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Ciclo XXXI

Post-kidney transplant malignancies affect graft survival: results from a time-dependent analysis

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Coordinatrice

Prof.ssa. Marisa Gariglio

Tutor

Prof. Corrado Magnani

Dottoranda

Dr.ssa Tiziana Cena

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Summary

Introduction and Aims.

Malignancies are a well-known complication following kidney transplantation (KTx): non-melanoma skin cancers (NMSC) are the most common, but the highest mortality is related to non-cutaneous malignancies (NCM), including solid and hematologic tumors. Indeed, a chronic use of immunosuppressive (IS) drugs -which are needed to prevent graft rejection- is associated with an increased risk of cancers, up to 20% at ten years after KTx.

However, oncologic active surveillance programmesin KTx recipients (KTR) resulted in an improved post-malignancy survival, which is as high as 71.3% at 10 years after NCM. Consequently, novel questions arise about the long-term outcomes of KTR with a post-transplant malignancy, such as the risk of graft failure in patients who survived a NCM. As malignancies develop in “overimmunosuppressed” patients they may be at a lower risk of graft failure, on the other side after an NCM, IS therapy is often reduced with therefore a higher risk of chronic rejection and graft failure.

Aim of this cohort study was to evaluate the impact of NMSCs and NCMs on death-censored graft survival in a cohort of KTRs from deceased donors. The association between malignancies and chronic rejection or other causes of graft failure was investigated. As NCM were associated with graft failure, it was checked how the reduction of IS therapy interacted with malignancies and graft failure. Lastly, thespecific post-malignancy risk factors for graft failure were looked for.

Patients and Methods

The study cohort includes 672 patients who have been transplanted in a single transplant center (Novara) from 1998 to 2013. Adult patients receiving their first

kidney transplant from a deceased donor have been included if they had a minimum follow-up of 6 months after KTx without any malignancy or graft failure.

The design was a cohort study. Outcome wasgraft failure for any cause, with primary endpoint defined as the need of chronic dialysis at any time after study entry. Graft failures were divided in chronic rejection (diagnosed with renal biopsy or clinically after excluding other plausible causes of renal damage) and "graft failure due to other causes", which was usually diagnosed by renal biopsy.

A modified Kaplan–Meier method was used to estimated cumulative hazard rates of graft failure according to the presence or absence of tumor(NMSC or NCM) diagnosed during patient follow up. To quantify the tumor effect in terms of hazard ratio, both univariable and multivariable- Cox models were fitted, adjustedby known risk factors for graft failure. The "final" model was validated by an internal Leave-One-Out Cross validation and by performance measures, such as C-statistic, time-dependent ROC curves and AUC function.

The heterogeneity of the effect of tumor occurrence on the cause-specific graft failure ("chronic rejection" versus "other causes") was assessed comparing the hazard ratios estimated from two time-dependent multivariable Cox models.To evaluate the joint effect of the reduction of the IS therapy and the occurrence of NCM on graft failure, the graft failure rate was analyzed dividing the cohort based on IS levels and NCM diagnosis.

Finally, to investigate which oncologic treatment or characteristic was associated with a worse graft prognosis among patients with a NCM, univariate survival analysis was performed for different post-malignancy variables and graft failure rates were estimated among different subgroups.

Results

A total of 59 graft failures were observed (39 due to chronic rejection and 20 for other causes) with a 5-year cumulative incidence of 7.5% (95%CI: 5.3–10.0). Among the 40 observed NCMs(5-yrs cumulative incidence of 5.6%), 29 were solid tumors and 11 were hematologic tumors, while 47 NMSC were observed (5-yrs cumulative incidence of 6.5%).

From the multivariable Cox model, the adjusted hazard ratio of graft failure associated with a NCM diagnosis was 3.27 (95%CI=1.44-7.44, p=0.005). The occurrence of a NMSC was, on the contrary, not associated with the graft failure risk(HR = 0.80; 95% CI = 0.30–2.14, p = 0.66).The model validation procedure showed a C-statistics value of 0.80 (95%CI: 0.72 - 0.88) for the cross-validated cohort, ruling out a possible model overfitting and validating the predictive ability of the estimated model.

Investigating the effects of NCM on cause-specific graft failure, a NCM diagnosis seemed to have a different association ($P = 0.002$) when considering graft failed due to chronic rejection (HR 0.55, 95% CI: 0.07–4.08) or for other causes (HR 15.59, 95% CI 5.43–44.76). Moreover, the yearly incidence rate of graft failure after NCM was not affected by a reduced IS, being 5.3% (95% CI 1.2–22.9) in NCM patients with a reduced IS and 6.8% (95% CI 1.8–25.3; ratio = 0.78) in NCM patients maintained on standard IS.

We were not able to identify any significant association between post-NCM variables and graft failure risk among patients with a NCM; nevertheless, the causes of graft failure in patients with an NCM included three “malignancy-related nephropathies”and two chronic pyelonephritides.

Conclusions

This study shows that in our cohort NCM are associated with a higher graft failure risk and might suggest that early after a NCM diagnosis the causes of graft failure may include paraneoplastic nephropathies and other otherwise “uncommon” nephropathies (ie: chronic pyelonephritides). Therefore, transplant physicians should be aware of these associations and should be careful in kidney function monitoring of KTRs with a NCM, which should include specific evaluations depending on the malignancy itself.

Even if this study has novel methodological approaches(time dependent survival analysis and individual survival risk estimates) and shows interesting results, we were limited by three main factors: cohort size, which is too small to perform further

analyses;a relatively low event rate in some tumor types;arelatively short follow-up time, particularly after malignancies.

Moreover, the findings from this cohort are consistent with the hypothesis by which some post-transplant malignancies are preventable and may be linked to an over-immunosuppression, even if drug levels are “on target”. Given that the best therapy for post-transplant malignancies is prevention, more efforts should be made to develop more reliable biomarkers of the overall IS burden of transplant recipients.

Riassunto

Introduzione e Obiettivi.

I tumori sono complicanze ben note del trapianto di rene (KTx): i tumori cutanei non melanomatosi (NMSC) sono i più comuni, mentre la maggior mortalità è dovuta ai tumori non cutanei (NCM), inclusi sia i tumori solidi che ematologici. Infatti, la terapia immunosoppressiva (IS) cronica, necessaria per prevenire il rigetto, è associata ad un aumentato rischio di tumore, che raggiunge il 20% a 10 anni dopo il trapianto.

Tuttavia, programmi di sorveglianza attiva oncologica nei pazienti trapiantati di rene (KTR) hanno portato ad un miglioramento della sopravvivenza dopo un tumore, che è pari al 71.3% a 10 anni. Di conseguenza, nuove domande sorgono riguardo gli outcomes a lungo-termine dei pazienti trapiantati di rene, come per esempio il rischio di fallimento del trapianto nei pazienti che sopravvivono ad un tumore non cutaneo. Poiché i tumori si sviluppano frequentemente in pazienti "troppo immunosoppressi", questi stessi potrebbero avere un rischio più basso di fallimentodel trapianto, mentre, d'altro canto, dopo un tumore la terapia IS è spesso ridotta con un conseguente maggior rischio di rigetto cronico e fallimento.

Lo scopo di questo studio di coorte è stato di valutare l'impatto di NMSC e NCM sulla sopravvivenza del rene in una coorte di pazienti trapiantati di rene da donatore deceduto. E' stata valutata l'associazione tra tumori e rigetto cronico e altre cause di perdita del rene. Poiché i tumori non cutanei erano associati con la perdita del rene, è stato valutato come la riduzione della terapia immunosoppressiva interagisse con tali tumori e con il fallimento del trapianto. Infine sono stati cercati fattori di rischio per fallimento del trapianto specificatamente dopo un tumore.

Pazienti e metodi

Lo studio di coorte include 672 pazienti che sono stati trapiantati in un singolo centro (Novara) dal 1998 al 2013. Pazienti adulti che hanno ricevuto il loro primo trapianto

da donatore deceduto sono stati inclusi se avevano un follow-up minimo di 6 mesi dopo il trapianto senza diagnosi di tumore o perdita del rene.

Il disegno dello studio è uno studio di coorte. L'outcome principale era la perdita del rene per qualsiasi causa, con l'endpoint primario definito come la necessità di dialisi a qualsiasi tempo dall'ingresso dello studio. Il fallimento del trapianto è stato riclassificato come rigetto cronico (diagnosticato con biopsia renale o clinicamente dopo aver escluso altre possibili cause di danno renale) o come "perdita di rene dovuta ad altre cause", le quali sono di solito diagnosticate con la biopsia renale.

Il metodo di Kaplan–Meier modificato è stato utilizzato per stimare il rischio cumulativo di perdita del rene in base alla presenza e assenza di tumori diagnosticati durante l'osservazione dei pazienti.

Per quantificare l'effetto del tumore in termini di hazard ratio, sono stati utilizzati modelli di regressione univariati e multivariati di Cox, aggiustando per i fattori di rischio noti per il fallimento del trapianto.Il modello "finale" è stato poi validato con una "Leave-One-Out Cross Validation" e con misure di performance, quali C-statistic, curve ROC tempo-dipendenti e AUC.

L'eterogenità dell'effetto di tumore sul fallimento del trapianto causa-specifico ("rigetto cronico" versus "altre cause") è stata valutata confrontando gli hazard ratio stimati dai due modelli multivariati tempo-dipendenti di Cox. Per valutare l'effetto congiunto della riduzione della terapia IS e della comparsa di tumore sulla perdita del rene, si è analizzato il tasso di fallimento del trapianto stratificando la coorte in base ai livelli di terapia IS e alla presenza di tumore.

Infine, per valutare quale caratteristica o trattamento del tumore fosse associato con un peggioramento della prognosi renale tra i pazienti con NCM, è stata eseguita una analisi di sopravvivenza univariata per differenti variabili post-tumore e si è calcolato il tasso di fallimento del trapianto tra differenti sottogruppi.

Risultati

Si sono osservati 59 fallimenti di trapianto (39 per rigetto cronico e 20 per altre cause) con un'incidenza cumulativa a 5 anni del 7.5% (IC95%: 5.3–10.0). Tra i 40 pazienti con NCM (incidenza cumulativa a 5 anni del 5.6%), 29 pazienti avevano un

tumore solido e 11 un tumore ematologico, mentre sono stati osservati 47 NMSC (incidenza cumulativa a 5 anni del 6.5%).

Dal modello di regressione multivariato di Cox, l'hazard ratio per il fallimento del trapianto associato alla diagnosi di tumore è stato di 3.27 (95%IC=1.44-7.44, p=0.005). Diversamente, la comparsa di NMSC non è stata associata con un rischio di perdita del rene (HR = 0.80; 95% IC = 0.30–2.14, p = 0.66).La procedura di validazione del modello ha mostrato una C-statistics di 0.80 (95%IC: 0.72 - 0.88) nella coorte di validazione, escludendo un possibile effetto di overfitting e validando la capacità predittiva del modello stimato.

Indagando l'effetto dei NCM sul fallimento del trapianto causa-specifico, una diagnosi di NCM sembra avere una differente associazione (P = 0.002) quando si considera la perdita del rene dovuta a rigetto cronico (HR 0.55, 95% IC: 0.07–4.08) rispetto a quando si considerano altre cause di perdita del rene (HR 15.59, 95% CI 5.43–44.76). Inoltre, il tasso annuo di incidenza di fallimento del trapianto dopo NCM non è stato influenzato dalla riduzione della terapia IS, essendo del 5.3% (95% IC 1.2–22.9) nei pazienti con NCM e una ridotta IS e del 6.8% (95% IC 1.8–25.3; ratio = 0.78) nei pazienti con NCM mantenuti con una IS standard.

Non è stato possibile identificare alcuna associazione significativa tra le variabili post-NCM e il fallimento del trapianto tra i pazienti con NCM; tuttavia, le cause di fallimento del trapianto nei pazienti con NCM includevano tre "nefropatie correlabili al tumore" e due pielonefriti croniche.

Conclusioni

Questo studio mostra come nella nostra coorte le neoplasie non cutanee siano associate ad un maggior rischio di fallimento del trapianto e che precocemente dopo un NCM le cause di perdita del trapianto possano includere alcune nefropatie paraneoplastiche e altre nefropatie altrimenti "poco comuni" (ad esempio le pielonefriti croniche). Pertanto, i medici del trapianto dovrebbero essere consapevoli di queste associazioni e dovrebbero prestare attenzione al monitoraggio della funzione renale nei pazienti trapiantati di rene a cui è stato diagnosticato un tumore, poiché potrebbero includere specifiche valutazioni in base al tipo di tumore.

Anche se questo studio presenta in parte aspetti metodologici nuovi (analisi della sopravvivenza tempo-dipendente e stima del rischio individuale) e risultati interessanti, tre sono stati i principali fattori limitanti: la dimensione della coorte, che è troppo piccola per eseguire ulteriori analisi; i tassi di evento relativamente bassi in alcuni tipi di tumore; il tempo di follow-up relativamente corto, in particolare dopo un tumore.

Inoltre, i risultati ottenuti da questa coorte sono coerenti con l'ipotesi per cui alcuni tumori post-trapianto potrebbero essere prevenuti poiché potrebbe essere associati ad una eccessiva IS, nonostante normali livelli circolanti dei farmaci IS. Dato che la miglior terapia per i tumori post-trapianto è la prevenzione, maggiori sforzi potrebbero essere impiegati al fine di sviluppare biomarcatori più affidabili sul carico complessivo di IS nei pazienti trapiantati.

1. Introduction

1.1 Chronic kidney diseaseand renal replacement therapies

Chronic Kidney Disease (CKD) is defined as a Glomerular Filtration Rate (GFR) lower than 90 ml/min/1.73m^2 lasting for longer than three monthsor the presence of a kidney damage, defined as urinary tract abnormalities (microhematuria and/or proteinuria), or macroscopic or microscopic morphologic abnormalities. Chronic kidney disease can be classified into five categories, according to renal function, as shown in Table 1.1.

Table 1.1 - Chronic Kidney Disease (CKD) Stages. GFR: glomerular filtration rate

CKD Stages	Definition
1	GFR ≥ 90 ml/min; urinary abnormalities or ultrasound-determined or histologically observed morphological alterations. for at least three months
2	GFR 60 – 89 ml/min
3	GFR 30 – 59 ml/min
4	GFR 15 – 29 ml/min
5	GFR < 15 ml/min

Chronic kidney disease represents a serious problem in both industrialized and developing countries, affecting millions of people in the world. According to the preliminary data of the Ca.R.H.E.S. study(Cardiovascular Risk in the Health Examination Survey), it is estimated that about 8% of the Italian population suffers from CKD and that 37% of them are in a CKD stage 3-5 (De Nicola et al., 2011).

When the GFR rate is lower than 15 ml/min/1.73m^2, renal function is extremely reduced and this stage is commonly called End Stage Renal Disease (ESRD). In 2015, in European Countries (Report of ERA-EDTA), ESRD had a prevalence of 801 patients per million people.

ESRD patients are likely to receive a replacement of their renal function (Renal Replacement Therapy – RRT). Renal function of a patient with ESRD can be

artificially substituted by dialysis (64% of prevalent RRT patients) or by a kidney transplant(36% of prevalent RRT patients).

According to the Italian Register of Dialysis and Transplant (RIDT) managed by the Italian Society of Nephrology (SIN), in 2015 (latest available data), the prevalence in Italy of patients undergoing dialysis was 770 per million people and the dialysis incidence rate was of 154 per million people (Report SIN-RIDT 2015), http://ridt.sinitaly.org/2017/10/09/report-2015/, lastly accessed in January 2017).

Every RRT has its pros and cons, so in the choice of the best RRT the patient's clinical conditions "in toto" should be considered, and not only his/her renal function.

There can be a change from a RRT to another one when the patients' clinical conditions have changed or when the patient develops unsolvable complications. Kidney dialysis techniques, haemodialysis or peritoneal dialysis, have the same depurative efficiency (about the 10% of the normal renal function of a healthy person) and can grant the same survival of the patient (Wolfe et al.,1999). For eligible candidates, kidney transplant (KTx) grants a better survival and an overall better quality of life.

1.2 Dialysis techniques

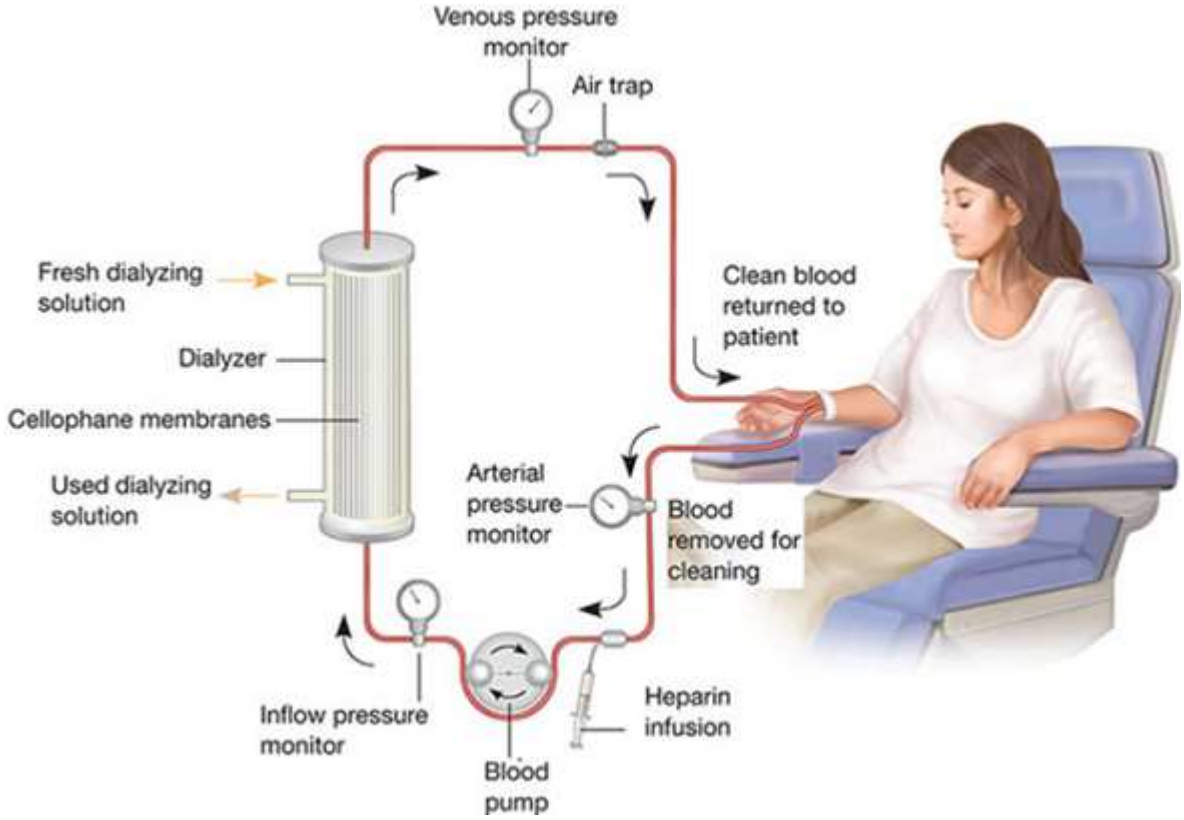
There are two main types of dialysis, haemodialysis and peritoneal dialysis.

Haemodialysis is the most frequently used technique for the treatment of ESRD: the patient undergoes three weekly sessions each lasting four hours, where is blood is circulated in an extra-corporeal circuit and uremic toxins are removed (Figure 1.1). The treatment is completely handled by highly qualified health staff and the patient is free when he/she is not in hospital for his/her session.

Poor blood pressure control and dialysis-related symptoms (hypotensive episodes, cramps, arrhythmias, extreme asthenia and headache), are the main disadvantages of haemodialysis. Besides, due to the alternation of sessions, patients must strictly follow prescribed diets to avoid potassium, phosphorus and sodium build-up between sessions. The patient must also firmly stick to the dialysis program, planning his/her

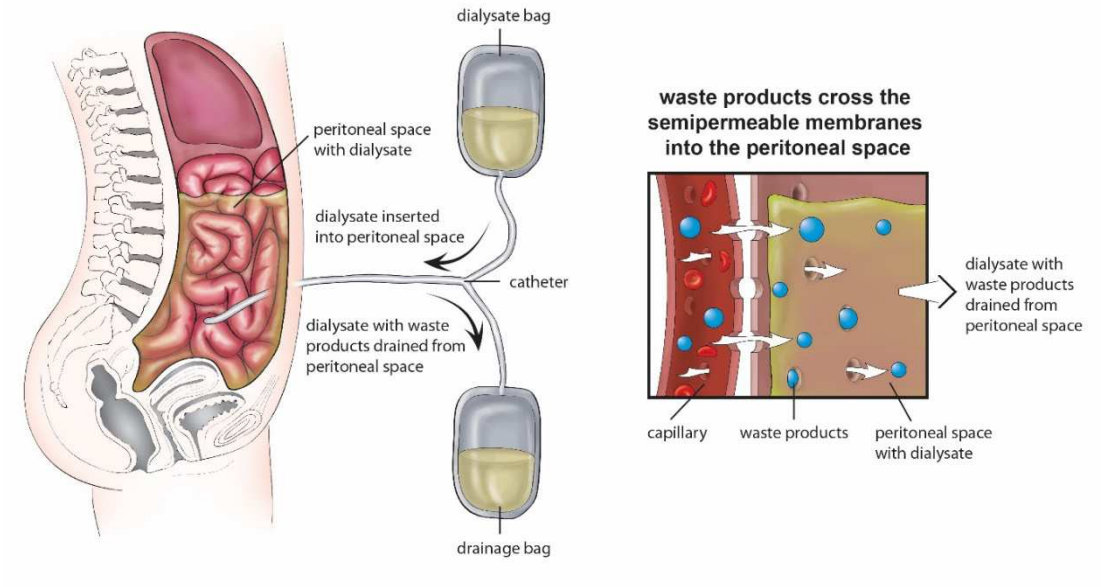
life according to the session schedule (Jablonski &Chonchol, 2014; Enam et al., 2014).

Figure 1.1 - The haemodialysis blood circuit. A dialysis machine pumps blood from the patients, through disposable tubing, through a dialyser, or artificial kidney, and back into the patient. Waste solute, salt and excess fluid is removed from the blood as it passes through the dialyzer.



The peritoneal dialysis is a continuous kind of dialysis and is performed at home, allowing for more comfortable transfers (for pleasure or for job) without the need to reach a Dialysis Centre (Figure 1.2). Unfortunately, not all patients are good candidates for peritoneal dialysis because it has to be performed at home: consequently, patients with cognitive and visual impairment or patients with Parkinson's disease or other severe neurological syndromes, cannot follow this procedure unless properly house assisted (Krediet, 2005).

Figure 1.2 - The peritoneal dialysis. A catheter is surgically placed in the abdomen and in used to fill the abdomen with dialysate. The dialysate usually stays in the abdomen for a few hours (dwell time) and during this dwell time wastes and extra fluid pass through the peritoneal membrane into the dialysate (right panel). After the dwell, the dialysate is removed into an empty bag and discarded.



1.3 Kidney Transplant

Kidney transplant is a surgical procedure that places a functioning kidney from a donor (either living or deceased) into a recipient with ESRD.

The nephrectomy of native kidneys is not usually performed, while the transplanted kidney is placed into the anterior part of the lower abdomen.

Kidney Transplant represents the treatment of choice for patients affected by a renal disease in terminal phase. It can offer the kidney transplant recipient a good psycho-physical recovery, a better quality of life and a longer life expectancy as compared to patients that receive a dialysis treatment. (Danovitch, 2005).

However, in order to adequately select patient that can benefit from transplantation it is essential to consider in detail his/her pathologies and clinical picture as a whole, including vascular, infective and oncological diseases, which can rapidly deteriorate during anti-rejection treatments.

The graft may come from a deceased or living donor. Usually, the pair of kidneys given by a deceased donor will be transplanted into two different recipients. However, it is possible to transplant both kidneys from a single donor to a single

recipient (dual or double transplantation) when these kidneys are both irreparably damaged and the supposed renal function of each single one might not be enough for a long-lasting transplantation (Davison et al., 2000).

Prior to organ retrieval from a deceased donor, it is necessary to evaluate the potential donor in order to verify his/her condition of brain death according to the Law's dictation, the immunological eligibility of the organs and the absence of pathological conditions which could involve a risk for the recipient, particularly for transmittable diseases (D.L. n. 91/1999).

As far as the donation from a living donor is concerned, once identified and evaluated both the donor and the recipient, surgery can be arranged.In Italy, it will be planned upon the evaluation and approval of the Judge of the place where the donor lives where the transplant center is located.

In order to increase the number of patients benefiting from KTx, in the past decades the selection criteria for donor selection have been expanded: organs from donors with an impaired -but still acceptable- renal function (ie: CKD stage 1 or 2 or older donors) are called as “from Expanded Criteria Donor” (ECD).

Expanded-criteria donors (ECDs) are defined as kidney donors older than 60 years or donors aged from 50 to 59 years and who have two of the following risk factors: hypertension, serum creatinine >1.5 mg/dl (acute rise), or death from cerebrovascular accident. Kidneys from diabetic donors or those with a reduced eGFR (ie< 60 ml/min) are usually not used for transplantation, unless they have a normal or semi-normal histology.

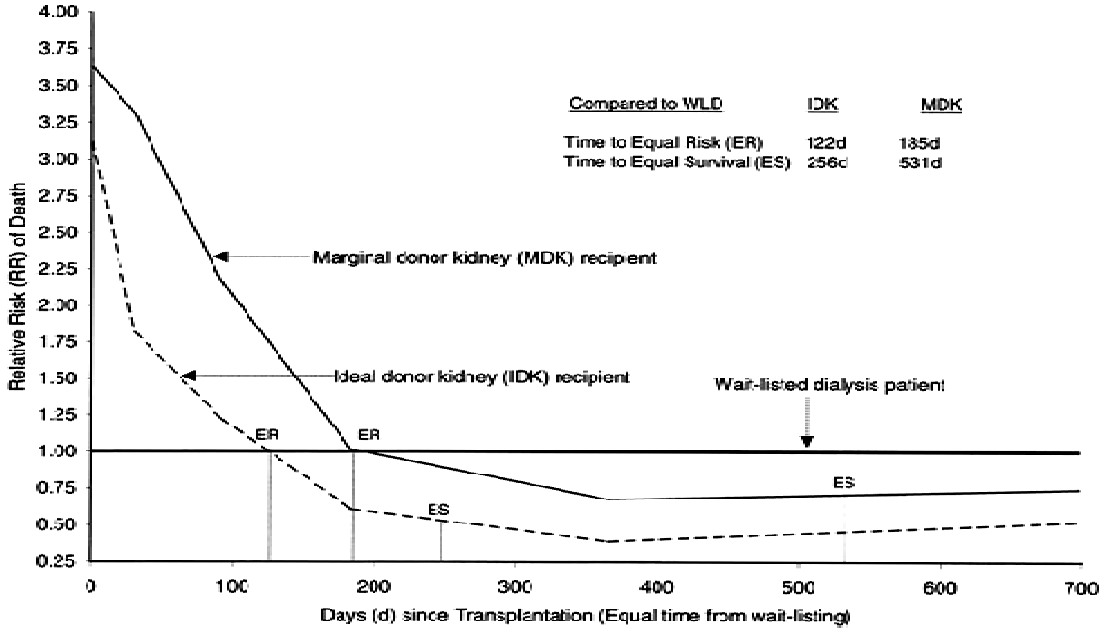
As it is shown Table 1.2, the annual mortality rate is lower (and life expectancy consistently higher) for Kidney Transplant Recipients (KTR) from both Ideal Donor Kidney (IDK) and Expanded-Criteria Donors (ECDs), as compared to Wait-listed Transplant Candidates, (WTCs) (Ojo et al., 2001). However, in the first post-surgery days and weeks the HR for death is higher in Ktx recipients (about 3) as compared to wait listed patients, while it decreases in the following months (Wolfe et al., 1999).

Table 1.2 - Annual mortality rate, average life expectancy and relative risk of death among patients on wait-listed on dialysis treatment with no transplant (WLCs), recipients of a marginal donor kidney transplant (MDK - KTRs) and from"ideal" or optimal donor Kidney transplantation (IDK - KTRs), Ojoet al., 2001.

	Wait-listed Transplant Candidates	**Extended Criteria Donor Kidney KTRs**	**Ideal Donor Kidney KTRs**
Annual mortality rate	6,30%	4,70%	3,30%
Average life expectancy in years	15,3	20,4	28,7
Relative Risk of death	reference	0.75	0.52

The best survival benefit is observed for patients receiving a transplant from a IDK (HR < 1 from the 122 post-operative day), while it is less in recipients from ECD (HR < 1 from the 185 post-operative day) (Figure 1.3) (Ojo et al., 2001).

Figure 1.3 - Mortality risks in two groups of cadaveric renal transplant recipients relative to wait-listed dialysis patients(Ojo et al., 2001)



1.4 Post-transplant anti-rejection therapy

Anti-rejection drugs will be given for the whole duration of the transplant to prevent acute rejection and try to preserve graft function (Figure 1.4). Therefore, these drugs must have a good oralbioavailability and few collateral effects (Danovitch, 2005; Danovitch, 2001).

Figure 1.4 - "Pill burden". On average, for the first month of transplantation, patients need 25 tablets a day: 6-12 are immunodepressive drugs, 3-5 for prophylaxis of infections and gastric ulcer, in addition to hypotensive drugs, diuretics, vitamin D, calcium and other "cardiovascular" drugs already present in therapy before transplantation (antiplatelet, anticoagulants, statins, insulin etc.)

Calcineurin inhibitors (CNIs), including cyclosporine (CSA) (Neoral, Gengraf, or the earliest form, Sandimmune) and tacrolimus (TAC; Prograf) have been the cornerstones of an immunosuppressive regimen, which usually includes two or more additional agents, such as glucocorticoids, a purine antagonist (mycophenolic acid [CellCept] or azathioprine [Imuran]) (Table 1.3). Sirolimus (SRL; Rapamune) has been used as a substitute for CNIs. The choice of agents is often protocol driven but is usually adapted to each recipient's risk profile. High-risk recipients treated with more intensive immunosuppression include those with increased levels of preformed antibody (panel-reactive antibody [PRA] >20%-50%), repeat transplantation after early immunologic loss of a previous graft, and African Americans. High-risk recipients typically receive induction therapy consisting of monoclonal or polyclonal antibodies administered intravenously beginning inthe perioperative period.

