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The struggle for personalized medicine: from genes to viruses in the modern transplant era

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In respectful memory of Luca,

Whose battle was prematurely halted:

May his courage be an example for us all,

Facing the darkest nights with serenity and glee.

We all miss you.

Abbreviations

KTx	kidney transplant
KTR	kidney transplant recipient
ESRD	end stage renal disease
CKD	chronic kidney disease
U-prot	urinary proteins
DGF	delayed graft function
IS	immunosuppressive
CNI	calcineurin inhibitor
mTORi	mammal Target Of Rapamycin inhibitor
CyA	Cyclosporine A
Tac	Tacrolimus
MMF	Mycophenolate
Aza	Azathioprine
Rapa	Sirolimus
Ever	Everolimus
ATG	anti-thymocyte globuline
BPAR	biopsy proven acute rejection
DSA	donor specific antibody (anti-HLA mismatches)
PTDM	post transplant diabetes mellitus
T2DM	type 2 diabets mellitus
PTLD	post transplant lymphoproliferative disorder
MACE	major adverse cardiovascular event
SNP	single nucleotide polymorphism
OR	odds ratio
HR	hazard ratio
95%CI	95% confidence interval
HPV	human papilloma virus
HPyV	human polyoma virus
ADPKD	autosomal dominant polycystic kidney disease

Introduction

Complications of Kidney Transplant

Kidney transplant (KTx) is the therapy of choice of end stage renal disease (ESRD), assuring a better and longer life than other renal replacement therapies (RRT). However –to date- KTx recipients (KTR) need a chronic antirejection therapy with immunosuppressive drugs (IS), which cannot be withdrawn to maintain a stable graft function.

Moreover most KTRs have some degree of chronic kidney disease (CKD) because only one kidney is transplanted, it is perfused from the iliac artery (smaller than abdominal aorta) and most of them develop a chronic rejection which impairs over time the kidney function.

Lastly they often have metabolic anomalies which pathogenesis has not been elucidated: the major hypothesis claim as culprits the chronic inflammation due to chronic rejection, rapid changes in the diet (from dialysis to KTx), IS drugs (like steroids) and long term alterations persisting after the ESRD phase.

Indeed KTRs have a lower life expectancy than the general population, mainly due to cardiovascular events, severe infections and malignancies. Moreover KTRs as a population are a "sick" population with a very high morbidity despite a relatively normal lifestyle and social rehabilitation. Interestingly these characteristics have dramatically reduced the resources devoted to the development of novel IS agents, as pharmaceutical company front-men say: "they [novel IS] all fail to achieve better outcomes than standard IS and get too many side effects, even if they are not likely to be related to the study drug". Therefore, research and development of IS are now targeting different "immunological" population, like multiple sclerosis and rheumatology patients.

Cardiovascular events

Cardiovascular disease is a leading cause of morbidity and mortality after KTx, accounting for 50-60% of post-transplant deaths. For instance the incidence of ischemic heart disease of approximately 1 per 100 person-years at risk.

The high rate of cardiovascular deaths in the transplant population is due in part to the large number of diabetic patients receiving a KTx and to KTR who develop post-transplant diabetes (PTDM). However, the cardiovascular risk among non-diabetic KTR is still higher than in the general population, due to both an exacerbation of traditional risk factors by IS drugs (Figure) and to nontraditional risk factors related to both IS and CKD.

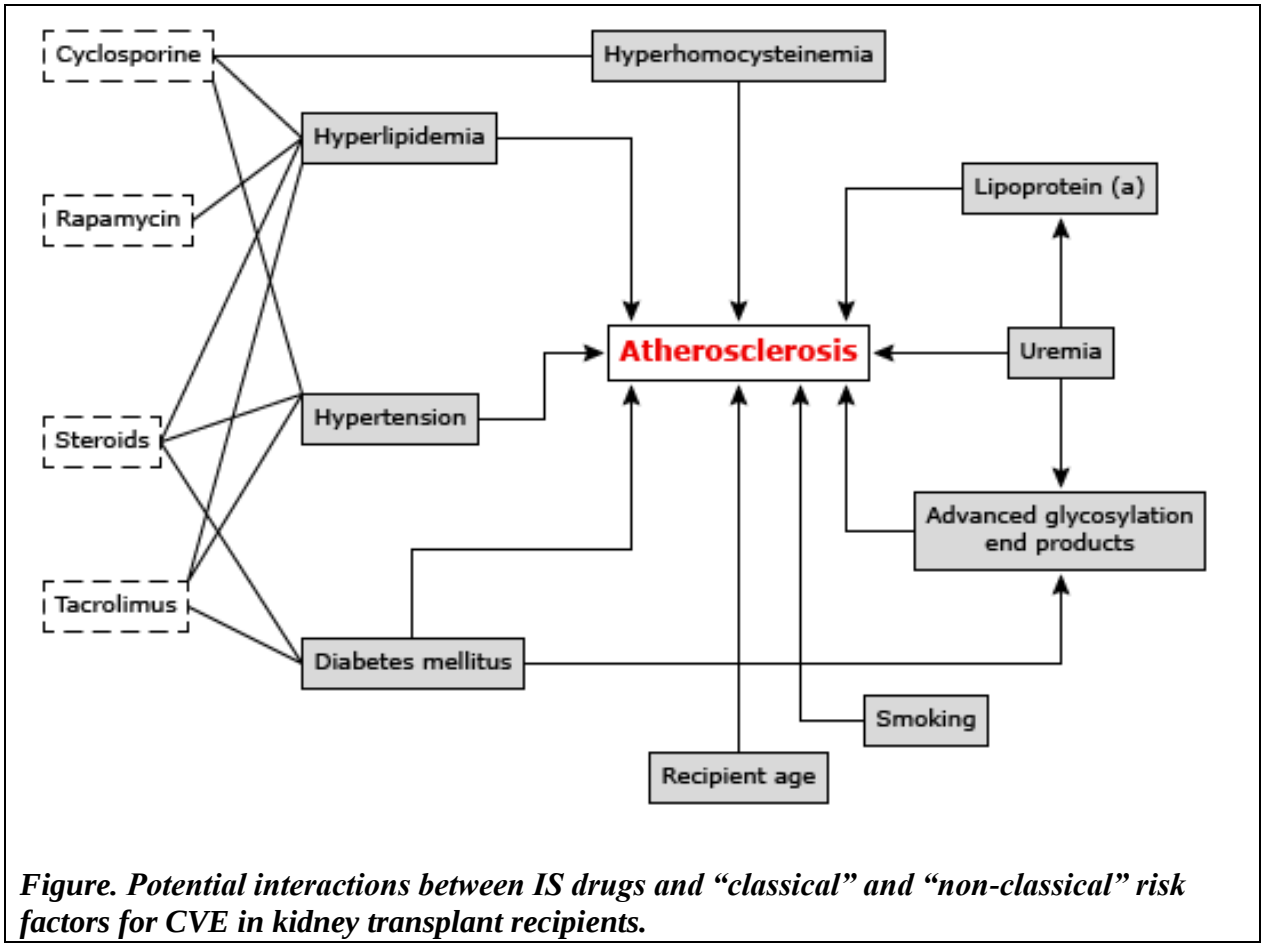


Figure. Potential interactions between IS drugs and "classical" and "non-classical" risk factors for CVE in kidney transplant recipients.

Traditional risk factors for major adverse cardiovascular events (MACE) include previous events, patient age, diabetes mellitus, male sex, hypertension and dyslipidemia. However the Framingham risk score greatly underestimates the risk of cardiovascular events among KTRs.

Although the individual Framingham risk factors are significantly associated with cardiovascular risk among KTRs, the effect sizes are increased among such patients, especially among patients at high risk.

Nontraditional risk factors that have been associated with increased cardiovascular risk in various studies include reduced kidney function following transplantation, dialysis vintage prior to transplantation, rejection, hyperhomocysteinemia, elevated levels of lipoprotein(a), elevated C-reactive protein and interleukin-6 levels, reduced homoarginine levels, proteinuria, and low levels of physical activity. Although not directly linked to cardiovascular disease, the presence of vascular calcifications detected radiographically prior to transplantation (a common finding) is also associated with increased cardiovascular and all-cause mortality posttransplantation.

Among MACEs, myocardial infarction may be a fatal event and is relatively common: among nearly 36,000 patients (USRDS data), the incidence of myocardial infarction at 6, 12, and, 36 months was 4.3%, 5.2%, and 11.1% respectively. KTx-related risk factors are older and deceased donors (worse post-KTx renal function), delayed graft function (DGF), PTDM, a failing graft, and previous vascular disease.

Even if not fatal, myocardial infarction may result in congestive heart failure (CHF), which is in the US the second most common cause of hospitalization of KTRs, having a cumulative incidence of 18.3% at 3 years after surgery. Independent risk factors for new-onset CHF after transplantation include age, diabetes, anemia, hypertension, obesity, and suboptimal graft function.

Interestingly an increasing dialysis vintage prior to transplantation is associated with a graded increase in the risk of cardiovascular death after KTx. A likely explanation for this is that accelerated atherogenesis is observed as part of the uremic syndrome. Proatherogenic factors that contribute to the progression of vascular disease prior to transplantation may include hyperhomocysteinemia, hyperfibrinogenemia, increased calcium ingestion, abnormalities of

mineral metabolism, dyslipidemia, and modification of low-density lipoproteins (LDL) by advanced glycosylation end-products (AGE), particularly in diabetic patients, but also in non-diabetic patients. These same factors become increasingly important with the onset of chronic allograft dysfunction that leads to progressive uremia.

Indeed, only one model(by Soveri et al) has been validated to predict the risk of MACE in KTRs and includes some "Framingham" variables, as well as the mostly associated "KTx-associated" variables.

Table. Model by Soveri et al (Transplantation 2012) predicting the 7-year cumulative incidence of major cardiovascular events (MACE) and death in kidney transplant recipients. *In the original paper the AUC of these models in the assessment and test samples was 0.735-0.740 and this model was validated in the BENEFIT and BENEFIT-EXT populations.*

Risk Factor	MACE Hazard Ratio	Death Hazard Ratio
Diabetes Mellitus	2.21	1.91
LDL-cholesterol (per 1 mmol/L)	1.29	-
Smoker		
Current	1.43	1.87
Previous	1.42	1.29
Coronary heart disease	1.97	1.32
# of Transplants	1.40	-
Total time on RRT (per 5 years)	-	1.02
Creatinine (per 1 mg/dL)	1.52	1.50
Age (per 10 year)	1.51	1.75
"Standard" 7-years cum. incidence *	3.1%	6.74%

** no risk factors, age=50 years, Creat=1.0 mg/dL, LDL=1 mmol/L, first transplant, 1-year on RRT*

At the end of the day, the reduction of cardiovascular risk requires an accurate assessment of risk in KTRs (which is not ease achieved) and the appropriate pharmacologic and nonpharmacologic interventions. Particularly there is considerable practice variation in the use of potentially cardioprotective medications in KTR, even among patients considered to be at high risk. Studies suggest that primary and secondary cardiovascular preventive measures (aspirin and lipid-lowering drugs) are underutilized among transplant recipients, probably because their utility is far from being proven in this population. For example the Long-Term

Deterioration of Kidney Allograft Function (DeKAF) study revealed that fewer than 60% of KTRs with a pre-existing cardiovascular disease received aspirin and about half received a statin.

Metabolic complications

Body mass index (BMI) was independently associated with CHF in several USRDS registry studies. In one study, BMI greater than or equal to 30 predicted up to 59 percent relative risk increase, compared with BMI less than 30. In another study of USRDS data, BMI greater than 28.3 independently predicted a 57 percent relative increase in the risk of hospitalized CHF, compared with lower BMI.

A portion of obesity-related cardiovascular risk may be mediated by impacts on metabolic health including diabetes. A meta-analysis of six studies including 4111 patients demonstrated an increased risk of post-transplant diabetes (PTDM) associated with BMI >30 compared with <30 kg/m^2 (pooled risk ratio 2.24, 95% CI 1.46-3.45).

Obesity trends in transplant recipients tend to mimic the general population: from the data of the United Network for Organ Sharing, among 27,372 individuals who underwent kidney transplantation between 1997 and 1999, 20% were obese (BMI>30), and an additional 30% were classified as overweight (BMI 25 to 29).

Moreover, weight gain after transplant is common and may be related to improved appetite with reversal of uremia and relatively high steroid doses in the peritransplant period, but is also associated with physical inactivity.

Obesity among transplant recipients is frequently associated with the metabolic syndrome. Obesity increases the risk of cardiovascular disease in the general population; among kidney transplant recipients, the presence of obesity, particularly in association with the metabolic syndrome, also appears to be associated with an increased number of adverse cardiovascular

events. In one study of 337 renal transplant recipients, one-third had metabolic syndrome by the first transplant anniversary, whereas only 20 percent had metabolic syndrome before transplant. Over eight years of follow-up, 42 percent of the sample developed atherosclerotic disease events, and the risk of these events was significantly increased among patients with metabolic syndrome by one year after transplant (HR 3.4, 95% CI 1.6-7.3).

High BMI has also been associated with an increased risk of cardiac death after kidney transplantation. In a study of nearly 52,000 patients in the USRDS registry, the adjusted risk of cardiac death increased at both low BMI (aHR 1.3 for BMI <20) and high BMI (aHR 1.2 for BMI 30 to 32; aHR 1.4 for BMI >36), compared with the reference group with BMI 22 to 24. Lifestyle changes based in diet and exercise, with supervision by a renal dietician as needed, are first-line strategies to achieve and maintain normal body weight among obese transplant recipients. Cases of bariatric surgery among morbidly obese transplant recipients have been reported, but surgical therapies warrant prospective evaluation to quantify risks and benefits in this population.

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.

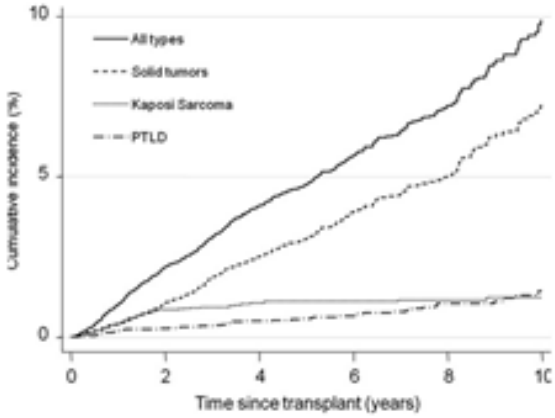


Figure. Cumulative incidence of malignancy in an Italian population of kidney transplant recipients, excluding non-melanoma skin cancers.
The bold line represents all solid and hematologic malignancies, the dotted line all solid tumors, and the dashed line hematologic malignancies (post-transplant lymphoprolipherative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

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%. As compared to the age- and sex- matched general population, a 3-to-5-f old 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.

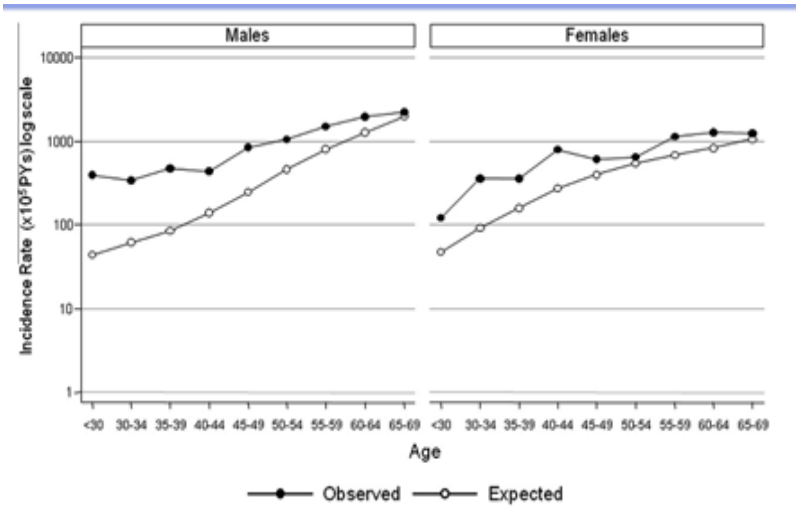


Figure. Age specific incidence of malignancies in the general population and in KTRs. Data from Italy, 1997-2009.

NMSCs are the most common cancers in renal transplant recipients. In contrast to the general population, there is a reverse relationship between the risk of basal cell and squamous cell carcinomas. The squamous cell/basal cell carcinoma ratio is approximately 5:1 as opposed to

1:4 in the general population. 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. Long-term immunosuppression 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. The incidence of melanomas is also increased, but to a lesser extent, in renal transplant recipients, with an excess risk of approximately 4 times than the general population.

For non-cutaneous malignancies (NCM), the 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, whereas non–viral-related solid organ tumors such as breast and prostate cancers are not increased.

Meta-analysis SIRs for cancers related to infections in transplant recipients

Cancers*	Meta-analysis SIR*
EBV-related cancers	
Hodgkin's lymphoma	3.89 (2.42–6.26)
Non–Hodgkin's lymphoma	8.07 (6.40–10.2)
HHV8-related cancers	
Kaposi sarcoma	208.0 (114–369)
HBV/HCV-related cancers	
Liver	2.13 (1.16–3.91)
HPV-related cancers	
Cervix uteri	2.13 (1.37–3.30)
Vulva and vagina	22.8 (15.8–32.7)
Penis	15.8 (5.79–34.4)
Anus	4.85 (1.36–17.3)
Oral cavity and pharynx	3.23 (2.40–4.35)
Nonmelanocytic-related skin	28.6 (9.39–87.2)

HBV indicates hepatitis B virus; HCV, hepatitis C virus.

For instance, more than 90% of cases of posttransplant 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. Recipients of older ages (> 65 years), with exposure to CNIs are at augmented risk. Evidence from the USRDS had demonstrated that risk for PTLD was highest for persons in the first post-KTx year and decreases thereafter. 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.

HPV infections, transmitted commonly through hetero- and homosexual contacts, are associated with various types of cancers such as NMSC, anogenital, and oropharyngeal cancers. The four major oncogenic strains, HPV 6, 11, 16, and 18, account for more than 70% of HPV-related ano-genital malignancies. Human papillomavirus infections usually remain dormant in sub-clinical states by an intact immune system, but could potentially be reactivated by IS. A higher prevalence of anogenital and cutaneous HPV infection s was observed among renal transplant recipients, suggesting a potential role of HPV infections in the etiology of these cancers.

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, but it is also seen in dialysis patients.

Numbers of 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.

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).

For KTRs, two important exposures that contribute to increased cancer risk are unavoidable: end-stage kidney disease (ESRD) itself is associated with increased cancer risk at many sites and after transplantation, and the additional burden of the immunosuppression necessary for continued graft function amplifies cancer risk further.

General lifestyle choice policies recommended for the general population such as healthy eating and stop-smoking campaigns have benefits in beyond cancer prevention, and it is generally agreed and 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 association between nonmelanocytic skin cancers (NMSC) and melanoma with ultraviolet radiation (UV) is well established. 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.

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. People on dialysis have a reduced response to vaccination, with a lower antibody titer and an inability to maintain adequate antibody titers over time. Antibody response after transplantation is usually even worse, particularly in the first posttransplant year, when the burden of iatrogenic immunosuppression is most intense.

Vaccination against Hepatitis B is currently recommended before the commencement of renal replacement therapy, along with Varicella zoster and Hepatitis A, to maximize overall response and seroconversion. Although primarily directed to prevent blood-borne infection and subsequent liver disease in people with ESRD, Hepatitis B vaccination has a role in reducing risk of hepatocellular carcinoma, as people with chronic infection with hepatitis B experience a greatly increased risk.

Emerging preventive measures of specific relevance to KTRs include human papillomavirus (HPV) vaccine against HPV-related cancers. HPV has been implicated in cancers of the anogenital tract (particularly cervical cancer in females) and the oropharynx and HPV DNA can be detected in up to 90% of skin cancers in transplant recipients (though a causative role of HPV infection is debated).

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). 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 based-IS.

However, despite considerable progress, long-term graft loss in renal transplant recipients remains substantial, with resulting high morbidity, mortality and costs. 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%. In addition, patients who require re-transplantation are quite 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. The costs associated with failed transplants with return to dialysis represent a considerable financial burden for health care systems (MoH) while decreasing the quality and length of life

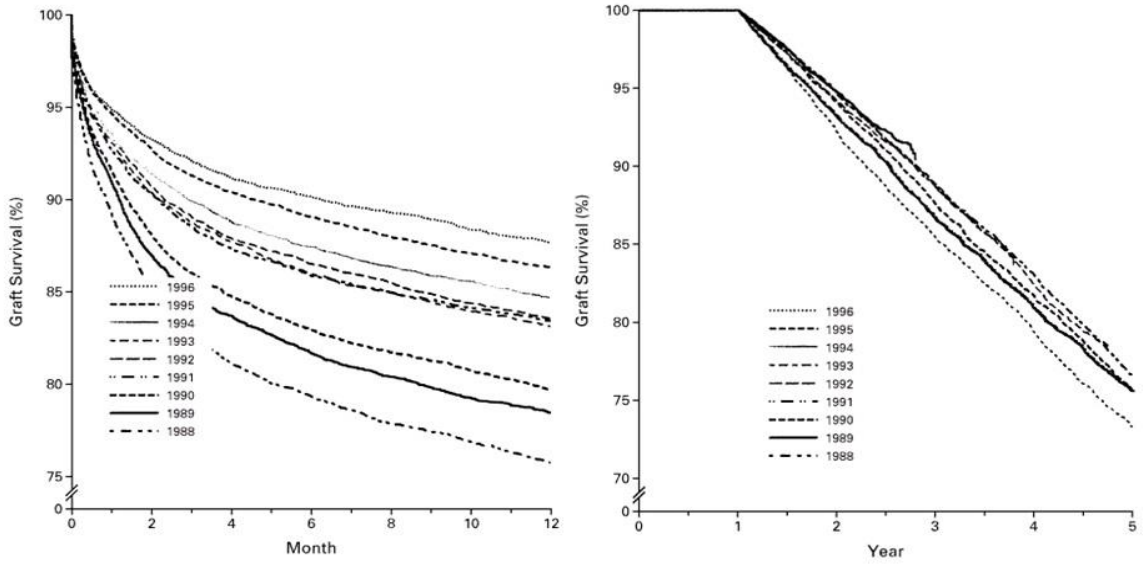


Figure 4 Graft survival over time: on the left 1-year graft survival between 1988 and 1996, which showed a dramatic improvement (from 76% to 88%); on the right, survival function after the first year: no significant difference can be noted.

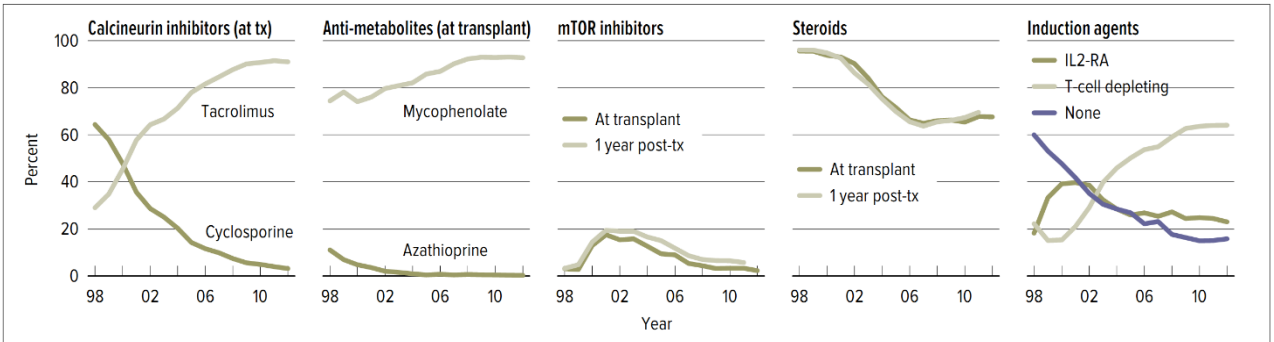
for affected patients. Clearly, the identification of critical pathologic pathways responsible for allograft loss, with the attendant development of therapeutic interventions to improve the duration and quality of allograft function, is one of the most important objectives of transplant medicine.

Over the past two decades, our thinking has changed from considering rejection as a primarily T-cell-mediated process (one that is now increasingly better managed), to the realization that insufficient control of the humoral arm of a recipient's immune system by current IS regimens is now the pathogenic factor primarily responsible for allograft dysfunction and loss. 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.

Monitoring parameters and Pharmacogenetics of Immunosuppressive drugs

The immunosuppressive therapy used in kidney transplantation is usually composed by a calcineurin inhibitor (CNI, ie: cyclosporine or tacrolimus) alone or in combination with either a purine-metabolism inhibitor (ie: azathioprine or mycophenolate) or a proliferation signal inhibitor (mTORi, ie: sirolimus or everolimus), with or without steroids.



KI 4.7 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 2. Common IS drugs and their associations.

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

Tacrolimus is the most commonly used IS drug in KTx for the prevention of allograft rejection. 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.

Eight oxidized tacrolimus metabolites have been identified: one monohydroxylated (M-IV), three mono-demethylated (M-I, M-II, M-III), and three di-demethylated (M-V, M-VI, M-VII)

metabolites, in addition to a complex metabolite (31-O-demethyltacrolimus or M-VIII). While M-II is equipotent to tacrolimus in terms of immunosuppressive activity, all other metabolites have a very weak effect. Enzyme immunoassay (EIA) techniques employ a monoclonal antibody against tacrolimus that also cross-reacts with its eight metabolites; however, although the strength of the reactivity is similar between M-II, M-III, and M-V, the remaining metabolites show almost negligible reactivity.

Tacrolimus has a narrow therapeutic index and large inter-individual variations in pharmacokinetics, which may partly be the consequence of metabolism by CYP3A5 and P-gp. The expression of both proteins is affected by genetic polymorphisms: for example, patients with one or two wild-type allele CYP3A5*1 express CYP3A5, whereas homozygotes for mutant allele CYP3A5*3 are considered to be non-expressors. Consequently, variability in tacrolimus pharmacokinetics depends not only on interactions with concomitant drugs acting on CYP3A (such as ketoconazole or rifampicin), but also on a complex genetic conditioning. Indeed, polymorphisms of CYP3A5 would have a significant impact on tacrolimus metabolism while the role of MDR1 variants is more controversial.

However, until genotypic profile data become available in daily clinical practice, tools to define the drug exposure profile other than the conventional and laborious dose-interval area under the concentration curve (AUC) would be very useful.

Tacrolimus requirement may be easily normalized by drug levels and expressed as the concentration/dose (C/D) ratio , a surrogate index of tacrolimus bioavailability and pharmacokinetics that tends to change over the first months after transplantation and then stabilize after 6 months. This calculation identifies different phenotypic profiles, ranging from very fast to very slow tacrolimus metabolizers. Therefore, for any targeted trough concentration, fast and slow metabolizers need completely a different tacrolimus daily weight-adjusted dose, leading to a low and high tacrolimus C/D ratio, respectively.

A relationship between the C/D ratio and the CYP3A5*1 polymorphism has been shown, as homozygous *3/*3 and *1/*1 patients have been shown to have the highest and lowest C/D ratio, respectively, and the *3/*3 genotype has been determined to represent an increased risk of nephrotoxicity.

Steroids are often administered after renal transplantation, and they share some common metabolic and transporter pathways, such as the cytochrome P450 and P-gp systems. Furthermore, corticosteroids may have an induction effect on CYP3A4 expression and reduce tacrolimus concentration by increasing tacrolimus clearance.

