKD transition and all starts from demonstrating that the worsening of renal function following nephrectomy is not a real AKI: we tested urine samples collected 7 days after donation for the cell cycle arrest biomarkers [TIMP-2]·[IGFBP7] (NephroCheck) and NGAL to validate the absence of structural tubular injury in the immediate postoperative period.

Interestingly, the amount of urinary extracellular vesicles and the expression of the renal stem cell markers CD133 and CD24 were found to be increased 7 days after nephrectomy in most of the donors. A cell population expressing CD133 and characterized by a progenitor phenotype has been identified in different segments of the human kidney including the proximal tubules, the glomerular Bowman capsule, the inner medullary papillar region (S3 limb segment and Henle’s loop). These progenitor cells express renal embryonic and stem-related transcription factors so they could differentiate into mature renal epithelial cells [47-49]. Previous studies demonstrated

that levels of urinary CD133+ EV are increased in the kidney cortex after AKI episodes and are reduced in patients with progressive end-stage renal disease [50, 51]. However, CD133+ levels were restored after recovery from acute glomerulonephritis or AKI patients, suggesting that the level of CD133+ uEVs might be used as a biomarker of normal renal physiology, providing more information on the regenerative potential of tubular cells [47-52]. After nephrectomy, the increased levels of CD133 and CD24 detected in the majority of the donors may reflect the involvement of progenitor cells in renal homeostasis, providing renal regenerative potential. Furthermore, the restoration of normal levels of CD133, comparable to those present before the donation, and the persistence of negativity of both NGAL and [TIMP-2]·[IGFBP7] (NephroCheck®) suggest a physiological adaptation of the remnant kidney.

Analyzing our data, we found a linear correlation between pre-donation RFR and the compensatory mGFR increase 1 year after donation that can be defined as moderate according to the guide that Evans [53] suggested for the value of r: r=0.58 for absolute mGFR increase (mL/min) r=0.56 for relative mGFR increase (%) vs the pre-donation mGFR of the single kidney.

The use of the glomerular stress test with protein load for the evaluation of RFR in kidney donors could provide more information about the quality of the kidneys (which usually don't undergo biopsy) and consequently about the suitability of living donor kidney transplantation. This is useful both for the donor in which RFR is a good predictor of the development of a compensatory post-donation GFR increase and in the recipient, in which kidney graft function can be affected by different factors (e.g., immunosuppressive drugs, infections, rejection, vascular and urological problems, recurrent disease…). For this reason, pre donation RFR is equally divided between the donor and the recipient [40].

To the best of our knowledge, this is the first study demonstrating the validity and potential utility of a test that provides a dynamic evaluation of pre-donation donors' renal function with prognostic information, also providing a RFR threshold. RFR assessment may represent a useful screening tool for living kidney donors, possibly increasing the number of donors that could be safely accepted for living kidney donation (elderly donors, donors with border-line GFR, medically complex donors).

Analyzing our data, the empirically proposed RFR threshold of 25 ml/min/1.73 m² identified two distinct populations of kidney donors with different degrees of of mGFR increase 1 year after donation: a median mGFR increase of 8.1 (RFR < 25 ml/min) vs 21.9 (RFR > 25 ml/min). Moreover, ROC curve identified a threshold RFR value of 18 ml/min as the best performing predictor of an adequate 1 year mGFR increase of 10 ml/min. Having an intact RFR (>20 ml/min/1.73 m²) is not highly predictive of the precise amount of the mGFR increase of the remnant kidney but if RFR is lower than 20 ml/min, it is highly probable that there will not be a significant compensatory increase. Therefore, we propose the threshold of 20 ml/min for a safer kidney donation. Moreover, this could be a useful tool for a more precise informed consent during living kidney donor evaluation, providing new elements to ensure kidney donors' safety.

After donation, the assessment of RFR with the same protocol could provide further important information during the clinical follow up after donation: it can be compared to the pre-donation RFR and express the susceptibility of the donor (with a remanent kidney) to develop potential renal injuries during his/her remnant life.

It is interesting to notice that 1 year after donation, kidney donors maintained a certain amount of RFR (median 1 year post-donation RFR was 9.7 mL/min/1.73m² vs median pre-donation of the same donors RFR 29 mL/min/1.73m²) but, if we consider that before donation, RFR is shared between two kidneys and assuming that the split function is equal (no evidence if a kidney can have more RFR than the other and no possibility to assess it with the modern technologies) the pre-donation RFR of the single kidney was about 14.5 mL/min/1.73m², the RFR of the single kidney is still maintained. Even more interesting is the observation that the single kidney of the same cohort of patients increased its basal mGFR 21.5 mL/min (median) one year after donation: thus, we should assume that the remnant kidney does not use its whole RFR to increase GFR accordingly. For this reason, we could speculate that other mechanisms of adaptation to maintain RFR occur and that RFR may represent a dynamic value that could even increase in certain conditions. However, further studies are needed to confirm this intriguing hypothesis.

We acknowledge some limitations of the present study: first of all, this is a single center cohort of kidney donors, all caucasians. Secondly, we used creatinine clearance to assess GFR during the stress test instead of inulin (since radioisotope GFR did not show differences between baseline mGFR and mGFR during stress test, as reported in the Results section) because it is the most non-invasive, cheap and repeatable tool. This study was focused on functional evaluation of renal function with radioisotope GFR, no assessment of kidney volume has been performed. We planned to analyze frozen plasma and urine samples collected during donors' kidney stress tests for Cystatin C and beta-trace-protein (BTP) to determine if they could be better biomarkers of glomerular filtration and might have a better correlation with post-donation mGFR and its compensatory increase. Novel techniques for GFR measurement such as use of visible fluorescent tracers such as rhodamine derivative and fluorescein carboxymethylated dextran could make RFR testing easier and more accessible to most kidney transplant centers.

The strengths of our study are the use of mGFR with 51Cr-EDTA or 99mTc-MAG before and one year after donation to assess the donors' kidney function, with the possibility to calculate the single kidney mGFR, using a concomitant sequential scintigraphy during pre-donation, and to correlate the RFR measurements during stress test with its compensatory increase. Moreover, to the best of our knowledge, this is the first study proposing a RFR threshold below which donation may not be safe, considering renal function outcomes of the remnant kidney. Finally, we characterized for the first time uEVs before and after donation and demonstrated that they increased in number and in expression of CD133, reflecting a possible renal regenerative potential.

Future perspectives include the search for new functional tests and biomarkers, which can better stratify "medically complex" donors in which clinical acceptance can be debated, with the final purpose to increase donor safety.

In conclusion, radioisotope measurement of GFR is feasible and allows a precise determination of renal function at different time points after nephrectomy in KD, in particular compensatory GFR increase after donation. RFR assessment with a kidney glomerular stress test with protein load has a good correlation with the compensatory GFR increase after donation and could become a valid

tool for living donor screening, in particular in “medically complex” cases due to the presence of multiple comorbidities. Analyzing our data, we established a RFR threshold of 20 ml/min as a reasonable guide to ensure an adequate compensatory mGFR increase (10 ml/min) 1 year after donation. Integration of this dynamic tool (RFR) with the baseline static radioisotope GFR could provide a more complete evaluation of the potential kidney donor. Moreover, urinary biomarkers of kidney injury are negative in the immediate postoperative period, whereas extracellular vesicles showed markers of regeneration (CD133, CD24). In addition, one year after donation, restoration of normal levels of CD133 and the persistent negativity of both NGAL and [TIMP-2]·[IGFBP7] suggested a physiological adaptation of the remnant kidney.

6. Disclosures

No conflicts of interest to declare.

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