Figure 1.5– Immunosuppression use in adult kidney transplant recipients. One year post-transplant data limited to patients alive with graft function one year post-transplant. Mycophenolate group includes mycophenolate mofetil and mycophenolate sodium

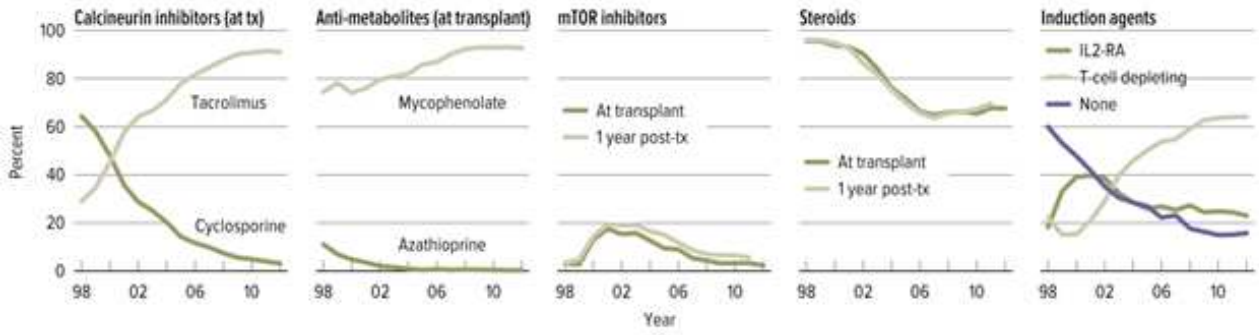


Table 1.3 -Common IS drugs and their associations

Main drug	Common combinations	Abbreviation
Calcineurin inhibitor *Cyclosporine* *Tacrolimus (FK506)*	**Purine metabolism antagonist** *Azathioprine* *Mycophenolate*	Cya-Aza FK-MMF
	mTOR-inhibitor *Sirolimus* *Everolimus*	Cya-Ever FK-Rapa
mTOR-inhibitor *Sirolimus* *Everolimus*	**Purine metabolism antagonist** *Azathioprine* *Mycophenolate*	Rapa-MMF

CalciNeurin Inhibitors (CNI), cyclosporine and tacrolimus, are the most commonly used agents in modern IS schemes. However, they have a reduced therapeutic range and they need a drug monitoring with blood through levels. Tacrolimus is the most commonly used IS drug in KTx for the prevention of allograft rejection (Figure 1.5). It is a lipophilic drug with high metabolic clearance and is almost completely metabolized in the liver and, to a lesser extent, in intestinal mucosa, via cytochrome P4503A (CYP3A) isoenzymes CYP3A4 and CYP3A5. Tacrolimus is also a substrate for P-glycoprotein (P-gp), a transmembrane efflux pump expressed in intestinal epithelial cells and biliary canalicular cells which affects drug absorption and excretion.

Steroids have been the cornerstone of antirejection therapies and are the mainstay particularly for acute phases, like at the time of organ transplantation or acute

rejection(Figure 1.5). However, their prolonged use at high doses has been recognized to lead to several chronic side effects (hypertension, diabetes, infections, osteopenia, skin and vascular frailty, psychosis, cardiovascular events), so in the past decades their use has been greatly reduced in chronic (maintenance) IS regimens (Augustine &Hricik, 2007).

The other "historical" class of IS are purine antagonists, which are also called "anti-metabolites" (azathioprine and mycophenolate). They have mainly a gastrointestinal and hematopoietic toxicity (Figure 1.5).

The last discovered class of IS is the inhibitors of the mammalian Target Of Rapamycin (mTOR-i), Sirolimus and Everolimus (Figure 1.5). They have an anti-prolipherative effect on most replicating cells and have shown good results also in the medical treatment of kaposi sarcoma and renal cell carcinoma.

Induction therapies include sera with specific lymphocyte-toxic effects, either on all CD3+ cells or on specific subpopulations. They are given in the first post-transplant days and are used to induce tolerance after the first contact with the graft.

1.5 Open problems in kidney transplant

The natural history of kidney transplant ends with either patient death (with a functioning graft) or graft failure. The most common cause of graft failure is chronic rejection, while mortality is attributed to cardiovascular events and malignancies. Indeed, malignancies might be related to the IS therapy, and so they are becoming a relevant issue in long term management of kidney transplant recipients.

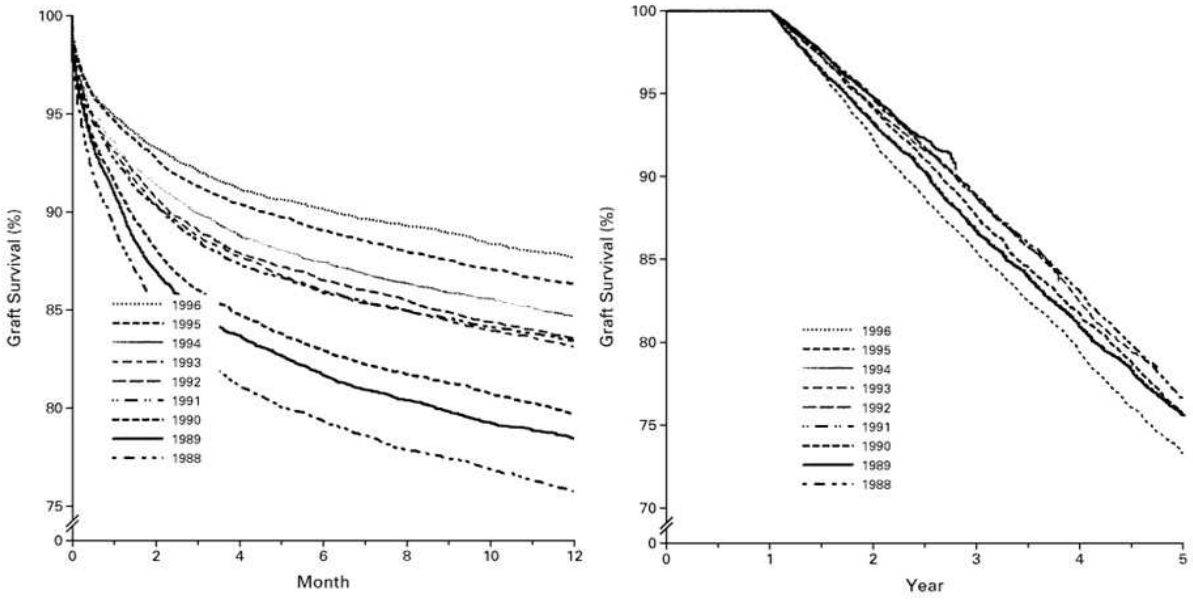
1.5.1 Graft failure

As compared to the '70s and '80, graft survival has dramatically improved: the median half-life was 7.9 years in that era, while it is as high as 13.8 years nowadays. However this improvement is mainly due to a better 1-year survival and to a lower incidence of early T-cell mediate acute rejection after the introduction of

CNIs (first cyclosporine use in 1978) (Britton & Palacios, 1982). Acute rejection rates dropped from about 50% at the time of azathioprine-steroid based-IS to less than 10% with the use of induction sera and FK-mycophenolate-basedIS. (Danovitch, 2005).

However, despite considerable progress, long-term graft loss in renal transplant recipients remains substantial, with resulting high morbidity, mortality and costs(Hariharan et al., 2000) (Figure 1.6). Indeed, in most centers the 1-year graft survival is as high as 95%, while the 10-years graft survival is between 50% and 65%(Matas et al., 2014; Report of CTS 2017). In addition, patients who require re-transplantation are likely to be sensitized to HLA antigens, which significantly hinders their chances for subsequent transplantation unless they are desensitized. Currently, more than 5,000 kidney transplants fail each year in the US. There are no epidemiological data on the early graft failure rate in Italy; however, given an estimated prevalence of 23467 KTR in Italy in 2015 (Report SIN-RIDT 2015) we can estimate that about 600 grafts fail each year (Report CNT 2014).
The costs associated with failed transplants with return to dialysis represents a considerable financial burden for health care systems (MoH) while decreasing the quality and length of life for affected patients.

Figure 1.6–Historical report showing graft survival between the '80s and the '90s: on the left 1-year graft survival between 1988 and 1996, which showed a dramatic improvement (from 76% to 88%); on the right, survival function after the first year: no significant difference can be noted (Hariharan, 2000).



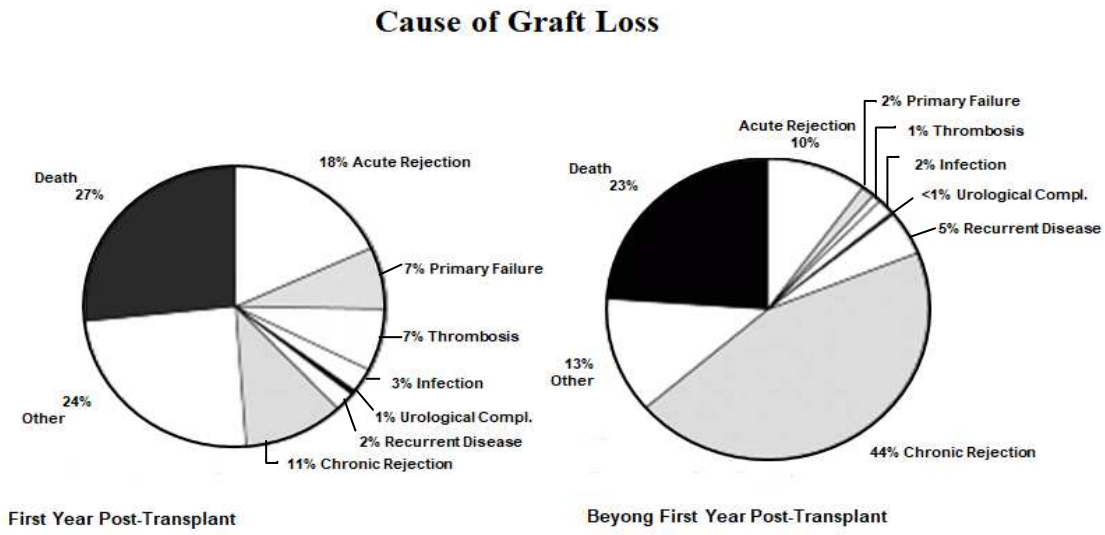
Clearly, the identification of critical pathologic pathways responsible for allograft loss, with the attendant development of therapeutic interventions to improve the duration and quality of allograft function, is one of the most important objectives of transplant medicine.

Over the past two decades, our thinking has changed from considering rejection as a primarily T-cell-mediated process (that is now increasingly better managed), to the realization that an insufficient control of the humoral arm of a recipient's immune system by current IS regimens may be the main pathogenic factor responsible for long term allograft dysfunction and failure. This notion is now progressively superseding the historical dogma that such allograft losses were caused by calcineurin inhibitor (CNI) toxicity and chronic allograft nephropathy (CAN).

The most important advances in the past decade have been the implementation of sensitive assays for the identification of anti-HLA antibodies, improved comprehension of the pathology of antibody mediated rejection (AbMR) and the growing implementation of molecular approaches. Together, these advances have increased our understanding of antibody-mediated graft deterioration. Although no relevant animal model for ABMR is available, assessment of ABMR in humans has made major contributions to our understanding of this entity.

As shown in Figure 1.7, the most common cause of long-term graft failure is chronic rejection/chronic transplant glomerulopathy. However, relapsing nephropathies and de novo nephritides are relatively common (Danovitch, 2005; Colvin, 2003, Weir et al., 2005). As shown in Figure, during the first year, surgical complications and primary non-function account for about 15% of graft losses, as they are usually a very early (ie: few days or weeks) complication of KTx. On the other side chronic/subacute rejection is uncommon in the first year (11%), while it is the leading cause beyond the first year (44%). Indeed, acute rejection might be a cause of graft failure in the first year, but it is different from chronic rejection. However, an acute rejection is a major risk factor for developing later a chronic rejection.

Figure 1.7 - Causes of graft loss in the first year (n = 10,464) and after the first year (n = 12,805) for adult primary cadaveric kidney transplants since 1995, as reported by the UNOS Registry (Medical Management of KidneyTransplantation, Weir, 2005)



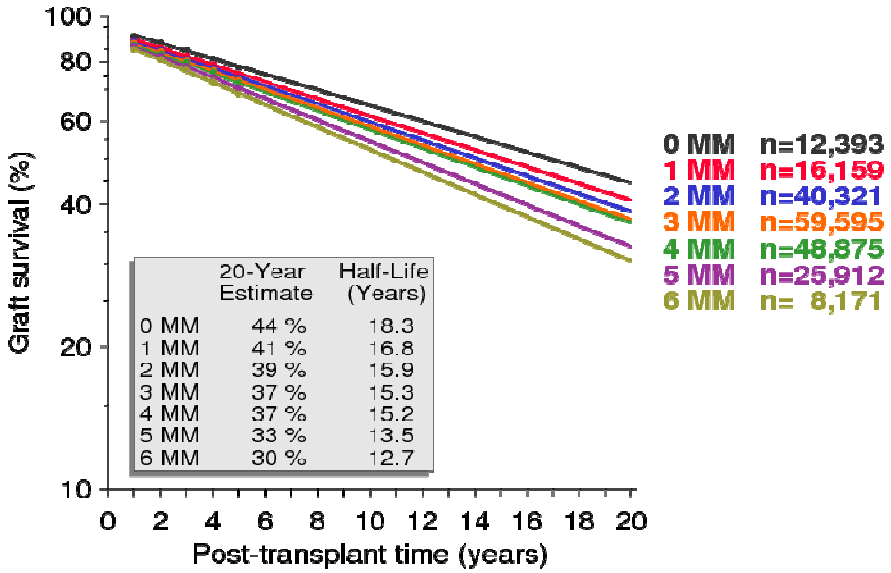
1.5.2 Chronic Rejection

For many years it was not clear if there was a direct connection between chronic anti-graft immune response and progressive loss of graft function, so the histological picture appearing before graft function deterioration has been called chronic transplant glomerulopathy (cTG). It is characterized by diffuse sclerosis, glomerular ischemia and vascular damage of medium and small vessels (Sis B, 2010). Independently from its causes this finding is associated with a severe renal prognosis, as within five years half of the grafts fail (Nankivell et al, 2003).

However, in the past decade there have been convincing evidences supporting a major role for immune response, through a mainly antibody-mediated chronic process. Indeed, among immunologic risk factors, HLA mismatches have a pivotal role: a better survival was observed in 0-mismatch transplant and worsened with the number of mismatched loci (Colvin, 2003; Geddes et al., 1998; Report of CTS 2017) (Figure 1.8). Moreover, many epidemiological studies have shown that acute rejection episodes, their number and also late acute rejection are a major risk factor for cTG (Archdeacon et al., 2011). Lastly the reduction of IS for any reason (like a

reduced compliance, recurring infections or malignancies) is a risk factor for both a late acute rejection (ie: after the first post-transplant year) and for chronic rejection, which is usually antibody mediated.

Figure 1.8 – Kidney graft survival by total HLA mismatches (A plus B plus DR) between donor and recipient in recipients of their first KTx from a deceased donor, between 1990-2016(CTS - Collaborative Transplant Study). Survival is expressed in a log scale.



There are also "non-immunological" risk factors for cTG: they are supposed to act through a reduction of functioning renal mass (like in aging) and exposure of graft antigens (mainly HLA class II and minor histocompatibily antigens). Actually kidneys from ECD and older donors have a higher risk of cTG and graft failure. Moreover a delayed graft function (DGF) and long ischemia times (from retrieval to transplantation) may cause glomerular deterioration and exposure of graft antigens. These also act also as pro-inflammatory events, favouring local inflammation and endothelial activation with a more efficient antigen presentation to the recipient's immune system (Tilney et al.,1998; Jindal & Hariharan, 1999).There is no specific therapy for transplant glomerulopathy, even if many different strategies have been tested (splenectomy, chronic induction, anti-B cell therapies, complement inhibition,

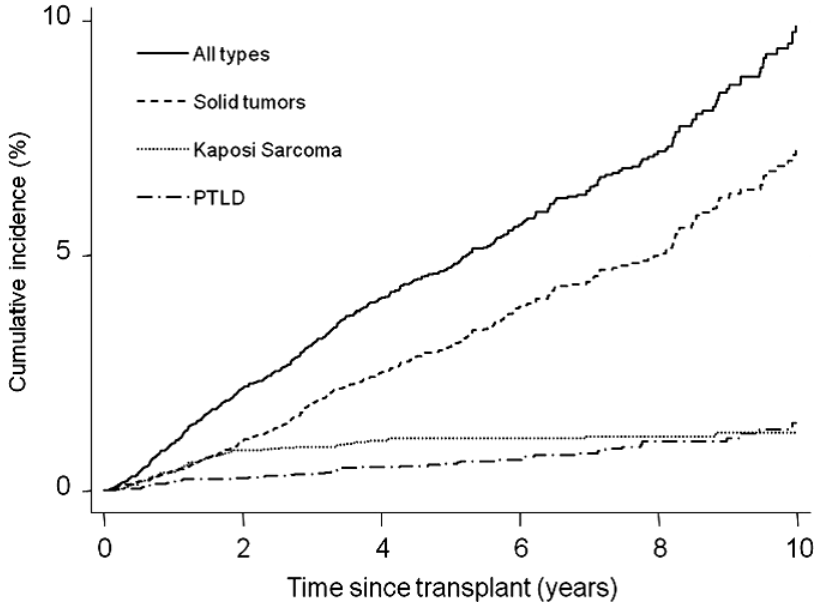
plasma exchange, etc), so the best way to "treat" cTG is its prevention, for example avoiding renal injuries, suboptimal immunosuppression and aggressively treating and preventing de novo donor-specific antibodies (DSA).

1.5.3 Post-transplant Malignancy

It has been known for many years that solid organ transplant recipients are at higher risk of cancer at most sites. Cancer is a major cause of morbidity after transplantation, with up to one-third of deaths with a functioning allograft due to cancer. Still, with an aging transplant population the presence of additional co-morbidity is increasingly common, and so, in aiming to optimize long-term patient outcomes, clinicians' advice must balance the prospect of graft failure and dialysis, with competing risk of diabetes, cardiovascular and cerebrovascular disease and the risk of malignancy.

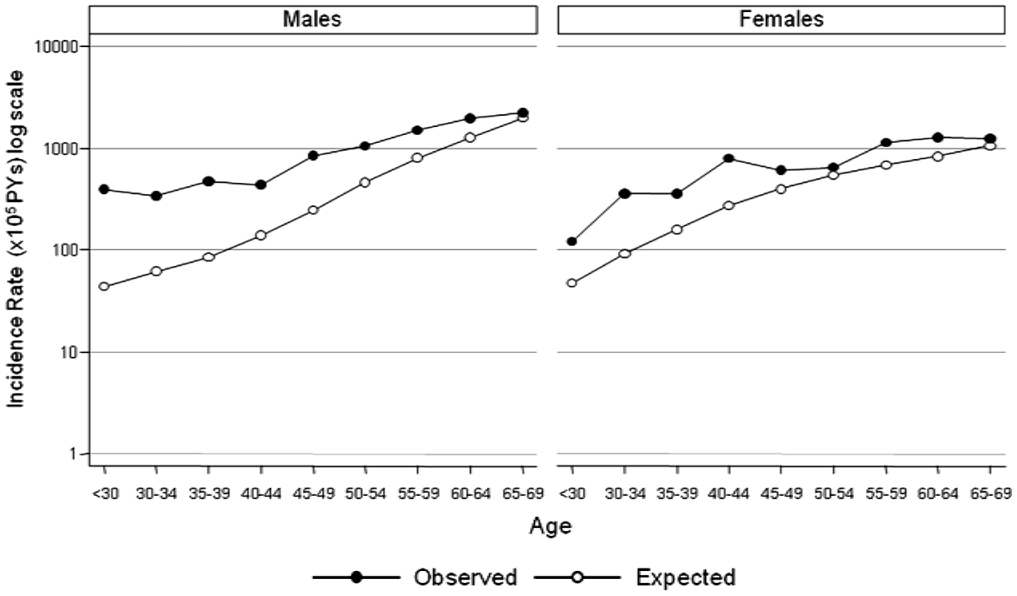
A large body of evidence, however, indicates that the chronic use of immunosuppressive drugs is associated with increased risks of opportunistic diseases, particularly cancers. After 10 years of immunosuppression, KTRs have a cumulative incidence of cancer as high as 20% (Figure 1.9).

Figure 1.9 - Cumulative incidence of malignancy in an Italian population of kidney transplant recipients, excluding non-melanoma skin cancers.The bold line represents all solid and hematologic malignancies, the dotted line all solid tumors, and the dashed line hematologic malignancies, post-transplant lymphoprolipherative disorders (Piselli et al, 2013b).



As compared to the age- and sex- matched general population, a 3-to-5fold increased risk was documented, among KTR, for NMSC and urological malignancies, while for some virus-related cancers such as non-Hodgkin lymphoma (NHL) or Kaposi sarcoma (KS) the risk was up to 100-fold higher. The increased risk of malignancies is particularly relevant in younger patients: indeed, the overall incidence of malignancy is almost the same for KTR and general population after 65 years, while it is higher for younger (< 30 years) patients (Figure.1.10)

Figure 1.10 -Age specific incidence of malignancies in the general population and in KTRs. Data from Italy, 1997-2009 (Piselli et al., 2013b).



NMSCs are the most common cancers in renal transplant recipients. Squamous cell carcinoma occurs at least 25 times more frequently in the transplant population than the general population. In Australia, the incidence of skin cancer is highest in the world:light skin (ie: Fitzpatrickphototype 1 or 2) and overexposure to ultraviolet light are the major causes of this increase in risk. There is also a cumulative dose-response relationship between duration of immunosuppressive agents used and incidence of NMSC. Compared with the general population, in KTRs NMSC develops at a younger age, and occurs more frequently at multiple sites. NMSCs also behave more aggressively, with more frequent recurrence after resection and metastasis, and can cause death, an event otherwise extremely rare.

Non-cutaneous malignancies (NCM) are malignant solid and hematologic tumors. Their risk is greatest among viral-related neoplasms: cancers related to infections, such as human herpesviruses 8 (HHV 8), Epstein-Barr virus (EBV), hepatitis B and C viruses, and HPV infection have been found to occur at a markedly increased rate (Piselli 2013a), whereas non–viral-related solid organ tumors such as breast and prostate cancers are not increased (Table 1.4).

Table 1.4 – Standardized incidence ratio for cancer related to infection in transplant recipients. EBV=Epstein-Barr virus. HBV=hepatitis B virus. HCV=hepatitis C virus. HHV8=human herpesvirus 8. HPV=human papillomavirus (Grulich et al., 2007)

Cancer	Meta-analysis SIR (95%CI)
EBV-related cancer	
Hodgkin's lymphoma	3.89 (2.42-6.26)
Non-Hodgkin's lymphoma	8.07 (6.40-10.2)
HHV8-related cancer	
Kaposi sarcoma	208.0 (114-349)
HBV/HCV-related cancer	
Liver	2.13 (1.16-3.91)
HPV-related cancer	
Cervix uteri	2.13 (1.37-3.30)
Vulva and vagina	22.76 (15.8-32.7)
Penis	15.79 (5.79-34.4)
Anus	4.85 (1.36-17.3)
Oral cavity and pharynx	3.23 (2.40-4.35)
Possibly HPV-related cancer	
Non-melanoma Skin	28.62 (9.39-87.2)

For instance, more than 90% of cases of post-transplant lymphoproliferative disease (PTLD) are of B-cell origin and associated with latent EBV infection. The overall risk, dependent upon the age of recipients, dose, and type of IS, increases by 3- to 10 –fold when compared with the age- and sex-matched general population. Evidence from the USRenal Data System (USRDS) had demonstrated that risk for PTLD was highest for persons in the first post-KTx year and decreases thereafter (Report of USRDS 2017). In Australia and New Zealand, and in Denmark, there was a bimodal distribution of the timing of the occurrence of

PTLD, with an early peak within 1-2 years and with a second peak after 5 to 10 years from transplantation (Maksten et al., 2016).

Moreover, a marked increase in the incidence of bladder and renal cell carcinomas by 3- and 8-fold was observed in the renal transplant recipients when compared with the age-and sex-matched general population (Figure 1.11), but it is also seen in dialysis patients: for instance, the incidence of RCC is 520/100,000 pt-year in patients on dialysis (Hurst et al., 2011) as compared to 15.3/100,000 pt-year in the general population (Ridge et al., 2014).

Figure 1.11 - Observed (Obs) and expected (Exp) cases of de novo malignancies in kidney transplant recipients, corresponding standardised incidence ratios (SIR), and 95% confidence intervals (CI). Italy, 1997–2009 (Piselli et al., 2013b).

Type/site	ICD-10	Obs No.	Exp No.	SIR (95% CI)	*p*-Value
Kaposi's sarcoma	C46	74	0.5	135 (106–169)	<0.001
Post-transplant lymphoproliferative diseases (PTLD)					
All[a]		52	18.3	2.8 (2.1–3.7)	<0.001
NHL	C82–C85, C96	40	8.9	4.5 (3.2–6.1)	<0.001
Leukaemia, all types	C91–C95	8	5.2	1.6 (0.7–3.1)	0.30
Hodgkin's lymphoma	C81	3	1.3	2.3 (0.5–6.8)	0.28
Solid tumours					
All		269	248.4	1.1 (1.0–1.2)	0.20
Lung & trachea	C33–C34	36	31.9	1.1 (0.8–1.6)	0.52
Kidney	C64	36	7.4	4.9 (3.4–6.8)	<0.001
Prostate	C61	35	21.0	1.7 (1.2–2.3)	<0.01
Breast female	C50	22	27.5	0.8 (0.5–1.2)	0.29
Colon-rectum	C18–C20	21	27.2	0.8 (0.5–1.2)	0.27
Bladder	C67	20	18.1	1.1 (0.7–1.7)	0.71
Stomach	C16	14	10.2	1.4 (0.8–2.3)	0.30
Melanoma	C43	11	6.0	1.8 (0.9–3.3)	0.08
Thyroid	C73	9	4.8	1.9 (0.9–3.6)	0.11
Oral cavity	C01–C10	8	5.0	1.6 (0.7–3.0)	0.20
Utherus (Corpus)	C54–C55	6	4.5	1.3 (0.5–2.9)	0.59
Pancreas	C25	5	5.8	0.9 (0.3–2.0)	0.97
Lip	C00	5	0.5	9.4 (3.1–22.0)	<0.001
Mesothelioma	C38, C45	5	1.2	4.2 (1.4–9.8)	<0.05
Testis	C62	5	1.2	4.1 (1.3–9.6)	<0.05
Liver	C22	4	9.4	0.4 (0.1–1.1)	0.09
Central nervous system	C70–C72	4	4.3	0.9 (0.3–2.4)	0.85
Salivary gland	C07–C08	3	0.5	5.8 (1.2–16.9)	<0.05
Oesophagus	C15	3	2.5	1.2 (0.3–3.6)	0.88
Larynx	C32	3	5.9	0.5 (0.1–1.5)	0.33
Ovary	C56	3	2.9	1.1 (0.2–3.1)	0.92
Others[b]		14	12.5	1.1 (0.6–1.9)	0.75
Total[*]		395	227.5	1.7 (1.6–1.9)	<0.001

ICD-10: International Classification of Diseases tenth revision.
[*] Non melanoma skin cancers are excluded from these analyses.
[a] It includes one case of multiple myeloma.
[b] It includes: invasive cervical cancers (2), small intestine (2), anus (1), breast-male (1), conjunctiva (1), connective and soft tissue (1), gallbladder (1), spermatic cord (1), hypopharynx (1), undetermined (3).

General lifestyle choice policies recommended for the general population -such as healthy eating and stop-smoking campaigns- have benefits beyond cancer prevention, and it is generally agreed that they should be encouraged and implemented in the transplant population. Modifiable life style risk factors known

to impact on cancer risk in the general population are also important in transplant recipients.

The pathogenic role of infections in carcinogenesis may offer opportunity to intervene to reduce risk. Although immunization against infections known to have oncogenic potential may seem an obvious preventive strategy for transplant recipients, achieving a protective immune response following vaccination is not always possible in wait-listed dialysis patients.

1.6 Study Rationale

Malignancies are an ominous complication followingkidney transplantation (KTx): their incidence is higherthan in the general population (Farrugia et al., 2014, Apel et al., 2013, Sampaio et al., 2012, Piselli et al., 2013b), and in KTR, their behaviour is usuallymore aggressive (Dantal et al., 2007, Vajdic et al., 2014). Therefore, in the past decades, screening andactive surveillance programmes have been implementedto perform early diagnoses (Asch et al,.2014, Ponticelli et al., 2012): These strategies havenot changed substantially cancer incidence (Tessari et al., 2013, Shu et al., 2014), buthave dramatically improved the survival. Indeed, in Italy, patient survival isas high as 71.3% at 10 years after the diagnosis of a NCM (Tessari et al.,2013). Consequently, novel questions arise aboutthe long-term outcomes of KTR with a post-transplantmalignancy, such as the risk of a second tumor (Viecelli et al., 2015; Tessari et al, 2013)and the risk of long-term graft failure in patients whosurvived a NCM.

However, it is not clear how the diagnosis of a malignancymay affect graft function as compared to patientswithout malignancy. Indeed, there are some studiesreporting death-censored graft survival rates after thediagnosis of some specific malignancies (particularly after post-transplant lymphoprolipherative disorders –PTLD and renal cell carcinoma – RCC (Melchior et al., 2011, Tsaur et al., 2011, Tillou et al., 2012), showinga worse renal prognosis for patients with a malignancyif compared to matched unaffected KTR (Rabot et al., 2014).However, it is difficult from these studies to quantifythe increase in risk of graft failure associated with thedevelopment of a tumor.Indeed, there could be at least two opposite situations.On the one side,

immunosuppressive (IS) therapyis often reduced after a malignancy diagnosis (Salesiet al., 2014, Serre et al., 2014)and exposure to chemotherapy and radiation therapy iscommon, which may trigger or favour chronic rejection,yielding eventually to a premature graft failure. On theother side, some patients may be particularly "susceptible"to IS and therefore develop some virus-associatedmalignancy (Piselli, et al., 2013a): these patients may be protected fromchronic rejection as they might be adequately immunosuppressedeven with a low-dose IS.

Given these premises, the aim of this cohort studywas to evaluate the impact of NMSCs and NCMs ondeath-censored graft survival in a cohort of recipient oftheir first KTx from a deceased donor. In detail,we checked whether and how much NMSC or NCM were associated with worse graft outcomes and we validated these associations by internal Leave-One-Out Cross-Validation; specifically,I evaluated if malignancies were associated to chronic rejection or other less common causes of graft failure. Moreover, as NCM were associated with graft failure, we checked how the reduction of IS therapy interacted with malignancies as a risk factor for graft failure. Lastly, even if the events were few, we tried to check if there was any particular post-malignancy risk factor for allograft dysfunction.

2. Patients and Materials

2.1 Database

We designed and developed a multi-level Access® database for all potentially useful variables for the proposed analyses. This database was firstly "conceived" in 2006 by Prof. Piero Stratta, Dr.ssa Caterina Canavese and Dr Claudio Musetti, and periodically updated in its structure to meet current clinical and research needs, including different biochemical and genetic parameters which have been recognized in the past decade as predictors of outcome in KTx.