Proposed project and specific aims

This pilot project aims to translate the recently acquired knowledge on genes and viral-induced oncogenesis in the current transplantation practice. As in the past decade many studies have been performed on different KTx complications, I will focus on the most common ones, being diabetes, cardiovascular events and malignancies. Indeed, before starting intervention or prevention studies on such complications, an in-depth risk stratification analysis is mandatory and this thesis project is aimed mainly to develop risk stratification models for each transplant complication. The main study cohort is composed by patients transplanted at the Novara Transplant Center (1040 KTx performed in the past 16 years).

The SNPs were selected from literature and previous works of our group: we focused for this project on four SNPs affecting tacrolimus metabolism and the rs7903146 SNP (TCF7L2 gene), which is the strongest associated SNP with type 2 diabetes in the general population, but may also interact with T-cell response. Viral detection and evaluation of replication was performed on small-DNA potentially oncogenic viruses, being human papilloma and polyoma viruses. Herpesviridae (EBV in particular) are already monitored in common clinical practice and their replication/reactivation was evaluated as a predictor of transplant complications.

The main projects I've been working on and their study rationale are here reported in detail.

Risk model for post-transplant diabetes mellitus

Specific Aim: to develop a post-transplant diabetes risk model based on pre-transplant variables, including genetic variants which might be associated with PTDM.

We analyzed the association of post-transplant diabetes (PTDM) with five previously selected SNPs in our cohort of KTx recipients (KTR). This study was also replicated for significant SNPs on a cohort from the Verona Transplant Center, which was indeed much different from ours, particularly for a shorter follow up time and a lower PTDM incidence rate.

Genome-wide association studies (GWAS) allowed detection of more than 100 polymorphic loci, which appear to modulate the risk of type 2 diabetes (T2DM) in the general population. While most of them appear to exert a small effect size (with OR between 1.10 and 1.20) and were identified in single ethnicities, a stronger effect was noted for variant rs7903146 (T allele) of *TCF7L2* gene. The TCF4 protein belongs to a T-cell transcription factor family which regulates cell proliferation and differentiation through Wnt signaling pathway, which controls pancreas development and maturation, and islet function. T allele has been associated with increased protein expression, impaired insulin secretion, impaired incretin effects and hepatic insulin resistance. As beta-cell dysfunction –rather than insulin resistance– is possibly a predominant pathophysiological mechanism for PTDM, some studies confirmed the role of *TCF7L2* rs7903146 C>T also in this setting. However other investigations, did not detect this association, making results inconclusive. Therefore, although association of this *TCF7L2* polymorphism with PTDM remains highly plausible from a biological point of view, its clinical role remains to unclear and it is not routinely genotyped to predict individual risk of PTDM before KTx.

As only the *TCF7L2* polymorphism was associated with PTDM among studied SNPs, we performed a meta-analysis combining the present results with data of previously published studies to clarify the relationship between rs7903146 and the risk of PTDM in renal transplant patients. As the meta-analysis found a significant association between PTDM and rs7903146 TT genotype, we built a predictive model of PTDM including this polymorphism and environmental parameters.

This analysis gives a powerful predictive model of PTDM based on pre-transplant variables, which may be helpful in the clinical management of KTRs, also in the first days after surgery, a time period in which glucose-toxicity may be very relevant. Moreover as *TCF7L2* rs7903146 T allele is associated with beta cell dysfunction, novel preventive strategies will be tested in high risk patients.

Cardiovascular events

Specific Aims: to evaluate the impact of different therapies (treated as "environmental" risk factors) on post-transplant thrombotic events (both early and late events).

To evaluate if SNPs associated with IS metabolism or complications may indeed alter the risk of thrombotic or cardiovascular events.

To develop a risk model for specific post-transplant CVE based on pre-transplant variables.

As detailed above, cardiovascular events (CVE) have a complex pathogenesis in KTRs. We first evaluated those "environmental" factors which can be modified, particularly immunosuppressive therapy and pharmacological prevention strategies, focusing on thrombosis and thrombotic events.

The management of antiaggregant therapy both in elective –such as living donor KTx- and in urgent surgery –as deceased donor KTx- is an evolving issue: international consensus and guidelines suggest to evaluate bleeding and thrombotic risk for decision making, but no specific recommendation is made for kidney transplant surgery. Actually KTR represent a very peculiar population in which baseline thrombotic and hemorrhagic risk are both elevated if compared with the general population, as ESRD is associated with complex and severe vascular alterations and sub-clinical inflammation and, on the other side, platelet aggregation defects. Therefore results inferred from different populations might not directly apply to KTx surgery or recipients.

On these basis, we performed a retrospective analysis on 911 consecutive KTx to analyze the impact of antiplatelet or oral anticoagulation therapy on thrombotic events, which might be related to their withdrawal after surgery, and to identify the main risk factors for early (within 90 days from surgery) events. Moreover, even if initial IS was not associated with CVE, I investigated the role of mTOR-inhibitors (mTORi), which are a relatively common maintenance IS therapy.

Moreover, as different IS medications have been involved in the pathogenesis of thrombotic events. Recently, Baas et al have shown that KTR receiving everolimus have higher levels of inhibitors of fibrinolysis, which may be associated with more thrombotic events. Indeed, some case reports described devastating thrombotic events in solid organ transplant recipients treated with mTORi, but randomized controlled trials did not describe an increased risk of vascular complications. Therefore we retrospectively studied the incidence of major thrombotic events (MTE) in a cohort of unselected KTRs to evaluate if patients experienced more MTE while on mTORi.

Lastly we investigated the association of the above studied SNPs with CVE and cardiac ischemic events (symptomatic ischemic heart disease and acute myocardial infarction (AMI) in particular, including non-diabetic KTRs followed-up at our Center. Indeed, only *TCF7L2* rs7903146 showed a significant association with cardiac ischemic events and a risk-prediction model was developed. The role of rs7903146 T allele in modulating cardiovascular risk in the setting of KTx is biologically plausible and could be mediated by a wide range of mechanisms. TCF-4 transcription factor is involved in the pathway of NF-kB: a dysregulation of this axis can alter bone remodeling, a key process leading to coronary artery calcification (CAC), and promote vascular wall inflammation, oxidation and endothelial dysfunction. Interestingly, we found a stronger association of the allele with CAD in KTRs than in the general population, as we showed also for PTDM. This increased strength of association could be explained by the fact that genetic predisposition can emerge more clearly in KTx setting due to a higher event rate caused by interaction of polymorphisms with transplant-specific environmental factors such as IS therapy and chronic renal failure.

Indeed, we could come up with a predictive model of acute thrombosis in the first post-transplant months, an integrated gene-clinical model for the prediction of ischemic heart disease and define the risk of thrombosis associated with a specific class of IS, which is used commonly without any further preventive strategy.

Genetics or viruses determine viral-related malignancies?

Specific Aims: to evaluate the role of active HPV on NMSC development in KTRs.

To evaluate the role of active HPyV or BKV-associated nephropathy on the development of tumors of the urinary tract in KTRs.

To evaluate if SNPs associated with IS metabolism or complications may indeed alter the risk of virus-related malignancies.

To identify risk factors and clinical biomarkers of PTLDs.

We investigated post-transplant malignancies from both susceptibility genes and viral determinants, including five pre-determined SNPs, clinical pharmacokinetic parameters, and viral replication indexes, focusing on viral-associated malignancies, particularly on NMSC, PTLD and urinary tract tumors.

Most of the cancers with an elevated standard incidence rate (SIR) are caused by reactivated viruses whose oncogenic potential is suppressed by immunological reactions in healthy individuals. A role for beta HPV in the development of NMSC has been repeatedly reported by a series of epidemiological, seroepidemiological and experimental data. A similar scenario can be envisaged for human polyoma virus (HPyV) whose causal association with human tumors is also difficult to be established because they may also be constituents of the human skin and urinary tract microbiome.

HPVs and HPyVs share a common morphology and structural organization, being small, circular DNA viruses. Cutaneous beta-HPVs cause widespread unapparent or asymptomatic infections in the general population: however, in immunosuppressed patients these viruses can spread unchecked and have been implicated in the development of skin cancer.

The aim of this retrospective study was to determine the incidence of virus-related cancer in a cohort of kidney transplant recipients, focusing on their association with HPV or HPyV reactivation.

Moreover previously studied SNPs affecting CNI metabolism were studied as potential risk factors for virus-induced cancer development, particularly PTLD: their interaction with EBV status and reactivation has been analysed in our large cohort. Lastly a large case-cohort study, involving nine transplant centers, on PTLDs was performed to identify risk factors for this rare, but severe complication, including EBV status, immunosuppressive therapy and pharmacokinetic parameters. As this study collected data retrospectively and most patients already died at the time of study initiation, genetic analysis could not be performed.

Patients and Methods

Data collection and cohort definitions

Novara "full" cohort

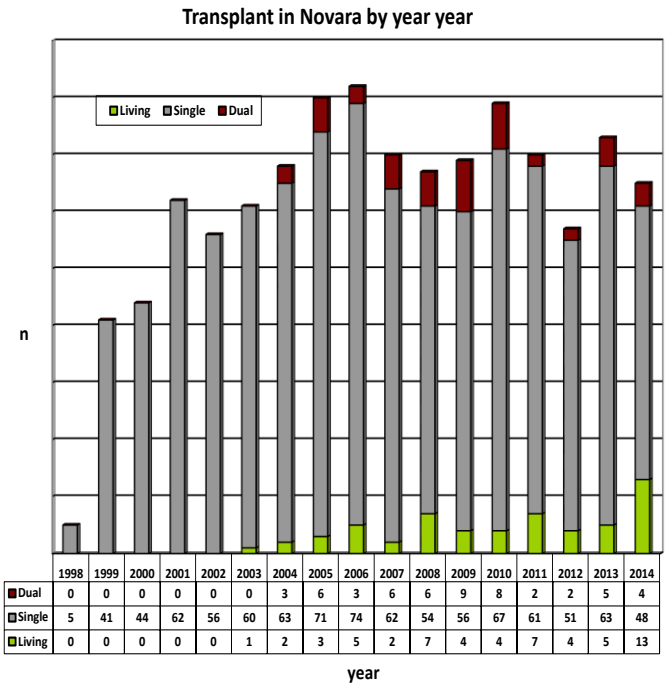
We conducted a retrospective analysis of all adult KTx performed in a single Kidney Transplant Centre from its opening in November 1998 up to December 2014, including deceased donor (94.6%) and living donor (5.4%) KTx. As the database is constantly updated and analysis were performed at different times, this same population in different analysis might vary. Moreover inclusion criteria may change depending on the specific aim which is investigated, as shown in Table 1.

Table 1. Analyses performed on the entire Novara cohort, reporting year, population selection criteria and numerosity of included population

Study	Year	Inclusion criteria	Exclusion criteria	N
Early post-KTx thrombotic events	2014	All patients	None	911
mTORi effect on thrombosis	2013	All patients with complete IS data	None	694
Malignancy incidence	2015	All patients	None	1040

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 TE, 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 malignancy and pre-malignant lesions is 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. Patient 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.



	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Dual	0	0	0	0	0	0	3	6	3	6	6	9	8	2	2	5	4
Single	5	41	44	62	56	60	63	71	74	62	54	56	67	61	51	63	48
Living	0	0	0	0	0	1	2	3	5	2	7	4	4	7	4	5	13

Note: in 1998 transplant activity started in november

Table 2. Baseline characteristics of the latest cohort analysis (1040 transplants in 1028 recipients). Variables are expressed as mean ± St dev or %.	
Age at KTx	51.3 ± 12.5
Sex (Male)	661/1040 (63.6)
Ethnicity (Caucasian)	1005/1040 (97.1)
Years on Dialysis	4.37 ± 3.71
Previous Transplants None 1 2	 941/1040 (90.5) 90/1040 (8.7) 9/1040 (0.9)
Living Donor	60/1040 (5.8)
HCV (Positive)	86/1040 (8.3)
Pre-Transplant Diabetes	65/1040 (6.3)
Body Mass Index	24.0 ± 3.5
Peak Reactive Antibodies > 0%	294/1040 (29.3)
Total HLA Mismatches	2.86 ± 1.04
Induction Therapy None Basiliximab rATG	 89/1040 (8.6) 797/1040 (76.6) 154/1040 (14.8)
Manteinance Therapy Tacrolimus Cyclosporine A Mycophenolate Azathioprine Sirolimus Everolimus	 911/1040 (87.6) 93/1040 (8.9) 934/1040 (89.8) 15/1040 (1.4) 46/1040 (4.4) 35/1040 (3.4)
Delayed graft function	234/1025 (22.8)
One or more acute rejection episodes	91/1040 (8.8)
Creatinine at hospital discharge (mg/dL)	2.05 ± 0.85

KTx: Kidney Transplant; HCV: Hepatitis C; rATG: Thymoglobulin

Novara "gene" cohort

We proposed this study to all patients transplanted in our Kidney Transplant Centre, of which 542 accepted to be included in the study. Patients were proposed to be included in the study at the time of KTx from 2009 to 2014 or at a follow up visit if they were transplanted earlier (the older transplants dating back to year 2000). No particular exclusion criteria was chosen for patient enrolment. However specific analyses were limited to each study's interest population. Enrolled patients underwent genetic analysis of five SNPs of four genes, as detailed below. Their main clinical characteristics (Table 3) are similar to those of the population from which they have been enrolled (Table 2). However as some patients have been enrolled during a follow up visit a selection bias (ie: patients alive with a functioning graft are more likely to be included) cannot be excluded.

Table 3. Clinical characteristics of the patients enrolled in the genetic studies (Novara-gene cohort)	
Age (years)	52.4 ± 12.2
Male	64.4%
Ethnicity (Caucasian)	98.4%
Years on dialysis	4.00 ± 3.68
Previous transplants	9.1%
Living Donor	4.7%
Pre-transplant Diabetes	6.6%
BMI (Kg/m^2)	24.1 ± 3.46
Peak PRA > 20%	8.3%
Total HLA mismatches	2.87 ± 1.04
Induction therapy: None Anti IL2 receptor ATG	 5.5% 81.3% 13.2%
IS therapy at discharge Tacrolimus Cyclosporine Mycophenolate Sirolimus or Everolimus	 90.9% 6.1% 94.4% 6.3%
Delayed Graft Function	21.9%
One or more acute rejection episodes	10.2%
Serum Creatinine at discharge (mg/dL)	2.06 ± 0.94
eGFR at discharge ($mL/min/1.73m^2$)	40.0 ± 19.6

Verona "gene" cohort

As confirmatory cohort for genetic study, we asked for a collaboration to the Transplant Center of Verona: this cohort includes kidney transplant recipients transplanted in Verona and who accepted to undergo genetic analyses (n=190). However this cohorts includes five kidney transplants in patients with already another transplanted organ (lung or heart). All of these patients were enrolled at a follow up visit in 2012-2013, which was from one to twenty years after transplantation: therefore this population is heterogeneous and selected as only patients alive and with a functioning graft in 2012 were proposed to enter the study.

Moreover the baseline characteristics of this cohort are quite different from the patients from the Novara-gene cohort (Table 4). Particularly this cohort has more KTx from living donors, less previous transplants, less immunized patients (defined as PRA > 0%) and their immunosuppression has much less use of tacrolimus in favour of cyclosporine.

Table 4. Clinical characteristics of the Verona cohort (n=190)	
Age (years)	50.0 ± 13.4
Male	64.4%
Ethnicity (Caucasian)	96.3%
Previous transplants	5.3%
Living Donor	11.7%
Pre-transplant Diabetes	5.8%
BMI (Kg/m^2)	24.5 ± 4.16
Peak PRA > 0%	4.0%
Total HLA mismatches	3.53 ± 1.30
Induction therapy: None Anti IL2 receptor ATG	 0% 98.4% 1.6%
IS therapy at discharge Tacrolimus Cyclosporine Mycophenolate Sirolimus or Everolimus	 44.7% 40.5% 95.8% 14.2%
One or more acute rejection episodes	5.7%

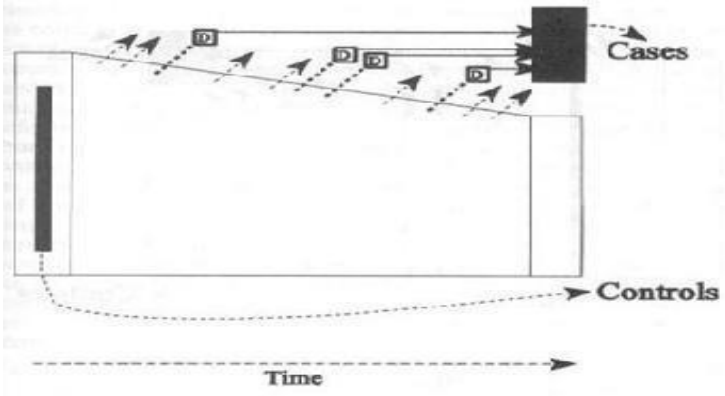
Moreover follow up data are lacking in some patients, particularly for older transplants. Indeed the more severe complications (leading to patient death or graft failure) were not reported as this cohort was enrolled cross-sectionally.

Parma case-cohort study

I've participated in a multicenter study coordinated by the Parma Transplant Center, started in 2010. Their main aim was to determine whether a post-transplant lymphoproliferative disorder (PTLD) affects death-censored graft survival. PTLD was defined as any lymphocyte malignant proliferative disease, including both lymphomas and leukemias. Involved transplant centers were Parma, Novara, Milano Policlinico, Pavia, Cagliari, L'Aquila, Catania and Matera. However, as they enrolled a large case-cohort population, we investigated together the risk factors for this rare complication (<1% of KTRs). They had no DNA for any patient, so this confirmatory cohort was used to determine which clinical and therapy variable are associated with an increased risk of PTLD: indeed, four of the SNPs we studied affect CNI metabolism and their effect might be estimated from phenotypes of KTRs.

We have enrolled 49 cases of PTLD, most of which (n=24) were diagnosed among KTRs referring to Parma. Each case was matched to four control KTR at the time of transplantation (case-cohort study) by age (± 5 years), sex, transplant center, year of transplantation (±2 years) and transplanted organ (living vs. deceased donor). Only patients at their first kidney transplant were elegible for inclusion in this study.

D: disease appearance (PTLD), cases; Arrow: lost to follow up



Data from cases included all relevant clinical and demographical characteristics. For all patients we included baseline characteristics and follow up data on anthropometric measures, renal function, rejections and IS therapy. The main clinical characteristics of cases and controls are reported in Table 5.

Table 5. Baseline characteristics of Cases and Controls of the Parma Case-cohort study

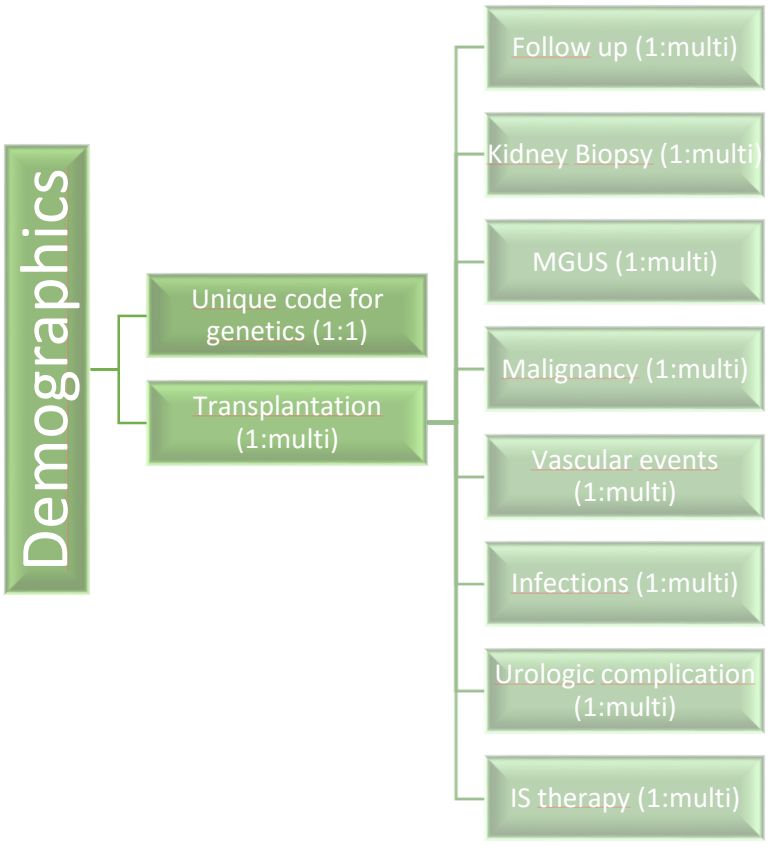
	Cases (n=49)	Controls (n=196)	p
Female	24,5%	25,0%	0,941
Age	42.0 ± 14.6	42.6 ± 13.6	0,814
Months on Dialysis	36.0 ± 32.7	49.9 ± 51.2	0,164
Mismatch HLA	3.12 ± 1.27	3.07 ± 1.18	0,808
ATG	85,7%	84,9%	0,783
Ab anti IL2R	63,3%	65,1%	
No induction	51,0%	47,6%	
Tacrolimus	38,7%	31,6%	0,253
MMF	40,8%	40,3%	0,792
m-TOR inib.	6,7%	10,3%	0,457

Clinical variable definitions and management

Main clinical and demographical variables were collected in an "ad hoc" database of the Novara cohort, focusing on pre-KTx history and therapy, cardiovascular risk factors and transplant-related variables. This database can he easy linked with the kidney donors' database managed in Torino (Immunologia dei Trapianti Regionale – ITR) and with the genetics database (Dipartimento Scienze del Farmaco – DSF) through a unique coding system.

Data management was performed by the Epidemiology Unit of our hospital. Internal and validation checks of the entered data were performed periodically twice a year.

The DB has a multilevel structure as represented in Figure.



Data of the Verona cohort were extracted from the local database of kidney transplants.

Data of the Parma case-cohort study were already recorded in two worksheets, the first including baseline data, while the second displayed follow up data (one line for each follow up

point). Data were collected from local patient records and entered in the central database by a local investigator and reviewed by two investigators from the coordinating Center.
The variable and event definitions are widely accepted and indeed all three databases used the same definition criteria for corresponding variables.

Events:

- Death is recorded from referring nephrology centers. The cause of death is determined through autopsy when available or by the caring physician otherwise. The initial main cause of death is reported in the database and grouped in 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 or clinically by the presence of a progressive renal function deterioration, increased urinary proteins and presence of donor-specific antibodies (DSA), after excluding other plausible causes of renal damage. No patient developed a graft failure due to a late-onset acute rejection. 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".
- Primary non function is 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).
- Diabetes was defined according to the 2013 American Diabetes Association Guidelines as a glycated hemoglobin (HbA1c) $\geq$ 6.5%, or a fasting plasma glucose $\geq$ 7.0 mmol/L (126 mg/dL), or a 2-h plasma glucose $\geq$ 11.1 mmol/L (200 mg/dL) during an oral glucose

tolerance test in two 30-days apart samples or a fasting plasma glucose of at least 11.1 mmol/L (200 mg/dL) with symptoms of hyperglycemia or use of any hypoglycemic agent for at least 30 days. This definition has been employed to define diabetes before KTx and after KTx; particularly the "therapy criteria" is important in the early post-transplant days, as sometimes patients need insulin for a few days (less than 30) during the high-dose pulse steroid therapy that is commonly used in the first days after surgery. Moreover a diagnosis of PTDM was made only once the transplant was stable and first evidence of hyperglycemia was employed as "event date". As we performed a diabetes-free survival analysis we did not include a post-transplant time cut-off to define post-transplant diabetes (PTDM), as recent literature suggests not to include a time-frame in the definition of PTDM.

- Thrombosis definition included death for cardiovascular causes, allograft loss for thrombosis, renal artery or vein thrombosis, AMI, stroke, transient ischemic attack and deep vein thrombosis with or without pulmonary embolism .
- Cardiac ischemic events included symptomatic ischemic heart disease and myocardial infarction. Symptomatic coronary artery disease (CAD) was defined as needing or prolonging an hospital admission for angina pectoris, including the ICD-9 diagnosis codes 411.1, 413, 413.0, 413.1 and 413.9. Acute myocardial infarction (AMI) was defined as needing or prolonging an hospital admission for an acute coronary syndrome, including the ICD-9 diagnosis codes from 410 to 410.9. All events required specific therapy, including medical therapy, percutaneous or surgical revascularization, or a combination of them.
- 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 to cancer screening as for the general population (breast, prostate, colon-rectum, cervix-uterus), and additionally they undergo to a yearly dermatologic evaluation, abdomen ultrasonography, and chest X-ray. 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. In the Parma case-cohort study we observed the following diseases: early lesion (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.

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 criteria is 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. The inclusion of a creatinine criterion improves the sensitivity of DGF, but reduces its specificity. Moreover if a patient is transplanted pre-emptively (ie: before starting dialysis) it is very unlike to start dialysis after transplantation. Lastly, patients with a follow up shorter than one week (death or primary non function) have this variable left as missing.
- 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. Sometimes -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).

- 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
- Parthormone has been divided in tertiles for analyses. This decision was taken as PTH in the general CKD population shows a U-shaped curve when related to cardiovascular risk.
- BKV nephropathy: diagnosed histologically by specific BKV-related cytopathic inclusions and changes and a positive SV40 staining. It has been rarely diagnosed clinically as BKV viremia greater than 10.000 copies/mL with a worsening renal function or with an increasing tubular proteinuria greater than 0,5 gr/24h
- Pharmacokinetic profile
    - Weight normalized daily dose: daily drug dose in mg (usually CyA or Tac) divided by patient's weight in kilograms. A high value means that the patient assumes a high dose of the IS drug.
    - Dose normalized through level: through level in ng/mL (usually measured for CyA or Tac) divided by the weight normalized daily dose. A high value means that the patient reaches a high through level given a fixed weight-adjusted IS drug dose, ie: he has a slower metabolism/higher absorption and might be more exposed to IS drugs.
    - Coeficient of variability: it can be calculated if a patient has at least three consecutive IS drug trough level. It is calculated as the ratio between the standard deviation of through levels and their mean value. It is usually expressed as a percentage. It has

been associated with a higher risk of graft failure probably because it might be sign of poor therapy compliance.

- Antiaggregant and anticoagulant management strategies. Patients on VKA underwent transplantation only after the PT-INR was lower than 1.7, which was achieved through transfusion of fresh frozen plasma and/or vitamin K. After surgery, if the indication to anticoagulation persisted (ie: it was not for vascular access patency) and there was no clinical or ultrasound evidence of active bleeding, they started enoxaparin within 12-24 hours from KTx, at a dose able to reach an anti-factor Xa activity target of 0.5 – 0.9 IU/mL. Patients on AAT stopped drug administration on the day of surgery, and they resumed it after one or two months, if still indicated. Meanwhile a bridge therapy with enoxaparin was given to reach an anti-factor Xa activity of 0.3 – 0.5 IU/mL. All the other patients received a deep venous thrombosis prophylaxis with anti-embolism compression stockings or enoxaparin (anti-factor Xa activity target of 0.3 – 0.5 IU/mL) up to two weeks after surgery. Anti-Xa activity measurement was available in our Center since 2004 and previously it was used at a dosage of 2000-4000 IU qd for deep vein thrombosis prophylaxis while for parenteral anticoagulation (ie: prosthetic heart valve) unfractionated heparin or enoxaparin 4000 IU bid have been used.

Laboratory methods

SNPs genotyping: we investigated the following SNPs: *CYP3A5* *1 and *3 (6986A>G), *CYP3A4* rs35599367C>T, *ABCB1* (*MDR1*, P-glycoprotein) rs1045642 (3435C>T) and rs1128503 (1236C>T), and *TCF7L2* rs7903146C>T.