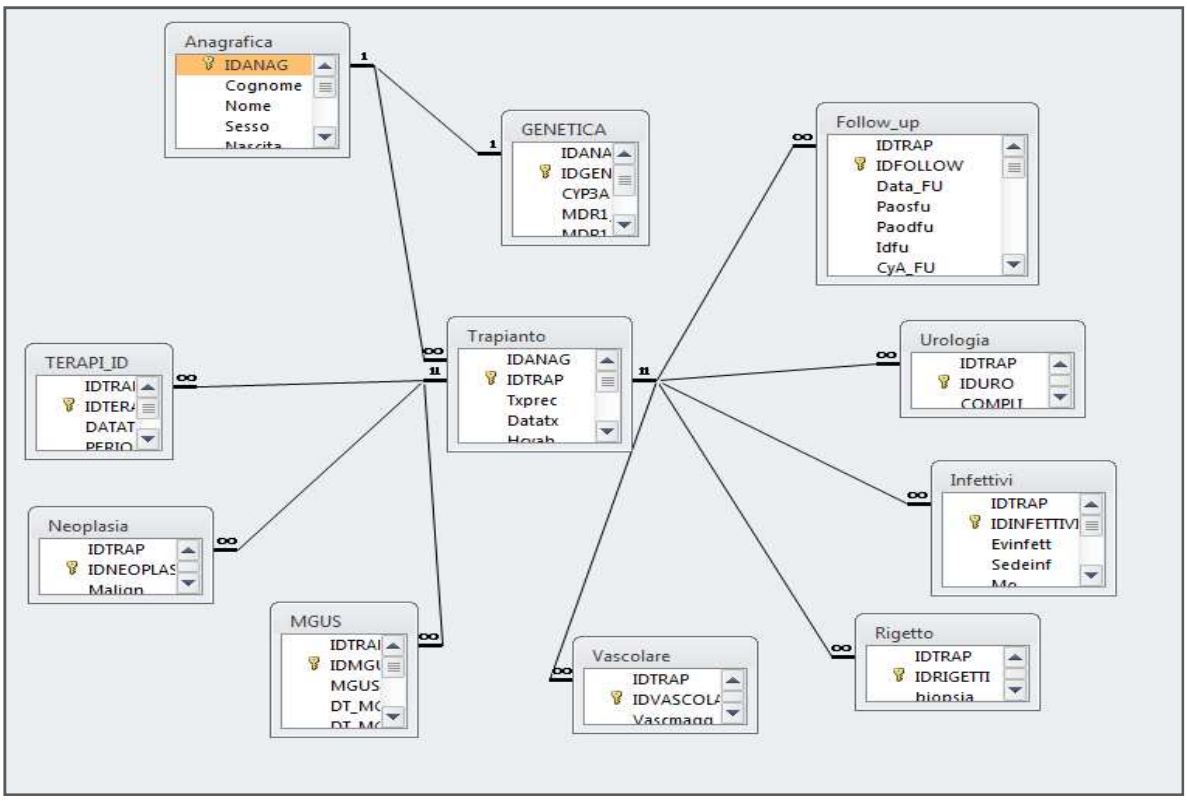
Each patient is recorded with a unique record, including information over their life status at the last observation (table name: *Anagrafica*). This table has one-to-many relationship with table "transplant" (table name: *Trapianto*) in which there are all the information about the transplant procedure and early complications (Figure 2.1). The transplant table is joined one-to-many with other tables, which included complication after transplant (table name: *Urologia, Infettivi, Rigetto, Vascolare, Neoplasia*) and the follow-up of the patients (table name: *Follow-up, Terapia_IS, Mgus*).

The pre-transplant data, transplant information and its complications, occurred during patient's admissions were found in discharge letters, in folders and outpatient hospital intranet. The information related to state of life and return to dialysis, in term of date and cause of deathor graft failure, have been looked for into *Registrodell'ImmunologiadeiTrapiantiRegionale* (ITR).

An important work was done to achieve a good quality of collected data, thank to an appropriated "ad hoc" check, that has evaluated all the variables considered.

Moreover, this database was linked with other databases, for example, the ITR's and the pharmacogenetics database

Figure 2.1 - Multi-level Access® database of the kidney transplant recipients of the hospital "Maggiore dellaCarità" in Novara



2.2 Study design

The design has been a cohort analysis, with the primary endpoint of death censored graft failure for any cause, defined as the need of chronic dialysis at any time after KTx. We retrospectively analyzed a prospective cohort of 672 patients who have been transplanted in a single KTx center between November 1998 and November 2013 and who had a minimum follow-up of 6 months after KTx.

The main aim of this cohort study was to evaluate the impact of NMSCs and NCMs on death-censored graft survival in a cohort of recipient of their first KTx from a deceased donor. Therefore, the primary endpoint was death-censored graft survival, while as secondary endpoint I considered graft failure divided by the cause of graft failure (see 2.4 Study events).

2.3 Study population

Adult patients receiving their first kidney transplant from a deceased donor at our transplant center have been included if they had a minimum follow-up of 6 months after KTx. In the same period in our center, 44 transplants were performed from a living donor and 103 patients received a second or third transplant and were not included in this analysis. Patients with a known active malignancy at the time of transplantation did not receive a KTx, and therefore, none of the included patients were known to carry a malignant disease at the start of observation.

Inclusion criteria

Adult patients receiving their first kidney transplant from a deceased donor at the transplant center (StrutturaComplessa a DirezioneUniversitaria di Nefrologia e Trapianto) of the AziendaOspedaliero Universitaria "Maggiore della Carità" - Università del Piemonte Orientale (Novara) have been included from the first transplant (November 4th, 1998) up to (March 24th, 2013).

Inclusion criteria:

- Surgery performed in Novara
- First transplant in the patient's medical history
- Deceased donor (either standard or ECD)
- Any sex
- Recipient older than 18
- At least six months of follow up with a functioning graft after transplantation.

Pre-transplant work up included medical history, echocardiography and Doppler-ultrasound evaluation of peripheral arteries and veins; if a patient was older than 50, or diabetic, or had had a previous cardiac event, a stress test (usually a nuclear medicine perfusion scan or a dobutamine stress echo) was performed and the patient was treated accordingly. Patients with previous multiple thrombotic events, including miscarriages, deep venous thrombosis, pulmonary embolism or vascular access thrombosis, were evaluated for genetic and acquired causes of thromobophilia and treated accordingly. Moreover, a strict pre-transplant screening

for malignanciesand pre-malignant lesions is always performed at our Center in order to exclude from transplantation patients with an active neoplasia, including dermatology evaluation, chest x-ray, abdomen CT, gastroscopy, thyroid US, protein electrophoresis, and specific sex- and age-related screenings like colonoscopy (patients older than 50), PSA and urological evaluation, mammogram and PAP test. Moreover, each patient with a particular risk factor or pre-malignant lesion (for example an MGUS) is evaluated with specific exams and visits. Patients with a previous malignancy but considered free from disease are re-evaluated at the Transplant Center and depending on the tumor histology and stage may be admitted to the KTx wait list after 2 to 5 years from the end of therapies.

Exclusion criteria

Patients with any of the following were excluded:

- Recipients from living donors:

  These patients were excluded because it is well known that grafts from living donors have a better survival as compared to those from deceased donors. Therefore, including these patients would have required a correction for "donor type": however, given that transplants from living donors were few during the study period (44 out of 982), this correction would have lowered the study power and made imprecise estimates.

- Previous kidney transplants:

  These patients were excluded because patients receiving a second transplant have a worse prognosis as compared to patients at their first transplant. Moreover, risk factors for graft failure of second and third transplants might be slightly different than those of first transplants. Lastly only a minority of patients is eligible for a second transplant and this "sub-population" should be considered as very selected, particular population due to their long history of renal failure and immunosuppression.

- KTR followed up in Novara, but who underwent surgery elsewhere

- Graft primary non-function, defined as the need of chronic dialysis within 3 days from surgery in the absence of any sign of graft function (ie: creatinine never going down after surgery).
- Graft failure within six months from surgery
- Patient death within six months from surgery
- Malignancy diagnosis within six months from surgery: indeed, ten patients developed a NCM within 6 months from transplantation and were excluded as they might have had an undiagnosed malignancy before surgery.

2.4 Study Events

- **Death** is recorded from referring nephrology centers. The cause of death is determined through autopsy when available or by the caring physician suspicion otherwise. The initial main cause of death is reported in the database and classified as cardiovascular death, malignancy, infection or other.
- **Graft failure** is defined as the need of any chronic renal replacement therapy after the KTx.
    - **Chronic rejection** was diagnosed with renal biopsy performed for a worsening renal function as defined by the Banff 2013 criteria or clinically by the presence of a progressive renal function deterioration (eGFR slope lower than -5 ml/min/1.73m^2/year), increased urinary proteins (>0.5 g/24h) and presence of donor-specific antibodies (MFI > 3000), after excluding other plausible causes of renal damage. No patient developed a graft failure due to a late-onset acute rejection. All patients with a malignancy were biopsied if they had a worsening renal function or increase in proteinuria and therefore their causes of graft failure are histologically defined.
    - **Graft failure due to other causes** was usually diagnosed by renal biopsy and included relapse of underlying nephropathy, new onset (“de novo”) nephropathies (including paraneoplastic nephropathies, like myeloma kidney), BK virus associated nephropathy, chronic vascular

nephropathy (including cardio-renal syndrome), chronic pyelonephritis, and chronic obstructive/reflux nephropathy: these cases have been included in the group of "graft failure not due to chronic rejection".

2.5 Covariates included in the study

In this study, we included some covariates to adjust risk estimates for graft failure and cancer diagnosis.

Main study variables:

Malignancy was diagnosed histologically or –rarely- on clinical bases, the latter case being relevant only for NMSC, which were sometimes treated with cryotherapy. All KTRs referring to our Center are proposed a cancer screening for breast, prostate, colon-rectum, cervix-uterus cancer, and additionally they undergo to a yearly dermatologic evaluation, abdomen ultrasonography, and chest X-ray; moreover, they undergo at least every three months a more general medical screening by physical examination and blood tests (complete blood count, renal and liver function and urinalysis). For every malignancy, we recorded the diagnosis (ICD9-CM code) and details (as free text), first diagnosis date (as first clinical recognition of disease), stage and therapy (which, dates, dose for chemo- and radiation therapy). Malignancies were divided into:

- **NMSC** included skin lesions with the ICD-9 code 173, being basal cell carcinomas and squamous cell carcinomas; no Merkel cell carcinomas were diagnosed.
- **NCM** included all other invasive malignancies, including both solid and hematologic tumors and excluding pre-cancerous lesions: the ICD-9 codes included 140 to 172 and 174 to 208.

  PTLD was defined as any malignant lymphocyte proliferation after KTx, including lymphomas and leukemias, such as early lesions (high grade EBV-related oligoclonal dysplasia), polymorphic lymphoma (oligoclonal lymphoma with various differentiations), large-B-cell diffuse lymphoma, monomorphic T cell lymphoma, other monomorphic B-cell lymphoma,

Hodgkin disease, large granular lymphocyte leukemia, chronic lymphocytic leukemia.

NMSC and NCM were considered as independent variables: for each variable (ie: NMSC and NCM) only the first event was analyzed among patients who had more than one neoplasm in either group. For example, if a patient had three NMSCs (and no NCM), only the first NMSC was considered to determine variable date. On the other hand, if a patient had both a NMSC and a NCM (ie: bladder cancer), both were considered, the first for the "NMSC variable" and the second for the "NCM variable".

Other variables:

- **Delayed graft function** is defined as the need for dialysis in the first week after transplantation, regardless of the indication, including dialysis for isolated hyperkaliemia. No creatinine criterionwas included in this definition, even if in the changing transplant population it has been advocated by some authors. This definition is highly specific for DGF and DGF defined according to this criterion has been shown to predict accurately long-term graft failure. However, this definition has a limit, which is that if a patient is transplanted before starting dialysis, even if the kidney transplant does not function immediately, it is very unlikely for him to start dialysis after transplantation.
- **Acute rejection** is usually defined histologically according to the Banff classification and subsequent revisions, including the 2013 revision which re-defines acute and chronic antibody mediated rejection. A few times -when a kidney biopsy is contraindicated or considered to be too risky- it was defined clinically as a rise in serum creatinine more than 2 times the baseline level or a persistent creatinine of more than 6 mg/dL in the presence of active urinary sediment (hematuria or proteinuria) and that recovered within one week of high-dose steroid pulses (more than 1000 mg cumulative iv dose).

- **Transplant year**was included as "summary" covariate of multiple possible confounders which have changed during the long enrollment time, including different donor and recipient selection criteria and IS schemes. The enrollment period was divided in 5-years groups: 1998-2003; 2004-2008; 2009-2013.
- **HLA Mismatches**. This number represents the number of HLA antigens (loci A, B and DR) of the donor against which the recipient may develop an immune response. It is well known that low-mismatch transplants have a better prognosis than high-mismatch transplants even with current IS therapies.
- **Previous immunization**. This data is a measure of pre-formed anti-HLA antibodies of the recipient, due to previous immunizing events (ie: pregnancy, blood transfusion). It is expressed as the percentage of HLA alleles against which there are antibodies: a Panel Reactive Antibody (PRA) of 0% means that there are no antibodies against any HLA allele, while a PRA of 100% means that there antibodies against all HLA alleles. This percentage has been associated with a higher risk for acute and chronic rejection, as well as graft failure.
- **Underlying nephropathy**. Some nephropathies have a known high relapse risk on the graft, leading to ESRD as they did on native kidneys. The most frequently relapsing nephropathies as focal glomerulosclerosis (FSGS, up to 40-70%), IgA nephropathy (almost 95%, but rarely a graft failure cause), atypical hemolytic uremic syndrome (up to 100% depending on underlying mutation), primary oxalosis (almost 100%) and membrano-prolipherative glomerulonephritis complex (including C3 deposit disease). Therefore, these diseases were reclassified as primary glomerulopathies/nephritides, secondary nephropathies (ie: renal involvement during other diseases, like diabetes) and unknown nephropathies. Indeed, as much as 40% of patient are diagnosed with an ESRD without previous medical history or events and their underlying nephropathy may not be determined.
- **Expanded criteria donor**. In order to increase the number of transplants, acceptance criteria for donors have been expanded: organs from donors with

an impaired -but still acceptable- renal function (ie: CKD stage 1 or 2 or older donors) are called as "from Expanded Criteria Donor" (ECD). They are defined as kidney donors older than 60 years or donors aged from 50 to 59 years and who have two of the following risk factors: hypertension, serum creatinine >1.5 mg/dl (acute rise), or death from cerebrovascular accident. Indeed, given that the major determinant of renal function and renal reserve capacity is age, donor age was included both in the ECD definition and as a separate covariate. Moreover, other kidney donor profiling scores (ie: Kidney Donor Profile Index) and tools have been developed, but still age is still the main determinant of long term graft function in all risk estimate models and donor evaluation models.

- **Cold Ischemia Time** is the time during which the graft is preserved in a cold electrolyte solution waiting to be transplanted. These variable is a risk factor for delayed graft function and long-term graft function even if there is probably a threshold effect.
- **Immunosuppressive therapy**. This variable was evaluated at the time of transplant as a category variable, and during the follow up including for each drug its mean dose and –if appropriated- blood through level. Baseline therapy was re-classified for analyses as "FK-based", which included tacrolimus, mycophenolate or azathioprine with or without steroids; "Cya-based", which included cyclosporine, mycophenolate or azathioprine with or without steroids; while other combinations have been classified as "other IS therapies".
- **A reduced immunosuppression** (Red-IS) included any single-drug therapy or a therapy with steroids and either an mTOR-inhibitor or mycophenolate (CNI-free). All other IS drug combinations (i.e., CNI-steroids, CNI-MMF, CNI-mTORi, three-drug therapy) were considered as "standard dose"IS regardless of drug doses and levels (Table 2.1). the date of IS reduction was the date of drug discontinuation.

Table 2.1. Definitions of "standard" and "reduced" maintenance immunosuppressive therapy adopted in this study. Any single drug therapy was considered "Reduced IS", any three-drug therapy was considered a "Standard IS", while patients on two different anti-rejection drugs were classified as for drug type and blood through levels. FK: tacrolimus; CyA: Cyclosporine A

Number of drugs	Drugs	Through Blood Level	Category
1	Any	Any level	Reduced IS
2	Any, not steroids	-	Standard IS
	FK + Steroids	>= 4 ng/mL	Standard IS
	CyA + Steroids	>= 300 ng/mL	Standard IS
	Steroids + any other	-	Reduced IS
3	Any	Any	Standard IS

- **Post-transplant renal function and proteinuria** were evaluated at time of study entry (ie: six months after KTx). At this time, renal function is stable in most patients and chronic rejection (which may be symptomatic for proteinuria) is unlikely to have started. Given that ESRD is reached when graft function is greatly reduced (ie: a glomerular filtration rate of less than 5-10 ml/min), both baseline renal function and proteinuria –which is associated to a faster renal function deterioration- are important predictors of graft survival.

2.6 Identification of baseline risk factors (potential confouders)

We looked for time-fixed risk factors for death censored graft failure, in order to adjust later estimates of the study variables, which are time-dependent covariates.

Risk factors were chosen by known and potential risk factors from literature. Among the included variables the main predictors of graft failure are expected to be collinear, as they often represent different measures of the same underlying biological process. For instance, recipient and donor age are usually matched as in our Center the allocation policy has -as a major criterion for allocation- a relatively good age match between donor and recipient. Moreover, there are different measures that might be related to the baseline or donor renal function, such as donor age, ECD (and type of transplant), DGF and -to some extent- post-transplant creatinine (which -if the KTx is "uneventful"- is mainly determined by the donor's renal function).

However, DGF has been associated in literature with cold ischemia time and need for blood transfusions, so -even if these variables will be associated with graft failure- only one will be chosen.

Therefore, the choice of the mostly significant covariates was based on the clinical representation of the underlying biological process and -if unable to determine which one was most representative- based on the strongest statistical association.
Moreover, if some “important” adjustment variables were excluded by this selection process, they were reintroduced one by one and checked if their inclusion changed the model or the estimates of the other covariates, and if so they were kept in the model independently by their association with the outcome.

3 Statistical Methods

Survival analysis was the main statistical technique applied in these cohort study.
The study of the relationship between the appearance of a post-transplant malignancy, defined as a time-dependent covariate, and the endpoint on death-censored graft survival was addressed by the extension of the Cox Proportional Hazard model, which is a powerful tool when time-dependent covariates are present.
Moreover, model validation is an important step in the model building process, because it provides opportunities to assess the reliability of models before their deployment. Predictive accuracy measures the ability of the models to predict future risks, and significant developments have been made in recent years in the evaluation of survival models (Changbin at al., 2017).
Some techniques to calculate overall concordance statistics and time-dependent receiver operator characteristic (ROC) curves for right-censored data will be performed in this work.

3.1 Survival Analysis

Survival analysis aims to study the time between a certain starting point, represented in our case by subjects undergoing renal transplantation, with functioning kidney for at least 6 months, and the onset of a certain event, the cessation of the vital functions of the kidney (death censored graft survival). Interest is not focused only on the occurrence of the event, but also on the underlying temporal process. A peculiar characteristic of the survival analysis is that for some subjects included in the study it is not possible to observe the event of interest due to the censoring of the observation period.
Right censoring occurs when a subject leaves the study before an event occurs, or the study ends before the event has occurred.
In our study, patient's death with a functioning kidney, patients alive without graft failure at the date of last available visit or patients transfer to another Center are considered as censored data.

In general, it is necessary that censored data are non-informative or independent.It essentially means that within any subgroup of interest, the subjects who are censored at time *t* should be representative of all the subjects in that subgroup who remained at risk at time *t* with respect to their survival experience. Informative censoring or dependent can lead to biased results regarding maximum likelihood estimation (Kleinbaum and Klein, 2012).

Survival data observed for each subject is represented by the pair of variables (T, δ) where *T*is time since entry into the study and δ is an indicator of failure, assuming values of $\delta=1$ if the event of interest is observed and $\delta=0$ if the time is censored.

Suppose U is the true survival time that it cannot be always observed, and V is the censoring time. Then, the observed time is $T = \min(U, V)$. If δ=1 then $U \leq V$ otherwise if δ=0 then $U > V$, that is $T = U$ only when the observation is uncensored.

Survival analysis methods are classified in non-parametric methods, semi-parametric and parametric, based on the assumptions that are made on the distribution of *T*.

In this work the analysis were carried out using non-parametric methods, and the most known non-parametric method for estimating survival probability is the limit product method, better known as Kaplan-Meier estimator, which also includes the contribution of censored data (Kaplan and Meier, 1958).

In the analysis of survival data, two functions that are dependent on time are the survival function and the hazard function. The survival function $S(t)$is defined as the probability of surviving at least to time t. The hazard function $h(t)$ is the conditional probability of dying at time t having survived to that time. The graph of $S(t)$ against t is called the survival curve.

The Kaplan–Meier method can be used to estimate this curve from the observed survival times without the assumption of an underlying probability distribution.

Let n be the total sample size, letY_i denote the follow-up time and let δ_i be an event indicator (1 if the patient had an event at time Y_i and 0 if the patient was censored at time Y_i), where the subscript, *i,* is a patient indicator. The covariate X_i defines the cohort of interest. If we let $t_1 < t_2 < \cdots < t_j < \cdots$ represent the distinct event times, then at each time t_j there are $n_{jk} = \sum_i (X_i = k)(Y_i \geq t_j)$ individuals in cohort k who

are at risk of an event, and $d_{jk} = \sum_i (X_i = k)(\delta_i = 1)(Y_i = t_j)$ individuals in cohort k with an event time t_j.

The usual Kaplan-Meier estimator for cohort k is:

$$\hat{S}_{k(t)} = \prod_{j:t_j \leq t} \left\{ 1 - \left(\frac{d_{jk}}{n_{jk}} \right) \right\} \qquad (3.1)$$

The 95% IC formula to estimate KM probability at any time point over follow-up is given by:

$$\hat{S}_k(t) \pm 1.96 \sqrt{\hat{Var}\left[\hat{S}_k(t)\right]} \qquad (3.2)$$

where Greenwood's formula is the most common approach to estimate the variance (Greenwood, 1926):

$$\hat{Var}\left[\hat{S}_k(t)\right] = \hat{S}_k(t)^2 \sum_{j|t(j) \leq t} \frac{d_{jk}}{n_{jk}(n_{jk} - d_{jk})} \qquad (3.3)$$

Comparison of two survival curves can be done using a statistical hypothesis test called the Log Rank Test (Mantel &Haenszel, 1959).

The Log-Rank test is used to test whether the difference between survival times between two or more groups is statistically different or not, but does not allow to test the effect of the other independent variables.

Cox Proportion Hazard model enables us to test the effect of other independent variables on survival times of different groups of patients, just like the multiple regression model.

Kaplan-Meier survival estimation, the Log-Rank test and the Cox regression model, rely on an assumption of independent censoring for valid inference in the presence or right-censored data.

To evaluate the cumulative incident of graft failure, patient's death was considered as a competitive risk. When there are competitive risk, the Kaplan-Meier survival curve

may not be very informative because it is based on an independence assumption about competing risk that cannot be verified.

If there is only one risk, the cumulative incident curve is given by $1 - \hat{S}_k(t)$, with competitive risk, however, the cumulative incident curve is derived from a cause-specific hazard function, provides estimates of the "marginal probability" of an event in the present of competing events, and does not require the assumption that competing risk are independent (Kleinbaum and Klein, 2012).

The %CIF macro SAS, which implements nonparametric methods for estimating cumulative incidence functions with competing risks data, was used (Lin & Johnston, 2012).

3.2 Cox Proportional Hazards model

In 1972 Cox introduced a model called semi-parametric as it does not assume any specific form about the distribution of the random variable *T*, but models the effect of prognostic variables in a parametric way (Cox, 1972). The basic model assumes that the hazard function for failure time *T* for an individual*i*with *P* covariate

$\boldsymbol{x}_i{}' = (x_{1i}, x_{2i}, \ldots x_{ki}, \ldots . x_{Pi})$ is:

$$\lambda(t; \boldsymbol{x}_i) = \lambda_0(t) exp\left(\boldsymbol{\beta}' \boldsymbol{x}_i \right) \qquad (3.4)$$

for i= *1*…….,*N*.

The hazard (3.4) depends on both λ_0(t), that is a function of time only, which is left arbitrary but is assumed to be the same for all subjects, and the individual covariate only through the $(P \times 1)$ vector $\boldsymbol{\beta}'$of regression coefficients.

The covariates are assumed to be constant in time (time-independent) and, in our case, they concern the personal and demographic variables, the history of nephropathy before transplantation, the distribution of transplants over time and the details of the transplant, including the description of the donor and the transplanted kidney.

The Cox regression model specifies the hazard ratio for any two individuals with covariate vectors x_1 e x_2, and this hazard ratio turns out not to depend on λ_0(t):

$$\frac{\lambda(t;\boldsymbol{x}_i)}{\lambda(t;\boldsymbol{x}_j)} = \frac{\lambda_0(t)exp(\boldsymbol{\beta}'\boldsymbol{x}_1\ \ )}{\lambda_0(t)exp(\boldsymbol{\beta}'\boldsymbol{x}_2\ \ )} = exp[\boldsymbol{\beta}'(\boldsymbol{x}_1 - \boldsymbol{x}_2)] \qquad (3.5)$$

The model in (3.4) is called proportional hazard (PH) regression model since it assumes that the failure rates of any two individuals are proportional, given that the ratio in (3.5) does not depend on time.

The estimate of $\boldsymbol{\beta}$, a vector of unknown regression parameters,allows us to quantify the relative rate of failure for an individual with covariate vector $\boldsymbol{x}_1$ with respect to an individual with vector $\boldsymbol{x}_2$, assuming that this risk varies proportionally in all the subjects characterized by different covariates.

In particular, if two individuals are taken to have covariate vectors $\boldsymbol{x}$and $\mathbf{0}$, the ratio of their hazards is:

$$\frac{\lambda(t;\boldsymbol{x}\ \ )}{\lambda(t;\mathbf{0})} = \frac{\lambda_0(t)exp(\boldsymbol{\beta}'\boldsymbol{x}_i\ \ )}{\lambda_0(t)} = exp(\boldsymbol{\beta}'\boldsymbol{x}) \qquad (3.6)$$

This shows that λ_0(t) may be regarded as the hazard function of an individual with all covariates of a value zero, and for this reason λ_0(t) is often termed the baseline hazard (Marubini& Valsecchi, 1995).

Since λ_0(t) is not specified parametrically, it is not possible to use an ordinary likelihood to estimate the regression coefficients $\boldsymbol{\beta}$**.** In his original work, Cox (Cox, 1972), estimates the regression coefficients $\boldsymbol{\beta}$**,**considering, in the likelihood function, $\lambda_0(t)$ as a nuisance function.

Let $R(t)$ be the set of subject, at risk at time t, the probability that an individual with covariate $\boldsymbol{x}$ fails in a small interval $(t, t+dt)$ is $\lambda(t;\ \boldsymbol{x})dt$. Thus, conditional on the fact that one individual is observed to fail at $t_{(j)}$, the probability that it is an individual with covariate $\boldsymbol{x}_j$ is:

$$L_j(\boldsymbol{\beta}) = \frac{\lambda(t_{(j)};\boldsymbol{x}_j)dt}{\sum_{i\epsilon R_{t(j)}}[\lambda(t_{(j)};\boldsymbol{x}_i)dt]} = \frac{exp(\boldsymbol{\beta}'\boldsymbol{x}_j)}{\sum_{i\epsilon R_{t(j)}} exp(\boldsymbol{\beta}'\boldsymbol{x}_i)} \qquad (3.7)$$

The formula for the Cox model likelihood function is actually called a "partial" likelihood function rather than a (complete) likelihood function. The term "partial" likelihood is used because the likelihood formula considers probabilities only for those subjects who fail, and does not explicitly consider probabilities for those subjects who are censored.

The partial likelihood can be written as the product of several likelihoods, one for each of *j* failure times. Thus, at the *j-th* failure time, L_j denotes the likelihood of failing at this time, given survival up to this time. Note that the set of individuals at risk at the *jth* failure time is called the "risk set," $R_{t(f)}$, and this set will change – actually get smaller in size – as the failure time increases (Kleinbaum and Klein, 2012).

The partial likelihood is defined by:

$$L_{PA}(\beta) = \prod_{j=1}^{J} \frac{exp(\boldsymbol{\beta}'\boldsymbol{x}_j)}{\sum_{i\epsilon R_{t(j)}} exp(\boldsymbol{\beta}'\boldsymbol{x}_i)} \qquad (3.8)$$

The regression coefficients $\boldsymbol{\beta}$ are estimated by the values $\widehat{\boldsymbol{\beta}}$which maximize the partial likelihood$\boldsymbol{L(\beta)}$ or equivalently its logarithm:

$$l_{PA}(\boldsymbol{\beta}) = logL_{PA}(\boldsymbol{\beta}) = \sum_{j=1}^{J} \left\{ \left(exp(\boldsymbol{\beta}'\boldsymbol{x}_j) \right) - log \sum_{i\epsilon R_{t(j)}} exp(\boldsymbol{\beta}'\boldsymbol{x}_i) \right\} \qquad (3.9)$$

An interactive process such as the Newton-Raphson one has to be adopted to solve this system of equations for $\boldsymbol{\beta}$.

The β's estimates make it possible to study the effect of each covariate on the risk of developed the event of interest.

Hazard ratios alone do not provide a complete picture of longitudinal survival. Survival estimates are a standard complement. The estimate cumulative hazard is given by:

$$\Delta(t;\boldsymbol{x}) = \int_0^t \lambda_o\,(u)\exp(\boldsymbol{\beta}'\boldsymbol{x})\,du = \exp(\boldsymbol{\beta}'\boldsymbol{x})\cdot\Delta_0(t) \qquad (3.10)$$

and the survival function is given by

$$S(t;\boldsymbol{x}\ ) = exp[-\Delta(t;\boldsymbol{x})] = S_0(t)^{\exp\,(\boldsymbol{\beta}'\boldsymbol{x})} \qquad (3.11)$$

where $S(t;\ \boldsymbol{x}\ )$ is the survival probability at time t for an individual with covariate values x, and $S_0(t)$ is the baseline survival function, that is, the survivor function for an individual whose covariate value are all 0. After estimating $\boldsymbol{\beta}$ by a partial likelihood it is necessary to have an estimator for di $S_0(t)$. One possibility is the estimator proposed by Breslow (Breslow & Day, 1980), defined as:

$$\hat{S}_0(t) = \prod\nolimits_{t(j)\le t}\left(1 - \frac{d_j}{\sum_{i\epsilon R_{t(j)}} exp(\boldsymbol{\beta}'\boldsymbol{x}_i\ \ )}\right) \qquad (3.12)$$

with $\hat{S}_0(0) = 1$

3.2.1 Extension of the Cox Proportional Hazard model for time-depended covariate

Until now, for the construction of the Cox model and the estimation of the parameters, we have considered only the presence of one or more fixed covariates over time (time-independent covariate).

In some cases it is necessary to consider time-dependent variables in the model. A time-depended covariate is an explanatory variable whose value may change over time, thus it has value $x_i(t)$ for individual i at time t.

In order for the Cox model can include this type of covariates, and therefore no longer be a "proportional risk" model the hazard function shall depend on the value $x_i(t)$ on time t.

Estimation of the corresponding regression coefficient can still be made on the basis of the partial likelihood function, suitably modified to account for the changing value of $x\ \ (t)$(Marubini and Valsecchi, 1995).

In this study we are interested in assessing whether the onset of a malignancy after a renal transplant is associated with a negative impact on renal function, resulting in graft failure.

It is necessary to adjust this estimate with the known risk factors for graft failure, which will be considered in Cox's model as time-independent covariates.

The onset of tumor will therefore be considered as a time-dependent covariate, $x\ \ (t)$, which assumes value 0 from the date of the transplant up to the date of onset of the tumor and assumes value 1 from date of onset of the tumor up to the date of last visit or up to the date of the event.

For a no tumor patient, x (t) has value 0 at date of transplant and does not change thereafter.

The Cox model with $x\ \ (t)$, taken to satisfy the log-linear dependence of hazard on covariate is:

$$\lambda(t; x(t)) = \lambda_0(t)\exp\left(\beta' x(t)\right) \qquad (3.13)$$

If a covariate x (t) is included in the model, all information from data at time t is taken conditionally on the actual value of the variable at t.

Indicating with x (t) a covariate vector, among which one or more are time-dependent, the partial log-likelihood is given by:

$$l_{PA}(\boldsymbol{\beta}) = logL_{PA}(\boldsymbol{\beta}) = \sum_{j=1}^{J}\left\{\left(exp(\boldsymbol{\beta'}\boldsymbol{x}_j\ \ (t_j\ \ ))\right) - log\sum_{i\epsilon R_{t(j)}} exp(\boldsymbol{\beta'}\boldsymbol{x}_i\ \ (t_j\ \ )\ \ )\right\} \qquad (3.14)$$

The sum is over the distinct failure times and the first term is the contribution of the subject failing at $t_{(j)}$ with $x_j(t_j)$ being his vector of covariate values at $t_{(j)}$. In the

second term, the sum runs over all subjects i whose failure times are equal to or greater than $t_{(j)}$. The likelihood collects the information only on the instantaneous failure rate, given the actual realization of the time-dependent variable at every $t_{(j)}$.