Genomic DNA was extracted from peripheral blood by using the QiaAmp DNA Mini Kit (Qiagen Valencia, California, USA). Genotyping was performed by real-time PCR using Applied Biosystems TaqMan Pre-Designed SNP Genotyping assays. Real-time PCR

amplification and detection was conducted on genomic DNA in 48-well PCR plates using a MiniOpticon PCR Detection System (Bio-Rad, Milan, Italy), as previously reported. Reaction was set up in a final volume of 10 μl containing 10 ng genomic DNA, 1× TaqMan Genotyping Master Mix (Applied Biosystems) and 1× SNP genotyping assay. Briefly, thermal cycling was initiated with a denaturation step of 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 90 s at 60 °C. After PCR was completed, allelic discrimination was analyzed using the Bio-Rad CFX Manager Software (Version 2.1, Bio-Rad). Genotype assignment was determined by plotting the end point relative fluorescent units (RFU) for one fluorophore (allele 1 on the x-axis) against the RFU for the other fluorophore (allele 2 on the y-axis) on the allelic discrimination plot. All reactions of real-time PCR were set up in a dedicated PCR area with dedicated PCR pipettes and reagents. For quality control purposes, each Real-time PCR included negative as well as positive controls for the three genotypes. For validation, about 15% of the samples were re-genotyped. The results were reproducible with no discrepancies in genotyping. Genotyping was performed blinded to all clinical data.

Antibodies, protein-protein (immunofluorescence) double detection, immunohistochemistry and fluorescent in situ hybridization (FISH) of HPV and polyomaviruses

Tissue sections were obtained from formalin fixed and paraffin-embedded blocks, previously collected and stored in the University Hospital medical material archives.

Antibodies: the polyclonal antibodies raised against the HPV β genus E4 and L1 proteins, and the HPV α genus E4 protein (namely PanHPVE4). The α-genus HPV L1 antibodies were obtained from DAKO Corp (Carpinteria, CA), minichromosome maintenance protein 7 (MCM7) from Neomarkers Fremont (Fremont, CA), p16 (clone E6H4) and Large T SV40 (clone MRQ-4) from Ventana Medical System (Tucson, AZ).

Consecutive 5 μm sections obtained from formalin-fixed paraffin-embedded tissues were processed for the immunofluorescent protein-protein double detection, and in immunohistochemistry using the automated immunostainers BenchMark ULTRA stainer

(Ventana Medical System, Tucson, AZ).

Large T– positive cases were further tested by DNA-fluorescent in situ hybridization (FISH) using a probe derived from nick translation of the entire BKHPyV genome (Biotin Nick Translation Mix, Roche, Basel, CH).

Images were acquired using a digital scanner (Pannoramic MIDI; 3D Histech Kft., Budapest, Hungary). For the assessment of histological features, the slides analyzed by α/β-HPVE4-MCM7 (or BKPyV DNA) were disassembled and stained with hematoxylin and eosin (H&E).

Data analysis

A p value<0.05 was considered statistically significant for all statistical analyses. Statistical analysis were performed with SAS 9.2 (Statistical Analysis System) software or MedCalc Version 13.3.3 (MedCalc Software, Mariakerke, Belgium) software.

Post-Transplant Diabetes

Chi square test was used to compare categorical variables; Wilcoxon Two Sample test and Kruskall-Wallis test were used to compare continuous variables as appropriate. ANOVA test was used for univariate analyses. Gene variations were tested for deviation from the Hardy-Weinberg equilibrium by use of the Pearson's chi-square test implemented in the online Finetti's program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Event-free survival analysis was performed with an actuarial Kaplan-Meyer method, defining the event as diagnosis of PTDM and censoring patients without event at the time of graft failure, death or last visit; time to event was measured from the day of KTx to the first diagnosis of PTDM or patient censoring.

The analysis of the risk factors associated with PTDM was performed using a multivariate Cox model with the same end-point as the event-free survival analysis.

We first used a forward stepwise inclusion method and automated selection of significant variables, including always age, sex and TCF7L2 genotype and, in order, body mass index (BMI)

at KTx, acute rejection episodes, delayed graft function, peak panel reactive antibodies (PRA), use of tacrolimus, ethnicity (Caucasian vs other), as well as those variables that were significantly associated at the univariate analysis (previous transplants and cold ischemia time divided in quartiles). For *TCF7L2* rs7903146, we considered either an additive, a dominant or a recessive mode of inheritance. For model parsimony, only variables with statistically significant hazard ratios were considered in the construction of predictive models for PTDM. The Cox's proportional hazard regression coefficients of each included parameter were converted into an integer risk score by rounding the quotient of dividing the regression coefficient by a single constant. To evaluate the predictive accuracy, the receiver operating characteristic (ROC) curve for each model was derived and the area under the ROC curve (AUROCs) was calculated.

Meta-analysis of TCF7L2 rs7903146 and risk of PTDM in renal transplant patients

We carried out a computerized literature search from Web of Knowledge and Pubmed databases (up to 30 June 2015) by using the Boolean combinations of the key terms: TCF7L2 AND (allograft OR transplantation OR transplant) AND (kidney OR renal) AND diabetes. We searched for all articles published in English that evaluated the association between *TCF7L2* rs7903146 and risk of PTDM in renal transplant recipient. The identified articles had to meet the following criteria: 1) sufficient genotype data to calculate the odds ratios (ORs) with 95 % confidence intervals (CIs). Exclusion criteria were as follows: 1) insufficient information, for example, genotype frequency or number not reported; 2) studies that were not published in English; 3) review articles and duplication of previous publication. The retrieved studies were then read in their entirety to assess their appropriateness for inclusion in the meta-analysis. If two or more studies shared part of the same patient population, the more complete or the one with the larger sample size was included. All references cited in the eligible studies were also

reviewed to identify additional published works that were not initially retrieved. A standardized form was used for each of the included studies, in which the following information was filled: the first author's last name, year of publication, number of patients, age, gender, ethnic background, proposed genetic contrasts and time of PTDM evaluation. All studies were independently analyzed by two of our collaborators and any discrepancies in data extraction were resolved through consensus. The strength of the correlation between *TCF7L2* rs7903146 and risk of PTDM was calculated using ORs, and the precision of the estimate was given by 95%CIs. When any zero cell occurred in the two-by-two contingency table, we added a Woolf–Haldane continuity correction of 0.5 to generate a finite OR. Data were combined using random-effects (DerSimonian and Laird) models which incorporate the between-study heterogeneity and allow for a different effect in each population. In case of lack of heterogeneity, the random-effect model coincides with the fixed-effect model. We estimated the between-study heterogeneity across all eligible comparisons by using the Cochran's Q chi-square test (significant for P <0.10). We also reported the I^2 index, which quantifies heterogeneity irrespective of the number of studies. Meta-analysis was performed with Open Meta-Analyst available at http://www.cebm.brown.edu/open_meta/ (Joseph Lau, Boston, Massachusetts). Publication bias was investigated using funnel plots, with a roughly symmetrical distribution on either side of the summary estimate suggesting a lack of bias. The presence of possible publication bias was evaluated graphically by drawing funnel plots for each outcome measure and statistically by means of Egger's standard regression test. Calculations of publication bias were performed using StatsDirect statistical software version 3.0.146 (Stats-Direct Ltd, Cheshire, UK) and p-values <0.10 were considered to indicate statistically significant publication bias.

Early thrombotic events

Only early thrombotic events were considered as outcomes, being defined as occurring within 90 days after transplantation.

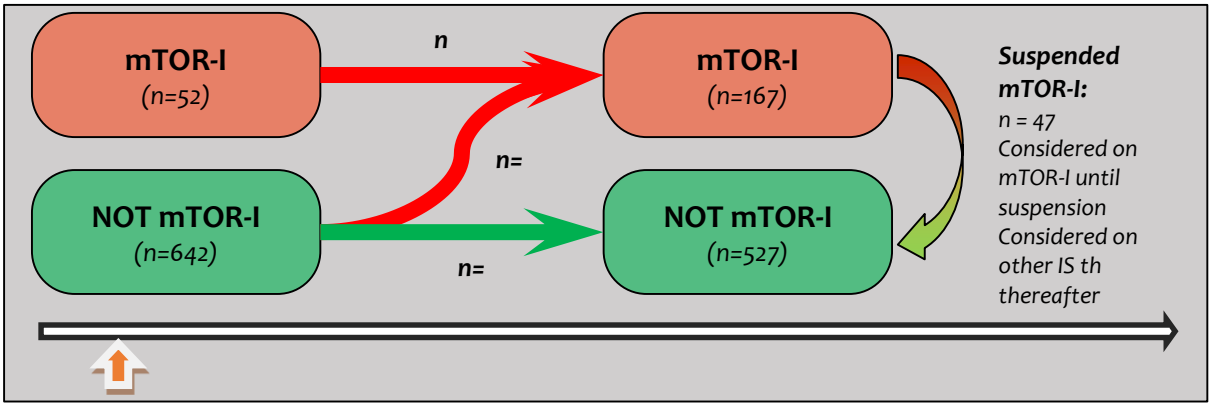
Chi square test was used to compare categorical variables; Wilcoxon Two Sample test and Kruskall-Wallis test were used to compare continuous variables as appropriate. ANOVA test was used for univariate and multivariate analyses. As the events were recorded in the first 90 days after KTx and all patients had a follow up longer than 90 days, logistic regression analysis were performed.

A multivariate logistic regression model for early CVE was developed by a step-wise forward procedure starting from the most strongly associated variables to assess the independent risk factors for CVE, taking into account at each step only the significant variables or those that significantly altered the estimated OR of previously included risk factors.

Thrombosis and mTORi

A total of 694 adult patients were enrolled fora total follow-up time of 3943 pt-years: patients who started an mTOR-I at the time of transplant (n 52) and those who were later switched to an mTOR-I (n= 115) were compared to patients who have never been on mTOR-I (n = 527).

The incidence rate of thrombotic events was calculated on the total time on therapy for immunosuppressive regimens including mTOR-I versus all the other immunosuppressive regimens, dividing the number of events by the total time at risk. Comparisons between groups were performed by t-Student test or chi square as appropriate.



Ischemic heart disease

Data were summarized and presented in the form of mean, standard deviation, and percentage as descriptive statistics. Comparisons between genotype groups were performed by one-way analysis of variance (ANOVA) for continuous variables and chi-square analysis for categorical variables. SNPs were tested for deviation from the Hardy-Weinberg equilibrium by use of the Pearson's chi-square test implemented in the online Finetti's program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

The effect of clinical variables on the risk of post-transplant CAD was first evaluated by univariate analysis, using the Chi square test for categorical variables and Wilcoxon Two Sample test or Kruskall-Wallis test for continuous variables as appropriate. Genotype frequencies were compared between patients with and without post-transplant CAD using the Armitage trend test for additive effect of rs7903146.

Event-free survival analysis was performed with an actuarial Kaplan-Meyer method, defining the event as diagnosis of CAD and censoring patients without event at the time of graft failure, death, diagnosis of post-transplant diabetes or last available visit; time to event was measured from the day of KTx to event or patient censoring.

We developed a multivariate Cox model using a backwards stepwise method, including always *TCF7L2* genotype and age, sex, previous ischemic cardiac events, time on dialysis, statin use as well as potential explanatory variables with a cutoff of P value <0.10 from univariate analyses (HLA mismatches and delayed graft function-DGF). Other variables that have been evaluated in the univariate analysis but did not result significantly associated with CAD at multivariate analysis were estimated glomerular filtration rate (eGFR, estimated with the CKD-EPI formula) at hospital discharge from KTx, antiaggregant therapy prior KTx, total cholesterol, LDL cholesterol, previous smoking, parathormone levels, previous parathyroidectomy, arterial hypertension, type of dialysis (hemodialysis, peritoneal dialysis,

pre-emptive transplantation), peak panel reactive antibodies (PRA), use of tacrolimus, presence of a monoclonal gammopathy of undetermined significance, and acute rejection episodes.

Malignancies: effects of viruses and genetics

NMSC included SCC and BCC, while no Merckel cell carcinoma was observed. All other solid and hematologic malignancies, including "*in situ"* carcinomas and malignant melanoma, were included as NCM at the time of their first diagnosis. In the analysis of genital alpha-HPV-related tumors we included both malignancies and precancerous lesions, such as high grade cervical dysplasia. As patients might have had multiple lesions, each group of tumors was treated as a separate variable such that a single patient could end up in several groups. Descriptive statistics were performed and results presented as means ± standard deviations or as events/patients (%). For incidence analysis, only the first tumor in each group was considered. The cumulative incidences of malignancies were calculated considering death and graft loss as a competing event. A univariate Cox-survival analysis was used to look for clinical risk factors for each malignancy.

To illustrate the effect of BKVAN occurrence over time on the risk of graft failure, we used a modified Kaplan-Meier method that estimated cumulative incidence of urinary tract malignancy according to the presence or absence of BKVAN. All patients at the beginning of the observations were included in the BKVAN-free group, and the assignment to the BKVAN group was updated at the time of the BKVAN diagnosis. To quantify the BKVAN effect in terms of hazard ratio, we fitted a univariable Cox model including BKVAN as a time-dependent covariate.

PTLD: case-cohort study

This is a retrospective case-cohort study. We defined as ‘‘index time’’ the time of PTLD diagnosis in the cases and the corresponding time from transplant in the controls. Because some controls did not reach the index time, some of them were lost before index time because of graft failure or death.

Then we evaluated risk factors for PTLD with an event-free survival, considering PTLD as the event and censoring patients if they died, returned to dialysis or last available visit. All available baseline variables were tested as predictors in the PTLD-free survival analysis, first in univariate models, and then we developed a multivariate Cox model including significant variables.

Moreover we performed a pilot analysis of post-transplant variables potentially associated with PTLD development, particularly IS therapy and its interaction with EBV serological status at baseline. As all cases reached the “index-time”, but not all controls did, we performed a multivariate logistic regression including patients who reached the “index time”, using PTLD as the dependent binary variable.

Results

Post-Transplant Diabetes

Specific Aim: to develop a post-transplant diabetes risk model based on pre-transplant variables, including genetic variants which might be associated with PTDM.

Population and risk factors for PTDM

We included 464 patients (52.4 ± 12.3 years, 65.3% males): they were mainly Caucasian (97.4%), received a KTx from a deceased donor (95.7%) and were on tacrolimus (90.7%), mycophenolate (94.0%) and steroid therapy (81.7%) (Table). The genotype frequency distribution of TCF7L2 rs7903146 was in accordance with Hardy-Weinberg equilibrium ($p=0.13$). In addition, according to dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=7903146), the minor allele frequency (MAF) of rs7903146 in the subset of Caucasian patients (39.0%) was similar to that reported for Tuscan Italians (HAPMAP-TSI, 38.6%).

Moreover *MDR1* polymorphisms were strongly associated each-other, with a concordance index of 81.3% ($p<0.001$), while no other interaction between SNPs was observed.

Description of the overall population and comparison between patients who developed diabetes after transplant (PTDM) and those who did not, stratified by genotype.

Parameter	Overall population (n=464)	Patients with PTDM (n=66)	Patients without PTDM (n=398)	p
TCF7L2 rs7903146 C>T				
CC	163 (35.1%)	14 (21.2%)	149 (37.4%)	
CT	237 (51.1%)	33 (50.0%)	204 (51.3%)	
TT	64 (13.8%)	19 (28.8%)	45 (11.3%)	<0.001
CYP3A5 Haplotypes				
1/1	2 (0.5%)	0	2 (0.5%)	
1/3	51 (11.7%)	4 (7.3%)	47 (12.4%)	
3/3	382 (87.8%)	51 (92.7%)	51 (87.1%)	0.466
CYP3A4 rs35599367 C>T				
CC	418 (89.9%)	49 (90.7%)	369 (89.8%)	
CT	46 (9.9%)	5 (9.3%)	5 (10%)	
TT	1 (0.2%)	0	1 (0.2%)	0.923
MDR1 3435C>T				
CC	110 (25.4%)	15 (27.3%)	95 (25.1%)	
CT	216 (49.9%)	29 (52.7%)	187 (49.5%)	
TT	107 (24.7%)	11 (20.0%)	96 (25.4%)	0.686
MDR1 1236C>T				
CC	102 (23.5%)	15 (27.3%)	87 (23.0%)	
CT	200 (46.1%)	22 (40.0%)	178 (47.0%)	
TT	132 (30.4%)	18 (32.7%)	114 (30.1%)	0.480

Table: MDR1 polymorphism correlation

MDR1	1236: CC	1236: CT	1236: TT	total
3435: CC	2	16	92	110
3435: CT	25	154	36	215
3435: TT	75	30	2	107
total	102	200	130	432

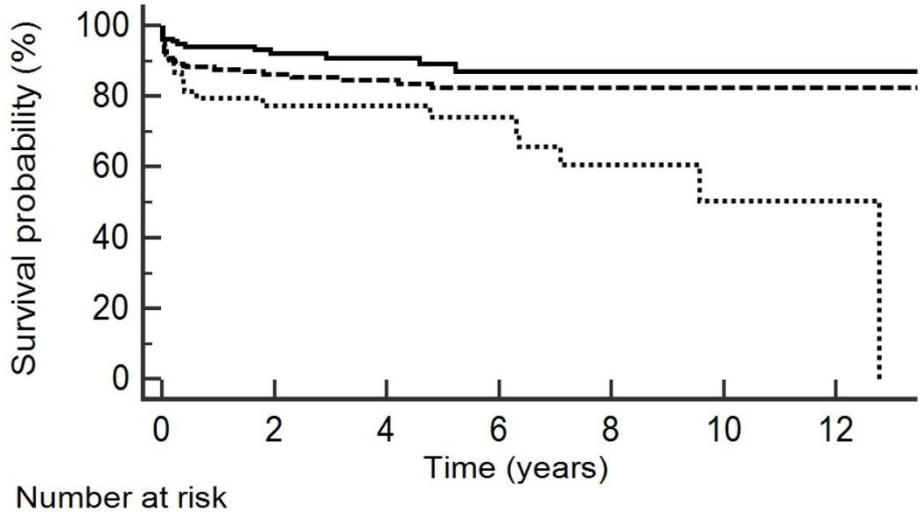
Sixty-six patients (14.2%) developed PTDM, after a mean time of 16.2±30.4 months from surgery. Incidence of PTDM was 9.25% and 11.03% at 1 and 2 years, respectively.

The comparison between patients who developed PTDM and those who did not showed that KTRs with PTDM were older, had a higher BMI at discharge from KTx and were less likely to carry TCF7L2 rs7903146 genotype CC, while no other major differences could be shown (Table). Indeed, none of the other study polymorphisms was associated with PTDM.

As for TCF7L2 rs7903146 genotype (Figure), the 1-year and 2-year-risks were respectively 5.82% and 7.83% in CC patients, 11.92% and 13.3% in CT patients and 20.47% and 22.74% in TT patients (p for trend <0.001).

However, restricting the analysis to 20/66 (30.3%) patients who were first treated for PTDM within 14 days of surgery, TCF7L2 rs7903146 genotype was not a significant risk factor, as 5/163 (3.1%) patients with a CC genotype, 12/237 (5.1%) with a CT genotype and 3/64 (4.7%) with a TT genotype developed this "early-onset" PTDM (p=0.619).

Figure 1. Diabetes-free survival analysis stratified by TCF7L2 rs7903146 genotype (censored for graft loss or patient death). Genotype CC is the bold line (n=163), CT is the dashed line (n=237) and TT is the dotted line (n=64).



Description of the overall population and comparison between patients who developed diabetes after transplant (PTDM) and those who did not. Values are expressed as mean±standard deviation or n (%); p values refer to the comparison between patients with PTDM vs. patients without PTDM.

Parameter	**Overall population** (n=464)	**Patients with PTDM** (n=66)	**Patients without PTDM** (n=398)	**p**
Age (years)	52.4 ± 12.3	55.4 ± 10.2	51.9 ± 12.6	0.008
Age > 40 years	385 (83.0)	61 (92.4%)	324 (81.4%)	0.027
Male	303 (65.3%)	39 (59.1%)	264 (66.3%)	0.252
Caucasian	452 (97.4%)	64 (97.0%)	388 (97.5%)	0.806
HCV Positive	31 (6.7%)	4 (6.1%)	27 (6.8%)	0.827
Ever Peritoneal Dialysis	112 (24.1%)	11 (16.7%)	101 (25.4%)	0.125
Ever Hemodialysis	388 (83.6%)	59 (89.4%)	329 (82.7%)	0.171
Peak PRA > 0%	115 (24.8%)	23 (34.8%)	92 (23.1%)	0.041
Previous transplants	44 (9.5%)	10 (15.2%)	34 (8.5%)	0.090
Deceased Donor	444 (95.7%)	66 (100%)	378 (95.0%)	0.063
HLA mismatches > 3	136 (29.3%)	21 (31.8%)	115 (28.9%)	0.634
Cold ischemia time (hours)	19.3 ± 5.7	20.4 ± 6.5	19.2 ± 5.5	0.085
BMI (Kg/m2)	24.1 ± 3.4	26.4 ± 3.8	23.7 ± 3.2	<0.001
BMI ≥ 25 Kg/m2	183 (39.4%)	42 (63.6%)	141 (35.4%)	<0.001
Delayed Graft Function	103 (22.2%)	19 (28.8%)	84 (21.1%)	0.164
One or more acute rejection episodes	32 (6.9%)	8 (12.1%)	24 (6.0%)	0.071
Induction therapy:				
None	27 (5.8%)	4 (6.1%)	23 (5.8%)	
Anti IL2 receptor	379 (81.7%)	53 (80.3%)	326 (81.9%)	
ATG	58 (12.5%)	9 (13.6%)	49 (12.3%)	0.716
IS therapy at discharge				
Tacrolimus	421 (90.7%)	63 (95.5%)	358 (89.9%)	0.159
Cyclosporine	29 (6.3%)	2 (3.0%)	27 (6.9%)	0.243
Mycophenolate	436 (94.0%)	62 (93.9%)	374 (94.0%)	0.992
Azathioprine	2 (0.4%)	0 (0%)	2 (0.5%)	0.564
Sirolimus or Everolimus	32 (6.9%)	5 (7.6%)	27 (6.8%)	0.814
Steroid withdrawal	85 (18.3%)	13 (19.7%)	72 (18.1%)	0.755
Follow up time (years)	3.97 ± 3.73	4.01 ± 3.93	3.96 ± 3.70	0.463

BMI: body mass index; KTx: kidney transplant; ATG: anti-thymocyte globulin; IS: immunosuppressive.

Multivariate analysis of risk factors for PTDM

Among analyzed risk factors, only age, sex, TCF7L2 rs7903146 polymorphism, BMI, previous transplants and CIT significantly affected the multivariate risk model (Table). Significant risk factors for PTDM were age (per year; HR = 1.029; 95%CI 1.005-1.054), BMI (25.0-29.9 vs <25.0, HR = 2.43; 95%CI 1.40-4.23; ≥30 vs <25.0, HR = 5.70; 95%CI 2.77-11.74), TCF7L2 rs7903146 (per each T allele; HR 1.807; 95%CI 1.261-2.590) and previous transplants (HR 2.798; 95%CI 1.388-5.638). When multivariate analysis was limited to Caucasian patients, the effect of rs7903146 remained significant. Comparison of multivariate analysis of risk factors for PTDM using a dominant and or a recessive model of inheritance of the TCF7L2 rs7903146 polymorphism showed that the strongest association was found under the recessive model (TT vs CC+CT, HR: 2.29, 95%CI: 1.30-4.04, p=0.004).

Multivariate analysis of risk factors for PTDM in renal transplant patients.

Risk Factor (reference group)	**HR**	**95% CI**	**p-value**
Age (per year)	1.029	1.005-1.054	0.017
BMI			
< 25.0	1 (Ref)		
25.0 – 29.9	2.429	1.396-4.226	0.002
≥ 30.0	5.698	2.766-11.738	<0.001
TCF7L2 rs7903146 (per each T allele)	1.807	1.261-2.590	0.001
Previous KTx (vs. first KTx)	2.798	1.388-5.638	0.004

Multivariate analysis adjusted by sex and cold ischemia time (CIT). Variables entered but not retained in the model: tacrolimus, cyclosporine A, delayed graft function, acute rejection, panel reactive antibodies, ethnicity. BMI: body mass index; KTx: kidney transplant; HR: Hazard Ratio; CI: Confidence Interval.

Comparison of multivariate analyses of risk factors for PTDM using a dominant or a recessive model of TCF7L2 rs7903146 in Caucasian patients.

	Dominant genetic model			Recessive genetic model		
Risk Factor (reference group)	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Age (per year)	1.029	1.005-1.054	0.019	1.029	1.005-1.054	0.019
BMI						
<25	1			1 (Ref)		
25.0- 29.9	2.239	1.278-3.922	0.005	2.484	1.409-4.378	0.0017
≥30	6.432	3.126-13.230	<0.0001	5.993	2.897-12.400	<0.0001
TCF7L2 rs7903146 (TT+CT vs. CC)	1.889	1.043-3.419	0.037	-	-	-
TCF7L2 rs7903146 (TT vs. CT+CC)	-	-	-	2.291	1.301-4.036	0.004
Previous transplants (vs. first KTx)	2.708	1.343-5.458	0.006	3.087	1.517-6.284	0.002

HR: Hazard Ratio; Ref: reference; CI: Confidence Interval; BMI: body mass index.
Multivariate analysis adjusted by sex and cold ischemia time (CIT).

Moreover TCF7L2 rs7903146 was evaluated as an effect modifier of the other risk factors by applying the same multivariate risk model in each polymorphism group (CC vs. CT vs. TT), and both the hazard ratio and strength of the associations remained the same, even if some risk factors lost statistical significance: therefore TCF7L2 rs7903146 polymorphism is not an effect modifier of the reported associations. The TCF7L2 rs7903146 polymorphism was also evaluated for collinearity with the other risk factors and was not associated with age (p=0.702), sex (p=0.618), BMI (p=0.415), previous transplant (p=0.079) and CIT (p=0.389).

Lastly, we developed a multivariate risk model for PTDM developing after 14 days from surgery: both the TT genotype (HR=3.30; 95%CI 1.69-6.44) and BMI (25.0-29.9 vs <25.0, HR=2.61; 95%CI 1.22-5.59; ≥30.0 vs <25.0, HR= 10.99; 95%CI 4.62-26.11) were confirmed to be a significant, independent risk factors, while age (HR 1.019; 95%CI 0.99-1.05) and previous transplants (HR 2.52; 95%CI 0.85-7.48) did not reach a statistical significance; however the parameter estimates were very similar to the general model for PTDM.

Comparison with the Verona Cohort

The Verona cohort consisted in 190 kidney transplant recipients who received their transplant in Verona and were included in a genetic study in Verona (50.0 ± 13.4 years, 64.4% males): they were mainly Caucasian (96.3%), and received a KTx from a deceased donor (88.3%). The genotype frequency distribution of TCF7L2 rs7903146 was in accordance with Hardy-Weinberg equilibrium (p=0.72). The comparison of risk factors for PTDM between the two cohorts is reported in Table: patients from Verona were younger, had less cold ischemia time, and were more likely to be obese (BMI > 30). The two cohorts received a different IS therapy, as for induction therapy, choice of CNI (much more Cyclosporine in Verona) and use of mTOR inhibitors (more common in Verona).

Table. Comparison between the "Novara gene cohort" and the "Verona cohort"

Parameter	**Novara cohort** (n=464)	**Verona cohort** (n=190)	**p**
Age (years)	52.4 ± 12.3	50.0 ± 13.4	0.033
Male	303 (65.3%)	122 (64.4%)	0.791
TCF7L2 rs7903146 C>T			
CC	163 (35.1%)	84 (44.2%)	
CT	237 (51.1%)	83 (43.7%)	
TT	64 (13.8%)	23 (12.1%)	0.094
Previous transplants	44 (9.5%)	14 (7.3%)	0.388
Prev. NOT-kidney Tx	0	4 (2.1%)	0.007
Cold ischemia time (hours)	19.3 ± 5.7	14.2 ± 5.6	<0.001
BMI (Kg/m2)	24.1 ± 3.4	24.5 ± 4.2	0.244
BMI ≥ 25 Kg/m2	183 (39.4%)	74 (38.9%)	0.907
BMI ≥ 30 Kg/m2	26 (5.6%)	21 (11.1%)	0.014
Acute rejection	32 (6.9%)	11 (5.7%)	0.604
Induction therapy:			
None	27 (5.8%)	0	
Anti IL2 receptor	379 (81.7%)	187 (98.4%)	
ATG	58 (12.5%)	3 (1.6%)	<0.001
IS therapy at discharge			
Tacrolimus	421 (90.7%)	85 (44.7%)	<0.001
Cyclosporine	29 (6.3%)	77 (40.5%)	<0.001
Mycophenolate	436 (94.0%)	182 (95.8%)	0.353
Azathioprine	2 (0.4%)	0	>0.90
Sirolimus or Everolimus	32 (6.9%)	27 (14.2%)	0.003
PTDM	66 (14.2%)	13 (6.8%)	0.008
Early PTDM (< 14 days)	20 (4.3%)	11 (5.8%)	0.419
Late PTDM (> 14 days)	46 (9.9%)	2 (1.1%)	<0.001

In the Verona cohort 13 patients (6.8%) developed PTDM, which is less than in the Novara cohort. However there was no difference between the rate of diagnosis of early diabetes (p=0.42), while the two cohorts were significantly different for late PTDM. Indeed, given this very low event rate in the Verona cohort (much less than expected), we were not able to identify any risk factor for PTDM. Moreover, as the Verona cohort was significantly different from the Novara cohort, particularly as for event rate and "late" event rate (only 2 events, instead of the expected 20), we were not able to replicate the results obtained from the Novara cohort.

As for the Novara cohort, TCF7L2 rs7903146 genotype was not a significant risk factor for early PTDM, as 4/84 (4.8%) patients with a CC genotype, 6/83 (7.2%) with a CT genotype and 1/23 (4.3%) with a TT genotype developed this "early-onset" PTDM (p=0.754).

Meta-analysis of TCF7L2 rs7903146 and risk of PTDM in renal transplant patients

The electronic search on Web of Knowledge and PubMed yielded a total of 15 articles. After exclusion of 10 studies, respectively for overlapping with another included study (n=5), irrelevance (n =1), not including renal transplant recipients (n=2), review (n=1) and nonresponse of authors to e-mail (n=1), a total of 5 studies fulfilled our inclusion criteria. These identified studies were published from 2009 to 2014, and their sample sizes ranged from 234 to 1076 KTRs, with a percentage of patients with PTDM ranging from 11.0% to 43.9%. In all studies, distribution of rs7903146 genotypes was found in Hardy-Weinberg equilibrium (all P>0.05). Other basic characteristics of identified studies are summarized in Table.

Synopsis of studies included in the meta-analysis of TCF7L2 rs7903146 and risk of PTDM in KTR.

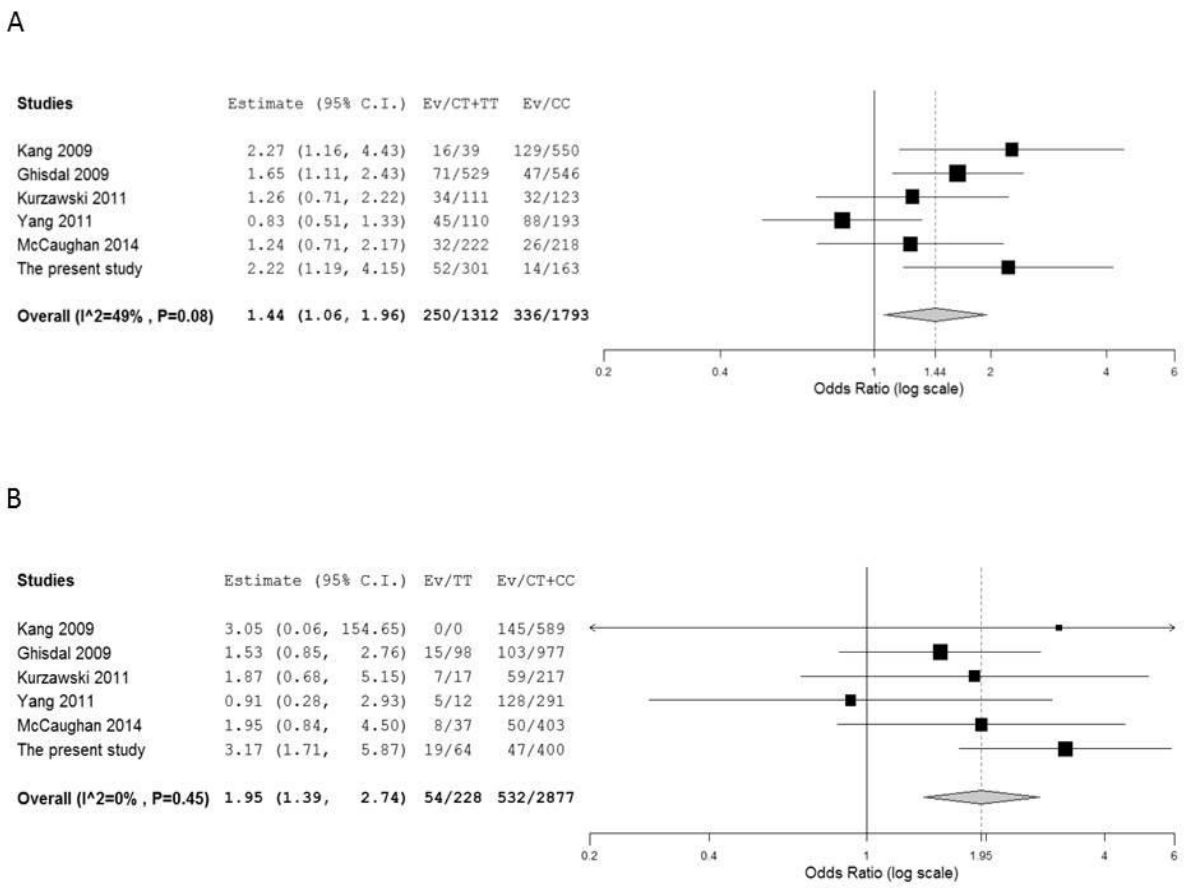
Authors	Year	N	Ethnicity	Age at transplantation (yr; mean ± SD)		M/F	Cases/ Controls	Time of PTDM diagnosis	Results	MAF %
				PTDM+	PTDM-					
Ghisdal et al (25)	2009	1076	Mainly Caucasian	52.8	46.7	1.74	0.12	within 6 months after Tx	Per each T allele, OR: 1.60 (1.18-2.25, p=0.002)	29.2
Kang et al (24)	2009	589	Korean	42.6 ± 9.1	37.4 ± 9.3	1.87	0.32	at 1 year after Tx	Per each T allele, OR: 2.20 (1.14-4.22, p=0.016) HR:1.71 (1.01-2.89, p=0.044)	3.3
Kurzawsky et al (26)	2011	234	Caucasian	47.7 ± 10.6	43.2 ± 13.0	1.16	0.39	within 1 year after Tx	All patients: NS Early onset*: NS Late onset[#]: CT+TT, OR: 1.88 (0.80-4.24, p=0.089)	23.0
Yang et al (28)	2011	303	Hispanic	44.3 ± 13.8	41.0 ± 13.1	1.29	0.78	up at least 1 year after Tx	NS	20.1
Mc Caughan et al (29)	2014	427	Caucasian	49.1 ± 13.2	42.4 ± 14.0	1.88	0.15	at 1 year after Tx	NS	29.4

NR, not reported; NS, not significant; MAF, minor allele frequency.*developed up to 14 days from transplantation; [#]developed later than 14 days from transplantation. †Test for departure of TCF7L2 rs7903146 from HWE.

Random-effect meta-analysis combining the present results with data from these previous studies (total patients, n=3105) showed a significant relationship between rs7903146 and risk of PTDM under the genetic dominant model (CT+TT vs CC, OR: 1.44, 95%CI: 1.06-1.96, p=0.019, Figure, panel A) which, however, was limited by the presence of heterogeneity among studies (I^2=49%, P=0.08). Conversely, a highly significant association was found with rs7903146 under the recessive model (TT vs CT+CC, OR:1.95, 95%CI 1.39-2.74, p=0.0001, Figure, panel B) in the absence of study heterogeneity (I^2= 0%, P=0.45). Given that minor allele frequency (MAF) of rs7903146 differs between European and Asian populations (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=7903146), we conducted a sensitivity analysis restricted to Caucasians which confirmed the stability and liability of the association (TT vs CT+CC, OR: 1.93, 95%CI, 1.32-2.80, p<0.001).

In addition, we also conducted a meta-regression analysis to assess the impact of MAF on the association of rs7903146. A trend towards a linear relationship was found between OR and MAF of rs7903146 under the recessive model (p=0.089). No evidence of publication bias was found under either the dominant (Egger's test p=0.55) or the recessive (Egger's test p= 0.72) contrast of rs7903146.

Figure - Forest plots for the association of TCF7L2 rs7903146 with risk of PTDM in the dominant (CT+TT vs CC, Panel A) or recessive (TT vs CT+CC, Panel B) mode of inheritance. Pooled estimates (OR, odds ratio) are from the random-effects model.



Predictive value of risk factors and models on PTDM

By using the area under the Receiver Operating Characteristic curve (AUC) and Youden´s index, age ≥56 was found as optimal cutoff value for PTDM prediction. Regarding BMI, patients were classified in three groups: BMI <25, 25≤ BMI <30 (this BMI interval defines an

overweight person), BMI ≥ 30 (this value of BMI defines an obese subject). Then, we compared three potential predictive risk models for PTDM based on the variables identified as significant predictors in the multivariate analysis (Table): Model 1 included only the three clinical factors (age, BMI and previous transplants), Model 2 additionally included rs7903146 in a dominant model (CT+TT vs CC) and Model 3 additionally included rs7903146 in a recessive model (TT vs CT+CC).

Table. Coefficients and risk points of each predictor of PTDM for risk prediction models.

Predictor	Beta	HR (95%CI)	P value	Score	Beta	HR (95%CI)	P value	Score	Beta	HR (95%CI)	P value	Score
	Model 1 (clinical factors only)				**Model 2 (clinical factors and dominant model of rs7903146)**				**Model 3 (clinical factors and recessive model of rs7903146)**			
Age, years												
<56	Ref	1		0	Ref	1		0	Ref	1		0
≥56	0.70	2.01 (1.21-3.34)	0.007	1	0.56	1.96 (1.19-3.24)	0.009	1	0.70	2.00 (1.21-3.33)	0.007	1
BMI												
<25	Ref	1		0	Ref	1		0	Ref	1		0
25-29.9	0.88	2.40 (1.39-4.13)	0.002	1	1.08	2.37 (1.37-4.08)	0.002	1	0.99	2.69 (1.54-4.67)	<0.001	1
≥30	2.07	7.91(3.90-16.03)	<0.001	3		7.64 (3.76-15.5)	<0.001	3	1.95	7.00 (3.44-14.27)	<0.001	3
Previous Transplants					Ref							
NO	Ref	1		0	0.82	1		0	Ref	1		0
YES	0.93	2.53 (1.27-5.03)	0.008	1		2.41 (1.21-4.79)	0.012	1	1.03	2.79 (1.40-5.60)	0.004	1
TCF7L2												
CC					Ref	1		0				
CT+TT					0.66	1.90 (1.06-3.43)	0.033	1				
TCF7L2												
CC+CT									Ref	1		0
TT									0.98	2.66 (1.53-4.60)	<0.001	1

Only Model 3 displayed a significantly better predictive ability than Model 1 (0.75 vs 0.71, P=0.013), suggesting that rs7903146 can improve the predictive ability of a model based on clinical variables in the recessive but not in the dominant model (Figure).

Indeed, when applying the score developed in Model 3, the proportion of patients with PTDM in each score group (from 0 to 5) generally showed an increasing trend from lower to higher scores (Figure): 4.0% (score 0), 7.5% (score 1), 27.2% (score 2), 38.5% (score 3), 40.0% (score 4) and 66.7% (score 5).

Figure - The area under the ROC curve (AUROC) for three PTDM risk-predicting models calculated by the risk score method. Model 1 includes clinical factors only (age, BMI and previous transplantation); Model 2 contains clinical factors and the dominant model of rs7903146 (CT+TT vs CC); Model 3 considers clinical factors and the recessive model of rs7903146 (TT vs CT+CC).

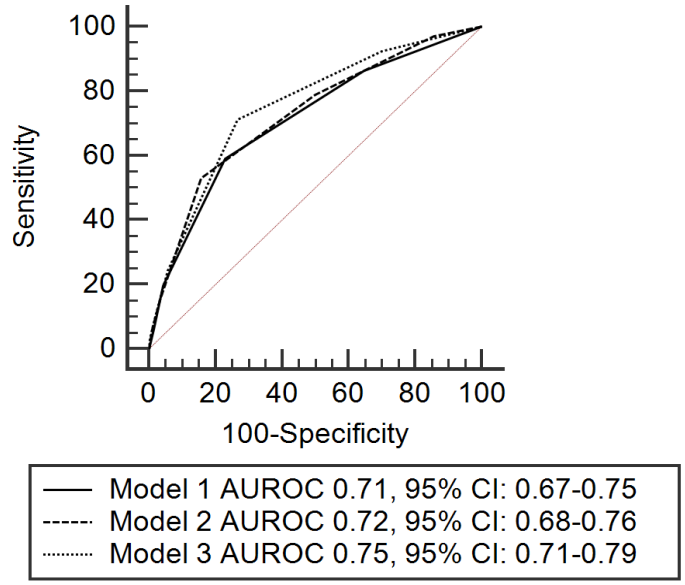
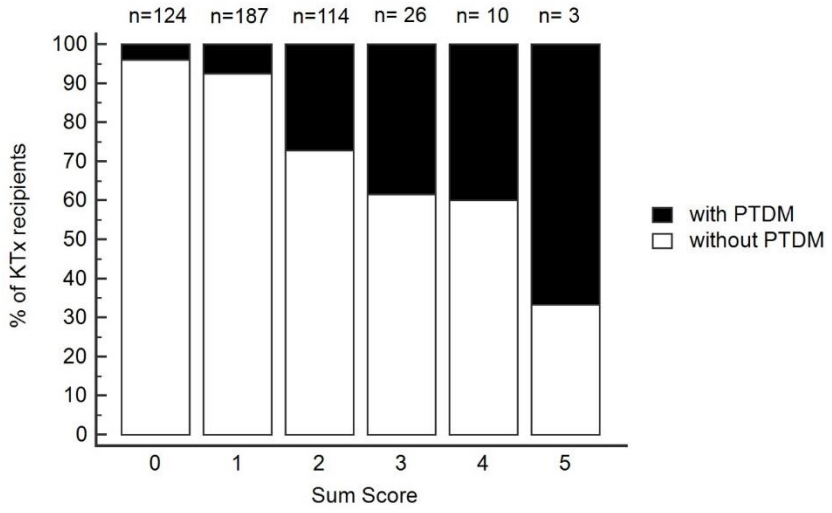


Figure - Proportion of patients with (black bar) and without (white bar) post-transplant diabetes at the end of follow up stratified by the developed risk score



Cardiovascular events

Specific Aims: to evaluate the impact of different therapies (treated as "environmental" risk factors) on post-transplant thrombotic events (early events).

Overall 911 patients (51.2 ± 12.5 years, 62.8% males) underwent KTx (Table): 326 of them (39.5%) were on single antiplatelet therapy at the time of surgery, of which 266 (81.6%) on aspirin, 40 (12.3%) on ticlopidine, 1 (0.3%) on clopidogrel and 19 (5.8%) on dipiridamole. Moreover 12 (1.5%) patients were on a dual antiplatelet therapy (aspirin plus ticlopidine) and 37 (4.5%) were on oral anticoagulant therapy, of which 2 were also on aspirin.

Secondary prevention of CVE was an indication for single AAT in 79/326 patients (24.2%), primary prophylaxis of CVEs in 192/326 (58.9%) and vascular access patency preservation in 55/326 (16.9%), while a dual AAT was used as secondary prophylaxis after a previous CVE in 8 (66.7%) and for vascular access patency in 4 (33.3%). VKA was indicated for atrial fibrillation (chronic or paroxysmal) in 15/37 patients (40.5%), as a secondary prophylaxis after repeated thrombotic events or known thrombophilia in 11/37 patients (29.7%), for the presence of a mechanic heart valve in 6/37 patients (16.2%) and for other indications (mainly vascular access patency) in 5/37 (13.5%). Immediately before surgery patients on VKA had a mean PT-INR of 1.41 ± 0.35.

Table - Description of the overall enrolled population. Values are expressed as mean±standard deviation or n/tot (%)	
Parameter	**Overall population** (n=911)
Age (years)	51.2 ± 12.5
Male	572/911 (62.8%)
Duration of dialysis (years)	4.5 ± 3.8
Deceased Donor	872/911 (95.7 %)
Dual deceased donor KTx	47/911 (5.1 %)
Transplant year 1998 – 2003 2004 – 2008 2009 – 2013	 268/911 (29.4%) 365/911 (40.0%) 278/911 (30.6%)
BMI (Kg/m2)	23.9 ± 3.5
PTH at transplant (pg/mL)	177.8 ± 178.2
Cinacalcet at transplant	56/893 (6.3%)
Parathyroidectomy before KTx	96/893 (10.7%)
HCV Positive	82/911 (9.0 %)
Diabetes at KTx	51/903 (5.6%)
Previous CVE Myocardial infarction Coronary artery disease Stroke Other	156/911 (17.1%) 46/911 (5.0%) 41/911 (4.5%) 15/911 (1.6%) 83/911 (9.1%)
Delayed Graft Function	215/896 (24.0 %)
One or more acute rejection episodes	84/635 (13.2 %)
Serum creatinine at discharge (mg/dL)	2.1 ± 0.9
eGFR at discharge (ml/min/1.73m^2)	39.3 ± 17.7
Induction therapy: None Anti IL2 receptor ATG	 89/910 (9.8%) 693/910 (76.1%) 128/910 (14.1%)
Maintenance therapy at discharge Tacrolimus Cyclosporine Mycophenolate Azathioprine Sirolimus Everolimus	 797/909 (87.7%) 83/909 (9.1%) 810/906 (89.4%) 15/906 (1.7%) 46/906 (5.1%) 28/905 (3.1%)

PTH: parathormone; BMI: body mass index; KTx: kidney transplant; CVE: cardiovascular event; eGFR: estimated Glomerula Filtration Rate (CKD-EPI equation); ATG: anti-thymocyte globulin

Patients on AAT or VKA were older and were more likely to have had a CVE before KTx; moreover, they had a higher incidence of delayed graft function, and received more blood transfusions after KTx. Immunosuppressive therapies were not different if the patients were stratified by AAT or VKA.

After surgery, AAT was resumed after a mean period of 240 ± 434 days in patient on primary prevention and 91 ± 119 days in patient on secondary prevention. Oral anticoagulation was resumed at

84.7 ± 87.5 days, but a bridge therapy with low molecular weight heparin (LMWH) was performed as detailed above.

Table - Comparison between patients on pre-transplant antiaggregant therapy, oral anticoagulant therapy or neither one. Values are expressed as mean±standard deviation or n/tot (%)			
Variables	**Antiaggregant therapy** (n=336)	**Oral Anticoagulant Therapy** (n=37)	**Neither AAT or VKA** (n=538)
Age (years)	55.0 ± 11.4 *	53.1 ± 10.4 *	48.9 ± 12.5
Male	221/336 (65.7%)	24/37 (64.9%)	327/538 (60.8%)
Duration of dialysis (years)	4.0 ± 3.6	7.6 ± 5.1 * *§*	4.4 ± 3.7
Deceased Donor	329/337 (97.6%) *	36/37 (97.3%)	507/538 (94.2%)
Transplant year 1998 – 2003 2004 – 2008 2009 – 2013	*§* * 65/336 (19.3%) 132/336 (39.3%) 139/336 (41.4%)	 13/37 (35.1%) 16/37 (43.2%) 8/37 (21.6%)	 190/538 (35.3%) 217/538 (40.3%) 131/538 (24.3%)
Diabetes at KTx	32/336 (9.5%) *	3/37 (8.1%)	16/538 (3.0%)
Previous CVE	87/336 (25.9%) *	29/37 (78.4%) * *§*	40/538 (7.4%)
Delayed Graft Function	95/336 (28.2%) *	16/37 (43.2%) *	104/538 (19.3%)
One or more acute rejections	35/253 (13.8%)	6/23 (26.1%) *	43/359 (12.0%)
Length of hospital stay (days)	25.0 ± 15.9	33.4 ± 21.9 * *§*	24.1 ± 17.3
Creatinine at discharge (mg/dL)	2.2 ± 0.8 *	2.4 ± 0.8 *	2.0 ± 0.9
eGFR at discharge (ml/min/1.73m^2)	35.8 ± 15.1 *	31.7 ± 11.0 *	42.1 ± 19.2
Blood units per patient	2.3 ± 2.8 *	4.0 ± 4.4 *	1.7 ± 2.6
Early hemorrhagic events	9/336 (2.7%) *§*	4/37 (10.8%) *	8/538 (1.5%)
Early post-transplant CVE	15/336 (4.5%) *§*	5/37 (13.5%) *	12/538 (2.2%)

*AAT: antiaggregant therapy; VKA: oral anticoagulation with vitamin K antagonist; eGFR: estimated Glomerula Filtration Rate (CKD-EPI equation); KTx: kidney transplant; CVE: cardiovascular event; ATG: anti-thymocyte globulin; * p < 0.05 vs. no AAT neither VKA;§ p < 0,05 of AAT vs. VKA.*

Over the first 90 days after KTx CVE occurred in 32/911 (3.5%), including 12 graft artery or vein thrombosis, 5 graft infarctions and 8 AMI.

Among them, 3 (9.3%) were fatal (2 cardiogenic shock in the early post-KTx hours, one mesenteric artery thrombosis) and 11 led to graft loss (34.4%) for graft thrombosis. The other 18 included 8 myocardial infarctions, 6 transplant infarctions, 3 angina episodes and one ischemic stroke. Among patients with a post-KTx CVE, 15/32 (46.9%) were on a single AAT (13 on acetylsalicylic acid and 2 on ticlopidine), 5/32 (15.6%) were on VKA; none was on dual AAT. Screening for genetic and acquired thrombophilia was performed in 16 patients with a post-KTx CVE and resulted positive in two: one patient has a heterozygous mutation of factor V Leiden and one has an anti-beta2 –glycoprotein I antibody.

At univariate analysis, risk factors for early CVE were age, KTx year, PTH, cinacalcet use at transplant, previous CVE and VKA at transplant, while AAT use was not associated with CVE (Table). Interestingly, even if PTH and cinacalcet were, parathyroidectomy was not significantly associated with CVE (OR=1.816; 95%CI=0.624-5.286; p= 0.270). Significant independent risk factors for CVE at multivariate analysis were previous CVE (OR 4.180, p = 0.0032) and cinacalcet use at time of KTx (OR 7.930, p < 0.0001) (Table 4).

Patients with a post-KTx CVE were more likely to have a DGF (42.9% vs. 23.5%, p = 0.041), to receive a higher number of transfusions during hospitalization (4±3.2 vs. 2.1±3.4, p < 0.0001) and to have a higher serum creatinine at discharge (2.6±1.1 vs. 2.0±0.8 mg/dL, p = 0.012).

Table - Univariate and multivariate logistic model for major early CVE (n=32) in the overall population (n=911).

Univariate analysis	**OR**	**95% CI**	**p-value**
Age (for 1 year increase)	1.035	1.003-1.067	0.032
Transplant year 2009-2013 (vs 1998-2003)	4.569	1.526 – 13.684	0.007
Transplant year 2004-2008 (vs 1998-2003)	1.859	0.577 – 5.993	0.299
PTH more than 187 pg/mL (vs less 187 pg/mL)	3.760	1.377 – 10.268	0.010
Cinacalcet use at transplant (vs no cinacalcet)	8.814	3.553 – 21.866	< 0.001
History of CVE (vs no previous CVE)	4.441	2.156 – 9.149	< 0.001
VKA at transplant (vs no VKA and no AAT)	4.813	1.730 – 13.389	0.003
Any AAT (vs no AAT and no VKA)	1.553	0.721-3.347	0.261
Multivariate analysis	**OR**	**95% CI**	**p-value**
Transplant year 2009-2013 (vs 1998-2003)	3.172	0.380 – 26.513	0.2865
Transplant year 2004-2008 (vs 1998-2003)	3.787	0.448 – 30.531	0.2183
Cinacalcet use at transplant (vs no cinacalcet)	7.930	3.002 – 20.945	< 0.0001
History of CVE (vs no previous CVE)	4.180	1.615 – 10.948	0.0032

PTH: parathormone; CVE: cardiovascular event; VKA: oral anticoagulation with vitamin K antagonist; AAT: antiaggregant therapy; OR: odds ratio; CI: confidence interval

Thrombosis and mTORi

Specific Aims: to evaluate the impact of different therapies (treated as "environmental" risk factors) on post-transplant thrombotic events (late events).

167 received an mTOR-I and were followed for a total time of 985 pt-years (mean 5.73±3.25 yrs), while the total time on mTOR-I therapy was 575 pt-years (mean 3.34±2.57 yrs). No major clinical differences were noted between patients on mTOR-I and those who never received them; however patient who received and mTOR-I were slightly older at the time of transplant (51.4 ± 10.8 vs 50.5 ± 13.4 years, p = 0.036). Among mTOR-I treated patients, the other most common IS was with tacrolimus (81/167=48.5%), followed by mycophenolate (65/167=38.9%). 47 pts (28.1%) suspended the mTOR-I, of which 23 (49%) due to major adverse events, while the others for infections or minor adverse events.

Table – Comparison between patients who ever received an mTORi and those who did not

	Study population (n=694)	**Never mTOR-I (n=527)**	**mTOR-I (n=167)**	**p-value**
Age (years)	51.06±12.78	50.62±13.41	52.45±10.78	0.036
Male (%)	442 (63.7)	334 (63.4)	108 (64.7)	0.762
Sirolimus, at any time	119 (17.1)	0	119 (71.3)	n/a
Initial therapy with SRL (%)	26 (3.7)	0	26 (15.6)	n/a
Initial therapy with EVER (%)	26 (3.7)	0	26 (15.6)	n/a
Initial therapy with TAC (%)	610 (87.9)	492 (93.4)	118 (70.7)	<0.001
Initial therapy with CsA (%)	60 (8.6)	27 (5.1)	33 (19.8)	<0.001
Follow up time (years)	5.68±3.76	5.61±3.92	5.73±3.25	0.355

During the follow-up, there were globally 59 MTE (8.5%), of which 35 (5.0% of patients) were arterial and 24 (3.5% of patietns) venous events. Among patients who ever received an mTOR-I, 16 events occurred during mTOR-I therapy (9.58%; M:F=11:5; males=68.8%, one lethal event), while 8 events occurred during the other mTOR-I free periods (4.79%; M:F=5:3; males=62.5%).

The overall incidence rate of MTE was of 1.496 events per 100 pt-year: 2.783 during mTOR-I therapy and 1.277 while not on mTOR-I. The incidence rate ratio was 2.180 (95% confidence interval: 1.228–3.870, p = 0.003) with a mean incidence rate difference of 1.507 more events per 100 pt-year during mTOR-I.

MTEs occurred at a mean age of 57.30±8.39 years and at 4.28±3.79 years after starting mTOR-I therapy (range: 7 days – 10.8 years). Among these patients, six were tested for acquired and genetically inherited causes of thromhophilia and one (1116 = 63%) resulted positive for an heterozygous mutation of factor V Leiden, as reported previously.