In the presence of time-depended covariates, the approach of conditioning on the realization $x\ (t)$ implies considering the updated value of *x* at *t* and neglecting the information on how the covariate value has been changing up to time *t*(Marubini& Valsecchi, 1995).

The association measure provided by these models is the hazard ratio (HR) that is obtained by exposing the estimate of the β parameter deriving from the model.

As previously seen for the Cox model with time-independent variables (3.4), it is possible to calculate the estimate the cumulative baseline hazard function, even when we are in the presence of time-dependent variables, using the Breslow estimator, whose formula for the calculation is given by:

$$\widehat{\Delta}_0(t) = \sum_{i=1}^{n} \frac{I(\tilde{T}_i \leq t)\Delta_i}{\sum_{j \epsilon R_{t(j)}} exp(\widehat{\boldsymbol{\beta}}' \boldsymbol{x}_j\ \ (\tilde{T}_i)\ \ )} \qquad (3.15)$$

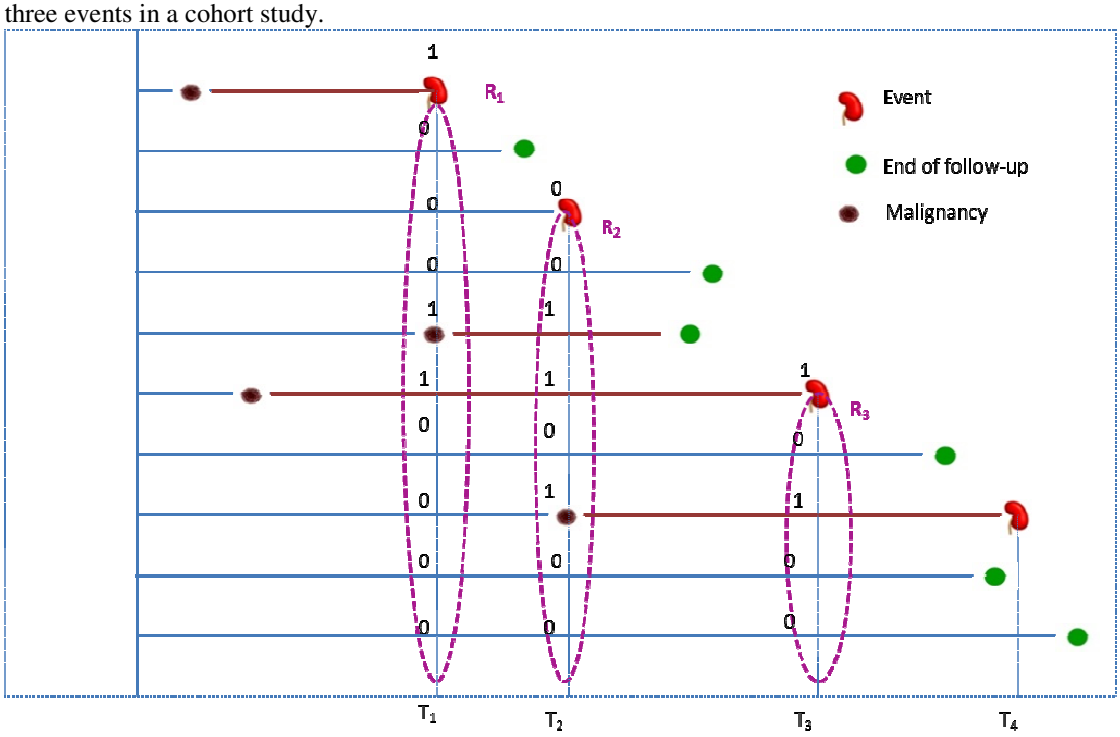
For any time at event t, in the numerator we have the number of events that occurred up to the time t included. $I(\tilde{T}_i \leq t)$ is the indicator function that is worth 1 if a subject has had the event or has been censored before time t and Δ_i is 1 if the subject has had an event and 0 if it has been censored.

In the denominator we find the sum of $\exp(\beta' x_j\ \ (\tilde{T}_i)\ \ )$ where β is the vector of parameters estimated by the Cox model and $x_j\ \ (\tilde{T}_i)$ is the vector of the value of the covariates of each subject measured at the time*t*, for all the subjects that are still in study at the time *t* (these subjects neither experience the event nor were they censored before t). At any time t there is a different value for the time-dependent covariates, while for the fixed covariates the value will remain constant.

The corresponding estimator for the conditional survival function is

$$\hat{S}(t; \boldsymbol{x}) = exp\left\{ - \int_0^t e^{\widehat{\boldsymbol{\beta}}' x(u)} d\widehat{\Delta}_0(u) \right\} \qquad (3.16)$$

Figure 3.1 - Representation of the variation of the set at risk and of the covariate time-dependent at three events in a cohort study.



The figure below is an example of a cohort, in which we want to show how the set at risk changes at 3 events, T1, T2, T3 and how it is considered a time-dependent variable that takes values 0 and 1 as shown in Figure 3.1

In Figure 3.1 it can be seen that the value of the covariate, diagnosis of tumor, is calculated at each event, for example at time T1, for all the persons belonging to the corresponding risk set, R1, ie the people who neither had event nor were they censored before T1.
At all times there is a different value for time-dependent covariates, while for fixed covariates the value remains constant.

To quantify the tumor effect in terms of hazard ratio, we fitted both univariable and multivariable- adjusted Cox models in which patient's status (with or without tumor) was similarly updated. We considered, as adjusting factors in the multivariable models, the following variables evaluated at baseline (i.e., 6 months from KTx): gender, donor age, year of transplant, underlying nephropathy, acute rejection episodes, creatinine, and proteinuria levels. These variables were selected among

known predictors of long-term graft failure that were significant risk factors at the univariate analysis in our cohort. If two covariates were associated (e.g., donor and recipient age), we maintained in the model the variable with the strongest association or the one with the highest clinical significance. The heterogeneity of the effect of tumor occurrence on the cause-specific graft failure (chronic rejection versus other causes) was assessed comparing the hazard ratios estimated from two time-dependent multivariable Cox models, using the methods described by Putter (Putter et al., 2007). In the two models, chronic rejection and failure from other causes were considered alternatively as the event of interest or as the censoring event.

3.3 Modified Kaplan–Meier method to take into account the tumor as time-depended covariate

To illustrate the effect of tumor (NMSC or NCM) occurrence over time on the risk of graft failure, we used a modified Kaplan–Meier method (Steven et al., 2005) that estimated cumulative hazard rates of graft failure according to the presence or absence of tumor. All patients at the beginning of the observation were included in the tumor-free group, and the assignment to the tumor group was updated at the time of the tumor diagnosis. In all standard Kaplan-Meier curves the size of the risk set diminished over time due to events and censoring, while in the extended Kaplan-Meier estimator the side of the risk set can increase or decrease over time.
The extended Kaplan-Meier estimator is calculated as:

$$\hat{S}_{k(t)} = \prod_{j:t_j \le t} \left\{ 1 - \left(\frac{d'_{jk}}{n'_{jk}} \right) \right\} \qquad (3.17)$$

where $n'_{jk} = \sum_i \big(X_i(t_j) = k\big)(Y_i \geq t_j)$ represent the size of the risk set for cohort k at event time t_j and $d'_{jk} = \sum_i \big(X_i(t_j) = k\big)(\delta_i = 1)(Y_i = t_j)$ represent the number of individuals in cohort k with an event at time t_j. This time $X_i(t_j)$ is a time-depended which indicates the cohort to which the patient belong at any point in time.

In our study the covariate of interest is if the patient developed a cancer after Kidney transplant and this covariate can only take the value 0 (if a patient did not developed cancer) or 1 (if the patient developed a cancer), and an individual's covariate value cannot change from 1 to 0 during the follow-up.

Just as the standard Kaplan-Meier curve can be considered to be a visual representation of the hazard ratio calculated from a Cox regression model with a time-invariant covariate, the extended Kaplan-Meier estimator can be considered to be a visual representation of the hazard ratio calculated from a Cox model with a time-dependent covariate.

Finally, to evaluate the joint effect of the reduction of the IS therapy and the occurrence of NCM on graft failure, the aggregate patient event rates was used. In analyzing aggregated event rates, the response or outcome variable is the number of events (person who lost their kidney) that occur divided by the number of accumulated patient-time of exposure to the study event which can be referred to as the incidence of the event. The event rates can then be compared between some covariate, in our case, diagnosis of NCM and IS therapy reduction.

Models for rates are considered in which the underlying rate at which events occur can be represented by a regression function that describes the relation between the patient characteristics and the unknown rate of occurrence (Frome 1983). When the events of interest follow the Poisson distribution, Maximum Likelihood Estimation is used. Poisson regression models are generalized linear models with the logarithm as the link function. To establish the relation between the dependent variable and the predictor variables a log-linear model is used.

In our analysis, the observation time was split into the following periods to calculate the person years at risk:

(i) free from NCM and treated with full dose of IS therapy,

(ii) free from NCM and treated with a reduced dose of IS therapy,

(iii) with a NCM and treated with full dose of IS therapy, and

(iv) with a NCM and treated with a reduced dose of IS therapy.

Eventually, for each time period, the graft failure rate was calculated. The Wald test was used to compare rates for the different categories of the variables considered in the model.

3.4 Evaluating predictive accuracy of survival models

Whatever model is used for studying survival, it is important to assess the performance of the model in two ways; its discrimination and calibration aspects. The first one is based on testing the ability of a predictive model to separate those who develop an event from those who do not. Calibration is the degree of correspondence between the estimated probability produced by the model and the actual observed probability. It can be argued that discrimination performance is more important than calibration since calibration can be adjusted whereas a model that cannot discriminate between the different groups can not be put into practice. On the other hand, poor calibration can occur in highly discriminating models when the output is transformed monotonically (Taktak et al, 2008).

The most appropriate method for assessing discrimination ability in survival analysis is based on the Harrell's Concordance index also known as C-statistic (Harrell et al., 1996). The concept underlying concordance is that a subject who experiences a particular outcome has a higher predicted probability of that outcome than a subject who does not experience the outcome. The C-statistic can be calculated as the proportion of pairs of subjects whose observed and predicted outcomes agree (are concordant) among all possible pairs in which one subject experiences the outcome of interest and the other subject does not. The higher the C-statistic, the better the model can discriminate between subjects who experience the outcome of interest and subjects who do not (Changbin et al., 2017).

Concordance measures usually take values between 0.5 and 1, where a value of 0.5 indicates no discrimination and a value of 1 indicates perfect discrimination.

In the context of survival analysis, various C-statistics have been formulated to deal with right-censored data, in this work will be shown Harrell's C-statistic (Harrell 1996).

Assessments of the discrimination ability of prognostic models have led to the development of several tools that extend the concept of discrimination as evaluated by the receiver operating characteristics (ROC) curve and the area under the receiver operating characteristic curve (AUC) of diagnostic settings. The next paragraphs will be devoted to briefly illustrating the methodology of the Harrell indicator, time-depended ROC curve and AUC function

3.4.1 Estimating Concordance Statistics

For the *i-th*individual ($1 \leq i \leq n$) in a sample, let T_i denote failure time and X_i the covariate value for subject *i*and $Z_i = \boldsymbol{\beta}'\boldsymbol{x}_i$ as a risk score or linear predictor computed from a Cox regression model.Let C_i denote the censoring time, Y_i=min(T_i, C_i) the follow-up time, and $\delta_i = 1$ if $T_i \leq C_i$, and $\delta_i = o$ if $T_i > C_i$.
We use the counting process $D_i(t) = 1$ if $T_i \leq t$ and $D_i(t) = 0$ if $T_i > t$ to denote failure (disease) status at any time t with $D_i(t) = 1$ indicating that subject i has had an event prior to time t.

For the ith individual, the Cox proportional hazards model assumes the hazard function to be

$$\lambda_i(t) = \lambda(t; \boldsymbol{Z}_i) = \lambda_0(t)exp\left(\boldsymbol{\beta}'\boldsymbol{x}_i\right) \qquad (3.18)$$

where $\lambda_0(t)$ is an arbitrary and unspecified baseline hazard function and $\boldsymbol{\beta}$ is the vector of true regression parameters that is associated with the explanatory variables. Let $\widehat{\boldsymbol{\beta}}$ denote the maximum partial likelihood estimates of $\boldsymbol{\beta}$.

Harrell's Concordance

Harrell (1996) defines the concordance probability as

$$C_H = \Pr\left(\boldsymbol{\beta}'\boldsymbol{x}_i \quad > \boldsymbol{\beta}'\boldsymbol{x}_j \quad | T_i < T_j, T_i < min\left(D_i, D_j\right)\right. \qquad (3.19)$$

Assuming that there are no ties in the event times and the predictor scores, $\hat{C}_H$ can be estimated as

$$\hat{C}_H = \frac{\sum_{i \neq j} \delta_i \, \mathrm{I}\left(y_i < y_j\right) \mathrm{I}\left(\hat{\beta}' x_i > \quad \hat{\beta}' x_j\right)}{\sum_{i \neq j} \delta_i \, \mathrm{I}\left(y_i < y_j\right)} \qquad (3.20)$$

For standard errors of $\hat{C}_H$, has been used the estimator derived based on the delta method (Kang et al. 2015).

3.4.2 Cumulative Dynamic ROC curve - cumulative sensitivity and dynamic specificity (C/D)

Besides the C-statistic, receiver operator characteristic (ROC) curves and AUC (area under the ROC curve) statistics are also commonly used to assess the discrimination ability of the model with binary outcomes. For survival models with time-to-event outcomes, ROC curves are computed at specific time points. Various definitions and estimators of time-dependent ROC curves and AUC functions have been proposed in the survival setting. Time-dependent ROC curves and AUC functions characterize how well the fitted model can distinguish between subjects who experience an event and subjects who do not. Whereas C-statistics provide overall measures of predictive accuracy, time-dependent ROC curves and AUC functions summarize the predictive accuracy at specific times. In practice, it is common to use several time points within the support of the observed event times (Changbin at al., 2017).

Heagerty and Zheng (Heagerty and Zheng, 2005) proposed three different definitions for estimating the above time-dependent sensitivity and specificity for censored event-times, namely cumulative/dynamic (C/D), incident/dynamic (I/D) and incident/static (I/S). In this work, cumulative/dynamic ROC curve will be shown. It is more appropriate to apply the C/D definitions when there is a specific time of

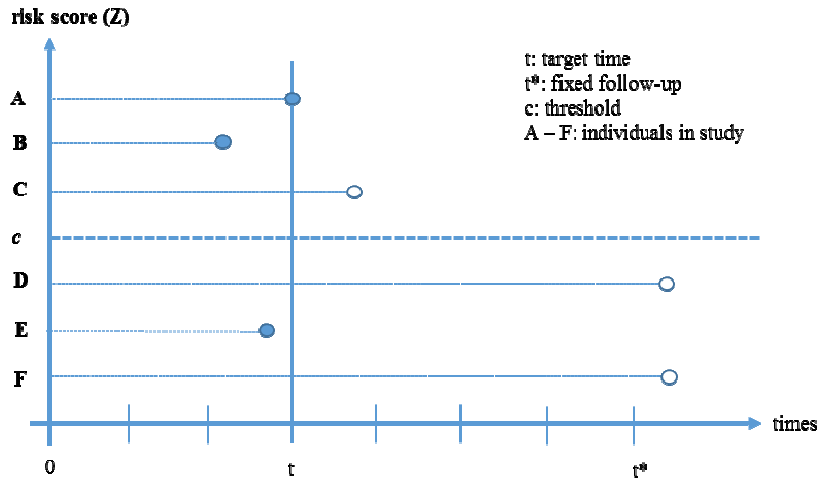
interest that is used to discriminate between individuals experiencing the event and those event-free prior to the specific time. This type of discrimination has more clinical relevance than the other definitions (I/D and I/S) and hence C/D definition has commonly been used by clinical applications (Kamarudin et al., 2017).

Recall that ROC curves display the relationship between a covariate X, and a binary disease variable $D_i$by plotting estimates of the sensitivity, and one minus the specificity, for all possible values threshold denoted by c (Heagerty et al., 2000).

At each time point t, each individual is classified as a case or control. A case is defined as any individual experiencing the event between baseline $t = 0$ and time t (individual A, B or E in Figure 3.2) and a control as an individual remaining event-free at time t (individual C, D or E in Figure 3.2). The cases and controls are changing over time and each individual may play the role of control at the earlier time (when the event time is greater than the target time, i.e. $T_i > t$) but then contributes as a case for later times (when the event time is less than or equal to the target time, i.e. $T_i \leq t$).

The cumulative sensitivity is the probability that an individual has a risk score computed from Cox regression model ($Z_i = \boldsymbol{\beta}'\boldsymbol{x}_i$) greater than c among the individuals who experienced the event before time t (individual A or B in Figure 3.2), and the dynamic specificity is the probability that an individual has a risk score computed from Cox regression model less than or equal to c among those event-free individuals beyond time t (individual D or F in Figure 3.2).

Figure 3.2 - Illustration for cases and controls of C/D: A, B and E are cases and C, D and F are controls (Kamarudin et al., 2017).



Thus, the sensitivity and specificity at time t and the resulting AUC(t) can be defined as

$$Se^{C}(c,t) = P(Z_i > c|D_i(t) = 1) \qquad (3.24)$$

$$Sp^{D}(c,t) = P(Z_i \leq c|D_i(t) = 0) \qquad (3.25)$$

$$AUC^{C,D}(t) = P\big(Z_i > Z_j \big| T_i \leq t, T_j > t\big), i \neq j \qquad (3.26)$$

The performance of the risk score or of the linear predictor computed from Cox regression model is evaluated by the area under the ROC curve (AUC) in which a higher AUC value indicates a better risk score performance (Kamarudin et al., 2017). The AUC is equal to the probability that the risk score results from a randomly selected pair of diseased and non-diseased individuals are correctly ordered. In a simpler way, the AUC is also equal to the probability of a diseased individual having a higher risk score than a healthy individual (Pepe, 2003).
Using these definitions, we can define the corresponding ROC curve for any time t, ROC(t).

Different methods have been proposed to estimate the time-dependent sensitivity and specificity, in this work,the estimator of Heagertywill be used(Heagerty et al.,2000).

In this work we will use the version of the estimator originally proposed by Heagerty, but later corrected by Akritas (3.27) to take into account and resolve some weaknesses as:

$$\hat{S}_{b_n}(t|Z = Z_i) = \prod_{s\epsilon T_n, s\leq t} \left\{1 - \frac{\sum_j K_{b_n}(Z_j, Z_i) I(Y_j = s)\delta_j}{\sum_j K_{b_n}(Z_j, Z_i) I(Y_j \geq s)\delta_j}\right\} \qquad (3.27)$$

Where $\hat{S}_{b_n}(t|Z = Z_i)$is a suitable estimator of the conditional survival function characterized by a parameter b_n (that is, a smoothed estimate of the conditional survival function) and T_n is the unique values of Y_i=min(T_i, C_i) for observed event, $\delta_i = 1$. Moreover,$K_{b_n}(Z_j, Z_i)$ is a kernel function that depends on a smoothing parameter b_n. Akritas (1994) uses a 0/1 nearest neighbor Kernel, $K_{b_n}(Z_j, Z_i) = I\{-b_n < \hat{F}_X(Z_i) - \hat{F}_X(Z_j) < b_n\}$, where $2b_n\varepsilon(0,1)$ represents the percentage of observations that is included in each neighborhood. The default value for b_n is 0.05.

3.5 Cross Validation to assessment performance of a survival prediction model

The purpose of a predictive model is to provide valid outcome predictions for new patients. Unfortunately, prediction models commonly suffer from a methodological problem, which is known as “overfitting”.

Since overfitting is a central problem in prediction modelling, we need to consider the validity of our model for new patients. There are statistical techniques to evaluate the internal validity of a model, i.e., for the underlying population that the sample originated from. Internal validation addresses statistical problems in the specification and estimation of a model “reproducibility”(Steyerberg et al., 2009).

Indeed, when learning from population samples, an important risk is that the data under study are well described, but that the predictions might not generalize to new subjects outside the study sample. We may capitalize on specifics and idiosyncrasies of that sample: this is referred to as “overfitting.”

Overfitting leads to a too optimistic impression of model performance that may be achieved in new subjects from the underlying population. Optimism is defined as true performance minus apparent performance, where true performance refers to the underlying population, and apparent performance refers to the estimated performance in the sample. Put simply: “what you see may not be what you get.” (Steyerberg et al., 2009).

Resampling methods are central techniques to correct overfitting and quantify optimism in model performance. They involve repeatedly drawing samples from a training set and refitting a model of interest on each sample in order to obtain additional information about the fitted model. For example, in order to estimate the variability of a linear regression fit, we can repeatedly draw different samples from the training data, fit a linear regression to each new sample, and then examine the extent to which the resulting fits differ. Such an approach may allow us to obtain information that would not be available from fitting the model only once using the original training sample.

The most common method used to controller overfitting is cross-validation (James et al., 2013). In general, there are two forms of validation, internal and external validation (Steyerberg et al., 2009).

Internal validation, involving training-testing splits of the available data or cross-validation, is a necessary component of the model building process and can provide valid assessments of model performance. External validation consists of assessing model performance on one or more datasets collected by different investigators from different institutions (Taylor et al., 2008).

Since we do not have an external validation cohort available, the internal validation, Leave One Out Cross Validation (LOOCV) approach, was used to evaluate the accuracy of predictive modeling of our survival data.

3.5.1 Leave-One-Out Cross-Validation (LOOCV)

LOOCV involves splitting the set of observations into two parts. A single observation is used for the validation set, and the remaining observations $(n - 1)$ make up the training set. In our case the multivariate Cox regression model is fit on the $(n - 1)$ training observations, and a linear prediction $(X'\hat{\beta})$, where a large value corresponds to shorter survival, is made for the excluded observation, using the value of its covariates. We can repeat this procedure by selecting the second subject for the validation data, training the Cox regression model on the $n - 1$ observations

(this time, reinserting the deleted subject in the previous step and removing the next one). A schematic of the LOOCV approach is illustrated in Figure 3.3.

Once the risk score of each subject has been calculated, the measures of performance (for instance: time dependent ROC Curve, AUCROC and C-statistic) were calculated on the dataset composed by the linear predictors of each left-one-out observation.Hight values of performance indexes in the validation cohort show that the model does not suffer of overfitting and that the parameter estimates from regression model can be used to predict the outcome in a new subject.

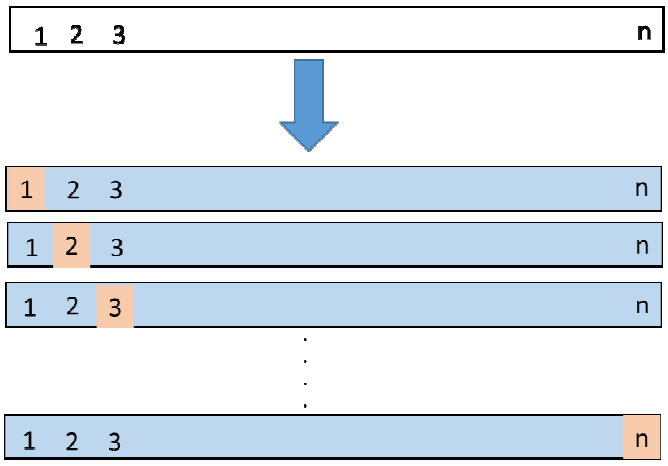


FIGURE 3.3 - A schematic display of LOOCV. A set of n data points is repeatedly split into a training set (shown in blue) containing all but one observation, and a validation set that contains only that observation (shown in beige). The first training set contains all but observation 1, the second training set contains all but observation 2, and so forth(James et al., 2013).

The advantages of LOOCV as compared to other methods are mainly two. First, we repeatedly fit the statistical learning method using training sets that contain $(n - 1)$ observations, almost as many as are in the entire data set. Consequently, the LOOCV approach tends not to overestimate the test error rate as much as the validation set approach does. Second, in contrast to the validation approach which will yield different results when applied repeatedly due to randomness in the training/validation sets, performing LOOCV multiple times will always yield the same results: there is no randomness in the training/validation set splits (James et al., 2013). The disadvantage could be an intensive computational work.

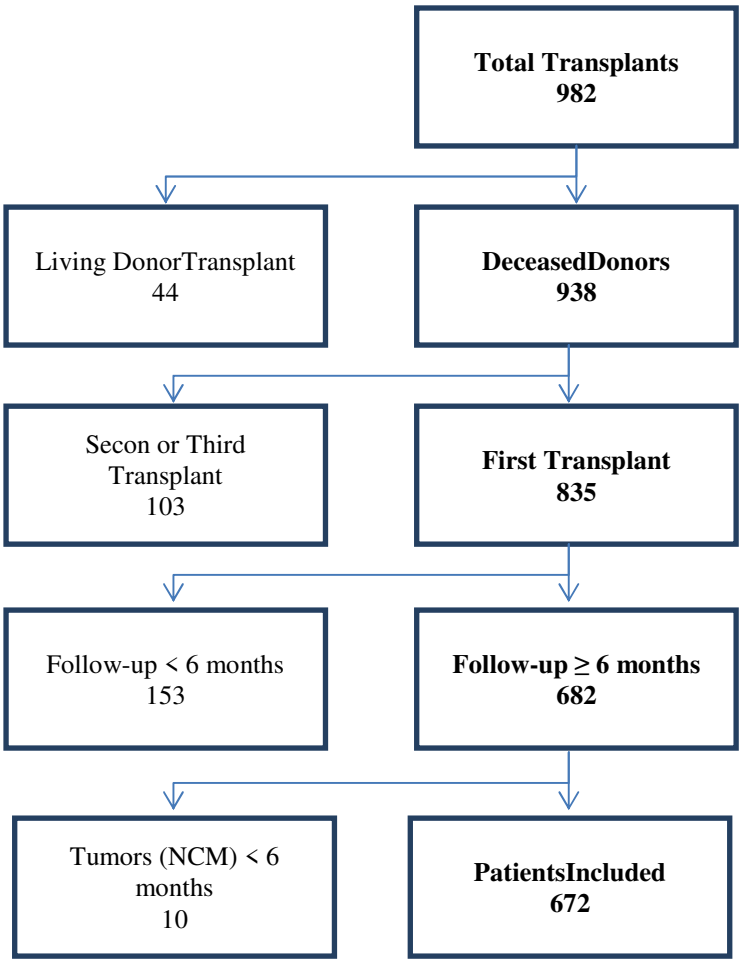
All statistical analyses were performed with SAS 9.4 (SAS institute, Cary, NC, USA) and R (R Foundation for Statistical Computing, Vienna, Austria) software.

4. Results

4.1 Population enrolled

We retrospectively analyzed a prospective cohort of 672 patients who have been transplanted in a single KTx center between November 1998 and March 2013. Adult patients receiving their first kidney transplant from a deceased donor at our transplant center have been included if they had a minimum follow-up of 6 months after KTx. In the same period in our center, 44 transplants were performed from a living donor and 103 patients received a second or third transplant and were not included in this analysis, as shown in flow-chart below (Figure 4.1).

Figure 4.1 - Flowchart of patient selection



Patients with a known active malignancy at the time of transplantation do not receive a KTx, and therefore, none of the included patients was known to carry a malignant disease at the time of kidney transplant. However, ten patients developed a NCM within 6 months from transplantation and were excluded as they might have had an undiagnosed malignancy before surgery, even if all KTx candidates underwent a strict pretransplant screening for malignancy and premalignant lesions.

Most of the enrolled patients were from Piedmont (66%), while most of the remaining patients came from Southern Italy and Isles (19% of study population).

Most enrolled patients received their transplant between 2001 and 2011: in the first years the transplant Center just opened and the total transplant volume was lower, while in later years (2012-2013) fewer patients reached the 6-months follow up with reliable data. In detail, we enrolled 210 (31.3%) patients who underwent kidney transplants between 1998 -2003, 277 (41.2%) between 2004-2008 and 185 (27.5%) between 2009-2013.

4.2 Outcome analysis: mortality and death censored graft survival (primary endpoint)

Of the 672 patients included in the study, median follow up since study entry (which is 6 months from KTx) was 4.70 years, from a minimum of 7 days to a maximum of 13.6 years. At the end of the follow up, patients on dialysis (ie: who developed graft failure, which is the primary endpoint) were 59 (8.8%), while 37 patients (5.5%) died with a functioning graft.

Moreover, 0.5% of patients were lost to follow up and 6% moved out (and therefore considered as lost to follow up) to other Transplant Centers (mainly Città della Salute, Ospedale San Giovanni Battista, Turin).

Mortality and graft failure incidence were estimated by competitive risk analysis and plotted in Figure 4.2. The causes of death are reported in Table 4.1, where the most common is cardiovascular accidents (32.4%). Among patients with a post-transplant malignancy, 25% died for malignancy during the follow up.

Figure 4.2 - Cumulative incidence of graft failure (bold line) and death (dashed line) adjusted using a competitive risk analysis; time is expressed in years after study entry (6 months after transplant).