Table – Follow up events of patients who ever received an mTOR-I and those who did not

	Never mTOR-I (n=527)	**mTOR-I (n=167)**	**p**
Follow up time (years)	5.61±3.92	5.73±3.25	0.355
Time on mTOR-I	-	3.34±2.57	n/a
Dead at the last follow up (%)	13 (2.5)	10 (5.9)	0.027
CV death (%)	8 (1.5)	3 (1.8)	0.802
Major thrombotic events (%)	35 (6.6)	24 (14.4)	<0.001
Venous events (%)	14 (2.7)	10 (6.0)	0.040
Arterial events (%)	21 (4.0)	14 (8.4)	0.024
CVE incidence rate (ev/100-pt-yrs)	1,277	2,783	0,003
Venous CVE inc. rate	0,511	1,160	0,023
Arterial CVE inc. rate	0,766	1,623	0,040
Incidence rate ratio	**1**	**2,180**	**0,003**

Pts on mTOR-I with MTE did not experience more malignancies needing switch to mTOR-I (33.3% versus 46.8%, p 0.520): particularly only one patient on mTOR-I developed a DVT during chemotherapy for lymphoma. Interestingly patients on mTOR-I who developed a MTE had a significantly longer duration of mTOR-I therapy (5.82±3.48 vs 3.19± 2.47 years; p=0.003), as compared to pts on mTOR-I without MTE, suggesting that they have been exposed to the same risk for a longer time.

Table – Comparison of patients on mTOR-i who developed a major thrombotic event (MTE) and those on mTOR-i who did not develop an MTE.

	MTE on mTOR-I (n=16)	**mTOR-I, no MTE** (n=151)	**p**
Age (years)	53.02±9.80	52.39±10.88	0.404
Male (%)	11 (68.8)	97 (64.2)	0.720
Follow up (years)	6.98±3.47	5.59±3.23	0.064
Initial therapy with SRL (%)	7 (43.8)	19 (12.6)	0.001
Initial therapy with EVER (%)	3 (18.8)	23 (15.2)	0.712
Malignancy or history of malign. (%)	2/6 (33.3)	51/109 (46.8)	0.520
Time of switch to mTOR-I (mo)	6.85±14.00	21.95±30.2	0.991
Sirolimus as maintenance (%)	12 (75.0)	107 (70.9)	0.728
Sirolimus dose (mg/day)	1.70±0.95	1.52±0.85	0.267
Sirolimus BTL (ng/mL)	6.64±1.58	6.73±1.48	0.574
Everolimus dose (mg/day)	1.25±0.66	1.84±0.70	0.95
Everolimus BTL (ng/mL)	4.7±1.21	5.59±1.52	0.917
Time on mTOR-I (years)	5.82±3.48	3.19±2.47	**0.003**

Ischemic heart disease

<u>Specific Aims</u>: To evaluate if SNPs associated with IS metabolism or complications may indeed alter the risk of thrombotic or cardiovascular events. To develop a risk model for specific post-transplant CVE based on pre-transplant variables.

We included 506 non-diabetic patients (age: 52.4 ± 12.2 years; 64.4% males), who received a KTx from a deceased donor (95.3%) and were on tacrolimus (90.9%), mycophenolate mofetil (MMF) or mycophenolic acid (MPA) (94.5%) and steroid therapy (82.6%) (Table).

Twenty-two patients (4.3%) developed CAD, after a mean time of 27.2 ± 40.1 months from surgery. Incidence of CAD was 3.1% and 5.3% at 1 and 5 years, respectively.

The univariate comparison between patients who developed a CAD and those who did not showed that KTR who developed a post-transplant ischemic heart event were more likely to have suffered from a cardiac ischemic event before KTx ($p<0.001$), to be on an anti-platelet therapy ($p=0.013$) and to have a higher number of total HLA mismatch ($p=0.041$) (Table). A non-significant trend of association was found for years on dialysis ($p=0.105$), statin therapy at KTx ($p=0.095$), and DGF ($p=0.095$). Even if non-statistically significant, 2/22 (9.1%) patients with a post-KTx CAD died during follow-up after the event, as compared to 8/484 (1.7%) of those who did not develop it ($p=0.315$). None of the other clinical parameters evaluated were found related in the univariate analysis to post-transplant CAD, including age, sex, renal function at hospital discharge after KTx, previous smoking and IS therapy (Table).

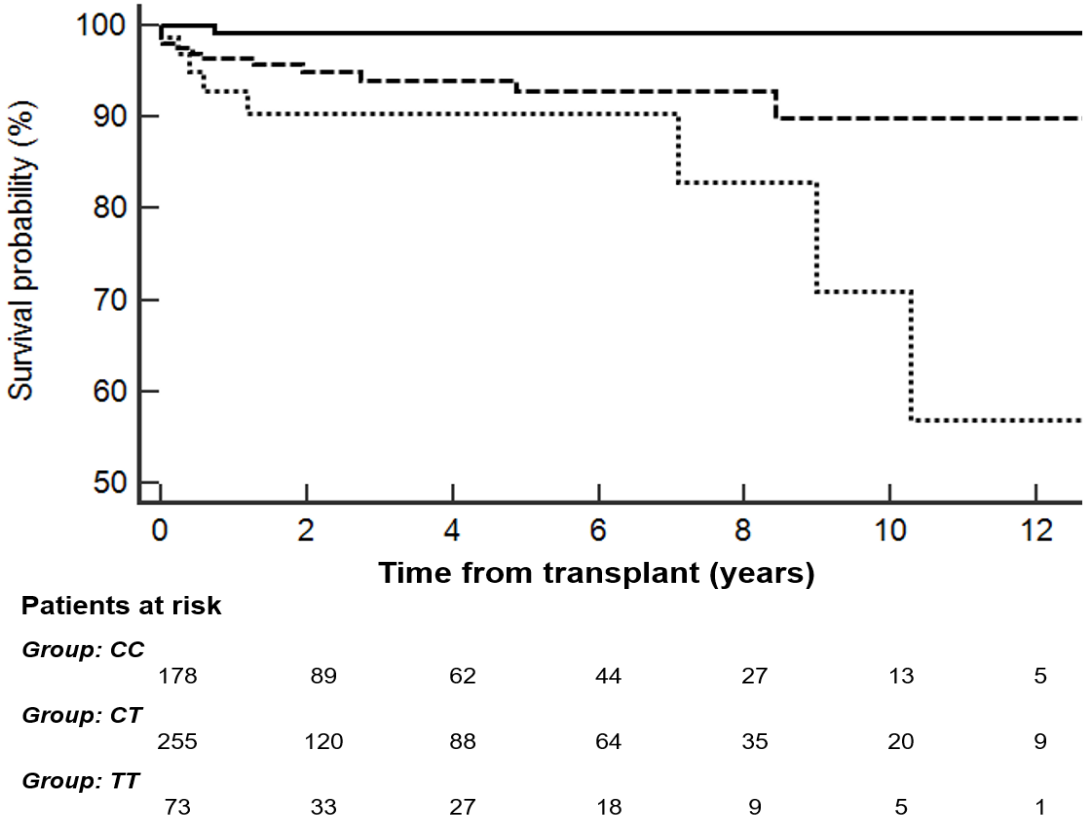
Description of the overall population and comparison between patients who developed post-transplant ischemic heart event (CAD) and those who did not. Values are expressed as mean±standard deviation or %				
Parameter	**Overall population** (n=506)	**Patients with post-transplant CAD** (n=22)	**Patients without CAD** (n=484)	**P**
Age (years)	52.4 ± 12.2	52.8 ± 9.4	52.4 ± 12.4	0.867
Male	64.4%	72.7%	64.0%	0.678
Years on dialysis	4.00 ± 3.68	5.57 ± 4.45	3.93 ± 3.60	0.105
Peak PRA > 20%	8.3%	9.1%	8.3%	0.891
Previous transplants	9.1%	9.1%	9.1%	1
Deceased Donor	95.3%	100%	95.0%	0.274
Total HLA mismatches	2.87 ± 1.04	3.18 ± 0.66	2.86 ± 1.05	0.041
BMI (Kg/m^2)	24.1 ± 3.46	24.5 ± 2.99	24.0 ± 3.48	0.536
Parathormone at KTx (pg/mL)	190.0 ± 186.0	223.2 ± 257.9	188.3 ± 182.3	0.547
Parathyroidectomy	10.7%	9.5%	10.9%	0.806
MGUS at KTx	3.4%	9.1%	3.1%	0.127
Ever smoker	56.3%	76.5%	55.1%	0.101
Hypertensive	83.8%	78.9%	84.1%	0.396
Any previous cardiovascular event	17.7%	42.9%	16.6%	0.003
Previous ischemic heart event	8.5%	38.1%	7.3%	<0.001
Previous stroke	1.6%	4.8%	1.5%	0.254
On antiaggregant therapy at KTx	42.8%	71.4%	41.6%	0.013
LDL-cholesterol (mg/dL)	114.6 ± 32.2	107.5 ± 36.7	115.0 ± 32.0	0.565
On statin at KTx	25.3%	44.4%	25.1%	0.095
Delayed Graft Function	21.9%	36.4%	21.3%	0.095
One or more acute rejection episodes	10.2%	11.1%	10.2%	0.875
Induction therapy:				
None	5.5%	9.1%	5.4%	
Anti IL2 receptor	81.3%	81.8%	81.2%	
ATG	13.2%	9.1%	13.4%	0.742
IS therapy at discharge				
Tacrolimus	90.9%	90.9%	90.9%	1
Cyclosporine	6.1%	0	6.4%	0.229
Mycophenolate	94.4%	90.9%	94.6%	0.118
Sirolimus or Everolimus	6.3%	13.6%	6.0%	0.553
Serum Creatinine at discharge (mg/dL)	2.06 ± 0.94	2.13 ± 0.75	2.07 ± 0.94	0.713
eGFR at discharge ($mL/min/1.73m^2$)	40.0 ± 19.6	37.1 ± 16.3	40.1 ± 19.8	0.410
Follow up time (years)	3.78 ± 3.73	5.01 ± 3.67	3.72 ± 3.72	0.121

BMI: body mass index; KTx: kidney transplant; MGUS: monoclonal gammopathy of unknown significance; ATG: anti-thymocyte globulin; IS: immunosuppressive.

Among studied SNPs, only the TCF7L2 rs7903146 seemed to be associated with the risk of post-transplant ischemic heart events. The genotype distribution of TCF7L2 rs7903146 was in accordance with Hardy-Weinberg equilibrium (p=0.23). As for TCF7L2 rs7903146 genotype (Figure), the 1-year and 5-year-risks were respectively 0.8% and 0.8% in CC patients, 3.6% and 7.2% in CT patients and 7.2% and 9.7% in TT patients. A significant association of rs7903146 was found with post-transplant CAD using the Armitage trend test for an additive effect (p=0.0002, Table).

SNP	**Overall population (n=506)**	**Patients with post-transplant CAD (n=22)**	**Patients without CAD (n=484)**	**P**
TCF7L2 rs7903146 C>T				
CC	35.2%	4.5%	36.6%	
CT	50.4%	59.1%	50.0%	
TT	14.4%	36.4%	13.4%	<0.001*
CYP3A5 Haplotypes				
1/1	2 (0.5%)	1 (5%)	1 (0.2%)	
1/3	51 (11.7%)	0	51 (12.3%)	
3/3	382 (87.8%)	19 (95%)	363 (87.5%)	0.090
CYP3A4 rs35599367 C>T				
CC	418 (89.9%)	17 (85%)	401 (90.1%)	
CT	46 (9.9%)	3 (15%)	43 (9.7%)	
TT	1 (0.2%)	0	1 (0.2%)	0.722
MDR1 3435C>T				
CC	110 (25.4%)	4 (20%)	106 (25.7%)	
CT	216 (49.9%)	12 (60%)	204 (49.4%)	
TT	107 (24.7%)	4 (20%)	103 (24.9%)	0.617
MDR1 1236C>T				
CC	102 (23.5%)	2 (10%)	100 (24.2%)	
CT	200 (46.1%)	11 (55%)	189 (45.7%)	
TT	132 (30.4%)	7 (35%)	125 (30.2%)	0.344

Ischemic heart event-free survival analysis of non-diabetic patients stratified by TCF7L2 rs7903146 genotype (censored for graft loss, patient death or PTDM diagnosis). Genotype CC is the bold line (n=178), CT is the dashed line (n=255) and TT is the dotted line (n=73). Log-rank test has a p<0.001.



Evaluation of collinear variables with respect to TCF7L2 rs7903146

We also investigated whether any of the known risk factors for CAD were associated with the TCF7L2 rs7903146 T allele in our population (Table). Indeed, cardiovascular events prior to KTx are not associated with this polymorphism in KTRs. Moreover, specific pre-transplant cardiovascular events and other traditional (hypertension, dyslipidemia, age, sex, BMI, previous smoking habit, renal function) and non-traditional risk factors (years on dialysis, parathormone, IS therapy, DGF, acute rejection episodes, HLA mismatches, and MGUS) were uniformly distributed among study groups. Moreover, patients carrying the TT genotype had actually a higher rate of PTDM (27.4% vs. 11.9%; p<0.001), but as these patients were censored from the main analysis at the time of PTDM diagnosis, PTDM cannot be considered a

confounder of the observed association. Moreover, PTDM risk associated with this *TCF7L2* polymorphism was not affected by the presence of a CAD event.

Table - Comparison between TCF7L2 rs7903146 genotypes in the study cohort. Values are expressed as mean±standard deviation or %; p values refer to the comparison between patients with PTDM vs. patients without PTDM.

Parameter	**CC** (n=178)	**CT** (n=255)	**TT** (n=73)	**P**
Age (years)	52.1 ± 13.3	52.6 ± 11.6	52.5 ± 11.8	0.908
Male	62.4%	65.1%	67.1%	0.736
Years on dialysis	4.05 ± 3.60	3.63 ± 3.34	4.09 ± 3.84	0.351
Ever on hemodialysis	82.0%	80.0%	84.9%	0.613
Ever on peritoneal dialysis	24.7%	25.9%	20.5%	0.648
Peak PRA > 20%	9.7%	7.3%	8.2%	0.687
Previous kidney transplants	5.6%	11.7%	8.2%	0.088
Deceased Donor	95.5%	94.5%	97.3%	0.610
Total HLA mismatches	2.96 ± 1.06	2.83 ± 1.04	2.82 ± 1.00	0.429
Cold ischemia time (hours)	19.0 ± 5.5	19.4 ± 5.6	19.6 ± 6.2	0.689
BMI (Kg/m^2)	24.0 ± 3.3	24.2 ± 3.5	23.6 ± 3.8	0.389
Parathormone at KTx (pg/mL)	185 ± 182	191 ± 185	198 ± 199	0.864
Parathyroidectomy	12.4%	9.8%	9.6%	0.623
MGUS at KTx	2.2%	4.3%	2.7%	0.477
Ever smoker	55.4%	56.5%	57.4%	0.966
Hypertensive	86.3%	80.5%	89.1%	0.113
Any previous cardiovascular event	18.5%	17.3%	15.7%	0.802
Previous ischemic heart event	11.2%	6.7%	8.2%	0.243
Previous stroke	1.1%	2.4%	0%	0.303
On anti-platelet therapy at KTx	46.9%	40.5%	40.8%	0.377
On statin at KTx	27.7%	21.3%	21.7%	0.399
LDL-cholesterol (mg/dL)	119 ± 31	111 ± 31	115 ± 37	0.250
Delayed Graft Function	21.9%	20.4%	24.7%	0.729
One or more acute rejection episodes	8.5%	8.3%	12.5%	0.616
Induction therapy:				
None	5.6%	4.3%	9.6%	
Anti IL2 receptor	83.7%	79.6%	80.8%	
ATG	10.7%	16.01%	9.6%	0.181
IS therapy at discharge				
Tacrolimus	93.3%	89.8%	89.0%	0.391
Cyclosporine	3.9%	7.1%	8.2%	0.296
Mycophenolate	94.9%	94.5%	93.2%	0.852
Sirolimus or Everolimus	3.9%	7.1%	9.6%	0.196
Serum Creatinine at discharge (mg/dL)	2.04 ± 0.94	2.08 ± 0.84	2.09 ± 1.20	0.885
eGFR at discharge ($mL/min/1.73m^2$)	35.4 ± 20.4	32.4 ± 15.6	32.5 ± 14.0	0.170
Follow-up time (years)	3.69 ± 3.66	3.71 ± 3.72	4.20 ± 3.90	0.579
PTDM	8.4%	14.1%	27.4%	<0.001

BMI: body mass index; KTx: kidney transplant; MGUS: monoclonal gammopathy of unknown significance; ATG: anti-thymocyte globulin; IS: immunosuppressive; PTDM: post –transplant diabetes mellitus.

Multivariate analysis of risk factors for CAD

In the multivariate Cox analysis, previous cardiac ischemic events (HR: 8.69, 95%CI: 3.57-21.16, p<0.001), TCF7L2 rs7903146 polymorphism (for each T allele, HR: 2.99, 95%CI: 1.62-5.52, p<0.001), DGF (HR: 2.42, 95%CI: 0.98-5.95, p=0.056) and HLA-mismatches (for each mismatch: HR: 1.55, 95%CI: 1.00-2.43, p=0.053) were independent predictors of post-transplant cardiac ischemic events. In addition, rs7903146 emerged as independent predictor of post-transplant ischemic cardiac events in both the subgroup of patients with (HR=4.10; 95%CI: 1.13-14.86; p=0.033) and without (HR=3.33; 95%CI: 1.44-7.69; p=0.005) previous cardiac events: therefore TCF7L2 rs7903146 polymorphism is not an effect modifier of the reported associations.

Table – Multivariate Cox model of risk factors for post-transplant ischemic heart events.

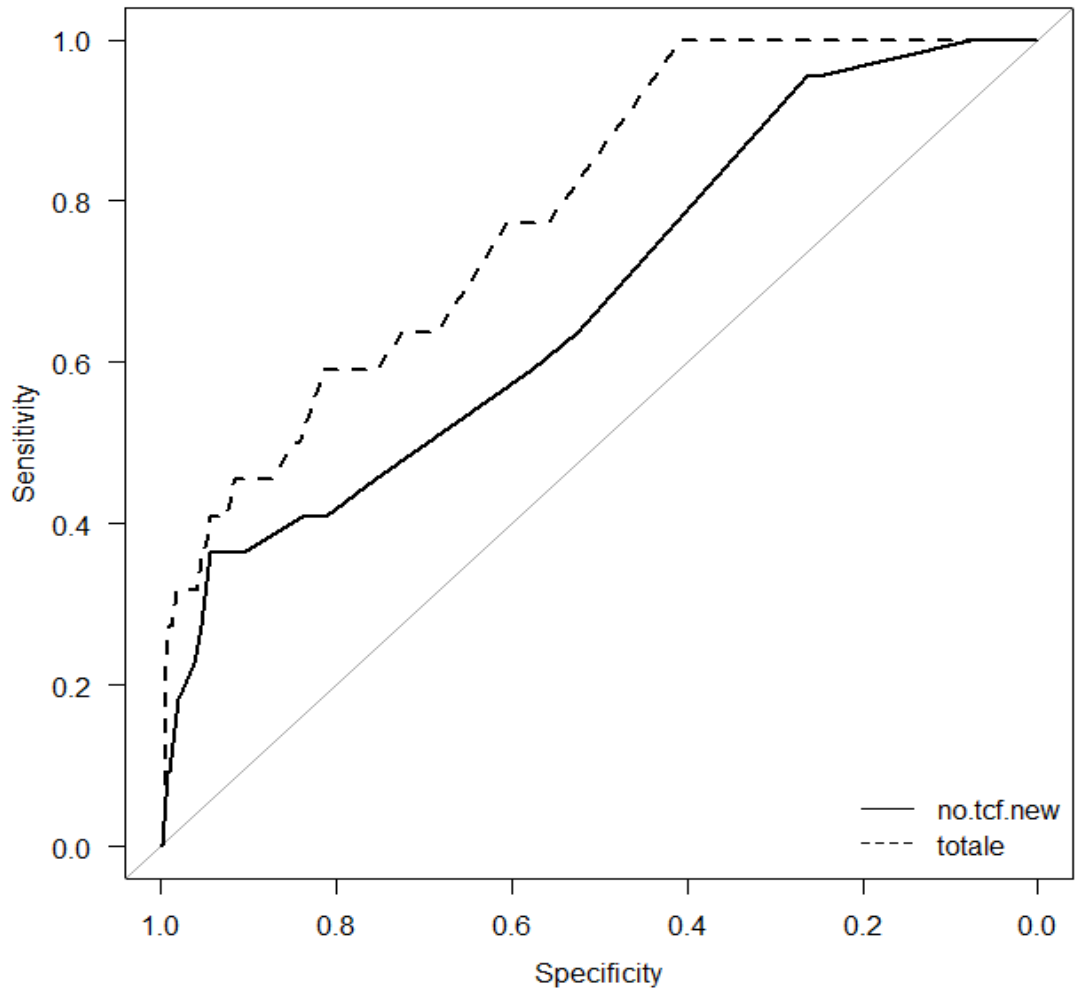
Risk Factor (reference group)	**HR**	**95% CI**	**p-value**
TCF7L2 rs7903146 (for each T allele)	2.99	1.62-5.52	<0.001
Previous cardiac ischemic event	8.69	3.57-21.16	<0.001
Delayed graft function	2.42	0.98-5.95	0.056
HLA-mismatches (for each mismatch)	1.55	1.00-2.43	0.053

HR: Hazard Ratio; CI: Confidence Interval.
Included but not retained in the model: age, sex, time on dialysis, statin use at time of transplantation.

Lastly, to evaluate the predictive accuracy of the multivariate model, we performed a receiver operating characteristic (ROC) curve analysis, using as independent variable the sum of beta-coefficients associated to each risk factor. This curve was compared to a similar "ad hoc" model including the same clinical variables (previous ICE, DGF and HLA mismatches), but without the TCF7L2 rs7903146 polymorphism. The area under the ROC curve (AUROC) of the model including clinical variables and TCF7L2 polymorphism was 0.790 (95%CI: 0.701-0.879; p=0.001). The mean sum of beta-coefficients of this model was 2.55 ± 1.13 (min-max range: 0-6.11). A model developed including the same clinical variables, but not the TCF7L2

polymorphism, showed a lower area under the ROC curve (0.678; 95%CI: 0.561-0.795) as compared to the model including the TCF7L2 polymorphism (p=0.003, DeLong's test).

Figure – ROC curves of the model including TCF7L2 (dashed line) and only clinical variables (bold line). The inclusion of TCF7L2 increases the area under the ROC curve of 0.112.



How much genes and viruses determine viral-related malignancies?

Overall, 1040 KTx mainly from deceased donors (94.2%) were performed in 1028 patients (51.3±12.5 year-old, 63.6% males). During a mean follow up of 4.45±3.9 years after KTx, we observed 130 NMSC and 80 non-cutaneous malignancies (NCM) in 66 and 71 patients respectively. Among NCM, 18 α-HPV related lesions were observed in 12 patients, who were mainly young female patients 84.6% (43.4 ± 7.5 year-old).

Table. Baseline characteristics of the cohort of 1040 kidney transplant recipients (KTRs). Variables are expressed as mean ± St dev or %.	
Age at KTx	51.3 ± 12.5
Sex (Male)	661/1040 (63.6)
Ethnicity (Caucasian)	1005/1040 (97.1)
Years on Dialysis	4.37 ± 3.71
Previous Transplants None 1 2	 941/1040 (90.5) 90/1040 (8.7) 9/1040 (0.9)
Living Donor	60/1040 (5.8)
HCV (Positive)	86/1040 (8.3)
Pre-Transplant Diabetes	65/1040 (6.3)
Body Mass Index	24.0 ± 3.5
Peak Reactive Antibodies > 0%	294/1040 (29.3)
Total HLA Mismatches	2.86 ± 1.04
Induction Therapy None Basiliximab rATG	 89/1040 (8.6) 797/1040 (76.6) 154/1040 (14.8)
Manteinance Therapy Tacrolimus Cyclosporine A Mycophenolate Azathioprine Sirolimus Everolimus	 911/1040 (87.6) 93/1040 (8.9) 934/1040 (89.8) 15/1040 (1.4) 46/1040 (4.4) 35/1040 (3.4)
Delayed graft function	234/1025 (22.8)
One or more acute rejection episodes	91/1040 (8.8)
Creatinine at hospital discharge (mg/dL)	2.05 ± 0.85

KTx: Kidney Transplant; HCV: Hepatitis C; rATG: Thymoglobulin

The incidence of NMSC was 1.1%, 7.1%, and 11.8% at respectively 1, 5, and 10 years after transplant. The cumulative incidence of malignancy of the kidney or urinary tract was 0.4%, 1.3%, and 1.6% at respectively 1, 5, and 10 years after transplant, while the cumulative incidence of the first alpha-HPV related tumor was 0.5%, 1.8%, and 1.8% at respectively 1, 5, and 10 years. The mean time from transplant to the first lesion was 4.2 ± 2.8 years for NMSC, 3.7 ± 3.3 years for NCM overall, 3.2 ± 3.1 years for alpha-HPV related lesions, and 3.4 ± 3.6 years for tumors of the kidney or urinary tract.

Figure – Cumulative incidence function (CIF) of non-cutaneous malignancies. X-axis: years from KTx

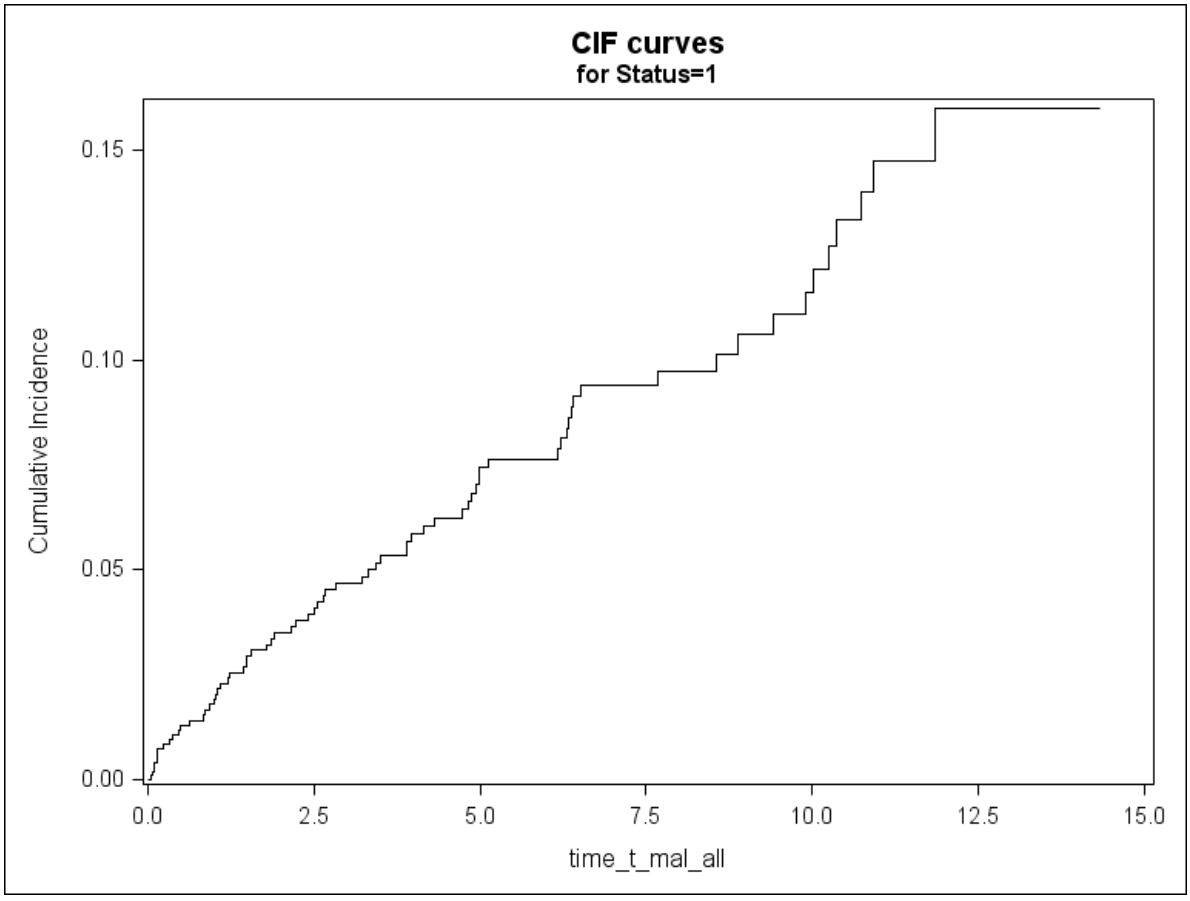


Figure – Cumulative incidence function (CIF) of NMSC. X-axis: years from KTx

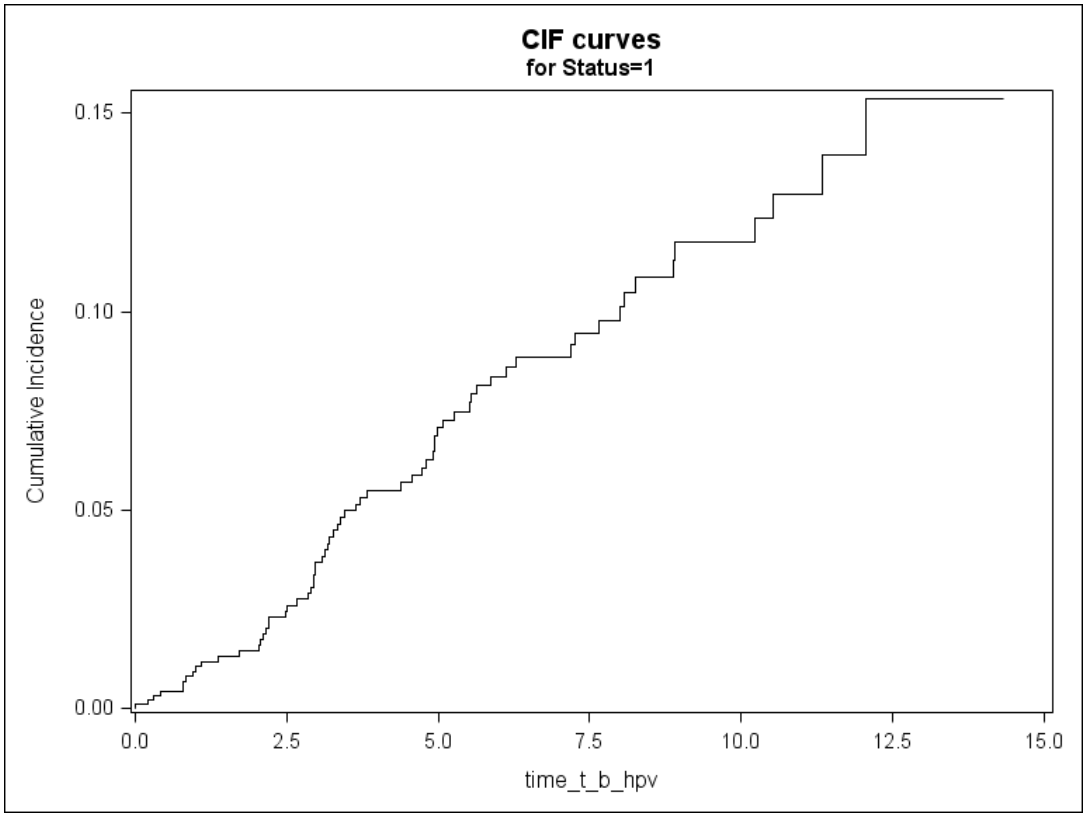


Figure – Cumulative incidence function (CIF) of alpha-HPV lesions. X-axis: years from KTx

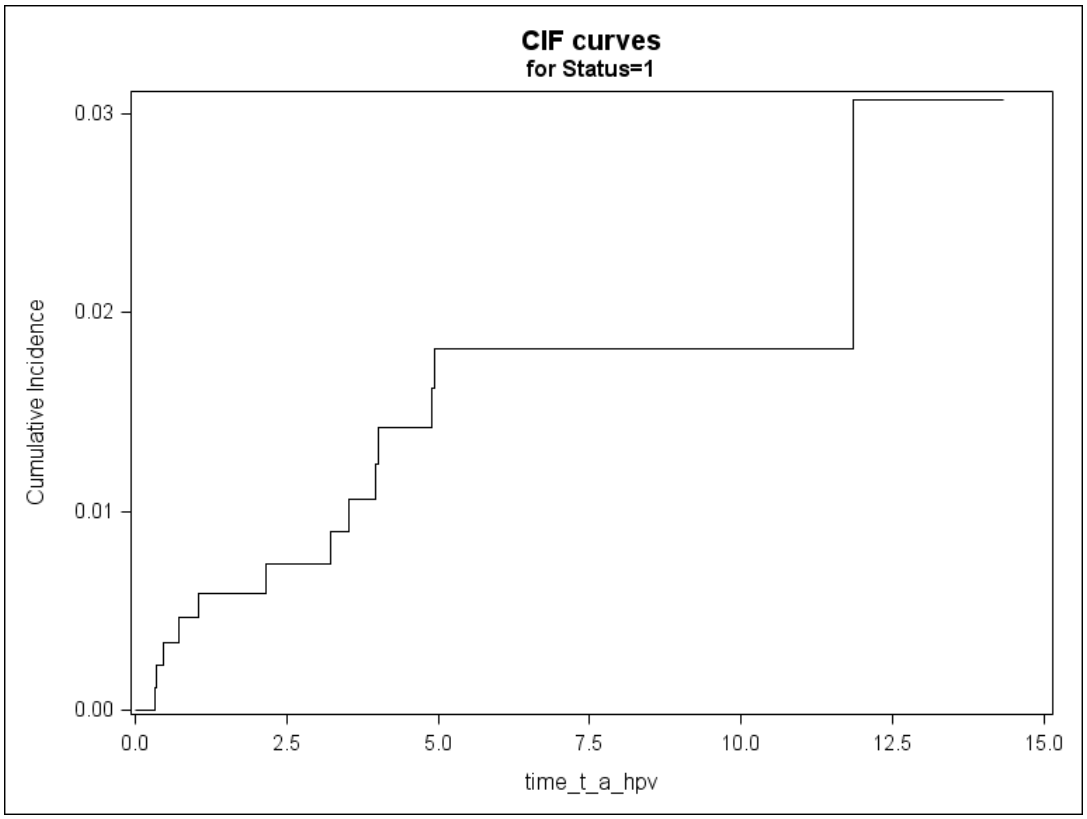
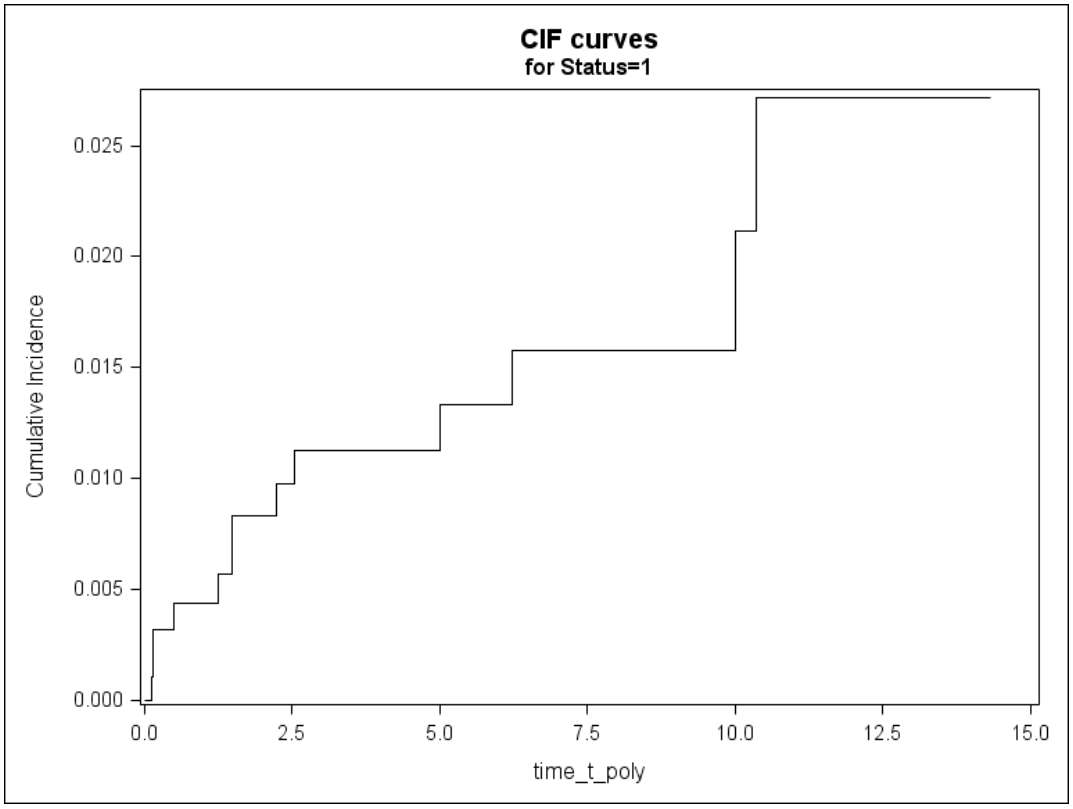


Figure – Cumulative incidence function (CIF) of urinary tract malignancies. X-axis: years from KTx



When comparing patients without any malignancy with those with malignancies, only age was associated with a greater risk of NCM (HR=1.305 for each 10-year increase; 95%CI: 1.072-1.583; p=0.007) and NMSC (HR=1.913 for each 10-year increase; 95%CI: 1.524-2.389; p<0.001), while no other major differences were revealed. Moreover, the use of azathioprine was associated with both alpha-HPV lesions (HR=6.38, 95%CI: 1.31-30.96; p=0.022) and NMSC (HR=2.76, 95%CI: 1.09-6.95; p=0.032). No other variable was associated with specific malignancies, but creatinine at discharge was significantly lower in patients with alpha-HPV lesions (p=0.029): this could reflect a young donor age (as donor and recipients are matched for age) and a predominance of female recipients in this group.

Table. Description of patients who developed different virus-related tumors after transplantation. Variables are expressed as mean +/- St dev or n (%).

Variable	Patients with alpha-HPV lesions (n=12)	Patients with kidney or bladder tumors (n=13)	Patients with NMSC (n=66)	Patients with any non-cutaneous malignancy (n=71)	Patients without any malignancy (n=969)
Age at KTx	42.7 ± 7.5	49.8 ± 10.6	57.1 ± 9.3	52.9 ± 10.9	51.1 ± 12.6
Sex (Male)	1/12 (8.3)	12/13 (92.3)	44/66 (66.7)	42/71 (59.2)	619/969 (63.9)
Ethnicity (Caucasian)	11/12 (91.7)	13/13 (100)	66/66 (100)	70/71 (98.6)	935/969 (97.0)
Years on Dialysis	4.00 ± 2.73	3.89 ± 1.95	4.34 ± 2.84	3.84 ± 2.75	4.41 ± 3.77
Smoke					
Never	3/7 (42.9)	6/11 (54.6)	17/41 (41.5)	19/43 (44.2)	249/568 (43.8)
Former	3/7 (42.9)	5/11 (45.4)	17/41 (41.5)	21/43 (48.8)	239/568 (42.1)
Active	1/7 (14.3)	0/11	7/41 (17.1)	3/43 (7.0)	80/568 (14.1)
Previous Transplants					
None	12/12 (100)	12/13 (92.3)	62/66 (93.9)	64/71 (90.1)	877/969 (90.5)
1	0	1/13 (7.7)	3/66 (4.6)	7/71 (9.9)	83/969 (8.6)
2	0	0/13 (0)	1/66 (1.5)	0/71 (0)	9/969 (0.9)
Living Donor	0/12 (0)	1/13 (7.7)	0/66 (0)	1/71 (1.4)	59/969 (6.1)
HCV (Positive)	0/12 (0)	0/13 (0)	2/66 (3.0)	5/71 (7.0)	81/969 (8.4)
Pre-Transplant Diabetes	0/12 (0)	0/13 (0)	3/66 (4.6)	3/71 (4.2)	62/969 (6.5)
Body Mass Index	22.7 ± 4.3	23.0 ± 3.1	23.8 ± 3.1	23.7 ± 3.2	24.0 ± 3.6
Peak Reactive Antibodies > 0%	6/12 (50.0)	4/13 (30.8)	21/65 (32.3)	23/69 (33.3)	271/969 (29.1)
Total HLA Mismatches	3.17 ± 1.17	3.31 ± 0.85	2.74 ± 0.92	2.93 ± 1.03	2.85 ± 1.04
Induction Therapy					
None	1/12 (8.3)	2/13 (15.4)	12/66 (18.2)	12/71 (16.9)	77/969 (7.9)
Basiliximab	10/12 (83.3)	9/13 (69.2)	42/66 (63.6)	45/71 (63.4)	140/969 (14.4)
rATG	1/12 (8.3)	2/13 (15.4)	12/66 (18.2)	14/71 (19.7)	752/969 (77.6)
Maintenance Therapy					
Tacrolimus	12/12 (100)	12/13 (92.3)	59/66 (89.4)	62/71 (87.3)	849/969 (87.7)
Cyclosporine A	0/12 (0)	1/13 (7.7)	4/66 (6.1)	8/71 (11.3)	85/969 (8.8)
Mycophenolate	10/12 (83.3)	11/13 (84.6)	52/66 (78.8)	58/71 (81.7)	876/969 (90.4)
Azathioprine	1/12 (8.3)	0/13 (0)	5/66 (7.6)	3/71 (4.2)	12/969 (1.2)
Sirolimus	0/12 (0)	1/13 (7.7)	7/66 (10.6)	3/71 (4.2)	43/969 (4.4)
Everolimus	0/12 (0)	0/13 (0)	0/66 (0)	1/71 (1.4)	34/969 (3.5)
Delayed graft function	0/12 (0)	2/13 (15.4)	17/66 (25.8)	13/71 (18.3)	221/954 (23.2)
One or more acute rejection episodes	3/12 (25.0)	1/13 (7.7)	4/66 (6.1)	10/71 (14.1)	81/969 (8.4)
Creatinine at hospital discharge (mg/dL)	1.48 ± 0.51	2.05 ± 0.62	2.10 ± 0.75	1.98 ± 0.69	2.05 ± 0.87

HPV: Human Papilloma Virus; NMSC: non-melanoma skin cancer; KTx: Kidney Transplant; HCV: Hepatitis C; rATG: Thymoglobulin

Pharmacogenetics of malignancies

Specific Aims: To evaluate if SNPs associated with IS metabolism or complications may indeed alter the risk of virus-related malignancies.

Among KTR of the Novara cohort, 430 were analyzed to evaluate the association between study SNPs and the occurrence of malignancies. Even if we included both newly transplanted patients and older prevalent KTRs (leading to a potential selection bias in this latter group), this sample is well representative of the entire Novara cohort. Even if age and immunosuppressive therapy are statistically different, the difference can not be considered as clinically significant (ie: 1.4 years older at the time of KTx).

Table. Comparison between the whole Novara cohort and the sample who was genotyped (Novara-gene cohort). Variables are expressed as mean ± St dev or %.

	Whole Novara cohort (n=1040)	Novara "gene" cohort (n=430)	P
Age at KTx	51.3 ± 12.5	52.7 ± 12.4	0.002
Sex (Male)	661/1040 (63.6)	282/430 (65.6)	0.255
Ethnicity (Caucasian)	1005/1040 (97.1)	416/430 (96.8)	0.869
Previous Transplants	100/1040 (9.6)	43/430 (10.1)	0.724
Living Donor	60/1040 (5.8)	19/430 (4.4)	0.117
Pre-Transplant Diabetes	65/1040 (6.3)	25/430 (5.7)	0.626
Body Mass Index	24.0 ± 3.5	24.2 ± 3.4	0.119
Total HLA Mismatches	2.86 ± 1.04	2.90 ± 1.02	0.295
Induction Therapy			
None	89/1040 (8.6)	32/430 (7.4)	
Basiliximab	797/1040 (76.6)	354/430 (82.3)	
rATG	154/1040 (14.8)	44/430 (10.3)	0.001
Manteinance Therapy			
Tacrolimus	911/1040 (87.6)	390/430 (90.6)	0.011
Mycophenolate	934/1040 (89.8)	400/430 (93.1)	0.004
Sirolimus or Everolimus	81/1040 (7.8)	30/430 (7.0)	0.412
Delayed graft function	234/1025 (22.8)	108/430 (25.0)	0.090
One or more acute rejection episodes	91/1040 (8.8)	55/430 (12.8)	<0.001
Follow up (years)	4.5 ± 3.9	4.3 ± 3.7	0.159

KTx: Kidney Transplant; HCV: Hepatitis C; rATG: Thymoglobulin

In this sub-cohort we observed 22 patients with a NMSC, 18 with a NCM, of which 8 had a PTLD, corresponding respectively to an affected patient rate of 5.1% (vs. 6.3% in the whole cohort), 4.2% (vs. 6.8%) and 1.9% (vs. 1.3%).

None of the study SNPs was significantly associated with NCM occurrence.

There was not any statistically significant association between study SNPs and NMSC; however a trend was observed as for CYP3A5 *1 carriers (fast CNI metabolism) did not develop any NMSC (p=0.077). Moreover, even if not statistically significant, CYP3A4 rs35599367 T carriers (slow CNI metabolism) had a more than double risk of NSMC as compared to CC homozygous (OR: 2.23; 95%CI: 0.716-6.93; p=0.131).

SNP	**Overall population (n=430)**	**Patients with NCM (n=18)**	**Patients with NMSC (n=22)**	**Patients with PTLD (n=8)**
TCF7L2 rs7903146 C>T				
CC	156 (36.4%)	5 (3.2%)	8 (5.1%)	1 (0.6%)
CT	219 (50.8%)	11 (5.0%)	10 (4.7%)	4 (1.8%)
TT	55 (12.8%)	2 (3.6%)	4 (7.3%)	3 (5.5%)
CYP3A5 Haplotypes				
1/1 or 1/3	52 (12.1%)	2 (3.8%)	0	0
3/3	378 (87.9%)	16 (4.2%)	22 (5.8%)	8 (2.1%)
CYP3A4 rs35599367 C>T				
CC	389 (90.5%)	17 (4.4%)	18 (4.6%)	8 (2.1%)
CT or TT	41 (9.5%)	1 (2.4%)	4 (9.8%)	0
MDR1 3435C>T				
CC	110 (25.4%)	5 (4.5%)	8 (7.3%)	4 (3.6%)
CT or TT	323 (74.6%)	13 (4.0%)	14 (4.3%)	4 (1.2%)
MDR1 1236C>T				
CC or CT	302 (69.7%)	12 (4.0%)	15 (5.0%)	2 (0.7%)
TT	131 (30.3%)	6 (4.6%)	7 (5.3%)	6 (4.6%)

Interestingly virus-related malignancies, including solid, hematologic and cutaneous virus-related malignancies, (n=32) were all observed among CYP3A5 *3/*3 homozygous (p=0.031). Moreover this group had more commonly CMV reactivations post-KTx (p=0.005) in patients not receiving an ATG induction therapy, which is so strongly associated to CMV reactivation that nowadays patients on this therapy undergo a six months CMV-prophylaxis with

valgancyclovir. Indeed CYP3A5 *3/*3 homozygous patients did not significantly differ from *1 carriers for all other clinical variables, as reported in table.

Table – Comparison of patients carrying a CYP3A5 *1 genotype (fast metabolizers) and those without (3/3)

	CYP3A5 1/x ***(n=42)***	**CYP3A5 3/3** ***(n=332)***	**p**
Male	57,1%	67.4%	0,22
Age	52,0±12,2	52,7±12,7	0,36
CMV reactivation	3,2%	22,5%	0,005
Creat 6 mo	1,72±0,56	1,78±0,66	0,29
BPAR	14.3%	13.0%	0.81
FK	85,7%	92,2%	0,12
MMF	88,1%	93,1%	0,20
mTORi	7,1%	7,5%	0,61
CMV Ab Positive	88,1%	82,5%	0,252
PTDM	7,7%	13,3%	0,255
Coron. A. Dis.	1,9%	5,9%	0,235
Stroke	1,9%	0,8%	0,418
Other CV Event	11,5%	14,6%	0,556

CMV: cytomegalovirus, BPAR: biopsy proven acute rejection; PTDM: post-transplant diabetes; CV: cardiovascular

As the most associated malignancy to these polymorphism were PTLDs and risk factors for PTLDs are not known we investigated more deeply this association. Indeed the following SNPs were associated with PTLDs, as shown in table:

Gene variant	**Reference**	**OR**	**95%CI**	**p**
TCF7L2 rs7903146:				
CT	CC	2.90	0.32-26.2	0.322
TT		8.94	0.91-87.9	0.024
TCF7L2 rs7903146: TT	CC or CT	4.26	1.00-18.3	0.035
CYP3A5 *1 carriers	*3/*3	2.24	0.13-39.7	0.290
CYP3A4 rs35599367 T carriers	CC	1.72	0.10-30.5	0.354
MDR1 3435: CC	CT or TT	3.00	0.74-12.2	0.107
MDR1 1236: TT	CC or CT	7.20	1.43-36.2	0.005

Interestingly in this cohort EBV seronegativity –the only well-recognized risk factor for PTLDs- at the time of KTx was not significantly associated with PTLDs (OR=4.70; 95%CI: 0.536-41.3; p =0.125).

As these SNPs act on CNI metabolism and a therapy with mTOR-i is being associated with less post-transplant virus-related malignancies, particularly NMSC and PTLD, we decided to focus our analysis on Caucasian patients who did receive a CNI, excluding few patients with a CNI-free immunosuppression. Indeed none of the patients who were on mTORi-MMF developed a PTLD (0/15, p: ns). The comparison between patients with a PTLD and those without was not able to identify any significant clinical risk factor. The associations of CYP3A5, TCF7L2 and MDR1 polymorphism were confirmed in this sub-population.

Table – Comparison between pre-PTLD characteristics of patients who later developed a PTLD and those who did not.

	PTLD (n=8)	***Other (n=407)***	***p***
Male	75,0%	66,1%	0,56
Age	57,9 ± 11,7	52,9 ± 12,4	0,13
EBV seroneg.	12.5%	2.9%	0.09
FK	100%	100%	-
mTORi	0%	0,5%	0,95
MMF	87,5%	97,1%	0,23
ATG	12,5%	11,5%	0,96
BPAR	12,5%	8.3%	0,51
6 mo Creat	1,70 ± 0,37	± 0,65	0,30
CMV react.	25%	17,0%	0,43

Therefore we were able to stratify the population by EBV serology, CYP3A5 haplotype, TCF7L2 rs7903146 and *MDR1* C1236T genotype in the categories reported in table. Indeed some subgroups of patients had few or no patients, so we re-grouped these subgroups according to their PTLD risk.

Table – Details of patients stratified by baseline risk factors and crude rate of PTLDs during follow up in each group.

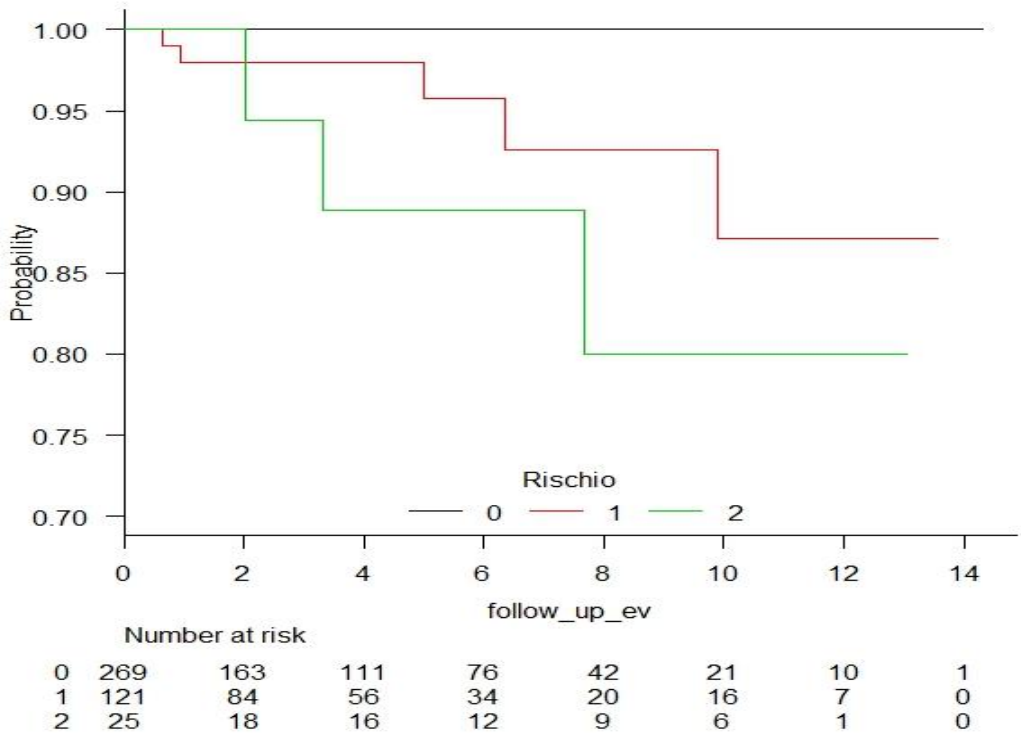
***CYP3A5* haplot**	**EBV serol.**	***TCF7L2* rs7903146**	***MDR1* C1236T**	**N (%)**	**Age at KTx**	**PTLD (%)**	**f/up**
*3/*3	Pos/miss	CT or CC	CT or CC	222 (53.5)	53.1±12.5	0	3.9±3.4
*3/*3	Pos/miss	CT or CC	TT	88 (21.2)	52.5±12.2	4 (4.5)	4.3±3.7
*3/*3	Pos/miss	TT	CT or CC	33 (8.0)	52.7±13.5	1 (3.0)	5.0±3.8
*3/*3	Pos/miss	TT	TT	14 (3.4)	50.4±9.9	2 (14.3)	5.4±4.0
*3/*3	Neg	CT or CC	CT or CC	6 (1.4)	43.7±16.9	1 (16.7)	5.5±4.6
*3/*3	Neg	CT or CC	TT	3 (0.7)	49.8±6.3	0	8.4±6.4
*3/*3	Neg	TT	CT or CC	1 (0.2)	72.4	0	5.1
*3/*3	Neg	TT	TT	1 (0.2)	32.2	0	11.5
*1 carr	Pos/miss	CT or CC	CT or CC	22 (5.3)	52.9±11.8	0	4.2±4.2
*1 carr	Pos/miss	CT or CC	TT	19 (4.6)	50.3±14.2	0	4.7±4.2
*1 carr	Pos/miss	TT	CT or CC	5 (1.2)	56.3±9.8	0	4.0±2.7
*1 carr	Pos/miss	TT	TT	0	-	-	-
*1 carr	Neg	CT or CC	TT	1 (0.2)	48.7	0	9.5
*1 carr	Neg	CT or CC	CT or CC	0	-	-	-
*1 carr	Neg	TT	any	0	-	-	-

From this data we can hypnotize that the *CYP3A5* *1 haplotype has a protective role, while risk factors for PTLD are a negative EBV serology, TCF7L2 rs7903146 TT genotype and MDR1 1236 TT genotype. Based on these definitions, we can define the a “low risk” group (n=269; 64.8%) including patients carrying the “protective” *CYP3A5* *1 haplotype (regardless of the

other risk factors, even if all patients had only one or none) or without any risk factor; an "intermediate risk" group (n=121; 29.2%) including patients with one genetic risk factor and a "high risk" group (n=25; 6.0%) including EBV-negative patients or those with both genetic risk factors.