Time from study entry	N. Event Graft loss	Graft loss (n=59) CIF% (CI95%)	N. Event Death	Death (N=37) CIF% (CI95%)
1 year	8	1.25 (0.59-2.37)	4	0.64 (0.21-1.56)
2.5 years	17	2.89 (1.74-4.48)	14	2.49 (1.42-4.04)
5 years	36	7.46 (5.30-10.1)	24	4.83 (3.17-7.01)
7.5 years	46	10.98 (8.07-14.40)	30	6.80 (4.62-9.38)
10 years	54	16.14 (11.83-21.04)	34	9.47 (6.32-13.37)

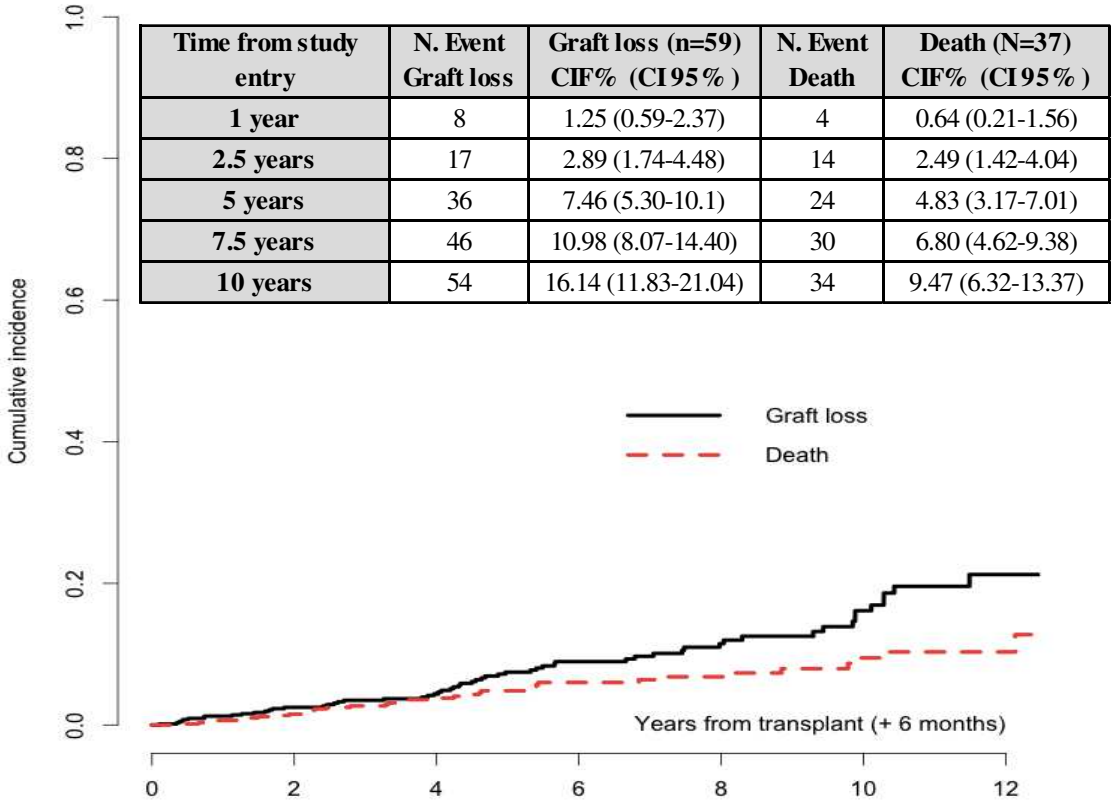
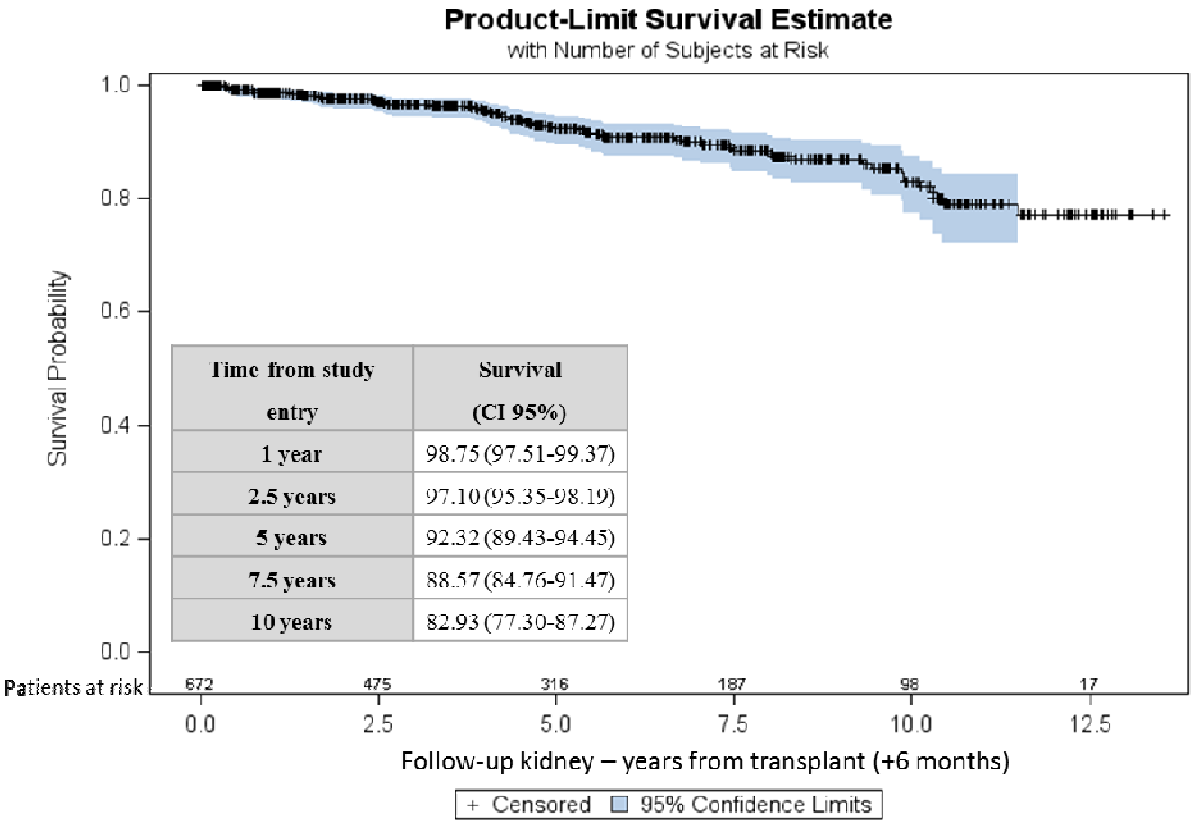


Table 4.1 - Causes of death stratified by the presence or absence of non cutaneous malignancy diagnosed after study entry (6 months from KTx)

	Overall (n=672)	**No NCM (n=632)**	**NCM (n=40)**
Deaths	**37 (5.5%)**	**27 (4.0%)**	**10 (25.0%)**
Causes of death			
Neoplasia	10/37 (27.0%)	-	10/10 (100%)
Major Cardiovascular event	12/37 (32.4%)	12/27 (44.4%)	-
Infectious disease	8/37 (21.6%)	8/27 (29.6%)	-
Other	7/37 (18.9%)	7/27 (22.2%)	-

Death censored graft survival was estimated and plotted with CIs in Figure 4.3 These results are in keep with other Italian cohorts (Report CNT 2014, Tessari et al., 2013, Piselli, et al., 2013a) and show a higher survival thanAmerican historical cohorts (Ojo et al., 2001, Report of USRDS of 2017).

Figura 4.3 - Death-censored graft survival, estimated over time since study entry (6 months from transplant), with 95% confidence intervals.



Time from study entry	Survival (CI 95%)
1 year	98.75 (97.51-99.37)
2.5 years	97.10 (95.35-98.19)
5 years	92.32 (89.43-94.45)
7.5 years	88.57 (84.76-91.47)
10 years	82.93 (77.30-87.27)

Causes of graft failure are reported in Figure 4.4 where the main one was, as expected, chronic rejection (62.7%). The stratification of causes of graft failure for presence of malignancy is reported in Table 4.2. As shown in Table 4.2, 17.5% of patients with a malignancy diagnosed after 6 months from transplant experiences graft failure, but only a minority (14.3%) for chronic rejection. Indeed, others causes in this group of patients are more common like “de novo” nephropathies and possibly malignancy-associated nephropathies (chemotherapy toxicity, infections, actinic nephritis, chronic obstruction and infections).

Table 4.2 - Causes of graft failure stratified by the presence or absence of NCM from study entry (6 months from KTx).

	Overall (n=672)	**No NCM (n=632)**	**NCM (n=40)**
Failed Grafts	**59 (8.7%)**	**52 (8.2%)**	**7 (17.5%)**
Cause of graft failure			
Chronic Rejection	37/59 (62.7%)	36/52 (69.2%)	1/7 (14.3%)
Relapsing nephropathy	3/59 (5.1%)	3/52 (5.8%)	-
De novo nephropathy	2/59 (3.4%)	-	2/7 (28.6%)
Acute Rejection	1/59 (1.7%)	1/52 (1.9%)	-
Chronic CNI Toxicity	1/59 (1.7%)	-	1/7 (14.3%)
Vascular/cardio-renal syndrome	3/59 (5.1%)	3/52 (5.8%)	-
Other	12/59 (20.3)	9/52 (17.3%)	3/7 (42.9%)

4.3 Cohort description and stratification by graft outcome

The main demographic and clinical characteristics of the cohort are described in the following tables. In order, there are variables describing recipients (Table 4.3, Table 4.4), donors (Table 4.5), transplant characteristics and admission (Table 4.6), and post transplant follow up at six months (Table 4.7).

Mean recipient age at six months from KTx was 51.7 ± 12.3 years, with a prevalence of male patients (61.9%) and a mean time on dialysis before transplant of 3.9 ±3.2 years (Table 4.3). In the entire cohort, there were 43/672 (6.4%) HCV positive patients, of which none received a graft from an HCV-positive donor and none of the recipients underwent a therapy with a direct-acting antiviral therapy before KTx (it was unavailable at that time). About a quarter of the enrolled patients had an unknown underlying nephropathy (23.5%) and their percentage of failed grafts (10.8%) looks in between the one of patients with a primary nephropathy (6.6%) and the one of those with a secondary nephropathy (13.2%; Table 4.4).Older donors (> 60 years) represent the 39% of overall donors, but as many 61% of donor meet the ECD criteria. However, KTR from a donor older than 60 years had a higher percentage of failed grafts (13.4%), as compared to KTR from donors between 40

and 60 (6.4%). Notably only 37/408 (9.1%) ECD were used for a dual transplant. (Table 4.5).

The median cold ischemia time (CIT) was 19 hours, but as many as 124/672 (18.6%) had a CIT greater than 24 hours (maximum 43.3 hrs). Indeed, among these 124 patients, the percentage of graft failure was 16.1% (vs. 7.2% in KTRs with a CIT < 24 hours). A delayed graft function was observed in 21.4% of transplants, and in these KTRs the percentage of graft failure was 16.0%. The main IS scheme used in the Novara Transplant Center was a basiliximab induction (antiIL2R 75.7%), Tacrolimus, Mycophenolate and Steroids (78%). Other schemes included for example mTOR inhibitors and were used in 105 patients (15.6%). Moreover, 35/672 (5.2%) patients needed an early (during the first two months from KTx) urological revision (for instance for urinary fistula or uretheral stenosis)and 15/372 (2.2%) a vascular revision (for instance for partial arterial thrombosis or renal artery stenosis) (Table 4.6).

Six months after KTx the acute rejection rate was 5.5%. The percentage of patients with a creatinine > 2 mg/dL was 30.3%, with a median creatinine of 1.6 (IQR: 1.3-2.0), while 16.0% of patients had urinary proteins greater than 0.5 g/24h: these patients had indeed a graft failure percentage of 18.9% (as compared to 6.2% of KTRs with a lower proteinuria)(Table 4.7).

Table 4.3 - Demographic characteristics of the recipients, stratified by graft outcome.

Recipients	**Total transplant 672**		**Functioning graft 613**		**Graft Failure 59**	
	N.	(% C.)	N.	(% R.)	N.	(% R.)
Sex						
female	256	38.1	228	89.1	28	10.9
male	416	61.9	385	92.6	31	7.5
Recipient age at KTx (years)						
0 - 40	135	20.1	126	93.3	9	6.7
>40 - 60	355	52.8	324	91.3	31	8.7
> 60	182	27.1	163	89.6	19	10.4
median (q_1-q_3) (min-max)	53 (43-61) (18-77)		52 (43-61) (18-77)		56 (49-62) (29-73)	
Previous renal replacement therapy						
Only hemodialysis	502	78.6	458	91.2	44	8.8
Only peritoneal dialysis	90	14.1	86	95.6	4	4.4
Both hemodialysis and peritoneal dialysis	47	7.4	39	83	8	17.0
missing	33		30		3	

Table 4.4 - Clinical and immunological characteristics of the recipients, stratified by graft outcome.

Recipients	Total transplant 672		Functioning graft 613		Graft Failure 59	
	N.	(% C.)	N.	(% R.)	N.	(% R.)
Total HLA mismatches						
0 - <4	474	70.5	426	89.9	48	10.1
>=4	198	29.5	187	94.4	11	5.6
HCV Serology (IgG)						
neg	629	93.6	574	91.3	55	8.7
pos	43	6.4	39	90.7	4	9.3
Underlying nephropathy						
Primary nephritis/nephropathy	393	58.5	367	93.4	26	6.6
Secondary nephropathy	121	18.0	105	86.8	16	13.2
Unknown	158	23.5	141	89.2	17	10.8
CMV Serology (IgG)						
negative	98	14.6	90	91.8	8	8.2
positive	566	84.2	515	90.9	51	9.0
missing	8		8		0	
Peak Panel Reactive Antibodies						
0	494	73.5	456	92.3	38	7.7
>0	167	24.9	146	87.4	21	12.6
missing	11		11		0	
median (q1-q3) (min-max)	0 (0-2.0) (0-100.0)		0 (0-0) (0-97.5)		0 (0-5.0) (0-100.0)	
EBV Serology (IgG)						
negative	36	5.4	33	91.7	3	8.3
positive	455	67.7	409	89.9	46	10.1
missing	181		171		10	
Body mass index (BMI)						
<= 25	413	62.1	380	92.0	33	8.0
> 25	252	37.9	227	90.1	25	9.9
missing	7		6		1	
median (q_1-q_3) (min-max)	23.9 (21.9-26.2) (15.9-40.5)		23.8 (21.8-26.2) (15.9-40.5)		24.5(22.5-26.6) (17.4-33.0)	

Table 4.5 - Demographic and main clinical characteristics of the donors, stratified by graft outcome.

Donor	Total transplant 672		Functioning graft 613		Graft Failure 59	
	N.	(% C.)	N.	(% R.)	N.	(% R.)
Sex						
female	339	50.5	309	91.2	30	8.9
male	332	49.5	303	91.3	29	8.7
missing	1		1			
Donor age at KTx (years)						
0 - 40	145	21.6	138	95.2	7	4.8
>40 - 60	265	39.4	248	93.6	17	6.4
>60	262	39.0	227	86.6	35	13.4
median (q_1-q_3) *(min-max)*	55 (42.5-67) (14-88)		54 (42-67) (14-88)		64 (50-71) (21-86)	
Extended criteria donors						
no	264	39.3	249	94.3	15	5.7
yes	408	60.7	364	89.2	44	10.8
Types of Deceased Donor Kidney						
single	635	94.5	581	91.0	54	8.5
dual	37	5.5	32	86.5	5	13.5
Donor CMV Serology (IgG)						
negative	95	14.8	88	92.6	7	7.3
positive	547	85.2	500	91.4	47	8.6
missing	30		25		5	

Table 4.6 - Transplant and KTx admission characteristics, stratified by graft outcome.

Transplant characteristics	Total transplant 672		Functioning graft 613		Graft Failure 59	
	N.	(% C.)	N.	(% R.)	N.	(% R.)
Cold ischemia time (hours)						
0 - <24	544	81.4	505	92.3	39	7.2
>=24	124	18.6	104	83.9	20	16.1
missing	4		4		0	
mediana (q_1-q_3) *(min-max)*	19 (16-22) (5.3-43.3)		19 (16-22) (5.2-43.3)		21.5 (18-24) (13.0-38.0)	
Induction therapy						
AntilL2R	509	75.7	471	92.5	38	7.5
ATG	85	12.7	69	81.2	16	18.8
none	78	11.6	73	93.6	5	6.4
Early surgical revision						
None	620	92.5	567	91.5	53	8.6
Urologic	35	5.2	31	88.6	4	11.4
Vascular	15	2.2	13	86.7	2	13.3
missing	2		2		0	
Blood transfusions during KTx admission (number)						
0	233	36.5	222	95.3	11	4.7
1 - 2	228	35.7	215	94.3	13	5.7
>2	178	27.9	147	82.6	31	17.4
missing	33		29		4	
mediana (q_1-q_3) *(min-max)*	1 (0.0-3.0) (0-26)		1 (0.0-3.0) (0-24)		3 (1-5) (0-26)	
Delayed graft function (DGF)						
no	528	78.6	492	93.2	36	6.8
yes	144	21.4	121	84.0	23	16.0
Maintenance IS therapy						
Tacrolimus – MMF/AZA ± steroids	524	78.0	487	92.9	37	7.1
Cyclosporine - MMF/AZA ± steroids	43	6.4	37	86.1	6	14.0
Other	105	15.6	89	84.8	16	15.2

Table 4.7 - Follow up characteristics before study entry (six months from transplant), stratified by graft outcome.

First 6 months post transplant (before study entry)	Total transplant 672		Functioning graft 613		Graft Failure 59	
	N.	(% C.)	N.	(% R.)	N.	(% R.)
One or more acute rejection episodes within 6 months						
no	635	94.5	588	92.6	47	7.4
yes	37	5.5	25	67.6	12	32.4
Urinary proteins at 6 months (g/24 h)						
0 - <0.5	557	84.0	519	93.2	38	6.2
>=0.5	106	16.0	86	81.1	20	18.9
missing	9		8		1	
median (q_1-q_3) *(min-max)*	0.20(0.1-0.4) (0-6.0)		0.20(0.1-0.3) (0-6.0)		0.35(0.2-0.8) (0-2.8)	
Serum creatinine at 6 months (mg/dL)						
0 - <2	466	69.7	446	95.7	20	4.3
>=2	203	30.3	165	81.3	38	18.7
missing	3		2		1	
median (q_1-q_3) *(min-max)*	1.6 (1.3-2.0) (0.6-6.0)		1.6 (1.3-2.0) (0.6-6.0)		2.25 (1.70-3.1) (1.0-4.6)	

4.4 Main study covariate: malignant tumors

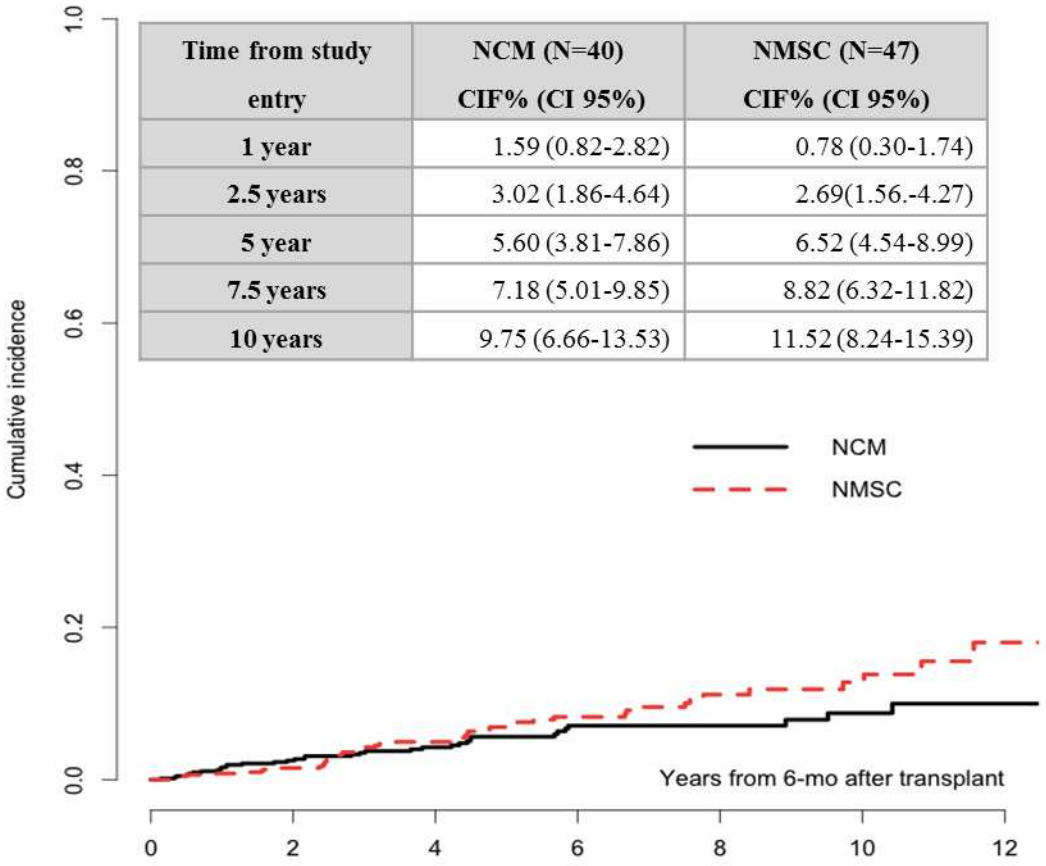
4.4.1 Malignancy incidence

Incident malignancies were re-classified as non-melanoma skin cancers (NMSC) and other malignant tumors (Non-Cutaneous Malignancies, NCM), including solid and hematological malignancies.

During the follow up, 47 patients developed a NMSC and 40 a NCM. Cumulative incidence function was estimated from study entry (six months from transplant) for NCM and NMSC, using a competitive risk analysis to adjust for patient death (Figure 4.4).

Figure 4.4 - Cumulative incidence of non-cutaneous malignancy (NCM, bold line) versus nonmelanoma skin cancer (NMSC, dashed line) from study entry; time is expressed in years after study entry (6 months after transplant)

Time from study entry	NCM (N=40) CIF% (CI 95%)	NMSC (N=47) CIF% (CI 95%)
1 year	1.59 (0.82-2.82)	0.78 (0.30-1.74)
2.5 years	3.02 (1.86-4.64)	2.69(1.56.-4.27)
5 year	5.60 (3.81-7.86)	6.52 (4.54-8.99)
7.5 years	7.18 (5.01-9.85)	8.82 (6.32-11.82)
10 years	9.75 (6.66-13.53)	11.52 (8.24-15.39)



4.4.2 Description of post-transplant malignancies

Malignancies were classified according to ICD-9 codes (Table 4.8): as expected PTLDs, breast and kidney tumors are the most common malignancies after KTx.

Table 4.8 - Details of observed malignancies and graft failure and death events.

Type of tumor	**Observed Malignancies N (%)**	**Graft Failure**	**Death with functioning graft**
151 - Malignant neoplasm of stomach	1 (2.5)	-	-
153 - Malignant neoplasm of colon	2 (5.0)	-	1
156 - Malignant neoplasm of gallbladder and extrahepatic bile ducts	1 (2.5)	1	-
162 - Malignant neoplasm of trachea. bronchus. and lung	2 (5.0)	-	2
163 - Malignant neoplasm of pleura	1 (2.5)	-	1
172 - Malignant melanoma of skin	2 (5.0)	1	-
174 - Malignant neoplasm of female breast	6 (15.0)	-	2
176 - Kaposi's sarcoma	1 (2.5)	-	-
185 - Malignant neoplasm of prostate	4 (10.0)	1	1
188 - Malignant neoplasm of bladder	2 (5.0)	-	-
189 - Malignant neoplasm of kidney and other and unspecified urinary organs	5 (12.5)	2	-
193 - Malignant neoplasm of thyroid gland	1 (2.5)	-	-
197 - Secondary malignant neoplasm of respiratory and digestive systems	1 (2.5)	-	1
200 - Lymphosarcoma and reticulosarcoma and other specified malignant tumors of lymphatic tissue	6 (15.0)	1	1
202 - Other malignant neoplasms of lymphoid and histiocytic tissue	1 (2.5))	-	1
203 - Multiple myeloma and immunoproliferative neoplasms	3 (7.5)	1	-
204 - Lymphoid leukemia	1 (2.5)	-	-
Overall - NCM	**40**	**7**	**10**
173 - NMSC	**47**	**4**	**1**

The characteristics of patients with a malignancy are reported in Table 4.9: patients who developed a NMSC and NCM were mainly males (68.1% and 62.5% respectively), as most KTx recipients are males (61.8%).NMSC were diagnosed at a

median age of 62.7 (median 3.2 years after KTx) and NCM at a median age of 59.0 years (median 3.0 years after KTx). Post-transplant immunosuppressive therapy was similar to our overall cohort, but patients with a NCM were more likely to reduce their maintenance IS after malignancy diagnosis: 19/40 (47.5%) of patients with a NCM reduced IS therapy versus 35/632 (5.5%) of the other patients (see below, chapter 4.7).

To be noted, the mean post-malignancy follow-up was 3.7 years for NMSC and 2.3 years after a NCM, which may be short to detect long term complications (ie: chronic rejection). However, the observed events of graft failure or death (after a malignancy) interestingly developed early after diagnosis of a NCM (1.1 and 1.0 years respectively), but "late" after a NMSC (3.9 and 5.6 years respectively). Indeed, these observations are consistent with diagnosis of NMSC at an earlier stage as compared to NCM.

Among NCM, solid malignancies (n = 29/40; 72.5%), which mainly were carcinomas, were characterized by a median age at tumor diagnosis of 59.4 years and a time from KTx of 2.2 years. Hematological malignancies (n = 11/40; 17.5%) were mainly lymphomas and were diagnosed at a median age of 58.3 years and 3.8 years from KTx. Among patients who developed a solid malignancy 24.1% did not receive any induction therapy (while this percentage was 11.6% in the entire cohort) and "only" 62.1% received a tacrolimus-mycophenolate based maintenance IS (while this percentage was 78.0% in the entire cohort). Among patients who developed an hematologic malignancy 27.3% received a lymphocyte-depleting induction therapy (while this percentage was 12.7% in the entire cohort) and 90.9% received a tacrolimus-mycophenolate based maintenance IS.

Among patients with a solid malignancy, 27.6% died at a median time from diagnosis of 1.0 year, while 17.2% had a graft failure in a median time of about one month. On the other side, patients with an hematologic malignancy, 18.2% died at a median time from diagnosis of 4.5 year, while 18.2% had a graft failure in a median time of 2.7 years. None of these failed grafts was due to acute rejection.

Table 4.9 - Main characteristics of patients who developed a malignancy after study entry. Data are expressed as n/N (%) or median (IQR)

Parameter	NMSC (n=47)	NCM (n=40)	NCM Solid (n=29)	NCM Hematological (n=11)
Age at tumor diagnosis (years)	62.7 (54.8-68.9)	59.0 (50.4-68.1)	59.4 (50.8-67.8)	58.3 (44.0-72.5)
Time from KTx to mal. diagnosis				
Median in years(q1-q3)	3.2 (2.4-5.7)	2.97(1.02-5.69)	2.16 (1.05-4.50)	3.81 (0.36-5.87)
Male Recipients	32/47 (68.1)	25/40 (62.5)	16/29 (55.2)	9/11 (81.8)
Induction therapy				
Anti IL2 receptor	27/47 (57.5)	24/40 (60.0)	17/29 (58.6)	7/11 (63.6)
ATG	9/47 (19.2)	8/40 (20.0)	8/29 (17.2)	3/11 (27.3)
None	11/47 (23.4)	8/40 (20.0)	7/29 (24.1)	1/11 (9.1)
Maintenance IS therapy				
Tacrolimus – MMF/AZA +/- steroids	31/47 (66.0)	28/40 (70.0)	18/29 (62.1)	10/11 (90.9)
Cyclosporine– MMF/AZA +/- steroids	2/47 (4.3)	3/40 (7.5)	3/29 (10.3)	0/11 (0.0)
Other	14/47 (30.0)	9/40 (22.5)	8/29 (27.6)	1/11 (9.1)
Acute rejection episodes within 6 months	4/47 (8.5)	3/40 (7.5)	3/29 (10.3)	0/11 (0.0)
Serum creatinine at 6 months				
≥2 mg/dL	19/47 (40.4)	17/40 (42.5)	12/29 (41.4)	5/11 (45.5)
Urinary proteins at 6 months				
≥0.5 g/24h	3/47 (6.4)	5/40 (12.5)	3/29 (10.3)	2/11 (18.2)
Last available status				
Alive with functioning graft	40/47 (85.1)	23/40 (57.5)	16/29 (55.2)	7/11 (63.6)
Graft failure	5/47 (10.6)	7/40 (17.5)	5/29 (17.2)	2/11 (18.2)
Death with functioning graft	2/47 (4.3)	10/40 (25.0)	8/29 (27.6)	2/11 (18.2)
Follow-up time after tumor				
Median in years (q1-q3)	3.70 (1.85-5.57)	2.29 (0.91-4.28)	2.13 (0.87-4.01)	2.72 (1.07-4.42)
Alive with functioning graft	3.4 (1.8-5.6)	3.52 (1.54-5.25)	3.61 (1.83-5.56)	2.72 (1.17-4.42)
Graft failure	3.9 (2.9-5.1)	1.1 (0.1-3.6)	0.1 (0.1-3.0)	2.7 (1.0-4.2)
Death with functioning graft	5.6 (4.6-6.7)	1.0 (0.4-1.2)	1.0 (0.6-1.2)	4.5 (0.02-8.9)

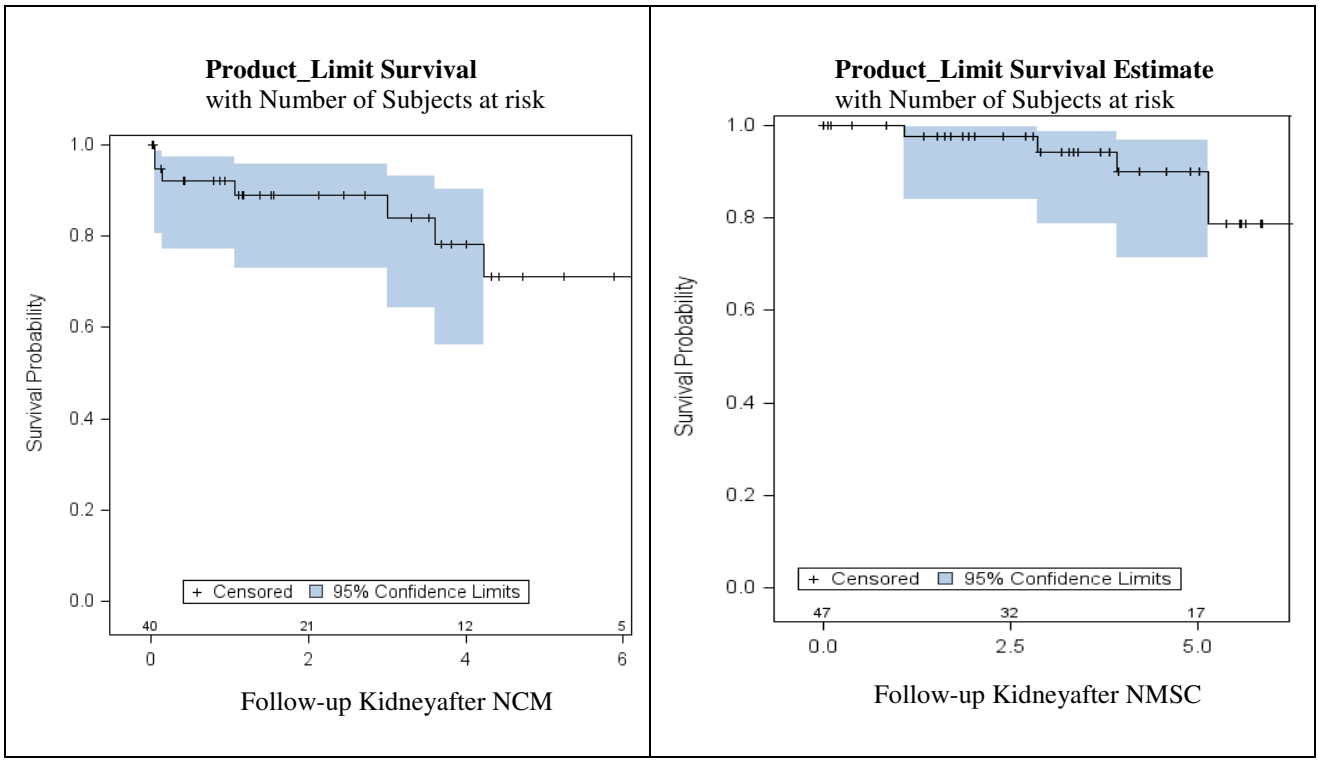
4.4.3 Post-Malignancy outcomes

The three-year patient survivals after a NMSC and NCM diagnosis were 100% (95%CI: 100%-100%) and 77% (95%CI: 59%-88%) respectively.

As the primary endpoint of this study was death-censored graft survival, the survival function was estimated in patients with a malignancy, starting from its diagnosis. This is a sub-optimal method to evaluate the impact of malignancies on graft survival and is because patients with early diagnoses and patients with late diagnoses -who might have a failing graft independently from malignancy- are put together: therefore, this analysis is not able to compare directly patients with a malignancy with those without one.

Interestingly, death-censored graft survival, at five years (Figure 4.5), was better in patients with a NMSC (90%) rather than patients with a NCM (71%): this may be explained by different reasons, but probably NMSC do not have a major impact on graft survival, as they are usually treated with minor surgery.

Figura 4.5 – Death censored graft survival after the diagnosis of NCM (left panel) and NMSC (right panel). The shaded areas represent the 95%CI.