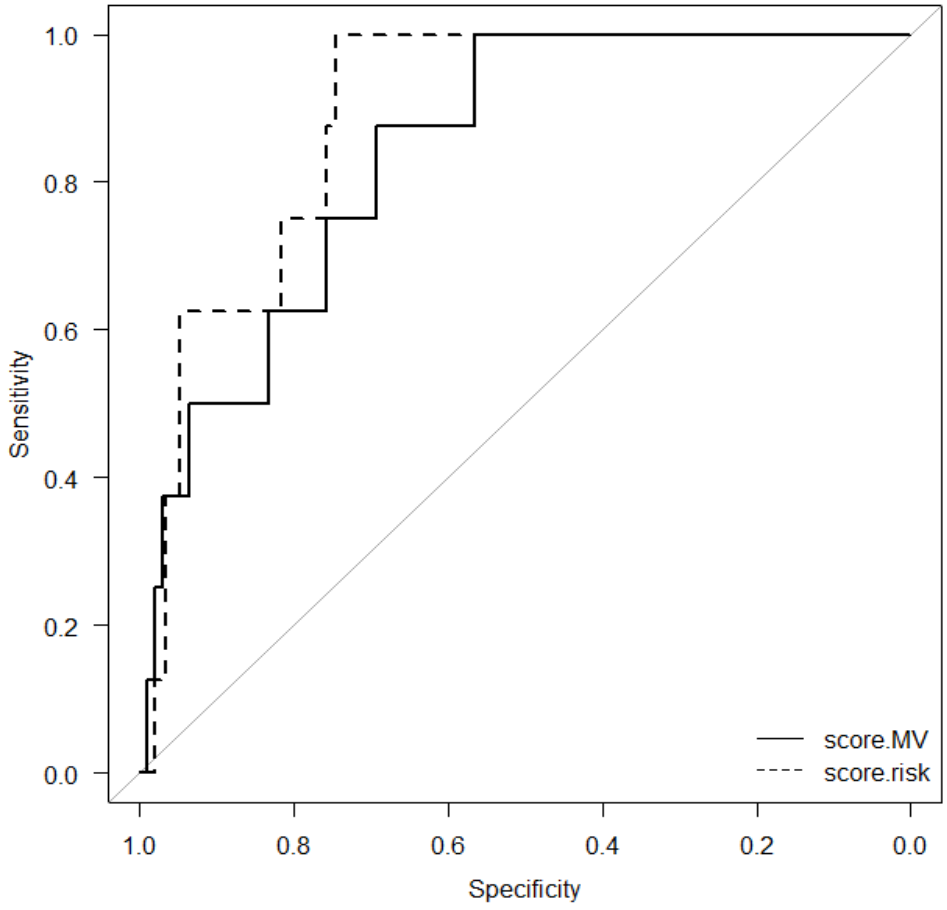
Indeed in "low risk" patients the mean PTLD incidence rate is 0 (95%CI: 0-3.34/100-pt-yr), while in "intermediate" and "high risk" patients it is respectively 9.15/100-pt-yr (95%CI: 2.95-21.34) and 19.87/100-pt-yr (95%CI: 4.00-58.07). When considering this risk stratification in a multivariate Cox model for PTLD giving to each risk increase a score of +1, this risk stratification accurately predicts the outcome (Model A), with narrower confidence intervals as compared to a model including automatically-selected significant variables (Model B). The AUROC of model A was 0.891 (95%CI: 0.818-0.964) and of model B was 0.841 (95%CI: 0.730-0.952), which are not statistically different, but model A seems to be more accurate.

Figure: Kaplan-Meyer PTLD-free survival stratified by risk class: low risk (black line), intermediate risk (red line), high risk (green line). Log-rank test < 0.001.



Comparison between the Cox proportional hazard models: Model A includes risk classes (low risk = 0; intermediate = 1, high = 2); model B only includes selected variables (backwards stepwise).				
Model	**Variable**	**HR**	**95%CI**	**p**
A	Age (per year)	1.083	1.011-1.160	0.024
	Risk class	6.219	2.225-17.38	<0.001
B	Age (per year)	1.087	1.010-1.171	0.027
	MDR1 1236: TT	7.190	1.392-37.14	0.019
	TCF7L2 rs7903146 TT	4.658	1.035-20.97	0.045

Comparison between the ROC curves of Model A (dashed line) and B (black line). Model A AUROC = 0.891 (95%CI: 0.818-0.964); model B AUROC: 0.841 (95%CI: 0.730-0.952),



PTLD: case-cohort study

Specific Aims: To identify risk factors and clinical biomarkers of PTLDs.

Overall, 49 PTLD index cases were identified in 9 transplant centers, to which 196 controls were matched. The mean index time (defined for each case-controls group as the time from KTx to PTLD occurrence) was 104±65 months: only 142 controls (2.90/case) reached the index time of their corresponding case.

The PTLD cases belonged to different clinic-histological categories, ranging from early lesions, to leukemia. Most of them were EBV positive, ie: EBV replication could be detected within pathologic tissue. However in most cases (29/49=59.2%) this evaluation was missing and could not be recovered.

PTLD Pathology of included cases	
Early Lesion	2 (4.1%)
Polymorphic Lymphoma	3 (6.1%)
B cell Monomorphic Lymphoma	32 (65.3%)
Large B Cell Diffuse Lymphoma	28 (57.1%)
T cell Monomorphic Lymphoma	4 (8.2%)
Hodgkin disease	4 (8.2%)
Large granula lymphocyte leukemia	1 (2.0%)
Unspecified	2 (4.1%)
EBV-replication within PTLD	14/20 (70%)
EBV-negative pathology	6/20 (30%)

Each case was matched to four control KTR at the time of transplantation (case-cohort study) by age (± 5 years), sex, transplant center, year of transplantation (±2 years) and transplanted organ (living vs. deceased donor). Matching was maintained more "free" as compared to the original study design, but still cases and controls had a good matching for the matching variables.

At baseline there were no significant differences between PTLD cases and controls.

Table – Matching verification of cases and controls from the "Parma" case-cohort study

	Cases (n=49)	Controls (n=196)	p
Female	24,5%	25,0%	0,941
Age at KTx	42.0 ± 14.6	42.6 ± 13.6	0,814
Year of KTx	1999 ± 5.9	1999 ± 5.8	0.952
Living donor	10.2%	4.6%	0.130
Center matching	*Confirmed in every control*		

Table - Baseline characteristics of Cases and Controls of the Case-cohort study

	Cases (n=49)	Controls (n=196)	p
ATG	7/49 (14.3%)	29/192 (15.1%)	
Ab anti IL2R	19/49 (38.8%)	63/192 (32.8%)	
No induction	23/49 (46.9%)	100/192 (52.1%))	0,783
Tacrolimus	19/49 (38.8%)	59/193 (31.6%)	0,253
MMF	20/49 (40,8%)	75/192 (40,3%)	0,792
m-TOR inib.	3/49 (6,7%)	18/196 (10,3%)	0,457
Initial IS drugs	2.65 ± 0.52	2.66 ± 0.51	0.918
CMV Serology (neg)	10/45 (22.2%)	37/169 (21.9%)	0.571
EBV serology (neg)	14/40 (35%)	26/114 (22.8%)	0.130
BPAR	7/49 (14.3%)	26/196 (13.3%)	0.983

EBV serology was not a significant risk factor for PTLDs overall (OR=1.822; 95%CI: 0.833-3.989; p=0.130), but EBV-related PTLDs showed a stronger trend towards being more common in EBV negative patients (OR = 2.567; 95%CI: 0.762-8.650; p=0.066) than EBV-unrelated PTLDs (OR of EBV negative = 1.415; 95%CI: 0.500-4.011; p=0.299).

Moreover, there was an unexpected interaction between EBV serology and ATG induction (lymphocyto-toxic): EBV negative patients who received ATG had a PTLD risk similar to the one of EBV positive patients (OR=0.971; 95%CI: 0.110-8.57; p>0.90), while EBV negative patients who did not receive ATG had an increased risk of PTLD as compared to EBV positive patients (OR=3.01; 95%CI: 1.381-6.560; p=0.004). Interestingly, among EBV-positive patients, the use of ATG was not associated with an increased risk of PTLD (OR=1.146; 95%CI: 0.432-3.037; p=0.785).

During follow up, data on therapy were available in all patients at one year and in most patients following the first post-transplant year: pharmacokinetic parameters have been calculated on these data. Interestingly no major differences could be noted for these parameters between cases and controls.

Table – Follow up characteristics of cases (PTLD) and matched controls

	Cases	**Controls**	**p**
Dose CyA follow up (mg/die)	226±52,2	212,1±62,7	0,28
Dose CyA f. up (mg/Kg/die)	3,43±0,88	3,29±1	0,51
C0 CyA f. up (ng/mL)	167,3±42,8	164,8±54	0,621
Dose FK f. up (mg/die)	5,1±2,8	5,3±2,3	0,695
Dose FK f. up (mg/Kg/die)	0,076±0,037	0,084±0,043	0,429
BTL FK follow up (ng/mL)	8,26±1,98	8,26±3,33	0,89
Dose MMF follow up (mg/die)	1119±388	1226±438	0,293
Dose AZA follow up (mg/die)	59,3±12	75,7±25,8	0,038
Dose Steroid f. up (mg/die)	6,3±2,5	6,1±2,2	0,532
Dose mTORi f. up (mg/die)	1,54±1,27	2,18±0,94	0,133
CoV Cyclosporine (%)	32,3±15,4	33,8±15,6	0,912
CoV FK (%)	32,7±19,9	27,1±10,6	0,107
CoV dei CNI (%)	32,6±17,5	31,4±14,3	0,262
DnTL CyA	53±19,6	54,4±25,6	0,788
DnTL FK	140,8±89,3	121,2±65,2	0,282

CoV: intrandividual coeficient of variability (on at least three BTL values; equals to st.dev(BTLi)/mean(BTLi)
DnTL: dose normalized trough level, calculated as BTL divided by weight-normalized drug daily dose
Cya: Cyclosporine; FK: Tacrolimus; CNI: CyA or FK; BTL: blood through level; C0: pre-assumption drug level.

However, we noted that patients on high doses of CNI were more likely to develop a PTLD (OR=2.890; 95%CI: 1.455-5.740; p = 0.002). A “High dose” of CNI was defined as a CNI dose greater than the median daily dose of the entire cohort, being a tacrolimus dose greater than 0.075 mg/Kg/day or a cyclosporine dose greater than 3.25 mg/Kg/day. Interestingly this variable interacted significantly (p=0.029) with EBV serology at the time of KTx. Particularly high dose CNI increased the risk only in EBV-negative patients (OR=3.451; 95%CI=0.811-14.67), but not in EBV-postive patients (OR=1.818; 95%CI=0.788-4.195).

Table - Crude PTLD rate in different subgroups of patients, stratified by CNI dose and EBV serology at KTx. Data are expressed as events/exposed patiets *(%)*

		EBV serology		Total
		Neg	**Pos**	
CNI Dose	**High**	11 / 20 *(55,0%)*	11 / 53 *(20,8%)*	22 / 73 *(30,1%)*
	Low	3 / 19 *(15,8%)*	17 / 135 *(12,6%)*	20 / 154 *(13,0%)*
Total		14 / 39 *(35,9%)*	28 / 188 *(14,9%)*	42 / 227 *(18,5%)*

Table – Five-years OR of PTLD in different subgroups of patients, stratified by CNI dose and EBV serology at KTx, as compared to EBV+ patients receiving low dose CNI

PTLD Odds Ratio (95%CI) *vs. EBV+LowCNI*		EBV serology	
		Neg	**Pos**
CNI Dose	**High**	8.48 (3.07-23.5)	1.82 (0.79-4.20)
	Low	1.30 (0.34-4.94)	1

HPV and NMSC

Specific Aims: to evaluate the role of active HPV on NMSC development in KTRs.

In a first step (2012-2014), our gourp analyzed 111 FFPE blocks obtained from 79 skin lesions of 17 patients from our cohort of KTRs for the presence of active β–HPV infection by anti-E4 and anti-L1 staining. Areas with E4-positive cells were found in 6 FFPE blocks from 4 patients that corresponded to 4 precancerous actinic keratosis (AK) lesions and the adjacent pathological area of a SCC and a BCC. Since then, 97 additional FFPE blocks from 70 skin lesions excised from 21 patients have been analyzed following the same procedure. Within this second group, an interesting case emerged of a female KTR who was evaluated for a second transplant due to the failure of her first graft after 25 years. Indeed, this new round of investigation allowed us to identify 3 additional blocks with βHPV-positive areas that were all derived from this case.

Her long clinical history reported the development of many proliferative skin lesions in different body sites, starting approximately 10 years after the first KTx. Fifteen FFPE blocks corresponding to the excised tumors from this patient were available that were diagnosed as SCC (n=7), BCC (n=4), and keratoacanthoma (KA) (n=4). E4-positive areas were found in 2 KA located on the leg and the pathological hyperplastic border of an SCC located on the hand. As expected for HPV infected tissues, E4 positive cells were clearly evident in the areas of the lesion with hyperkeratosis and parakeratosis; some of them were trapped in the keratin layers where L1-positive nuclei were also observed. The cellular proliferation marker MCM7 was more evident in the E4-positive area compared with the adjacent epithelium, and was most apparent in the basal and suprabasal layers, indicating that cells were stimulated to enter the cell cycle. This increase in MCM7 expression was even higher in the adjacent pathological epithelium of a BCC from another KTR reported in Figure where both E4 and L1 expression was evident.

Figure - Clinical and histological findings from the skin of a KTR patient with 25 years of immunosuppression. Flat whitish and reddish papular lesions on the forearm (left) and crusty/erythematous lesions on the hand (**Panel A**). Detection of the βHPV E4 and L1 proteins, or cellular minichromosome maintenance protein 7 (MCM7) in a keratoacanthoma (KA, leg) (**Panel B**). The top picture shows hematoxylin and eosin (H&E) staining in the tissue section (scale bar: 1000 μm). The region shown in the lower panels corresponds to the blue square highlighted in the overall H&E image, magnified in the left-hand lower panel. In the middle picture, the same section was subjected to double staining using antibodies to E4 (green) and MCM7 (red) (middle picture), and a serial section was stained with antibodies to L1 (red) and E4 (green)(right picture). The white arrows indicate nuclear L1 staining. All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue) to visualize cell nuclei. Scale bars: 100 μm.

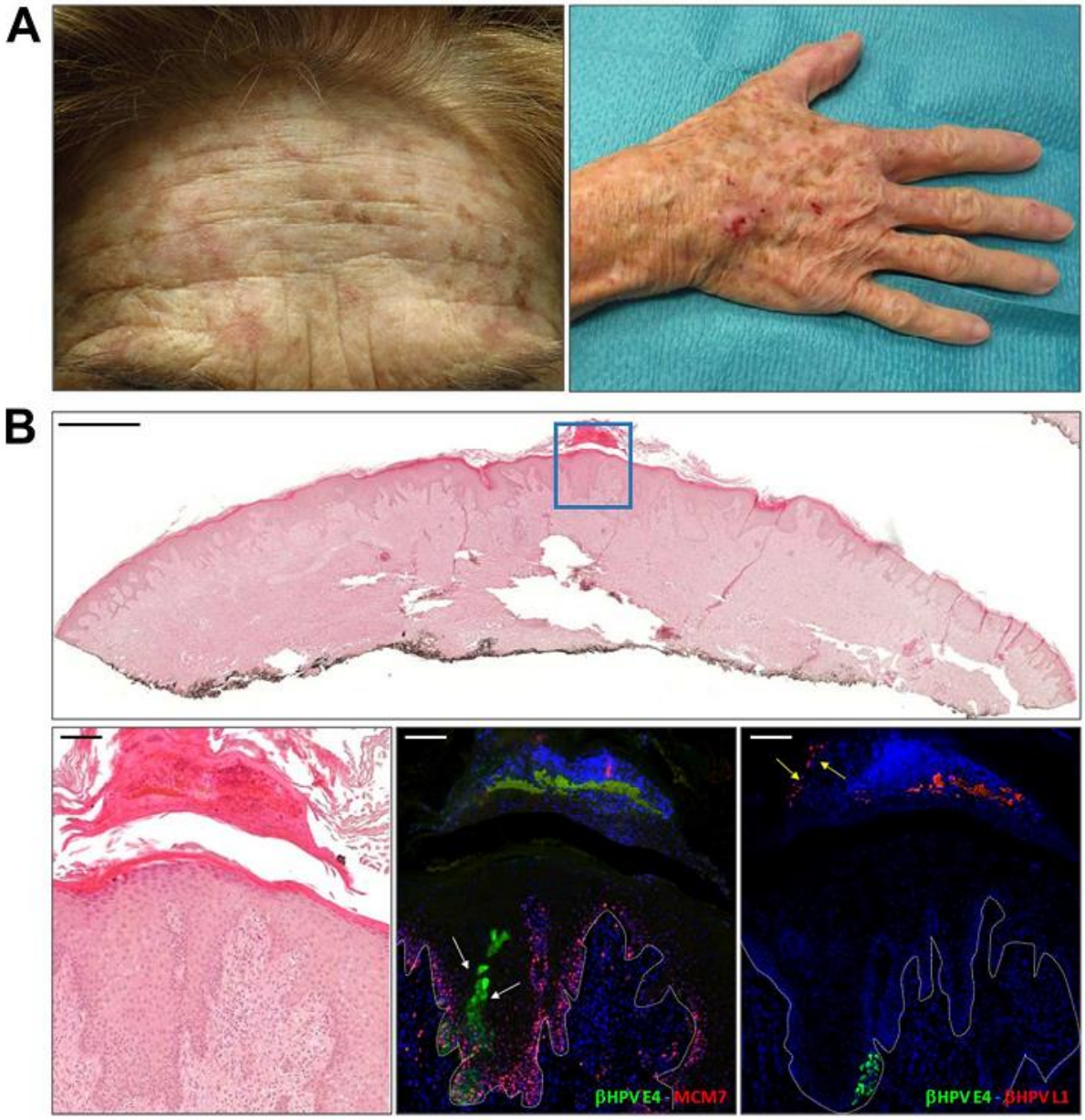
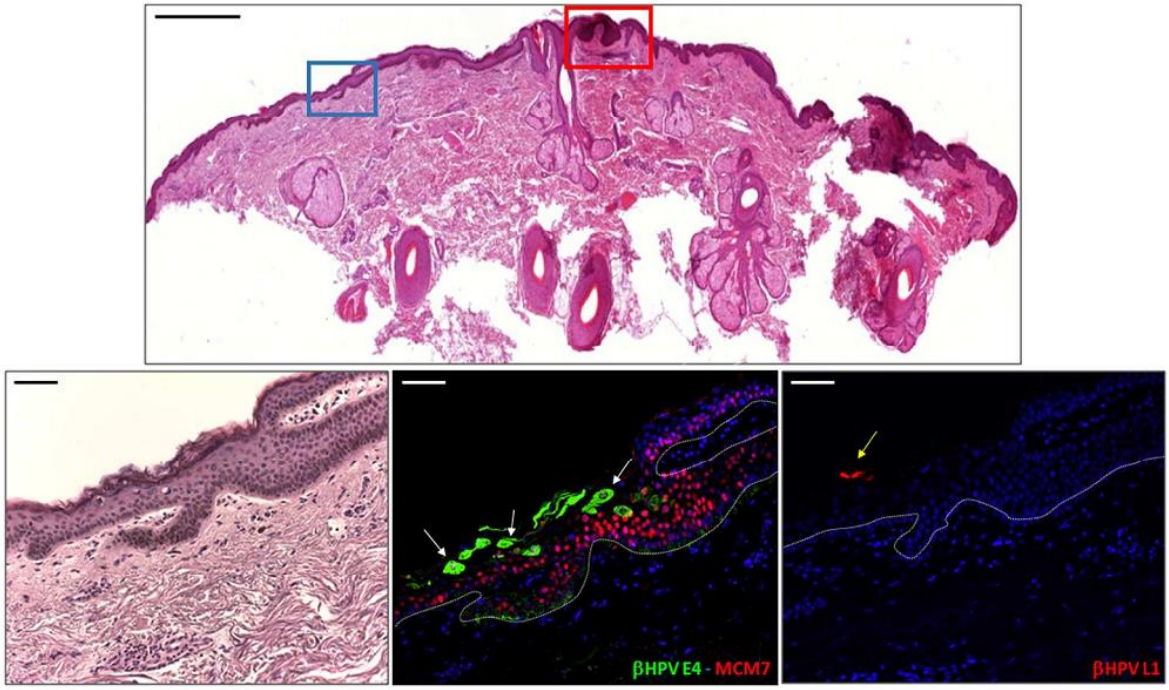


Figure - Immunohistochemical staining for the βHPV E4 and L1 proteins, or cellular minichromosome maintenance protein 7 (MCM7) in a basal cell carcinoma (BCC) located on the neck of a KTR. The top picture shows hematoxylin and eosin (H&E) staining of the tissue section (scale bar: 1000 μm). The red square shows the basal cell carcinoma and the blue square indicates the area of interest in the adjacent epithelium. The region shown in the lower panels corresponds to the blue square highlighted in the overall H&E image, magnified in the left-hand lower panel. In the middle picture, the same section was subjected to double staining using antibodies to E4 (green) and MCM7 (red), and a serial section was stained with antibodies to L1 (red) (right picture). The white arrow indicates nuclear L1 staining. All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue) to visualize cell nuclei. Scale bars: 100 μm.



As "positive control", we included 12 patients who developed genital cancer. Among these, 4 were vaginal and vulvar intraepithelial carcinoma, usually rare in the general population. Another intriguing observation is that 3 of them suffered from graft pyelonephritis that constitutes a rare infective complication in the general KTR population (<5%). Staining with anti-αHPVE4 of the VAIN from patient 9, diagnosed as grade 1 and 2, revealed high expression levels of E4 in the differentiating superficial layers, with small patchy negative areas, where p16

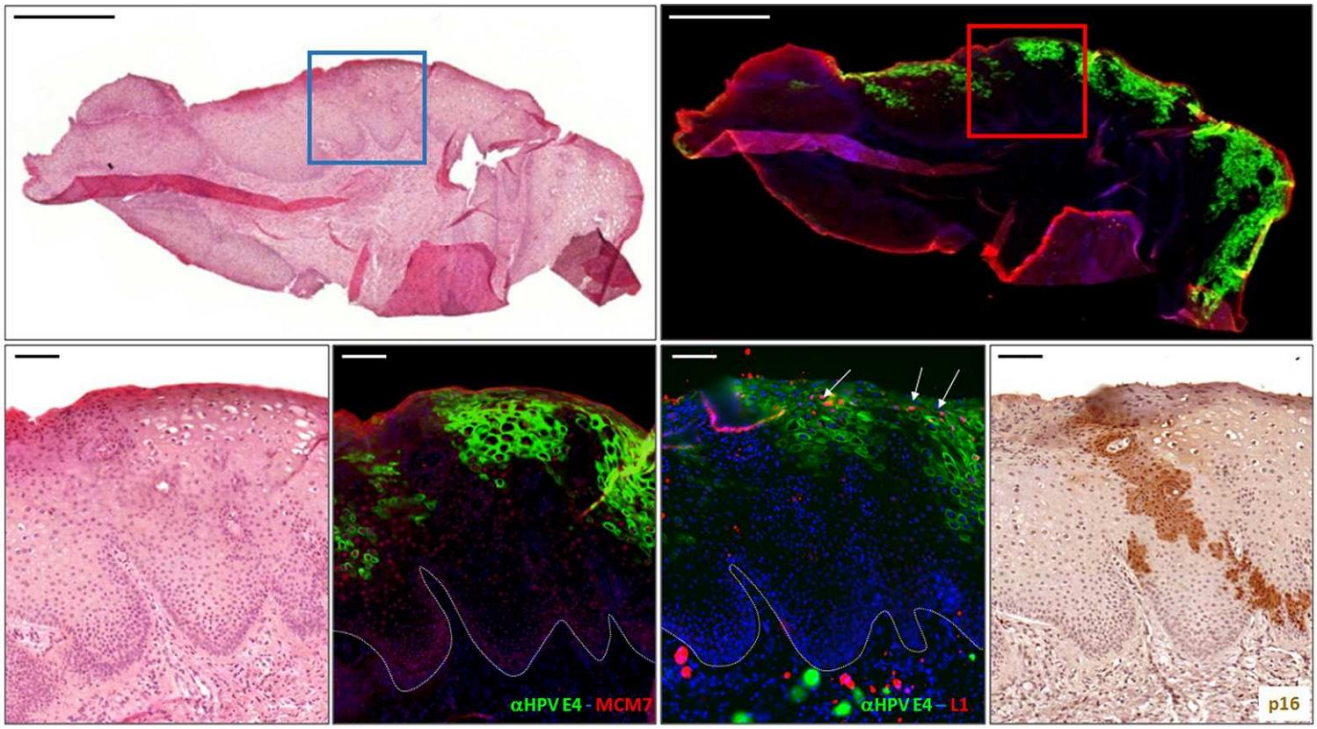
expression appeared in some of them. As expected for a low-grade productive lesion, the late protein L1 was clearly evident in the nuclei of the more superficial layers. Routine testing for HPV revealed its presence in nearly all lesions; all concerning high-risk genotypes and often presenting multiple infections (data not tabulated).

Table: clinical characteristics of the patients with an alpha-HPV associated lesion

Pt	Birth date	Tx Date	Lesion	Notes
1F	1961	2004	2008: Hysterectomy (CIN1, 2)	
2F	1954	2009	2010: Hysterectomy (atypical hyperplasia of endometrium)	2009: CMV reactivation
3M	1955	2005	2009: Condilomas	2009: BCC shoulder
4F	1945	2001	2006: Atypical hyperplasia of cervix	2005: Acute pyelonephritis (graft) 2005: CMV reactivation
5F	1962	2006	2009: VAIN2 2010: Vaginal, cervical koilocytosis	2006-2015: Multiple UTI 2012: Pyelonephritis with abscess (graft)
6F	1969	2006	2007: CIN2 2011: CIN1	2007: PyVAN 2011: Graft RCC
7F	1966	2011	2009: Esocervix metaplasia 2012: CIN1, 2	
8F	1952	2009	2010: VAIN1	
9F	1948	1999	2003: VIN2 2003: CIN1 2004: Hysterectomy (CIN1, 2, 3) 2005: VAIN1, 2	1999-2015: Multiple UTI
10F	1968	2001	2006: Atypical, high grade dysplasia on plain condiloma	
11F	1959	2000	2003: CIN3	2002: Acute pyelonephritis (graft) 2004: Acute pyelonephritis (graft)
12F	1967	2006	2007: CIN2 2007: CIN3	2008-2014: positive BK-viruria

KTx: Kidney transplantation; CIN: Cervical intraepithelial neoplasia; VIN: Vaginal intraepithelial neoplasia, VIN: Vulvar intraepithelial neoplasia, CMV: Cytomegalovirus; BCC: Basal cell carcinoma; UTI: Urinary tract infection; PyVAN: Polyomavirus-associated nephropathy; RCC: Renal cell carcinoma; BKPyV: BK virus.