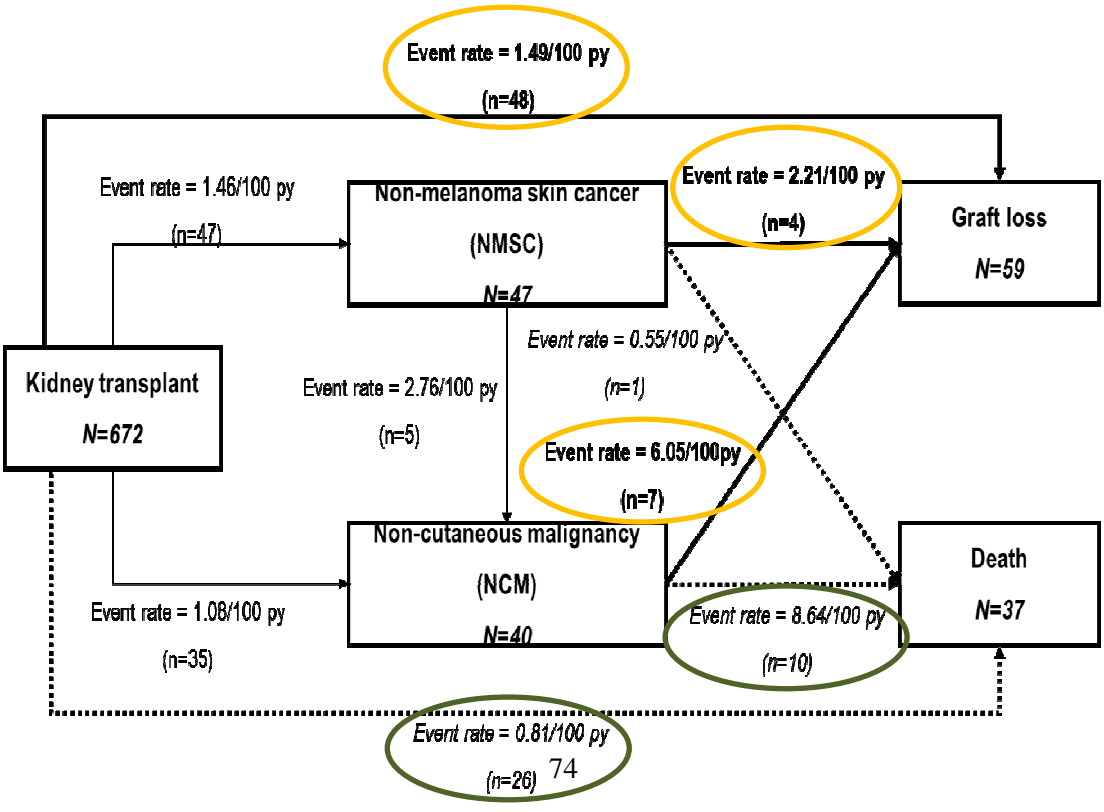


We developed a multi-state description of the potential transplant complications (development of NMSC, NCM, graft failure or death) and mean event rate for the switch from one state to another (Figure 4.6). For instance, the mean event rate of developing a NCM was 1.08/100pt-yr: 35 patients had a NCM as the first event, while 5 had had a NMSC before the NCM. Among 40 patients with a NCM, 7 developed graft failure (6.05 ev/100 pt-yr) and 10 died (8.64 ev/100pt-yr).

The rate of death in patients who never had a known malignancy was 0.81/100pt-yr, the one of patients who had only a NMSC was 0.55/100pt-yr, while the one of patients who developed a NCM was 8.64/100pt-yr: as expected, NMSCs are not associated with mortality, while NCMs are associated with an increased mortality rate.

Interestingly, the rate of graft failure after NCM is higher (6.05/100pt-yr) than after a NMSC (2.21/100pt-yr) or without any malignancy (1.49/100pt-yr). This analysis might be more accurate in describing the relationship between graft failure and malignancies than post-malignancy graft survival itself. However, it is not able to determine the extent of the association between malignancies and graft failure, so we performed a survival analysis with time-dependent covariates adjusted by baseline risk factors (see section 4.5).

Figure 4.6 - The multi-state process for graft failure. Diagram of the observed transitions: bold lines are transitions to graft failure and dotted lines are transitions to death with a functioning graft.



4.4.4 Univariate analysis of tumor diagnosis on death-censored graft survival

In time-to-event analyses, the primary event of interest was graft failure, while deaths with a functioning graft were censored observations. Six months after KTx was considered as the baseline time for all the analyses.

To illustrate the effect of tumor (NMSC or NCM) occurrence over time on the risk of graft failure, we used a modified Kaplan-Meier method that estimates cumulative incidence of graft failure according to the presence or absence of tumor. All patients at the beginning of the observations were included in the tumor-free group, and the assignment to the tumor group was updated at the time of the tumor diagnosis (Figure 4.7-4.8). This is different from a standard Kaplan-Meyer method, in which patient stratification is performed at baseline.

Figure 4.7 - Cumulative incidence of graft failure in patients with (dashed line) and without nonmelanoma skin cancer (NMSC, bold line); time is expressed in years after study entry (6 months after transplant).

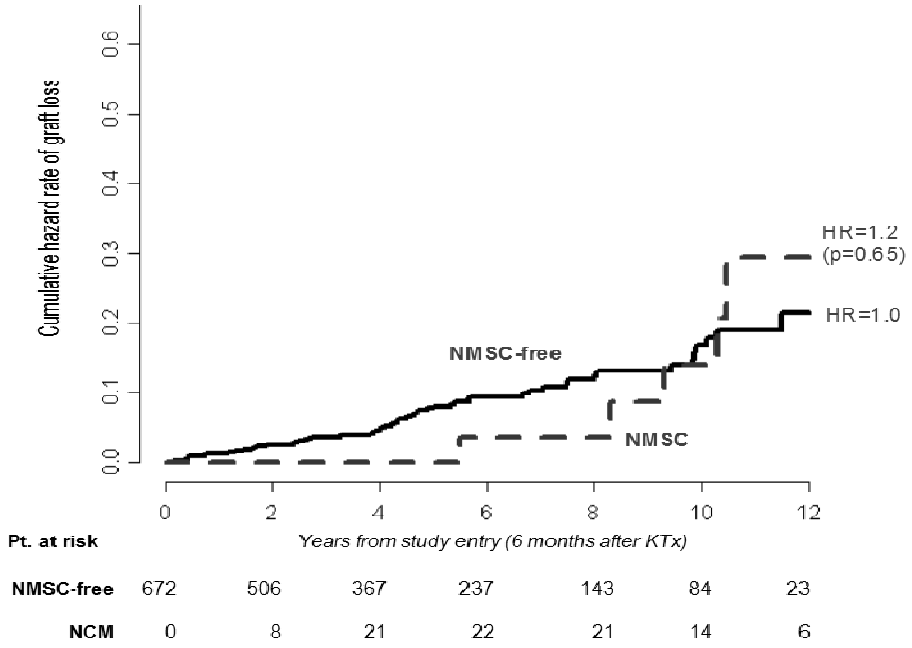
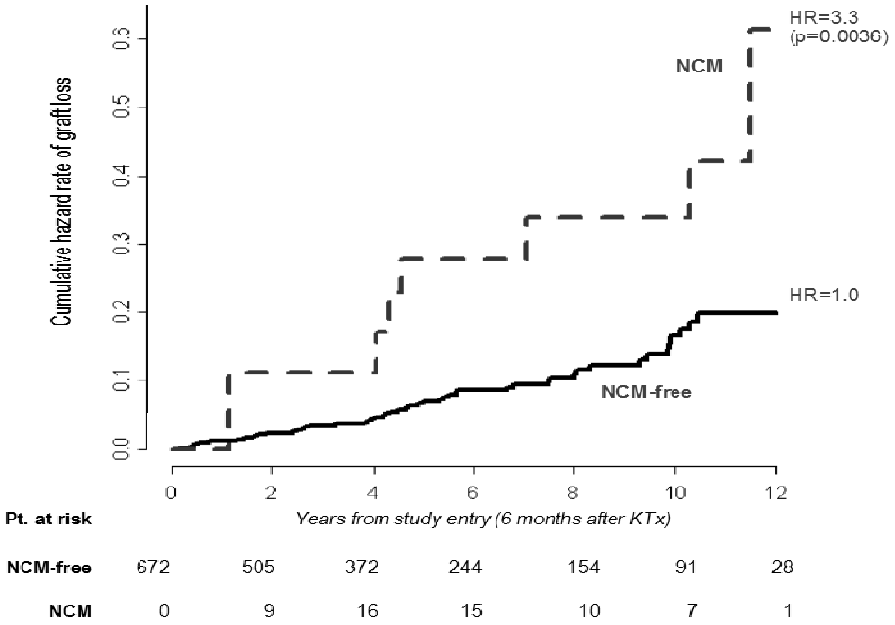


Figure 4.8 -Cumulative incidence of graft failure in patients with (dashed line) and without non cutaneous malignancies (NCM, bold line); time is expressed in years after study entry (6 months after transplant).



To quantify the tumor effect in terms of hazard ratio, we fitted a univariable Cox model in which patient's status (with or without tumor) was updated at the time of diagnosis. The hazard ratio from the Cox regression model where NCM was treated as a time-dependent covariate, was 3.31 (95% CI: 1.48-7.42, p=0.004). The occurrence of a NMSC was, on the contrary, not associated with the graft failure risk (HR=1.24, 95% CI 0.49-3.18, p=0.7). This observation is in keep with the baseline and outcome measures in NMSC and NCM patients, in which NMSC seem to have a much smaller impact than NCM on post-transplant complications.

Even if the association between NCM and graft failure seems to be strong, an adjustment for time-fixed confounders must be performed.

4.5 Impact of malignancies on death censored graft failure adjusted by known risk factors

4.5.1 Identification of baseline risk factors (univariate analysis)

We looked for time-fixed risk factors for death censored graft failure, in order to adjust later estimates of the study variables (NMSC and NCM).We performed a univariate Cox survival analysis considering graft failure as the outcome event and censoring patients at the time of last visit or death with a functioning graft.

Risk factors were chosen by known and potential risk factors from literature.

The covariates with the most significant association with graft failure (Tables 4.10 and 4.11) were recipient age ($p=0.0009$), donor age ($p<0.0001$), extended criteria donor ($p<0.0005$), DGF ($p<0.0001$), blood transfusions in the post-operation days ($p<0.0005$), acute rejection episodes ($p<0.0001$), urinary proteins ($p<0.0001$), creatinine ($p<0.0001$), cold ischemia time ($p<0.006$) and underlying nephropathy ($p<0.003$).

Among them, recipient and donor age are usually matched as in our Center kidneys are allocated with a relatively good age match between donor and recipient (linear correlation between donor and recipient age: $R=0.67$; $p<0.0001$). Indeed, most of the confirmed risk factors in this cohort may be related to the donor renal function, including donor age, ECD (and type of transplant), DGF and -to some extent- post-transplant creatinine. Moreover, DGF has been associated in literature with cold ischemia time and need for blood transfusions: in this cohort these associations were confirmed ($p = 0.0002$ and $p < 0.0001$ respectively).

Table 4.10 – Univariate Cox regression model results for pre-transplant characteristics

Recipients	p-value	HR (IC 95%)
Sex		
male		1.00
female	0.19	1.41 (0.84- 2.34)
Recipient age at KTx (years)		
0 - 40		1.00
>40 - 60	0.10	1.84 (0.88-3.9)
> 60	0.008	3.96 (1.32-6.60)
age as continuous covariate	0.0009	1.04 (1.02-1.06)
Transplant year		
1998 - 2003		1.00
2004 - 2008	0.07	1.81 (0.94-3.47)
2009 -2013	0.05	2.71 (1.02-8.85)
Types of Deceased Donor Kidney		
Single		1.00
Double	0.02	2.93 (1.15-7.44)
Total HLA mismatches		
0 - <4		1.00
>=4	0.26	0.68 (0.35-1.32)
Underlying nephropathy		
Primary nephritis/nephropathy		1.00
Secondary nephropathy	0.001	2.75 (1.47-5.16)
Unknown	0.013	2.17 (1.17-4.00)
Peak PRA		
0		1.00
>0	0.73	1.09 (0.64-1.87)
Peak PRA in countinuous	0.74	1.00 (0.99-1.02)
Donor		
Donor age		
0 - 40		1.00
>40 - 60	0.13	1.96 (0.81-4.74)
>60	0.0001	5.56 (2.44-12.63)
Donor age in countinuous	0.0001	1.04 (1.02-1.06)
Donor ECD		
no		1.00
yes	0.0005	2.84 (1.57-5.12)

Table 4.11– Univariate Cox regression model results for transplant and post-transplant characteristics

Transplant characteristics	p-value	HR (IC 95%)
Cold ischemia time (hours)		
0 - <24		1.00
>=24	0.006	2.14 (1.25-3.67)
Cold ischemia time (hours) in countinuous	0.006	1.06 (1.02-1.11)
Induction therapy		
AntilL2R		1.00
ATG	0.18	1.49 (0.83-2.70)
none	0.008	0.27 (0.1-0.71)
Early surgical revision		
None		1.00
Urologic	0.5	1.42 (0.51-3.91)
Vascular	0.6	1.41 (0.34-5.81)
Blood transfusions during KTx admission (number)		
0		1.00
1 - 2	0.64	0.82 (0.37-1.85)
>2	0.003	2.85 (1.43-5.70)
Blood transfusions during KTx admission in continuous	0.0005	1.09 (1.04-1.15)
Delayed graft function (DGF)		
no		1.00
yes	0.0001	3.03 (1.79-5.13)
Maintenance IS therapy		
Tacrolimus – MMF/AZA ± steroids		1.00
Cyclosporine - MMF/AZA ± steroids	0.67	0.82 (0.34-1.99)
Other	0.39	1.30 (0.71-2.4)
One or more acute rejection episodes within 6 months		
no		1.00
yes	0.0001	4.15 (2.20-7.84)
Urinary proteins at 6 months (g/24 h)		
0 - <0.5		1.00
>=0.5	0.0001	3.65 (2.12-6.30)
Urinary proteins at 6 months (g/24 h) in continuous	0.0001	2.45 (1.96-3.06)
Serum creatinine at 6 months (mg/dL)		
0 - <2		1.00
>=2	0.0001	4.80 (2.79-8.26)
Serum creatinine at 6 months (mg/dL) in continuous	0.0001	2.9 (2.27-3.70)

4.5.2 Death censored graft survival analysis of post-transplant malignancies adjusted by baseline confounders

Among the significant baseline covariates at the univariate analysis, there were a lot of collinear variables and regressors that are referred mainly to the same biological characteristic (see above). Therefore, we chose the mostly significant covariates based on clinical representation of the underlying biological process: given that the study entry was at six months from kidney transplant, we chose *baseline creatinine* as the measure of baseline graft function (which is influenced by donor renal function, ischemia time, DGF, and post-transplant complications), *urinary proteins* and *acute rejection episodes* as a marker of immunological activation and graft ongoing degenerative processes, *underlying nephropathy* (which was not associated with other covariates), and *donor age* as a "summary variable" for donor characteristics and as it is strongly related to recipient age. When adding DGF to the model including creatinine, acute rejections and urinary proteins, this variable was not significantly associated to graft failure and did not change the parameter estimates of the other variables: this observation is in keep with the good representation of early transplant events by the 6-months creatinine, which is indeed the strongest predictor of graft survival in literature.
Moreover, few not significant adjustment variables were added because they were considered a mandatory adjustment by the clinician (recipient sex and transplant year).

We therefore performed the primary outcome analysis for the main study variable (death censored graft survival) adjusted for the potential confounders. NCM and NMSC were studied separately as they represent two different subsets of malignancies (from risk factors for their development to their prognosis). Both study variables were included as time-dependent variables in the multivariate Cox regression model (Tables 4.12, 4.13).

From this analysis, the main results are:

- Non-cutaneous malignancies were statistically associated with graft failure (HR 3.27, 95%CI 1.44-7.44, p-value 0.005), and its HR was similar to the one from the univariate analysis (HR 3.3 95%CI 1.48-7.42, p-value 0.004)
- Non melanoma skin cancers were not statistically associated with graft failure (HR 0.80, 95%CI 0.30-2.14, p-value 0.66), and its HR was similar to the one from the univariate analysis (HR 1.24, 95%CI 0.49-3.18, p-value 0.7)
- Parameter estimates did not change after adjustment for potential confounders.

Moreover, the "adjustment" model seems to be solid (see validation section 4.8), confirming the risk factors known from literature.

As expected, patients with a NMSC are different from those with a NCM (risk factors, mortality, therapy) and given the lack of association between NMSC and graft failure in both univariate and multivariate analyses, this variable was not further investigated.

Table 4.12 - Multivariable Cox regression analysis for death-censored graft failure. Model A: multivariate Cox model including only adjustment-variables; Model B: multivariate Cox model including non-cutaneous malignancy and adjustment variables. The diagnosis of a NCM was included as a time-dependent covariate in the Cox model.

	Model A			**Model B**		
Covariate	$\beta \pm SE(\beta)$	**p-value**	**HR (IC 95%)**	$\beta \pm SE(\beta)$	**p-value**	**HR (IC 95%)**
Non-cutaneous malignancy *(time dependent)*	-	-	-	1.19±0.42	0.005	**3.27 (1.44-7.44)**
Creatinine (mg/dL) >=2 vs <2	1.10 ± 0.31	0.0005	3.00 (1.62-5.55)	1.08±0.32	0.0006	2.95 (1.59-5.47)
Proteinuria (g/24h) >=0.5 vs <0.5	0.83 ± 0.29	0.004	2.30 (1.30-4.08)	0.83±0.29	0.005	2.28 (1.28-4.06)
Acute rejection episode Yes vs No	1.12 ± 0.34	0.001	3.04 (1.56-6.00)	1.15±0.34	0.0008	3.14 (1.61-6.14)
Donor age 10-years increase	0.28 ± 0.10	0.005	1.33 (1.09-1.62)	0.29±0.10	0.004	1.34 (1.10-1.64)
Gender Female vs Male	0.72 ± 0.27	0.009	2.05 (1.20-3.51)	0.79±0.28	0.004	2.21 (1.28-3.81)
Year of transplant	0.31 ± 0.26	0.24	1.36 (0.81-2.27)	0.29±0.26	0.27	1.34 (0.80-2.25)
Underlying nephropaty						
Secondary vs Primary	0.62 ± 0.33	0.06	1.87 (0.99-3.53)	0.59±0.33	0.07	1.80 (0.94-3.43)
Unknow vs Primary	0.54 ± 0.33	0.09	1.72 (0.91-3.26)	0.58±0.33	0.07	1.79 (0.94-3.40)

Table 4.13 Multivariable Cox regression analysis for death-censored graft failure. Model A: multivariate Cox model including only adjustment variables; Model B: multivariate Cox model including non melanoma skin cancer and adjustment variables The diagnosis of a NMSC was included as a time-dependent covariate in the Cox model.

	Model A			**Model B**		
Covariate	$\beta \pm SE(\beta)$	**p-value**	**HR (IC 95%)**	$\beta \pm SE(\beta)$	**p-value**	**HR (IC 95%)**
NMSC *(time dependent)*	-	-	-	-0.22 ± 0.50	0.66	0.80 (0.30-2.14)
Creatinine (mg/dL)						
>=2 vs <2	1.10 ± 0.31	0.0005	3.00 (1.62-5.55)	1.10 ± 0.31	0.0004	3.01(1.63-5.56)
Proteinuria (g/24h)						
>=0.5 vs <0.5	0.83 ± 0.29	0.004	2.30(1.30-4.08)	0.82 ± 0.29	0.006	2.26 (1.27-4.02)
Acute rejection episode						
Yes vs No	1.12 ± 0.34	0.001	3.04 (1.56-6.00)	1.13 ± 0.34	0.001	3.09 (1.58-6.03)
Donor age						
10-years increase	0.28 ± 0.10	0.005	1.33 (1.09-1.62)	0.29 ± 0.10	0.005	1.33 (1.09-1.64)
Gender						
Female vs Male	0.72 ± 0.27	0.009	2.05 (1.20-3.51)	0.70 ± 0.28	0.01	2.00 (1.17-3.46)
Year of transplant	0.31 ± 0.26	0.24	1.36 (0.81-2.27)	0.30 ± 0.26	0.25	1.35 (0.81-2.26)
Underlying nephropaty						
Secondary vs Primary	0.62 ± 0.33	0.06	1.87 (0.99-3.53)	0.63 ± 0.33	0.05	1.88 (0.99-3.56)
Unknow vs Primary	0.54 ± 0.33	0.09	1.72 (0.91-3.26)	0.54 ± 0.33	0.09	1.72 (0.91-3.26)

4.6 Risk factors for graft failure by cause of graft failure

To investigate the cause-specific graft failure, we divided grafts failed for chronic rejection and those failed for other causes, including infectious (BK virus, etc), vascular and relapsing or "*de novo"* nephropathies. Overall 59 patients failed their graft, of which 39 (66.1%) due to chronic rejection and 20 due to other causes (33.9%).

Among the 40 patients with a NCM, 7 patients developed graft failure. One of them (1/7 = 14%) failed his graft due to chronic rejection and 6/7 (86%) due other causes, including two "de novo" nephropathies, two chronic pyelonephritides, one graft nephrectomy and one chronic CNI toxicity

Among patients without an NCM (n=632), 52 developed graft failure: 38/52 graft failed due to rejection (73.1%) and 14/52 for other causes (26.9%), including a relapsing or "de novo" nephropathy (6/52 = 11.5%), cardio-renal syndrome (4/52 = 7.7%) and 4 for other causes (4/52 = 7.7%).

Even if only seven grafts failed after a NCM, we tried to investigate the effects of NCM on cause-specific graft failure, by stratifying the previous multivariate Cox regression model by cause of graft failure.

We therefore developed two models, one including graft failure due chronic rejection as the event, and one including as event only graft failure due to other causes, considering in each model the alternative cause of graft failure as censoring event. The heterogeneity of the effect of tumor occurrence on the cause-specific graft failure (chronic rejection versus other causes) was assessed comparing the hazard ratios estimated from two time-dependent multivariable Cox models (Table 4.14). Indeed, NCM was not a risk factor for graft failure due to chronic rejection while it was a major risk factor for graft failure due to other causes ($p < 0.001$) and this different effect was actually statistically different (heterogeneity test between HR p=0.002).

The hazard ratios associated with baseline creatinine and donor age were not particularly different between the two models (Table 4.14), as they represent the baseline renal function which impacts on the prognosis of any further kidney damage. On the other side baseline proteinuria and rejection episodes were

statistically associated with graft failure due to chronic rejection (as expected), while they did not show an association with graft failure due to other causes.

Table 4.14 - Multivariable-adjusted Cox model, considering malignancies as a time-dependent variable stratified by cause-specific graft failure. Model A: Multivariable-adjusted Cox model for chronic rejection (N=39); Model B: Multivariable-adjusted Cox model for other causes (N=20)

	Graft failure due to chronic rejection			**Graft failure due to other causes**		
Covariate	$\beta \pm SE(\beta)$	**p-value**	**HR (IC 95%)**	$\beta \pm SE(\beta)$	**pvalue**	**HR (IC 95%)**
NCM si vs no	-0.60± 1.02	0.56	0.55 (0.07-4.08)	2.75 ± 0.54	0.0001	15.59 (5.43-44.76)
Creatinine (mg/dL) >=2 vs <2	0.95 ± 0.38	0.01	2.59(1.23-5.47)	1.40 ± 0.58	0.02	4.06 (1.31-12.59)
Proteinuria (g/24h) >=0.5 vs <0.5	1.01 ± 0.35	0.004	2.73 (1.37-5.46)	0.43 ± 0.52	0.40	1.54 (0.56-4.27)
Acute rejection episode Yes vs No	1.25 ± 0.40	0.002	3.49 (1.57-7.76)	1.03 ± 0.66	0.12	2.79 (0.77-10.14)
Donor age	0.25 ± 0.12	0.04	1.28 (1.01-1.62)	0.41 ± 0.19	0.03	1.52 (1.05-2.2)
Gender Female vs Male	0.85 ± 0.34	0.01	2.35 (1.21-4.56)	0.58 ± 0.51	0.25	1.79 (0.66-4.88)
Year of transplant	0.13 ± 0.34	0.69	1.14 (0.58-2.25)	0.56 ± 0.45	0.21	1.75 (0.73-4.19)
Underlying nephropaty						
Secondary vs Primary	0.72 ± 0.39	0.07	2.06 (0.96-4.45)	0.26 ± 0.61	0.67	1.90 (0.39-4.24)
Unknow vs Primary	0.48 ± 0.41	0.24	1.61 (0.73-3.59)	0.65 ± 0.57	0.25	1.92 (0.63-5.81)

Then we tried to investigate why NCM were associated with more graft failures due to other causes: indeed, a possible explanation is that patients with a NCM commonly undergo to a reduction of their maintenance IS and to some local or systemic therapies to treat the malignancy, which may be nephrotoxic.

4.7 Role of IS therapy reduction on graft failure in patients with and without NCM

Overall, 54/672 (8.0%) patients had a significant reduction of their overall IS burden, of which 19 had a NCM (47.5% of patients with an NCM) and 35 had not (5.5% of patients without an NCM). After reduction of IS none of the patients returned to a full dose IS. The causes of IS reduction in patients with a NCM was the diagnosis of

malignancy itself or the need of a chemotherapy with similar side effects. On the other side, for patients without NCM, IS was reduced because of infections in 11/35 patients (31.4%), CNI nephrotoxicity in 6/35 (17.1%), other side effects in 14/35 (40.0%) and for a minimization protocol in 4/35 (11.4%). Indeed, only in a minority of these cases IS was reduced due to an "overimmunosuppression" (recurrent infections, 31%);among the other patients (69%) IS was reduced for side effects, while having an adequate immunosuppressive burden.

Among patients with a NCM, 3 grafts failed in patients who reduced IS (3/19 = 15.8%) and 4 grafts failed in patients with a standard IS (4/21 = 19.0%), while 6 patients died after an IS reduction (6/19 = 31.6%) and 4 on standard IS (4/21 = 19.0%). Even with a small numerosity the number of patients who developed graft failure was not different between NCM patients with and without an IS reduction.

Among patients without a NCM, 7 grafts failed in patients who reduced IS (7/35 = 20.0%) and 45 grafts failed in patients with a standard IS (45/597 = 7.5%), while 2 patients died after an IS reduction (2/35 = 5.7%) and 25 on standard IS (25/597 = 4.2%). In patients without a NCM, the reduction of IS seemed to be associated with more graft failures, therefore we evaluated the interaction between the reduction of immunosuppression and the occurrence of NCM on graft failure, by measuring the mean graft failure rate in different timeframes (Figure 4.9): without malignancy (blue line) with standard IS (left column), without malignancy (blue line) with a reduced IS (right column), after malignancy diagnosis (red line) with standard IS (left column), and after malignancy (red line) with a reduced IS (right column).

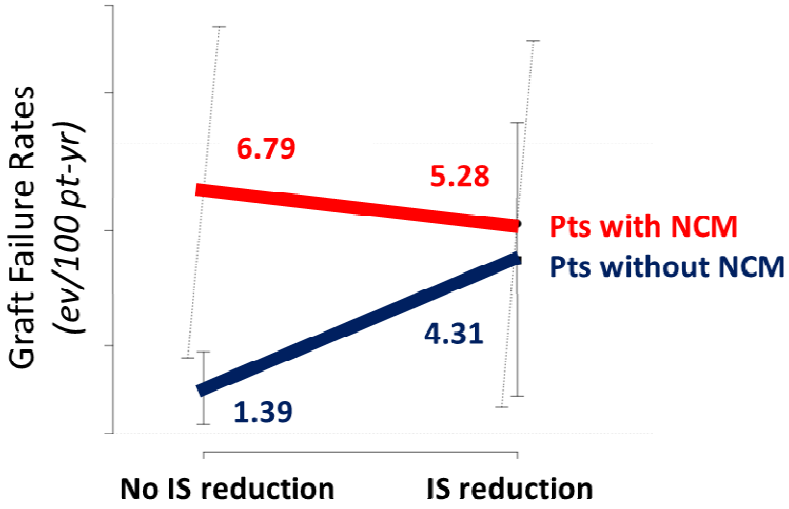


Figure 4.9: Graft failure rate in different timeframes, according to reduction of the IS therapy and the occurrence of NCM. For each patient the observation time was split into the following periods:

(v) free from NCM and treated with full dose of IS therapy (1.39 ev/100 pt-yr)

(vi) free from NCM and treated with a reduced dose of IS therapy (4.31 ev/100 pt-yr)

(vii) with NCM and treated with full dose of IS therapy (6.79 ev/100 pt-yr)

(viii) with NCM and treated with a reduced dose of IS therapy (5.28 ev/100 pt-yr)

The incidence rate of graft failure after NCM was not affected by a reduced IS, being 5.3/100pt-yr (95% CI 1.2– 22.9) in patients with a reduced IS and 6.8/100pt-yr (95% CI 1.8–25.3; ratio = 0.78) in patients maintained on standard IS. However, an IS reduction seemed to be associated with a higher rate of graft failure in patients without a NCM: in patients who reduced their IS, it was 4.3/100pt-yr (95% CI 1.45–12.84) and in patients on a standard IS, it was 1.4/100pt-yr (95% CI 1.0–1.9; ratio = 3.12). The test for interaction between IS reduction and NCM diagnosis on the risk of graft failure, calculated from a Poisson regression model, gave a P-value of 0.11. This finding is consistent with the distribution of graft failure causes between patients with and without NCM, being chronic rejection the main cause in 29% of failed graft in NCM-patients and 73.1% in non-NCM patients. Moreover, in patients without NCM only a minority (31.4%) reduced IS without being "over-immunosuppressed", but for drug toxicities.

4.8 Risk factors for graft failure among patients with a NCM

Given that NCMs were associated to graft failure, but not to graft failure due to chronic rejection, and that other risk factors associated to graft failure in the entire cohort were not associated to graft failure in patients with a NCM, we tried to identify which tumor-specific or tumor-related risk factors were associated to graft failure. These variables are evaluated only in patients with an NCM diagnosis, so the analysis was limited to these 40 patients, in which only 7 grafts failed. The specific cause of graft failure in NCM patients were one chronic rejection, one myeloma kidney, one immunotactoid glomerulonephritis, two chronic pyelonephritides, of which one after a radical prostatectomy, one graft nephrectomy for a renal cell carcinoma of the transplanted kidney and one chronic CNI toxicity.

Among them, 26/40 (65%) underwent open surgery, with either radical/therapeutic or palliative indication, 10/40 (25%) underwent radiotherapy, with either a adiuvant, therapeutic or palliative indication, and 17/40 (41.5%) were treated medically, as an adiuvant or therapeutic (ie: PTLDs) indication.

Therefore, we checked if there was any significant association between NCM therapies and graft failure among patients with a NCM, grouping therapies as chemotherapy (HR 0.86; 95%CI 0.19-3.84), radiation therapy (HR 0.44; 95%CI 0.05-3.68), and surgery (HR 0.46; 95%CI 0.10-2.10), using a univariate Cox regression model.

We estimated event rates of graft failure and death among NCM-patients stratified by single cancer therapies and categories, albeit sample size was small (Table 4.15).

Table 4.15 - Event rates of graft failure and death among NCM-patients stratified by single cancer therapies

		Total Event	Follow-up yy	graft faliure	Rate of Graft failure (ev/100pt-yr)	Death	Rate of death (ev/100pt-yr)
Tumor category							
	Urinary Tract	6	15.38	1	6.5 (0.3-32.1)	1	6.5 (0.3-32.1)
	Virus-related	12	54.90	3	5.5 (1.4-14.9)	1	1.8 (0.1-9.0)
	All other	22	45.46	3	6.6 (1.7-18.0)	8	17.6 (8.1-33.4)
Drug therapy							
	Cytotoxic	7	33.44	1	2.9 (0.1-14.8)	1	2.9 (0.1-14.8)
	Hormone therapy	6	19.82	1	5.0 (0.3-24.9)	1	5.0 (0.3-24.9)
	Other drug therapy	3	3.12	0	0.0	1	32.1 (1.6-158.1)
	None	21	55.21	5	9.1 (3.3-20.1)	5	9.1 (3.3-20.1)
Drugs							
	Cyclophosphamide	5	29.17	1	3.4 (0.2-16.9)	1	3.4 (0.2-16.9)
	Rituximab	5	25.73	0	0.0	1	3.8 (0.2-19.2)
	Triptoreline	4	11.45	1	8.7 (0.4-43.1)	0	0
	Tamoxifen	4	16.68	0	0.0	1	5.9 (0.3-29.6)
	Adriamycin	4	24.93	0	0.0	1	4.0 (0.2-19.8)
Radiotherapy							
	Not abdominal nor pelvic	6	12.39	0	0.0	3	24.2 (6.2-65.9)
	Local (brachytherapy)	2	9.26	0	0.0	1	10.8 (0.5-53.3)
	Pelvic	3	7.86	1	12.7 (0.1-6.3)	0	0.0
	No radiation therapy	29	86.23	6	6.9	6	6.9 (2.8-14.5)

The most common drug therapies were cyclophosphamide and rituximab (5 patients), followed by triptorelin, tamoxifen and adriamycin; indeed, four patients received an R-CHOP for a non-Hodgkin lymphoma. Interestingly patients receiving rituximab did not develop any chronic rejection: this drug is now used not only for B-cell malignancies, but also as an IS agent in transplant recipients.

Three patients underwent pelvic radiotherapy, of which one developed graft failure (12.7 ev/100-pt-yrs), while none of the patients treated with radiotherapy in other districts developed graft failure.

4.9 Model Validation

A critical task in the model building process is accessing the model's predictive capability systematically. Two important aspects of a prediction model are

calibration, ability of the model to correctly rank the individuals in the sample by risk, and discrimination, model ability to correctly classify subjects for their actual outcome. There are a variety of methodologies to assess the performances of a prediction model.

The concordance statistic (or C-statistic) is the most commonly used discrimination measure: it is the proportion of pairs of subjects whose observed and predicted outcomes agree (concordant pair) among all possible pairs in which one subject experiences the outcome of interest and the other subject does not. In the context of survival analysis, various C-statistics have been formulated to deal with right-censored data, we have used Harrell's Concordance index.

Besides the C-statistic, receiver operator characteristic (ROC) curves and AUROC (area under the ROC curve) statistics are also commonly used to assess the discrimination ability of the model. For survival models with time-to-event outcomes, ROC curves are computed at specific time points. Time-dependent ROC curves and AUC functions characterize how well the fitted model can distinguish between subjects who experience an event and subjects who do not. Whereas C-statistics provide overall measures of predictive accuracy, time-dependent ROC curves and AUC functions summarize the predictive accuracy at specific times.

For both C-statistics and AUROC, concordance measures are between 0.5 and 1, where a value of 0.5 indicates no discrimination and a value of 1 indicates perfect discrimination.

The "final" model was tested for death-censored graft survival including NCM as time-dependent covariate (Table 4.12, Model B), which is the primary aim of this project.

The concordance (C) statistics

The value of the Harrell's c-statistics, which is an overall measure of predictive accuracy of the model, in the study cohort is 0.82 ± 0.03 (95%CI: 0.76 - 0.88): there are 16569 concordant pairs, 3599 discordant pairs, 6 pairs that are tied in the linear predictor, and 0 pairs that are tied in the follow-up time. This result shows a good measure of the predictive accuracy of the model (ie: c-statistic > 0.80).

AUROC

The corresponding time-dependent ROC curve and the time-dependent area under the ROC curve (AUC) at different times (yearly, from 1 to 6 years) are represented by the green ROC curves in Figure 4.10. The AUROC of the study cohort was 0.93 at the first year, and 0.78 at five years.

We can conclude that our model has good predictive accuracy measurements as shown also by the AUROC indicator, whose value - at the different time points- are well over 0.70, which is considered as a good threshold to identify reliable models. As expected the accuracy of the model worsens a little as the time goes by, because of a reduced sample size of the cohort and because most variables are measured at the baseline.

4.9.1 A leave-one-out cross validation to assess performance of a survival prediction model.

Since prediction model is typically developed based on a single study, model validation often refers to the assessment of predictive performance. The generalizability consisted of reproducibility (internal validity) and transportability (external validity). Predictive performance (or accuracy) should be evaluated based on the patients that are not included in the model development. Validation hence is an important aspect of the process of predictive modelling. To assess the potential overfitting of our prediction model, we used an internal cross-validation method: indeed, if performance indexes of the validation cohort (ie: C-statistic and time-dependent ROC curves) are high (ie: greater than 0.80), the prediction model developed in the study cohort is unlikely to be overfitted.

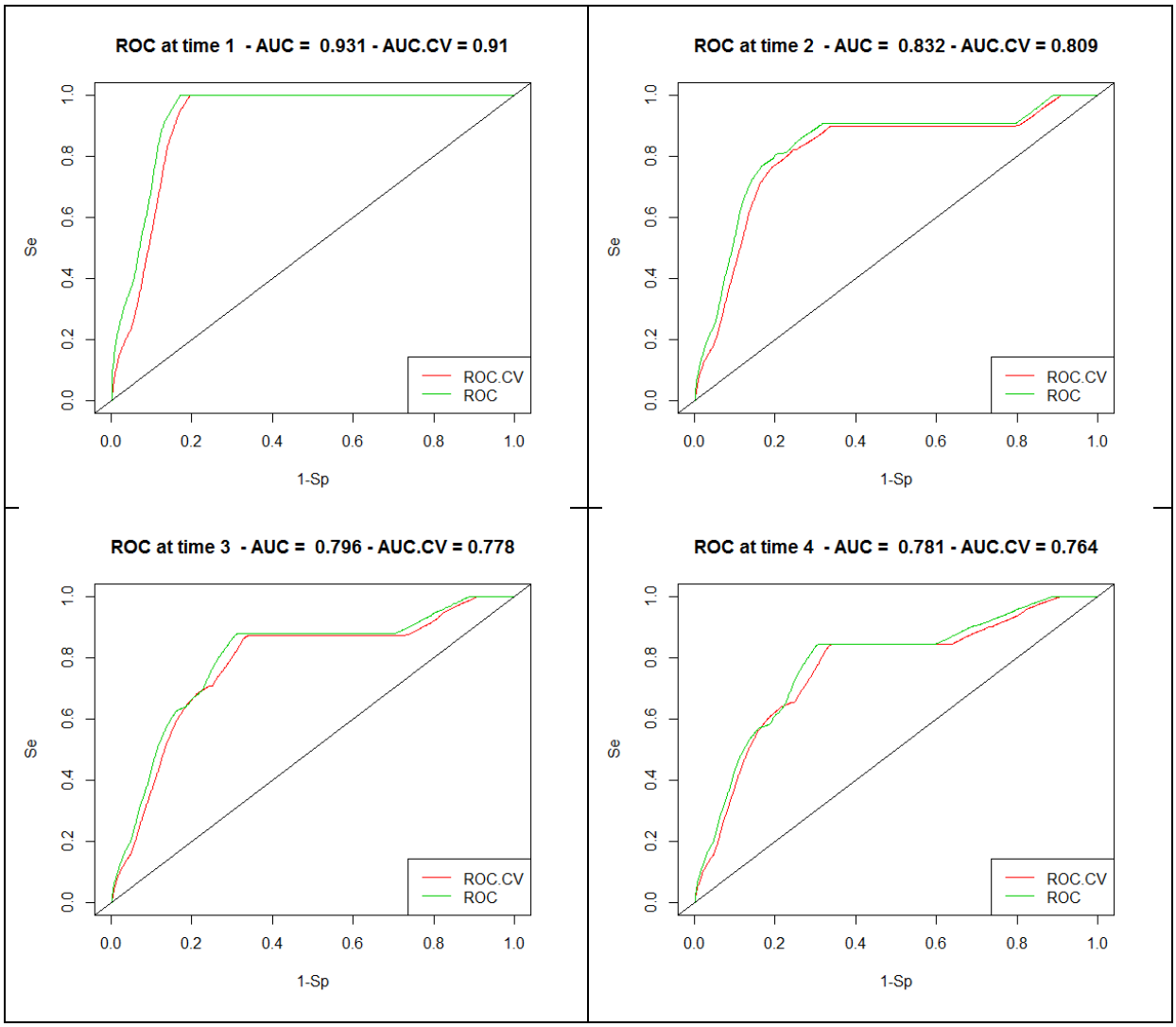
The value of C-statisticswas 0.80 (95%CI: 0.72 - 0.88) for the cross-validated cohort with 16177 concordance pairs, 3997 discordance pairs, 0 pairs that are tied in the linear predictor, and 0 pairs that are tied in the follow-up time.

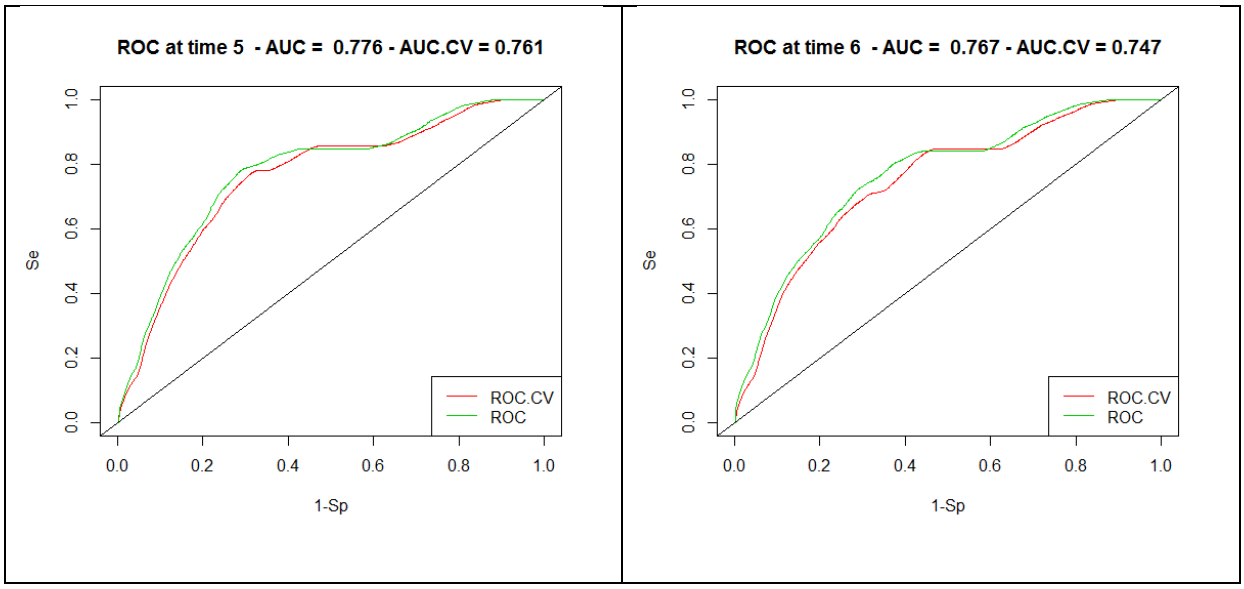
For the validation cohort, the corresponding time-dependent ROC curve and the time-dependent area under the ROC curve (AUC) at different times (yearly, from 1

to 6 years) are plotted in Figure 4.10. The values of AUROC over time in the validation cohort are between 0.75 and 0.91, with lower values for later years.

Therefore, we can conclude that this model is not likely to be overfitted, as shown from performance measures after internal cross validation and so we can use the parameter estimates from this Cox regression model to predict the outcome in a new subject (see Section 4.10).

Figure 4.10 - ROC and AUROC values of study (green) and validation (red) cohort at different time points (1, 2, 3, 4, 5, 6 years from study entry).





4.10 Estimating individual Survival from Cox Regression Models

The risk of long term graft failure is difficult to estimate for each individual transplant. The Cox proportional hazard model is a nonparametric model, which does not require knowledge of the underlying distribution of predictors, so it can estimate the hazard ratios of baseline and time-dependent risk factors.

However, it also shows the average survival rate during a specific survival time with the mean of covariates. Thus, it is possible to create a survival curve for each individual patient using the results of the Cox proportional hazard model developed in the study cohort.

To give an example of this approach applied to our study, we estimated the individual survival function in four different "prototypic patient profiles": low risk patient with a young donor (A), low risk, but older donor (B), high risk and very old donor (C), and high immunological risk, but with a "low risk" donor (D). These profiles are not necessarly those encountered in the everyday clinical practice, but may be helpful in the understanding of individual survival estimates. The NCM status (ie: whether or not the patient has developed a malignancy) will be added with further analysis (section 4.10.2).

Table 4.16: Definition (by predictors included in the Cox regression model) of the four profiles of prototype-patients.

Patient	Creatinine	Proteinuria	Acute rejection episode	Donor Age	Underlying nephropathy	Sex
A – young, low risk	<2	<0.5	No	50	Primary	Male
B – Low risk, older	<2	<0.5	No	65	Primary	Male
C – high risk, bery old	>=2	>=0.5	No	75	Unknown	Male
D – young, low risk, but immunologically active	<2	<0.5	Yes	50	Unknown	Female

4.10.1 Individual survival estimation

The individual survival function can be estimated by baseline regression coefficients and the estimated survival function of an hypothetical patient whose covariates are all "null" (ie: reference group or "0" for continuous variables). Therefore, the individual survival function can be derived by the following formula:

$$\hat{S}(t,X) = \left[\widehat{S_0}(t)\right]^{e^{\sum_{i=1}^{p}\widehat{\beta}_i x_i}}$$

where $\left[\widehat{S_0}(t)\right]$ *is defined as baseline survival of a hypothetical subject with variable scoring of zero at a given timepoint; and where Prognostic Index (PI) is the sum of regression coefficients, each multiplicated by the scoring of the corresponding variable.*

$$PI = \sum_{i=1}^{p}\widehat{\beta}_i x_i$$

Therefore, we could evaluate for each patient a Prognostic Index (PI), which is defined as the sum of regression coefficients, each multiplied by the scoring of the corresponding variable. For instance, a male patient who had a creatinine < 2,0 mg/dL, urinary proteins < 0.5 g/24h, no acute rejection episodes, and with a primary underlying nephropathy, who has received a transplant from a 50-yrs old donor, has a PI of: (0 x 0.72) + (0 x 1.10) + (0 x 0.83) + (0 x 1.12) + (0 x 0.54) + (50 x 0,028) = 1.4 (Table 4.17)

Table 4.17: Sample of calculation of prognostic index in the four prototypic patient profiles

Covariate	Parameter Estimate	Profile A	Profile B	Profile C	Profile D
Creatinine (mg/dL) >=2 vs <2	1.10	0	0	1	0
Proteinuria (g/24h) >=0.5 vs <0.5	0.83	0	0	1	0
Acute rejection episode Yes vs No	1.12	0	0	0	1
Donorage	0.028	50	65	75	50
Gender Female vs Male	0.72	0	0	0	1
Year of transplant	0.31	0	0	0	0
Underlying nephropaty					
Secondary vs Primary	0.62	0	0	0	0
Unknow vs Primary	0.54	0	0	1	1
Prognostic Index		**1.4**	**1.82**	**4.57**	**3.78**

We could estimate the expected death-censored graft survival for every given PI at different time points, using the formula $\hat{S}(t,X) = \left[\widehat{S_0}(t)\right]^{e^{\sum_{i=1}^{p}\widehat{\beta}_i x_i}}$

This procedure is able to give an estimate of the individual patient risk of graft failure, which is the probability of having a functioning graft after a given time from known covariates.

The survival probability for the four "patient-prototypes" are shown in Figure 4.11.

For instance, a low risk, young patient (profile A) has a 5-years death censored graft survival of 99%, and even if the donor is slightly older (profile B) the survival is almost the same. However, if a similar patient has had an acute rejection (profile D), her 5-yrs survival worsens to 91%.

On the other side, a high risk older patient, with a baseline residual chronic kidney disease (profile C) has a lower survival at five years (80%).

Figure 4.11 - Survival function estimate of the four different patient profiles.

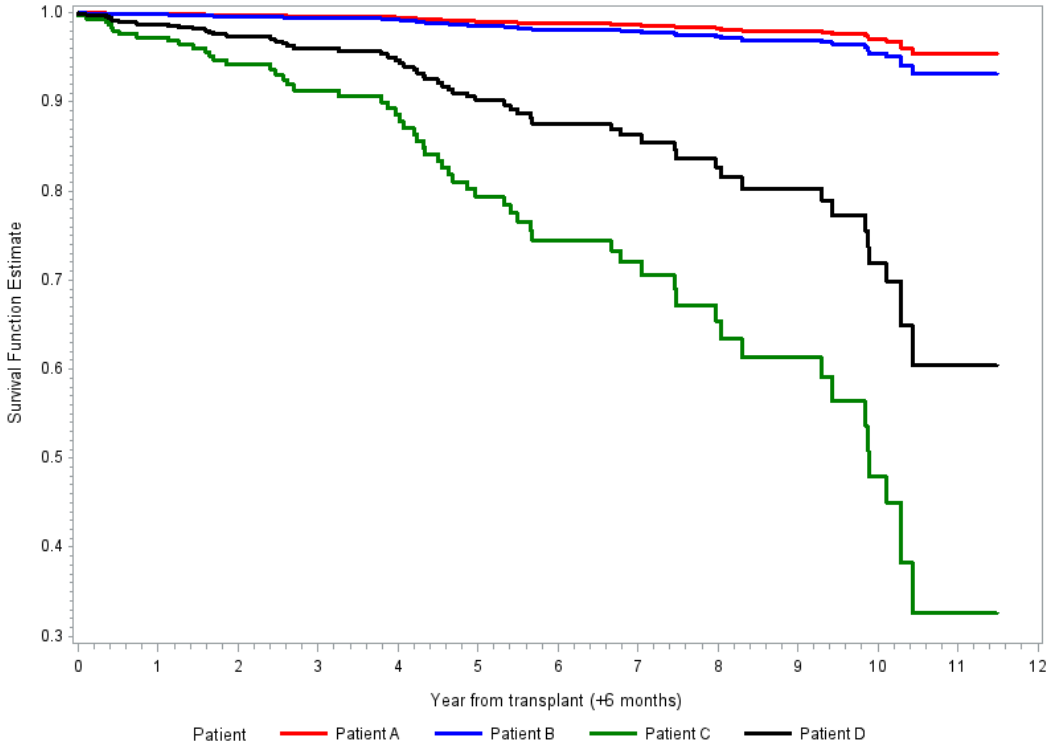


Table 4.18 – Probability of Surviving after 6 months from KTx

Patient	1 year	3 year	5 year	7 year
A	1.00 (1.00-1.00)	1.00 (1.00-1.00)	0.99 (0.98-1.00)	0.99 (0.97-1.00)
B	1.00 (1.00-1.00)	0.99 (0.99-1.00)	0.99 (0.97-1.00)	0.98 (0.96-1.00)
C	0.98 (0.94-1.00)	0.91 (0.81-1.00)	0.80 (0.61-1.00)	0.73 (0.50-1.00)
D	0.99 (0.97-100)	0.96 (0.91-1.00)	0.91 (0.81-1.00)	0.87 (0.74-1.00)

4.10.2 Survival estimates including a time-dependent covariate (diagnosis of non-cutaneous malignancies)

Indeed, a strength of this study is the inclusion of a time-dependent covariate and the use of the Cox model including this variable is particularly interesting. However, the inclusion of a time dependent covariate is particularly difficult because the prognostic index (PI) has to be evaluated at different time-points, ideally at the time of tumor diagnosis. However, given the relatively small numerosity of the sample

size, we needed to group NCM diagnoses by time of diagnosis and sample numerosity: we choose to create four time-varying covariates based on having a NCM diagnosis on year 0-1, 1-3, 3-5 or greater than 5. The time-varying covariates are defined as follows:

```
if stop > t2ncm_td and status_ncm=1 and 0<=t2ncm_td<=1then status_ncm1=1; else
status_ncm1=0;
if stop > t2ncm_td and status_ncm=1 and 1<t2ncm_td<=3then status_ncm2=1; else
status_ncm2=0;
if stop > t2ncm_td and status_ncm=1 and 3<t2ncm_td<=5then status_ncm3=1; else
status_ncm3=0;
if stop > t2ncm_td and status_ncm=1 and t2ncm_td >5then status_ncm5=1; else
status_ncm5=0;
```

where:

- *stop stands for year of follow-up;*
- *t2ncm_td stands for time of diagnosis of NCM*
- *status_ncm=1 identifies a patient with a cancer*

The 4 time-varying covariates therefore represent:

- *status_ncm1 (Non-cutaneous malignancy0-1) as those patients with a malignancy before the first follow up year (n=10)*
- *status_ncm2 (Non-cutaneous malignancy1-3) as those patients with a malignancy diagnosed between the first and the third follow up year (n=10)*
- *status_ncm3 (Non-cutaneous malignancy3-5) as those patients with a malignancy diagnosed between the third and the fifth follow up year (n=9)*
- *status_ncm5 (Non-cutaneous malignancy>5)as those patients with a malignancy diagnosed later than the fifth year of follow up (n=11)*

Then, we used the above described design variables to calculate the beta-coeficient associated with an NCM diagnosis at different time-points. These four "dummy variables" were included in the "final" survival COX regression model instead of the time-dependent covariate NCM. As in this model there are four design-variables, the beta coefficients are slightly different from the model presented in the study.

Table 4.19 - Cox regression survival model for death censored graft survival, including NCM diagnosis as a dummy variable divided for time of diagnosis.

Parameter	$\beta \pm SE(\beta)$	p-value	HR (IC 95%)
Non-cutaneous malignancy 0-≤1	1.02954±0.75	0.1674	2.80 (0.65-12.08)
Non-cutaneous malignancy 1>-≤3	1.21590±0.75	0.1055	3.373 (0.77-14.70)
Non-cutaneous malignancy 3>-≤5	1.57867±1.04	0.1290	4.849 (0.63-37.23)
Non-cutaneous malignancy>5	1.16782±0.78	0.1332	3.215 (0.70-14.77)
Creatinine (mg/dL) >=2 vs <2	1.09711±0.32	0.0006	2.996 (1.60-5.60)
Proteinuria (g/24h) >=0.5 vs <0.5	0.83342±0.30	0.0050	2.30 (1.29-4.12)
Acute rejection episode Yes vs No	1.13730±0.35	0.0010	3.118 (1.58-6.14)
Donor age	0.02924±0.01	0.0048	1.030 (1.01-1.05)
Gender Female vs Male	0.79284±0.28	0.0044	2.21 (1.28-3.81)
Year of transplant	0.29625±0.26	0.2632	1.35 (0.80-2.26)
Underlying nephropaty			
Secondary vs Primary	0.59374±0.33	0.0752	1.81 (0.94-3.48)
Unknow vs Primary	0.58667±0.33	0.0755	1.80 (0.94-3.43)

The next step was to estimate the PI at different time-points at which a NCM may be diagnosed. Obviously, as only the first malignancy was analyzed in this project, every patient could only be included in one of these categories and patients without any NCM have all these dummy variables (ie: NCM0-1 ; NCM1-3 ; NCM3-5 ; NCM>5) equal to 0 (Table 4.20). Clearly, the PI of a patient with a NCM within 1 year may be defined only after the first year: the patient has to be alive and with a functioning graft to the time of NCM diagnosis, otherwise he would have been excluded from the analysis.

Overall, in our cohort the median PI is 3.2 (IQR 2.4-4.1), ranging from 1 to 6.

We calculated the PI of the four "prototype patients" (as defined in Table 4.16) with a NCM at different time-points (Table 4.20). For instance, a male patient at one year of follow up (alive, with a functioning graft), who has developed an NCM during the first year, and had a creatinine < 2,0 mg/dL, urinary proteins < 0.5 g/24h, no acute

rejection episodes, and with a primary underlying nephropathy, who has received a transplant from a 50-yrs old donor, has a PI of: (0 x 0.793) + (1 x 1.0295) + (0 x 1.097) + (0 x 0.833) + (0 x 1.137) + (0 x 0.5937) + (50 x 0,0292) = 2.49

Table 4.20 - Sample prognostic index (for death censored graft survival) of the four different patient profiles, including non-cutaneous malignancies at different time-points. *NB: PI can be estimated only after all variables have been defined: for istance the PI of a patient with a NCM at the third year can be estimated only after the NCM diagnosis (ie: three years).*

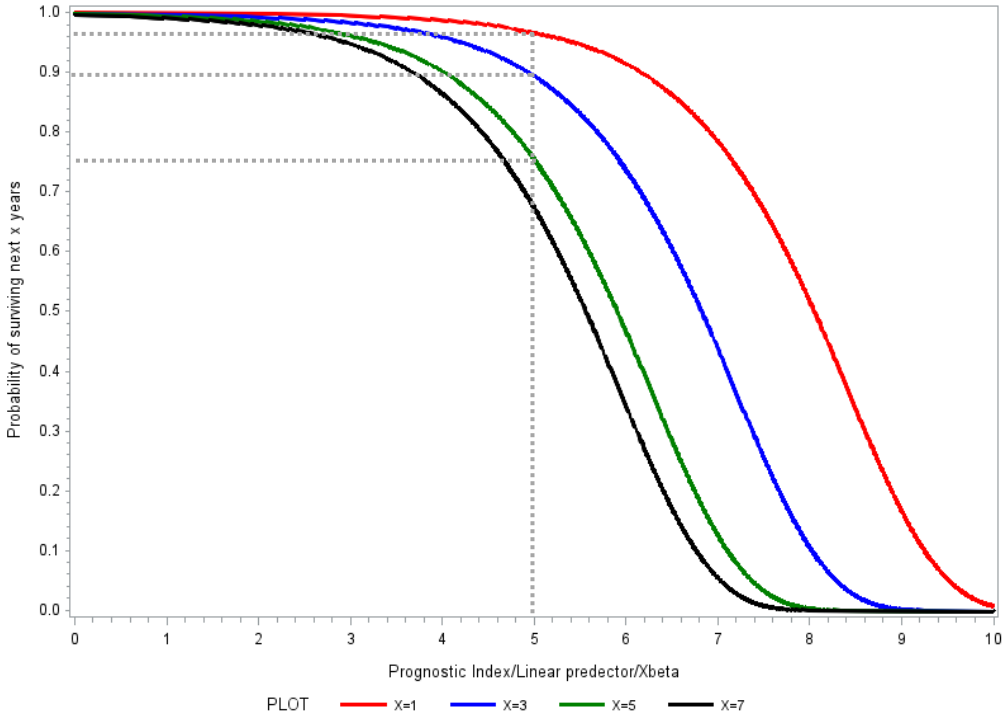
Covariate	Parameter Estimate	Patient A				Patient B			
		No NCM	NCM < 1	NCM 1>-≤3	NCM 3>-≤5	No NCM	NCM < 1	NCM 1>-≤3	NCM 3>-≤5
NCM 0-≤1	1.0295	0	1	0	0	0	1	0	0
NCM 1>-≤3	1.2159	0	0	1	0	0	0	1	0
NCM 3>- ≤5	1.5787	0	0	0	1	0	0	0	1
NCM >5	1.1678	0	0	0	0	0	0	0	0
Creatinine (mg/dL) >=2 vs <2	1.0971	0	0	0	0	0	0	0	0
Proteinuria (g/24h) >=0.5 vs <0.5	0.8334	0	0	0	0	0	0	0	0
Acute rejection episode Yes vs No	1.1373	0	0	0	0	0	0	0	0
Donorage	0.0292	50	50	50	50	65	65	65	65
Gender Female vs Male	0.7928	0	0	0	0	0	0	0	0
Underlying nephropaty									
Secondary vs Primary	0.5937	0	0	0	0	0	0	0	0
Unknow vs Primary	0.5867	0	0	0	0	0	0	0	0
Prognostic Index		**1.46**	**2.49**	**2.68**	**3.04**	**1.90**	**2.93**	**3.12**	**3.48**

Covariate	Parameter Estimate	Patient C				Patient D			
		No NCM	NCM < 1	NCM 1>-≤3	NCM 3>-≤5	No NCM	NCM < 1	NCM 1>-≤3	NCM 3>-≤5
No NCM									
NCM 0-≤1	1.0295	0	1	0	0	0	1	0	0
NCM 1>-≤3	1.2159	0	0	1	0	0	0	1	0
NCM 3>- ≤5	1.5787	0	0	0	1	0	0	0	1
NCM > 5	1.1678	0	0	0	0	0	0	0	0
Creatinine (mg/dL) >=2 vs <2	1.0971	1	1	1	1	0	0	0	0
Proteinuria (g/24h) >=0.5 vs <0.5	0.8334	1	1	1	1	0	0	0	0
Acuterejection episode Yes vs No	1.1373	0	0	0	0	1	1	1	1
Donorage	0.0292	75	75	75	75	50	50	50	50
Gender Female vs Male	0.7928	0	0	0	0	1	1	1	1
Underlying nephropaty									
Secondary vs Primary	0.5937	0	0	0	0	0	0	0	0
Unknow vs Primary	0.5867	1	1	1	1	1	1	1	1
Prognostic Index		**4.71**	**5.74**	**5.93**	**6.29**	**3.98**	**5.01**	**5.19**	**5.56**

However, in a "real-life" environment, the PI of each patient has to be determined individually: to be able to estimate the expected survival of a real patient we need to calculate the expected survival at different time-points for each possible PI and plotted as a graph (Figure 4.12). From these curves you can extrapolate the expected survival (at 1, 2, 3, …, n years) for each PI.

For instance, in the graph below the red curve represents the expected survival for a given PI (x-axis) after one year from the determination of the PI itself: so a patient with a PI of 5, has an estimated survival probability of 0.97 after 1 year. Reading the graph the other way around, a patient with a PI of 5 has a 0.89 survival at 3 years (blue line) and 0.77 at 5 years (green line), since the determination of the PI.

Figure 4.12 - Probability of surviving the next "x" years from the determination of the prognostic index (PI), based on different PIs. On the y-axis the survival probability; on the x-axis the PI. The red curve is the survival estimate at 1 year; the blue curve is the survival estimate at 3 years; the green curve is the survival estimate at 5 years; the black curve is the survival estimate at 7 years.



To be noted that the survival estimate is from the time of PI definition and not from the start of observation/study entry (ie: 6 months from KTx). For example, for a patient with a NCM diagnosed during the first year, the NCM variable (and thus the PI) can be defined only at the first year because it is after the NCM diagnosis. So, the PI including the NCM diagnosis has to be defined at the first year (ie: after NCM diagnosis) and the survival estimate calculated with this procedure starts from the first year (ie: the patient was alive with a functioning graft at the time the PI was calculated which is the first year).

This procedure is able to give an estimate of the individual patient risk of graft failure, which is the probability of having a functioning graft after for given time from known covariates at any time-point.

As a simulation, we applied this procedure to the previously defined "patient profiles", as shown in the table below.

Table 4.21: Simulation of different graft failure-free survival function estimates based on prognostic indexes (PI) of some hypothetical patient profiles (see text) without or with non-cutaneous malignancies (NCM) at different time points after study inclusion.
NB: the graft failure-free survival function estimate is the probability of "surviving" *n* years after the definition of the PI (ie: after NCM diagnosis)

	PI	Probability of Surviving next 1 year, after definition of risk (ie: tumor diagnosis)	Probability of Surviving next 3 years, after definition of risk (ie: tumor diagnosis)	Probability of Surviving next 5 years, after definition of risk (ie: tumor diagnosis)	Probability of Surviving next 7 years, after definition of risk (ie: tumor diagnosis)
Patient A noNMC *	1.46	1.00	1.00	0.99	0.99
Patient A NMC 0-≤1 **	2.49	1.00	0.99	0.98	0.97
Patient A NMC 1>-≤3 ***	2.68	1.00	0.99	0.97	0.96
Patient A NMC 3>-≤5 ****	3.04	1.00	0.98	0.96	0.95
Patient B noNMC *	1.90	1.00	1.00	0.99	0.98
Patient B NMC 0-≤1	2.93	1.00	0.99	0.97	0.95
Patient B NMC 1>-≤3	3.12	1.00	0.98	0.96	0.94
Patient B NMC 3>-≤5	3.48	0.99	0.98	0.94	0.92
Patient C noNMC	4.71	0.98	0.92	0.81	0.75
Patient C NMC 0-≤1	5.74	0.93	0.79	0.56	0.44
Patient C NMC 1>-≤3	5.93	0.92	0.75	0.49	0.37
Patient C NMC 3>-≤5	6.29	0.89	0.67	0.36	0.24
Patient D noNMC	3.98	0.99	0.96	0.90	0.87
Patient D NMC 0-≤1	5.01	0.97	0.89	0.75	0.67
Patient D NMC 1>-≤3	5.19	0.96	0.87	0.71	0.62
Patient D NMC 3>-≤5	5.56	0.94	0.82	0.61	0.50

* : patient without any NCM; the PI is constant over time and defined by time-fixed covariates
** : patient with an NCM diagnosed during the first follow up year; the PI has to be calculated after the NCM diagnosis and is constant thereafter.
*** : patient with an NCM diagnosed between the first and third follow-up year; the PI has to be calculated after the NCM diagnosis and is constant thereafter.
**** : patient with an NCM diagnosed between the third and fifth follow-up year; the PI has to be calculated after the NCM diagnosis and is constant thereafter.

5. Discussion

5.1 Main results

The primary outcome of this study was to evaluate the impact of NMSCs and NCMs on death-censored graft survival of KTRs: using a time dependent analysis, we were able to define the HR associated with the development of the first NMSC (HR = 0.80, p-value= 0.66) or the first NCM (HR = 3.27; p-value = 0.005), adjusted for known risk factors (Tables 4.12-4.13). Indeed, this issue is relevant to the transplant physician because even if the incidence of post-transplant malignancies is higher than in the general population (Farrugia et al., 2014, Apel et al., 2013, Sampaio et al., 2012, Piselli et al., 2013b), KTR-oriented specific screening programmes (Asch et al,.2014, Ponticelli et al., 2012) have dramatically improved post-malignancy survival in KTRs (Shu et al., 2014), being as high as 71.3% at 10 years after an NCM diagnosis (Tessari et al., 2013). This result from our cohort was validated by a Leave-One-Out Cross-Validation and eventually used to develop a method to estimate the individual probability of survival.

Patients with a NMSC are different from those with a NCM, as for risk factors for their development, mortality, therapy (usually a minor surgery for NMSC – Lvand Sun 2017) and therefore prognosis (Samarasingheand Madan 2012; Report of Canadian Cancer Society), it was observed in our cohort that NMSC and NCM behave in a completely different manner also as a risk factor for death censored graft failure (Tables 4.12 and 4.13). Given the lack of association between NMSC and graft failure (HR = 0.80), this variable was not further investigated.

Moreover, given the increased risk of graft failure associated with NCM (HR = 3.27), it was checked how the reduction of IS therapy interacted with malignancies as a risk factor for graft failure and it was foundthat a reduced IS was related with a higher rate of graft failure in patients without malignancy (graft failure rate ratio of 3.12), but not for patients with a NCM (graft failure rate ratio of 0.78) (Table 4.10). Indeed, clinicians often wonder if after a tumor diagnosis the IS therapy should be tapered and how much (Rama et al., 2010; Hope et al., 2015): from our observational

study, a small reduction of the maintenance IS therapy seems to be adequately safe for patients with an NCM.

Interestingly, in our cohort, among patients with a NCM with a failed graft, only 1/7 (14%) failed due to chronic rejection and 6/7 (86%) due other causes, including two "de novo" nephropathies, two chronic pyelonephritides, one graft nephrectomy and one chronic CNI toxicity. This observation is consistent the other results of this study, and may be somewhat unexpected: still it might have some interesting clinical implications, particularly in the management of kidney transplant recipients after a tumor diagnosis (see paragraph 5.3 – Clinical Relevance).

5.2 Discussion and comparison with literature

Some of our results confirm what is known about post-transplant malignancies: they are more common than in the general population, NMSC are not associated with worse outcomes, and NCM somehow worsen post-transplant survival.

Some other findings are actually unexpected, for instance the strength of association between NCM and graft failure, and the causes of graft failure among patients with a NCM.

Lastly, there were some interesting trends that given our limitations could not be confirmed or denied, but might be investigated in larger registries, such as the interaction between NCM and IS reduction and the identification of cancer-specific risk factors for graft failure, including cancer therapies.

The *NMSC and NCM incidence*, using a competitive risk analysis to adjust for patient death (Figure 4.5), in our cohort, is similar to other Italian and international cohorts (Piselli et al., 2013b, Tessari et al., 2013, Ma et al., 2014), even if there are different inclusion criteria among studies (Table 5.1). For instance, in our study, only patients with a 6-months follow-up were included, while Piselli et al. included patients since the day of transplant, as well as in the report by Engels et al. which included first and subsequent transplants and is a registry study (Engels et al., 2011).

Table 5.1 - Comparison of NCM incidence in other Italian cohorts

Case	Novara cohort	Piselli 2013	Tessari 2013	Wisgerhof (2011)	Engel 2011*	Ma 2014*a
NCM/pts	40/672	382/7217	253/3537	142/1906	10656/175732	308/3949
Country	Italy	Italy	Italy	Netherlands	US	Austr./NZ
Years	1998-2013	1997-2007	1980-2011	1966-2006	1987-2008	1997-2009
Median follow up	4.7	5.2	6.9	9.2	4.4 (mean)	4.4
Median age (min-max)	53 (18-77)	47 (18-80)	45 (18-68)	43.9 (3.8-77.5)	47 (nr)	47.2 (mean)
Male	61.9%	64.2%	65.3%	61.6%	60.9%	63.2%
NMSC incidence						
5 years	6.5% (4.5%-9.0%)	n/r	3.3%	3.0%	n/r	n/r
10 years	11.5% (8.2%-15.4%)	n/r	8.8%	8.7%	n/r	n/r
NCM incidence						
1 year	1.6% (0.8%-2.8%)	1.1%	0.3%	n/r	1.4%	1.6 %
3 years	3.4% (2.2%-5.2%)	3.2%	1.1%	n/r	4.1%	4.7 %
5 years	5.6% (3,8%-7.9%)	4.8%	2.3%	5.3%	6.8%	7.9%
10 years	9.8%(6.7%-13.5%)	9.9%	7.5%	13.1%	13.6%	14.9%

* the study reports the mean annual incidence rate: the n-years cumulative incidence was estimated as (mean annual incidence rate) * (n-years)
[a] reported values for the standard criteria donor sub-population

Besides, the *lack of association between NMSC and graft failure* (HR 0.80; 95%CI 0.30-2.14) could be expected from previous epidemiological and laboratory studies. Indeed, Christenson et al. (Christenson et al., 2011) showed on 46,216 KTRs that NMSC had a protective effect on graft failure (HR = 0.55; 95% CI 0.44–0.68). However, in their study, only 1.6% of KTRs developed a NMSC at 5-years (versus 6.5% in our cohort), reflecting different diagnostic and inclusion criteria: transplant year was between 1996 and 2001, but patients with multiple types of skin cancers, for instance more than one lesion with different histology (ie: basal cell carcinoma and squamous cell carcinoma) were excluded. Therefore, a slightly different association could be expected but actually their estimate is included in our 95% CI, confirming that our study may be underpowered to detect such small associations.
Moreover, NMSC have been associated with a chronic replication of beta-HPV in KTRs (Borgogna et al., 2014, Conolly et al., 2014): probably, these patients are particularly susceptible to chronic IS and in them certain strains of HPV -which usually are latent- are instead actively replicating causing eventually NMSC (Borgogna et al 2014). These same patients, who are likely particularly

immunocompromised (ie: have a greater effect of the IS therapy), are indeed at a lower risk for acute and chronic rejection, which is the main cause of graft failure.

Nevertheless, the *association of post-transplant NCM with graft failure* (HR 3.27 95%IC 1.44-7.44) has not yet been investigated directly (as in our study): however, this result is not completely unexpected as most studies on post-transplant malignancies presented a relatively low death censored graft survival after NCM diagnosis.

Only a case-cohort study was able to estimate the different death censored graft survival after a post-transplant malignancy (Rabot et al., 2014). Indeed, in this study the 5-year graft survival was 63% after a diagnosis of PTLD, which was much less than the one of matched patients from the DIVAT cohort (Données Informatisées et VAlidéesen Transplantation) which is 80-85%. Other studies (Table 5.2) included different types of malignancy with more favorable results, for instance a graft failure rate of 11.5% is reported at 5 years after a RCC of native kidneys (Tsaur et al., 2011).

Table 5.2: comparison of different graft survivals after a diagnosis of malignancy. PTLD: post-transplant lymphoprolipherative disorders, HCC: hepatocellular carcinoma; RCC: renal cell carcinoma

Study	Malignancy	n	Time from KTx	5-yr Graft Surv since NCM diagnosis
Serre (2014)	PTLD	101	9 yrs	76 %
Rabot (2014)	PTLD	104	4.4 yrs	63 %
Chuang (2008)	HCC	15	6.9 yrs	67 %
Tsaur (2011)	RCC	26	8.9 yrs	89 %
Novara Cohort	Any	40	2.97 yrs	71 %

Moreover, Salesi et al. investigated the incidence of graft failure after any post-transplant malignancy in recipients of living donor kidney grafts (Salesi et al., 2014). Even if they did not compare this result with similar recipients without any NCM, the incidence of graft loss was relatively high (4.4 of 100 patient-year) if compared with other cohorts of KTRs from living donors, in which the graft failure rate is about 2–3 of 100 patient-year in the first 5 to 10 years after KTx (Report of ERA-EDTA 2012).

Indeed, in our cohort, which includes transplants from deceased donors, the crude graft failure rate was 6.05 per 100 pt-years after a NCM, while it was only 1.49 per 100 pt-years in patients without any tumor.
Lastly, Hope et al. investigated patient and transplant outcomes after IS reduction for a diagnosis of post-transplant malignancy. They did not estimate a death censored graft survival function, but -among patients who survived 6 months from the malignancy- 6 / 55 (10.9%) experienced graft failure (median survival of 4.3 years), as compared to 7/40 in our cohort (17.5%): indeed, these crude rates are more than expected in patients with a normal renal function (median creatinine 113-116 µmol/L) at the time of cancer diagnosis.

Indeed, our study is actually the *first one in transplant medicine considering post-transplant malignancies as a time-dependent covariate* and its HR has not yet been defined. There are many different studies that investigated graft survival since the diagnosis of a NCM (see above), but they usually included both patients with early diagnoses –who likely have a good graft function- and patients with late diagnoses, who might have a failing graft independently from malignancy (Salesi et al., 2014, Rocha et al., 2013, De Biase et al., 2014). Therefore, such studies are not able to compare directly patients with a NCM with those without a NCM and may not be able to adjust for all known malignancy-independent risk factors.
A different approach might be an estimate of the rate of graft failure that might be more accurate in describing the relationship between graft failure and malignancies than post-malignancy graft survival itself, as performed by Salesi et al., 2014. However, it is not able to determine the extent of the association between malignancies and graft failure, so we performed a survival analysis with time-dependent covariates adjusted by baseline risk factors.
Indeed, our approach was able to include patients with early and late diagnoses (patients' prognosis is different regardless of malignancies) and reliably evaluate the HR of a NCM diagnosis in a time-dependent manner. Moreover, we could compare graft prognosis directly between patients with and without malignancies, which previous studies were only partially able to perform and only in a case-cohort design (Rabot et al., 2014; De Biase et al., 2014). Lastly, we were able to adjust the risk

estimates for all other known risk factors (at the time of transplant, or later on) and eventually we were able to determine an individual risk estimate based on our model (after internal validation).

Interestingly, in our cohort, *the causes of graft failure after a malignancy were different from chronic rejection*, which is the most common cause of graft failure in KTRs. Indeed, the specific cause of graft failure in NCM patients were one chronic rejection, two "de novo" nephropathies (myeloma kidney and immunotactoid glomerulonephritis), two chronic pyelonephritides (of which one after a radical prostatectomy), one graft nephrectomy for a renal cell carcinoma of the transplanted kidney and one chronic CNI toxicity.

Even if only seven grafts failed after a NCM, we tried to investigate the effects of NCM on cause-specific graft failure, finding an apparently different effect of a NCM diagnosis (p-value = 0.002) when considering graft failed due to chronic rejection (HR 0.55, 95% CI: 0.07–4.08) or for other causes (HR 15.59, 95% CI 5.43–44.76). To be noted, only two patients had a neoplasm on their graft, of which one was treated conservatively and the other underwent a transplant nephrectomy. However, this single subject did not impact by himself on the increased HR of graft failure in our cohort: actually, after censoring this event the HR was still 2.29 (95%CI 0.95-5.56) as compared to 3.27.

A plausible explanation for this finding is that, after an IS reduction, an acute rejection may be an early event (up to 5-10%; Hope et al., 2015, De Biase et al., 2014), but hardly leads to graft failure; however, it may take few years from an IS reduction to a chronic rejection and eventually graft failure (Terasaki et al., 2003). In our cohort the median post-malignancy follow-up is "only" 2.29 years: particularly grafts failed after a malignancy had a median follow up of only 1.1 years; therefore, it may have happened that some patients who reduced their IS therapy developed a chronic rejection, without -yet- experiencing a graft failure. However, in the study by Hope et al. (who had a median post-malignancy follow up of 3.9 years, up to more than 20 years), only 3/10 (30%) grafts failed due chronic rejection (of which 2 were pre-existing the malignancy diagnosis), while the other KTxhad other causes of graft

failure, for instance graft nephrectomy, recurrent nephropathies, and "de novo" nephropathies.

Therefore, post-malignancy nephropathies could be a major determinant of -at least- early graft failures. Indeed, in our cohort, three cases of graft failure were directly associated with a malignancy-associated event (myeloma kidney, immunotactoid glomerulonephritis, transplant nephrectomy) and two more cases could be attributed to the therapy of the malignancy (chronic pyelonephritides, one after developing a chronic reflux following a radical prostatectomy). Even if these data are very preliminary, they seem to confirm the observations by Hope et al. and might shift the focus of the clinicians towards other nephropathies rather than chronic rejection.

However, in our cohort, *NCMs seem to act as an effect modifier of the relationship between IS reduction and graft failure*. Actually, a reduced IS was associated in our cohort with a higher rate of graft failure in patients without malignancy (graft failure rate ratio of 3.12), but not for patients with a NCM (graft failure rate ratio of 0.78), despite the fact that almost half of the patients with a NCM reduced their IS burden. This finding could be due to a relatively aggressive policy of our center, in which, for example, no patient had a severe reduction of their maintenance IS and even those who reduced their IS were at least on a CNI regimen at full dose. This observation might be consistent with the hypothesis speculating that patients who develop a malignancy are particularly susceptible to chronic IS at "standard doses".

Indeed, also Hope et al found that there was no increase in the rate of graft failure in those with a dose reduction as compared to those without dose reduction after a diagnosis of malignancy. Moreover, Taylor et al showed that, in 24 cases of PTLD (9.2 years post-transplant) who ceased their IS while on chemotherapy, the time to creatinine increase was not significantly different (HR 1.19, 95% CI 0.44-3.23) with matched controls (age, sex, transplant year and renal function) (Taylor et al., 2015).

However, it is known that some therapies adopted to treat the malignancy might be directly or indirectly associated to renal toxicities (ie: chemotherapy and radiation therapy), worsening the decline of renal function due to causes different from chronic rejection.

Therefore, we tried to investigate which *"malignancy-associated" variables* could explain the increased risk of graft failure, but we were not able to find any significant association, due to the low event rate in the subgroup of KTRs with an NCM diagnosis. Speculatively there were some interesting "difference" in graft failure rates of different tumor sub-groups but we could not perform a direct comparison (see Limitations 5.4). For instance, none of the five patients treated with rituximab developed graft failure (overall follow up of 25.7 pt-yrs): this drug was first used in the therapy of non-Hodgkin lymphomas, but was later adopted as an IS drug for chronic antibody mediated rejection, so, even if there was an IS reduction, a rituximab "add-on" therapy could have prevented a chronic rejection in these patients. Moreover, a patient (out of three) who underwent pelvic radiotherapy had a graft failure due to chronic pyelonephritis: this complication might be expected (and possibly prevented) after an irradiation of the bladder and urinary tract.

5.3 Clinical and Research Relevance

We have shown that malignancies worsen renal function of KTRs, but registry-based studies are hard to perform as this is a late and relatively rare complication of an uncommon procedure: for instance, in Italy in 2017, 1934 KTx were performed accounting for an annual KTx rate of 32 per million people.

Indeed, in our cohort the association between a worsen graft prognosis and NCM does not seem to be mediated by an IS reduction or chronic rejection, but probably due to other concurrent (or malignancy-associated) nephropathies.

Therefore, *a different surveillance of kidney function* and nephropathies might be suggested for KTRs who develop a NCM. Transplant physicians should be aware that renal function might deteriorate after an NCM diagnosis, even if "safe" oncologic therapies are used. Indeed, chronic rejection in KTR with a NCM is a problem as much as in the KTRs without a NCM, but also other nephropathies might be on their way after a NCM, with different presentation times and symptoms. Particularly in the first months/years after diagnosis some otherwise rare nephropathies might be found (tubulo-interstitial, obstructive, vascular or

monoclonal gammopathy associated). Therefore, renal function monitoring and prevention of graft loss could include specific evaluations depending on the malignancy itself. But further studies on larger cohorts with specific NCMs are warranted to better to develop strategies to preserve kidney function after each tumor type.

Another clinically significant finding is that it seems *safe to taper immunosuppressive agents in patients with a NCM*. Clinicians often wonder if the maintenance of IS is safe or harmful for disease progression or relapse and whether or not it could be associated with worse graft outcomes, as it happens in the "general" transplant population. Thus, a randomized controlled trial would be desirable but it would not be easy to perform on large cohorts and with a wide variety of IS maintenance therapy and of clinical characteristics of KTRs. Moreover, as the available data from literature are few, nowadays the decision is made mainly on clinical and "expert-opinion" bases. Actually, the largest observational study (Hope et al. 2015) was published in 2015, and found that only "2/36 (6%) of KTR who underwent a dose reduction suffered acute rejection and that dose reduction of IS did not impair graft function, but also did not affect cancer free survival". Therefore, a reliable observational study, as ours, that included adjustments for the most common risk factors for graft failure, was needed and might be able to give clinicians some interesting information: indeed, the graft failure rate was not different between patients with a NCM who reduced their IS and those who didn't. Actually, none of the observed patients had an IS withdrawal or severe reduction of IS, but 19/40 had a significant IS reduction, defined as any "full-dose", single-drug therapy, or a therapy with steroids and either an mTOR-inhibitor or mycophenolate (CNI-free). Therefore, this intriguing observation paves the way to larger cohort studies, in which the same or a different statistical methodology (ie: matched by a propensity score index) could be applied.

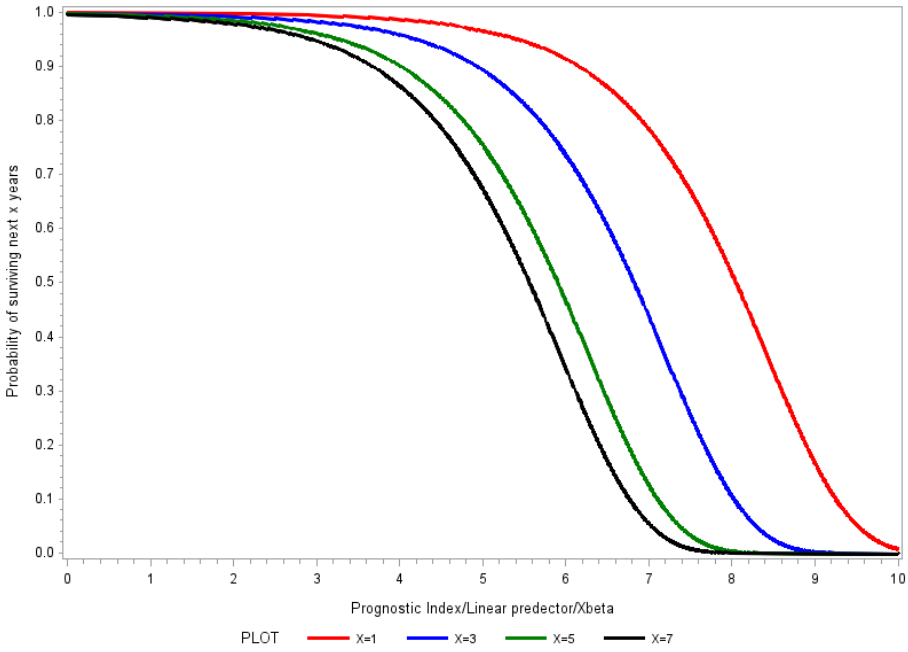
Lastly, at the moment,there are few tools to predict graft survival, while there are some scores/calculators to predict death or cardiovascular events (Soveri et al, Transplantation 2012). Therefore, with the results of these analyses, we were able to

develop a *reliable predictive survival model for death censored graft survival*. Indeed, for allocation purposes, there are "donor-evaluating" scores, like the KDPI (Kidney Donor Profile Index- https://optn.transplant.hrsa.gov/media/1512/guide_to_calculating_interpreting_kdpi.pdf), and patient survival scores, like the EPTS (Estimated Post Transplant Survival - https://optn.transplant.hrsa.gov/media/1511/guide_to_calculating_interpreting_epts.pdf). These were developed from the UNOS-OPTN (United Network for Organ Sharing - Organ Procurement and Transplantation Network) database which includes more than 100,000 donors and recipients, but are limited to pre-transplant variables. Besides, the Leuven risk score includes baseline histology -which is not always available- and has a AUROC of at best 0.81 in predicting the 5 years death censored graft survival. In our study, including only clinical covariates, the 5 years AUROC is actually 0.77. Indeed, given an individual prognostic index (calculated as detailed in Results 4.10.2), the 1-, 3-, 5-, 7-years graft survival can be estimated by the following graph. This evaluation can be made at six months from transplant (ie: study entry), but also later on, including baseline and time-dependent covariates: if this is the case, the expected survival is from the moment of calculation of the prognostic index.

Table 5.3 – Prognostic index calculation: Prognostic index is the sum of regression coefficients, each multiplied by the scoring of the corresponding variable. In the table are shown the Regression coefficients for death censored graft survival, including NCM diagnosis as a dummy variable divided for time of diagnosis.

Covariate	Regressioncoeficients
Non-cutaneous malignancy 0-≤1	1.03
Non-cutaneous malignancy 1>-≤3	1.22
Non-cutaneous malignancy 3>-≤5	1.58
Non-cutaneous malignancy>5	1.17
Creatinine (mg/dL) >=2 vs <2	1.10
Proteinuria (g/24h) >=0.5 vs <0.5	0.83
Acute rejection episode Yes vs No	1.14
Donor age	0.03
Gender Female vs Male	0.79
Year of transplant	0.30
Underlying nephropaty Secondary vs Primary Unknow vs Primary	0.59 0.59

Figure 5.1 - Probability of surviving the next "x" years from the determination of the prognostic index (PI), based on different PIs. On the y-axis the survival probability; on the x-axis the PI. The red curve is the survival estimate at 1 year; the blue curve is the survival estimate at 3 years; the green curve is the survival estimate at 5 years; the black curve is the survival estimate at 7 years.



Overall, our *hypothesis is that some patients with a NCM have probably been too much immunosuppressed after KTx* and therefore some malignant tumors might be considered transplant-associated. This association is well known for some specific malignancies, like kaposi's sarcoma, PTLDs, and kidney carcinoma (Tessari et al., 2013, Piselli et al., 2013b). Often this association is thought to be mediated by a chronic activation of viruses (Piselli, et al. 2013a; Engel et al., 2011). This hypothesis is supported by the fact that an IS reduction after a NCM is not associated with an increased risk for chronic rejection or graft loss, meaning that NCM patients might need less IS drugs than most of the other patients. Actually, to be noted that the biological effect of IS therapy on a single patient basis cannot be measured directly, not even measuring blood levels of immunosuppressive drugs. But it is much dependent on the "health" of the immune system of the patient and how it is influenced by anti-rejection drugs. Indeed, the "total burden" of IS might only be clinically estimated as scores and also *in vitro* lymphocyte function test do not seem to be adequately reliable. Given this "technical" issue on measuring the overall burden of IS, we are not able nowadays to determine which patients are too much immunosuppressed. However epidemiological observations (like incidence of IS-associated malignanciesand viral reactivations/infection incidence) might confirm this hypothesis.

As confirmed in our cohort, after a cancer diagnosis, the IS therapy is commonly reduced in order to improve patient's prognosis.There could be two different hypotheses: on one side, this reduction could bring the patients to a "correct" IS, without increasing his/her risk of chronic rejection; on the other side, IS could be reduced too much, increasing his/her risk of graft failure due to chronic rejection. Even if the interaction between NCM and IS reduction was not statistically significant, the trend showing a similar graft failure rate in both groups is consistent with our hypothesis that at least some patients with a post-transplant malignancy have been immunosuppressed too much.

Lastly, our results may stimulate further analyses on the *relationship between malignancies and kidney function in the general population* (Christensson et al., 2013), particularly in patients with a reduced renal function at the time of diagnosis

(i.e., eGFR < 45 ml/min/1.73 m2), like KTRs commonly are. Indeed, among KTRs a worse kidney function is associated with a higher incidence of NCM (Ma et al., 2014) and this association is true also in the general population, but it has not been investigated thoroughly. Indeed there might be a worsening renal function after a malignancy also in the general population (as observed in KTRs in our cohort): post malignancy survival is increasing in recent decades in the general population, particularly for youngerpatients(Report of CPO 2017) and in a few years more questions -rather than only patient survival- are likely to arise.

5.4 Limitations

Even if this study has a relatively novel methodological approach and shows interesting results, we were limited by three main factors: cohort size -which is too small to perform further analyses-, event rate -particularly on single tumor types-, and the relatively short follow-up time -particularly after malignancies.

Cohort studies on malignancies in KTRs are usually much wider than ours (Piselli, Serraino et al., 2013, Tessari et al., 2013, Ma et al., 2014), and we only had 40 patients with a NCM. Therefore, each single tumor site has only few cases (no malignancy with more than six affected patients) and the specific impact of high-risk localizations (like cancer of the lower urinary tract) or paraneoplastic nephritides (like myeloma kidney) could not be estimated. Still most “single-center” studies that investigate post-malignancy outcomes, have less than 100 patients with a post-transplant malignancy (Hope et al.,2015; Taylor et al.,2015; Bates et al., 2003, De Biase et al., 2003). Therefore, given the detail of the data, it is not completely unexpected that a time dependent analysis (as it is our study) has not yet been performed on graft outcomes following a post-transplant malignancy. Moreover, the coverage of cancer registries may not be as accurate and complete as needed for such analyses and thus we decided to perform an “ad hoc” analysis. Registry-based analysis would be actually feasible in smaller countries in which registries have been implemented in late ‘90s as performed by Maksten et al in Denmark (Maksten et al, 2016).

Besides, our median follow-up after the sixth post-transplant month was 4.7 years per patient, which is relatively short for studies on long-term graft failure, which is expected to happen about 10 years after KTx. This issue is particularly relevant for patients with a NCM: their median follow-up time after NCM diagnosis was 2.29 years, which is relatively short to be able to observe graft failure due to a chronic rejection arising after IS reduction.

Indeed, among seven grafts failed after an NCM, two were due to paraneoplastic kidney diseases diagnosed early after the malignancy and one was a transplant nephrectomy for a RCC of the graft. As we could not find any significant association with potentially biologically relevant causes of graft failure in our cohort due to the low event rate, the association between NCM and graft failure may not be considered a causal relationship at this point. We have adjusted our estimates for the known potential confounders (age, renal function, year of transplant), but we cannot exclude "*a priori*" that other still-unknown confounders might play a role.However, given the very limited event rate in patients with a NCM, we could not exclude a random effect: still, the interactions between NCM, IS, and graft failure could be investigated in larger cohorts, possibly investigating biomarkers of "excessive" IS which might be more accurate than drug through levels.

Lastly, the diagnosis of chronic rejection was sometimes an exclusion diagnosis, defined as the presence of a worsening renal function with proteinuria and without any other plausible cause of renal damage: this definition might have led to a relatively overestimation of chronic rejection diagnosis. On the other hand, the histological and clinical definition of chronic antibody mediated rejection is relatively recent (mid-late 2000s) and in the past, even in the presence of a kidney biopsy, this diagnosis could be missed or misinterpreted, leading to a relatively underestimation of chronic rejection diagnosis. Our cohort includes graft failed from late '90s and some of the uncertain diagnosis could be instead chronic rejections: however, among patients with a NCM, the first graft failures were observed in late 2000s when the diagnosis of chronic rejection was integrated in the everyday clinical practice. All patient records were reviewed and the included diagnoses are as accurate as a retrospective study is able to determine: in such studies some missing or uncertain data are commonly included (Hope et al., 2015, De Biase et al., 2014)

5.5 Conclusion

In conclusion, this study shows that in our cohort NCM are associated with a higher graft failure risk and might suggest that early after a NCM diagnosis the causes of graft failure may include paraneoplastic nephropathies and other "uncommon" nephropathies, such as chronic pyelonephritis and reflux nephropathy. Therefore, transplant physicians should be aware of these associations and should be careful in kidney function monitoring of KTRs with a NCM, which should include specific evaluations depending on the malignancy itself.

Moreover, our findings are consistent with the hypothesis by which some post-transplant malignancies are preventable and may be linked to an over-immunosuppression, even if drug levels are "on target". Given that the best therapy for post-transplant malignancies is prevention, more efforts should be made to develop more reliable markers of the overall IS burden of transplant recipients.

5.6 Future perspective

It would be interesting in the next few years, to confirm these results in other larger cohorts to be able to increase the number of patients and events, so that our observations would be confirmed and possibly to be able to perform some tumor-specific evaluations.

Moreover, it would be interesting to include some continuous and categorical time-dependent covariates, such as renal function, proteinuria and appearance of donor specific antibodies. Indeed, clinicians in their practice have often access to these information and they are known to be associated with the subsequent renal function. Therefore, the prediction model might be more reliable in long term predictions: indeed, the proposed model is more accurate in predicting early graft failures (ie: AUROC at 1 year of …) than late events (ie: AUROC at 6 years of …). Lastly these covariates -particularly renal function- have been associated with a slightly increased risk of renal cancer: even if creatinine at six months is a good marker of long term renal function, an over-time evaluation of renal function is probably more accurate.

This approach could also be applied to other common (and often not-fatal) post-transplant complications, like cardiac or vascular events, severe infections or viral infections.

Lastly, it would be interesting to look for genetic markers for post-transplant complications and how these pathways could be modulated by different IS or supportive therapies.

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