Figure - Immunohistochemical staining for the αHPV E4 and L1 proteins, or cellular minichromosome maintenance protein 7 (MCM7) and p16 ^{INK4a} in a vaginal intraepithelial neoplasia (VAIN) from patient 9 of Table 1S. The top pictures (scale bar: 1000 μm) show the hematoxylin and eosin (H&E) staining of the tissue section (left) and the same section following double staining using antibodies raised against E4 (green) and MCM7 (red). The region shown in the lower panels corresponds to the blue square highlighted in the overall H&E image and to the red square in the picture of the E4-MCM7 staining (first and second pictures). A serial section was stained with antibodies to L1 (red) (third picture). The white arrows indicate nuclear L1 staining. All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue) to visualize cell nuclei. The fourth picture shows a serial section stained for the cellular protein p16^{INK4a} by means of immune-enzymatic staining. This section was counterstained with hematoxylin to visualize cell nuclei. Scale bars: 100 μm.



BKV and urinary tract malignancies

Specific Aims: To evaluate the role of active HPyV or BKV-associated nephropathy on the development of tumors of the urinary tract in KTRs.

In the study cohort, 13 urinary tract malignancies arose in 13 KTRs as follows: 8 renal carcinomas in the native kidney and 2 in the transplanted kidney, and 3 carcinomas of the bladder (Table). Three of these patients (23.1%) experienced a PyV-associated nephropathy (PyVAN, polyomavirus-associated nephropathy) before cancer diagnosis, while none had a PyVAN after cancer development. Interestingly, the percentage of patients with urinary tract cancer for whom a diagnosis of PyVAN was reported before the development of this malignancy was significantly higher (3/16; 18.75%) in comparison with those who never experienced PyVAN (10/1024; 0.98%).

Figure. Cumulative incidence of urinary tract malignancies (renal cell carcinoma or bladder carcinoma), stratified by a time-varying indicator of diagnosis of a PyVAN. Death and graft failure were considered as competing events. HR = 23.9 (95%CI: 6.02-95.2; p < 0.001). *Red line: patients with a PyVAN; Blue line: patients without a PyVAN.*

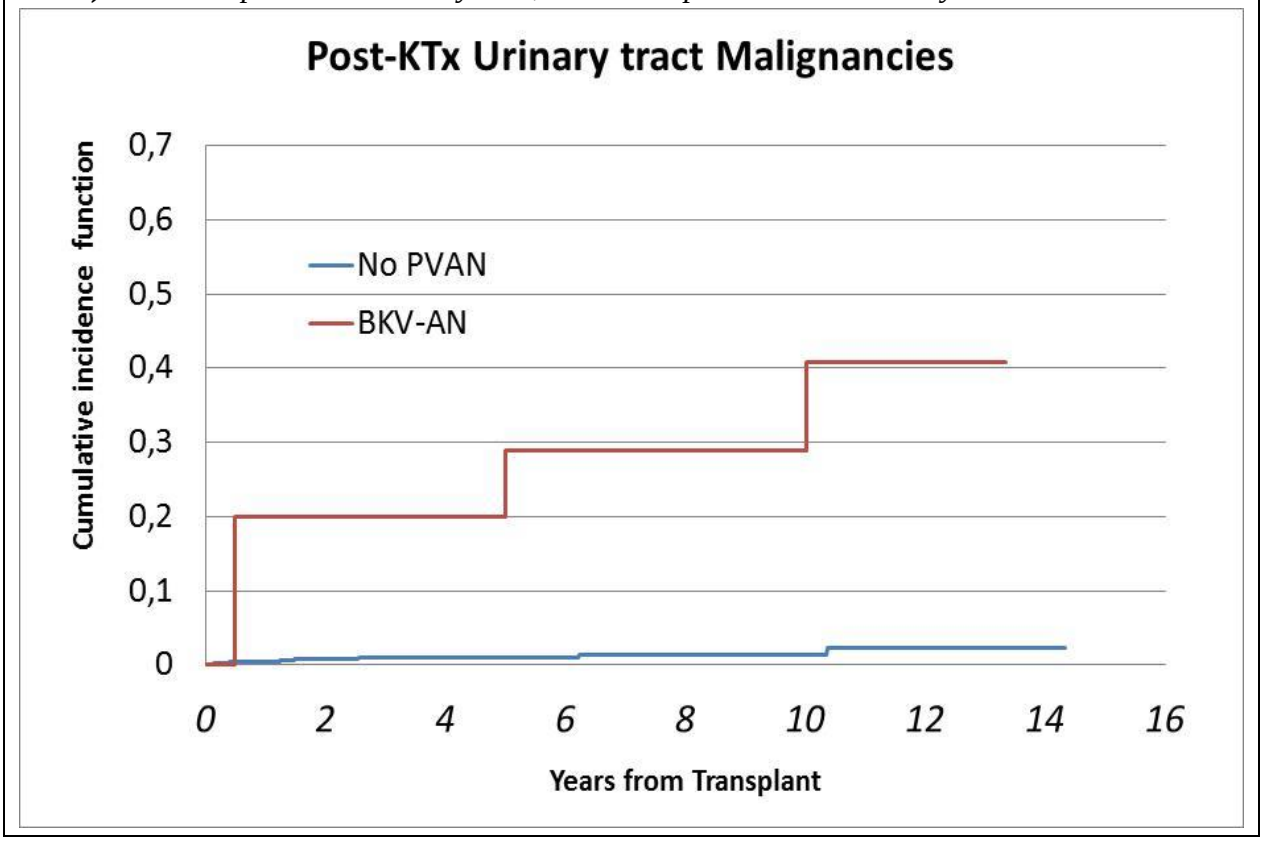


Table: clinical characteristics of the patients with a malignancy of the kidney or urinary tract

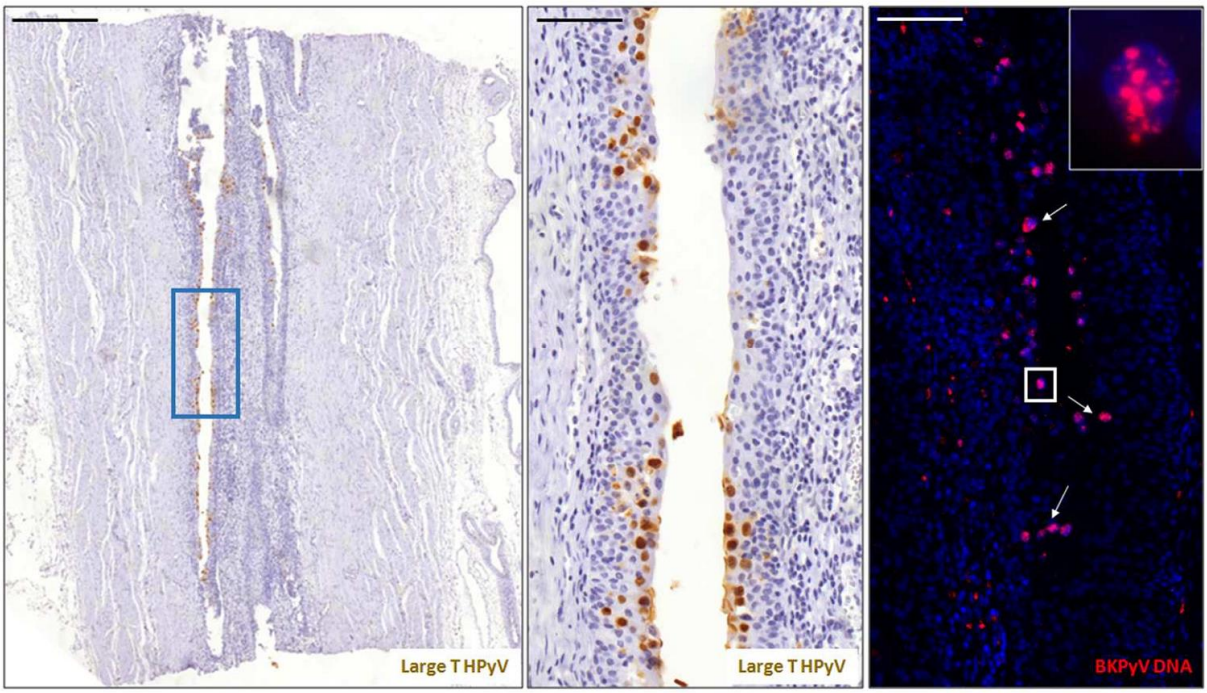
Pt	Birth date	Tx Date	Tumour type and grade	Pathologic stage	Notes
1M	1952	2008	2010: Clear cell RCC FG 2	T1a	2010: Graft failure /Second Transplant 2010: Acute promyelocitic leukemia
2M	1969	2008	2010: Clear cell RCC FG 3	T1b	
3M	1948	2003	2004: Clear cell intracapsular RCC FG 2	T1a	2003: Graft failure
4M	1954	2008	2008: Papillary RCC FG 2	T1a	
5F	1969	2006	2011: Clear cell RCC (KTx)	T3a N0	2007; 2011: CIN (Pt 6 Table 1S) 2007: PVAN
6M	1965	2005	2006: Clear cell RCC FG 3	T1a	2011: Acute HBV infection
7M	1954	2007	2009: Clear cell RCC FG 2	T3a	2009: Graft failure
8M	1969	2001	2007: Clear cell RCC FG 2	T1a	2006: Viral enteritis
9M	1949	2003	2004: Clear cell RCC FG 2	T1a	2004: PVAN (cause of graft failure) 2010: Death
10M	1942	2006	2006: Clear cell RCC FG 2	T1a	2006: Pneumonia; CMV reactivation 2006: Death
11M	1948	2000	2010: LG Bladder urothelioma	Ta	2001: CMV disease (hematological) 2002: AK; VZV reactivation 2006: SCC; AK (neck) 2007: MGUS IgG-k (plasmacells at BMB: 5%) 2014: Sepsis
12M	1940	2001	2001: LG In situ transitional carcinoma		2005: Prostate cancer (Gleason 4) 2009: Graft failure
13M	1954	2000	2010: LG Bladder urothelioma, MD	T1	2001: PVAN 2010: Early tumor relapse (Ta)

KTx: Kidney transplantation; RCC: Renal cell carcinoma; FG: Fuhrmann grade; CIN: Cervical intraepithelial neoplasia; PVAN: Polyomavirus-associated nephropathy; HBV: Hepatitis B virus; CMV: Cytomegalovirus; SCC: Squamous cell carcinoma; AK: Actinic keratosis; VZV: Varicella zoster virus; LG: low-grade; MGUS: Monoclonal gammopathy of undetermined significance; BMB: Bone marrow biopsy; MD: moderately differentiated.

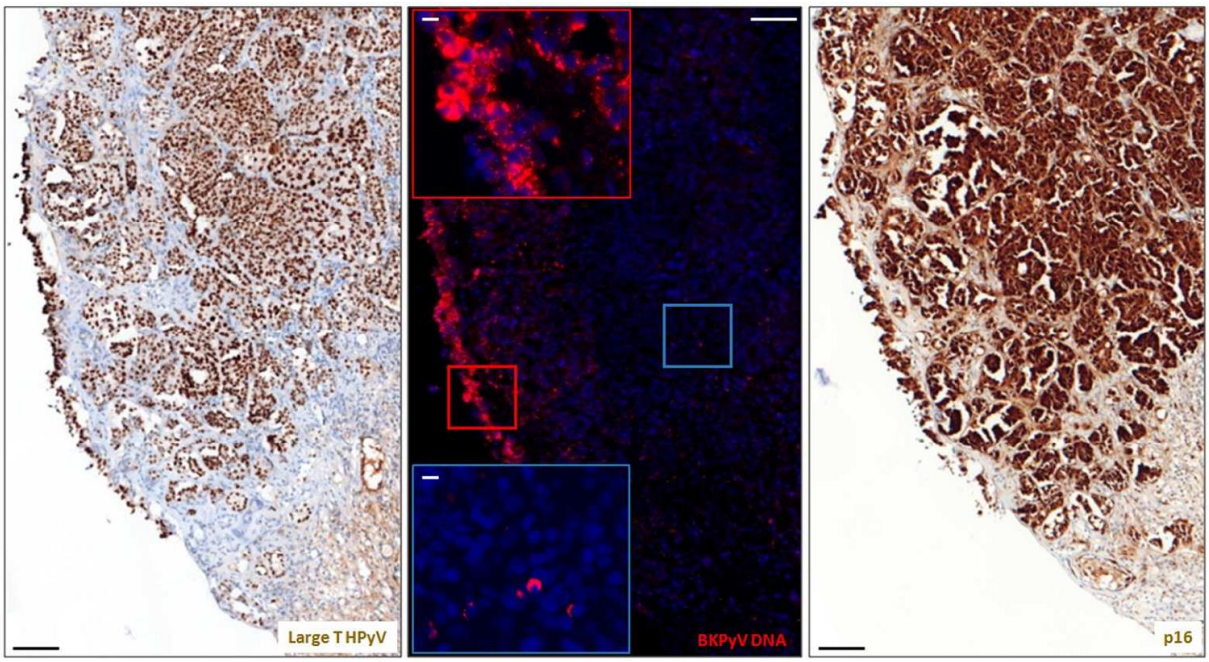
These epidemiological associations led us to investigate whether it was possible to detect HPyV infection/reactivation in the tumor mass or surrounding non-pathological tissues from the surgical specimens. To this regard, tissue sections from the available FFPE blocks (including blocks presenting the tumor core as well as those presenting non-neoplastic areas, such as the renal pelvis and ureter) were stained with anti-large T antibodies (pan-polyomavirus antigen) by immunohistochemistry. Staining was only detected in patient 9, who underwent nephrectomy for RCC 3 weeks after the diagnosis of PyVAN. Consistent with the SV40-positive staining observed for the routine diagnosis of PyVAN (data not shown), numerous large T-positive cells were found in the urothelium lying the ureter, while the tumor cells were negative. FISH analysis was performed using the BKPyV genome as a probe, since genotyping of a urinary sample performed at the time of PyVAN diagnosis revealed the presence of BKPyV. A very strong nuclear signal overlapped the large T staining, indicating viral genome amplification in these urothelial cells.

Out of the 3 urinary bladder carcinomas, only one turned out to be positive for the anti-large T-antigen staining. This patient had a documented PyVAN 9 years before the onset of the bladder carcinoma in 2001. The majority of the tumor cells from this moderately differentiated carcinoma displayed a very strong staining for the large T antigen. By contrast the FISH signal with the BKPyV genome (BKPyV II genotype was found in the urine at the time of PyVAN) was less evident and mostly in the more superficial cells. Consistent with the observed dysregulated expression of the large T antigen in dysplastic cells, sustained p16INK4a expression was also found. Since this carcinoma quickly relapsed after endoscopic removal, a decision was made to change his IS therapy from tacrolimus-steroids to sirolimus-steroids. At the time of the patient's last follow up visit (in December 2015), he was still being treated with sirolimus and steroid and there was no sign of urinary tract malignancy, although low levels of BKPyV viral loads were still present in the urine (640 copies/ml).

Detection of polyomavirus large T antigen and BKPyV DNA in the ureter of the native kidney removed for a clear cell carcinoma from patient 9 of Table 2S. The left picture shows the scan of the tissue section stained for the large T antigen (scale bar: 1000 μm). The region shown in the middle picture corresponds to the blue square highlighted in the left image (scale bar: 100 μm). This section was counterstained with hematoxylin to visualize cell nuclei. The right picture shows a serial section stained for the presence of the BKPyV genome by fluorescent in situ hybridization (FISH) (red) (inset: magnification of the white square). The white arrows indicate FISH-BKPyV positive nuclei. This section was counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue) to visualize cell nuclei (scale bar: 100 μm).



Detection of polyomavirus large T antigen, BKPyV DNA, and the cellular protein p16 ^{INK4a} in the urinary bladder carcinoma from patient 13 of Table 2S. Left picture shows the scan of the tissue section stained for the Large T antigen (scale bar: 100 μm). Right picture shows the scan of a serial section stained for p16^{INK4a} (scale bar: 100 μm). This section was counterstained with hematoxylin to visualize cell nuclei. The middle picture shows a serial section stained for the presence of the BKPyV genome by fluorescent in situ hybridization (FISH) (red) (scale bar: 100 μm). The regions shown in the insets correspond to the red and blue squares (scale bar: 10 μm) This section was counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue) to visualize cell nuclei.



Discussion

This work on long term kidney transplant complications was divided into different sub-studies: indeed, long term complications are the major issue of current clinical transplantation and a "personalization" of therapy is an unmet clinical need in this field. The most common (and life-threatening) complications are post-transplant vascular events and malignancies.

Still, personalization in transplant medicine is an intriguing choice: we can choose among at least four classes of immunosuppressants (CNI, mTORi, antimetabolites, steroids) and novel therapies are being developed and implemented ("chronic" induction, anti-immunoglobulin therapies, anti-citokine therapies). Moreover, trials on single "non-immunosuppressant" drugs (like statins, ACEi. ARBs) have shown inconsistent results, probably because the "transplant mileau" is different from the general population and because the same events might have a different pathogenesis in KTRs than in the general population. Lastly, "over-the-counter" drugs (like vitamin D) and life habits changes are being widely prescribed, but without solid proofs of their efficacy or safety: given that there is a strong rationale to adopt these drugs/measures, the doses might be very different in KTRs due to the interaction with IS and the residual CKD.

This thesis was performed with the aim to find some "cornerstones" which may be able to guide clinical practice and future research: the first step of modern "personalized medicine" is to develop risk stratification tools for hard endpoints. Then, we will be able to identify among non-reponders the causes/pathways of a reduced response and act on them specifically. Indeed, personalization of medicine is the personalization of care based on measurable patient's genetics, molecular or cellular analyses: therefore, diagnostic testing is not performed to "diagnose" a pathological condition, but rather to guide therapy, such as drug monitoring was proposed several decades ago, which was very effective on short-term outcomes, but yet is not able to accurately predict long term complications.

Which gene variants are useful?

We have investigated five SNPs which were previously associated with post-transplant complications, including two SNPs affecting IS metabolism (*CYP3A5* *1, *CYP3A4* rs35599367), two SNPs of the MDR1 affecting IS biological effect (rs1045642 and rs1128503) and a SNP affecting "induced" inflammation, apoptosis and fibrosis (*TCF7L2* rs7903146). Indeed for specific endpoints there might be more and different genetic variants: for example there are about 30 variants which have been associated with the risk of acute rejection and almost 20 SNPs affecting cardiovascular risk, but with inconclusive and conflicting results in different populations.

CYP3A4 rs35599367 was not found associated with any of the investigated endpoints. This is consistent with previous reports showing an association with "acute", peak-dependent side effects, particularly for cyclosporine, such as a delayed graft function (doi: 10.1097/FPC.0b013e328351f3c1). Indeed tacrolimus pharmacokinetics is affected by this variant, having T carriers a 33% lower BTL than CC homozygous (doi: 10.1373/clinchem.2011.165613), which could be translated to a 30-40% higher dose normalized through level. Even if it seems a large change in pharmacokinetics it is much smaller than the one associated with the *CYP3A5* *1 genotype, which induces a an increase in the DnTL of about 100% (doi: 10.1016/j.transproceed.2013.11.084).

Moreover the prevalence of T carriers in the white caucasian population of European ancestry is relatively small, with a minimum allele frequency of 0.0497: indeed, in our cohort it was 10.1% in white caucasians. As for power calculation, we could expect that we would be able to detect an OR of at least 5.5 (5% alpha error, 20% beta error), and such a large OR is unlikely to be associated with a single SNP affecting drug metabolism, given its small effect on pharmacokinetics.

CYP3A5 *1 genotype carriers were found to be less likely to develop a virus-related malignancy, particularly we focused our analyses on PTLDs.

This haplotype is relatively uncommon in western European patients, ranging between 10% and 15% (in our cohort it was 12.2%), while it is more common in Asian patients (up to 30%-35%). This polymorphism has been associated with high CNI requirements (tacrolimus daily dose > 0.1 mg/Kg, DnTL < 100 ng*Kg/(mL*mg)) and a relatively low drug exposure (doi: 10.1097/FPC.0b013e3283557c74). Indeed, even if DnTL could be a useful marker of the AUC of tacrolimus levels, genetic predisposition can be known before KTx and preventive measures could be implemented early after transplantation. In a previous paper by our group, *CYP3A5* *1 carriers had a higher risk to be "fast metabolizers" (doi: 10.1007/s00228-011-1150-0), and actually had less infections. Interesting the rate of CMV reactivation in our cohort was much higher rate in *3/*3 patients, regardless of CMV serostatus at the time of transplantation. These observations are consistent with a "zero-rate" of virus-associated malignancies in *CYP3A5* *1 carriers, confirming that patients with higher drug requirements may indeed suffer from less infective complications given a target BTL. As shown for CMV -for which surveillance programs are standard clinical care- a reduced rate of EBV reactivation could be the reason why we did not observe any PTLD among *1 carriers.

Interestingly non-immunological events, like PTDM and CAD, had a similar incidence in CYP3A5 *1 carriers and *3/*3: no simple explanation can be given, however, as PTDM was not associated with tacrolimus levels, we could speculate that the effects on glucose metabolism are not dose dependent within therapeutic range.

Knowing this genetic variant before transplantation may indeed help clinicians in different settings: first the CNI dose in the first post-transplant days could be better defined, as up-to-date guidelines only recommend a weight-normalized daily dose, exposing *1 carriers to be under-immunosuppressed in the very first days after transplantation. Indeed, during these days donor's antigens are widely exposed and -as CNIs mainly inhibit the response to unknown

antigens- these are the days in which an “on target” IS may be helpful in reducing long-term immunization. Moreover, patients with a high immunological risk and a *1 CYP3A5 could be maintained on higher BTL without exposing them to a significantly increased risk of infections or malignancies. Lastly, theoretically, screening for virus-induced malignancies (genital, PTLD, NMSC, kidney and urinary tract) and common viral reactivations (CMV, BKV) could be less intense in CYP3A5 *1 patients.

MDR1 rs1045642 and rs1128503 were strictly correlated (concordance > 80%) and even if some associations were more evident for rs1128503, they were present for rs1045642 too. The most interesting association is between rs1128503 TT genotype and PTLD risk: this variant has not been associated with different CNI pharmacokinetic profiles (doi: 10.2217/pgs.11.70; PMID: 16415525), but with several drug side effects (doi: 10.1097/MD.0000000000001315). Indeed, p-glycoprotein (PgP), the transcript of *MDR1*, is expressed in almost every cell, particularly on enterocytes (luminal side), kidney tubule, liver, endothelium, neurons and gonads, both on cell membrane and on intra-cellular membranes. As it is an efflux pump, its role on drug pharmacokinetics is well understood: a more efficient PgP reduces drug absorption (the drug is secreted in the intestinal lumen) and favors the renal and biliary clearance of the drug and its metabolites. Thus, *MDR1* polymorphisms affect mainly drugs with a highly variable drug absorption and with a mainly renal clearance, like digoxin (PMID: 12698307), but not CNIs and steroids.

On the other side, the effects of *MRD1* polymorphisms on drug pharmakodynamics have not been extensively studied in “physiological” settings, but mainly in malignant tissues. Some data are available on drug distribution to different tissues, but the effects on the intracellular drug distribution and effects are lacking. Therefore we cannot directly predict which will be the consequences of slow and fast transporters on the overall drug effects: epidemiological data are

consistent with the hypothesis supporting that slow transporters (ie patients with the TT genotype) might have more "drug effects" on PgP-expressing tissues (PMID: 12831320). Nevertheless, in the Novara cohort slow transporters (rs1128503 TT) had an increased risk of a long-term, virus-related complication: PTLDs. We could speculate that these patients had a greater drug absorption and reduced clearance, but this does not seem the case in our cohort, confirming literature data on the effect of this SNV on CNI pharmacokinetics (PMID: 14517192). However a longer intracellular CNI persistence may have had two effects: on the one side it might have inhibited more the anti-viral immune response, and the other hand it may have reduced the activation-dependent apoptosis of B cells. Therefore, given that activation induces PgP expression on lymphocytes (PMID: 10773966), *MDR1* variants could explain some CNI effects on activated lymphocytes even if they do not alter their pharmacokinetics. Indeed, in the Parma case-cohort study, we were not able to identify any single therapy-related risk factor for PTLDs even if the study power was adequate to find risk factors associated with an OR of 2 or more.

The observed association (slow PgP with PTLD) may thus be explained by a higher susceptibility to chronic, subclinical activation of EBV because these patients have a slower viral clearance. However in this work we did not monitor EBV reactivation and its duration, so we are not able to confirm this hypothesis. Moreover they are more likely to fail to "shut off" immune response (slower activation-dependent apoptosis) and therefore be more likely to acquire mutations inducing malignant diseases. Future studies could be aimed to confirm or confute these hypothesis, for example with periodic EBV viremia measurements and with "*ex vivo*" apoptosis tests of lymphocytes during CNI therapy.

TCF7L2 rs7903146 was confirmed as an optimal marker for PTDM and it was tested also for CAD and PTLDs.

TCF7L2 rs7903146 T variant was found to confer a higher risk of PTDM in an allele dose-dependent manner in our cohort and the pooled analysis of the present data with those of previously published studies provide evidence of strong association between rs7903146 TT genotype and risk of PTDM in KTRs. Furthermore, it appears to improve risk stratification for PTDM when added into a model including the main relevant clinical variables. Taken together these findings yield new insights into the genetic conditioning of PTDM and suggests the possibility of predictive models of PTDM integrating clinical and genetic factors.

The association of rs7903146 with PTDM is biologically plausible given that most immunosuppressive drugs (tacrolimus, steroids and mTOR inhibitors) both promote the apoptosis of pancreatic beta-cells and increase insulin resistance. However recent data support the hypothesis that impaired insulin secretion plays a greater role in PTDM than in T2DM (doi: 10.1093/ndt/gfs583): consequently, genes involved in beta-cell proliferation and apoptosis (such as TCF7L2) are excellent candidates to play a role in PTDM, probably to a larger extent than in T2DM. Therefore our results are consistent with the hypothesis that a genetically conditioned susceptibility to beta-cell dysfunction plays a significant role in the pathogenesis of long-term PTDM. Beta-cell dysfunction (affected by the interaction of environmental factors such as immunosuppressive drugs with genetic background) and the degree of insulin-resistance both likely modulate the risk of this multifactorial condition. Consistently, multivariate analysis shows that TCF7L2 rs7903146 T allele significantly increases the risk of PTDM but other variables related to insulin-resistance, such as higher BMI and previous KTx, are even stronger risk factors. Further studies are needed to elucidate the relative contribution of genetic and environmental factors and also to define their impact at different phases of KTx.

Moreover, *TCF7L2* rs7903146 C>T polymorphism seems to be strongly and independently associated with post-transplant CAD in non-diabetic KTRs in an allele dose-dependent manner. These findings might provide new insight into the genetic conditioning of ischemic heart disease and suggest the possibility of including this SNP in risk stratification models for post-transplant CAD.

The role of rs7903146 in modulating cardiovascular risk in the setting of KTx is biologically plausible and could be mediated by a wide range of mechanisms. TCF-4 transcription factor, encoded by *TCF7L2*, is involved in the pathway of NF-kB; a dysregulation of this axis can alter bone remodeling, a key process leading to coronary artery calcification (CAC), and promote vascular wall inflammation, oxidation and endothelial dysfunction, pathophysiological features which have usually already been active since moderate stages of CKD, long before KTx. *TCF7L2* rs7903146 has actually been associated with isolated fasting hyperglycaemia (IFH) and isolated postprandial hyperglycaemia (IPH) (doi: 10.1371/journal.pone.0071399), which may increase cardiovascular risk in carriers of TT genotype even without diabetes (PMID: 22820698). Repetitive glucose spikes have actually been demonstrated to accelerate atherosclerosis in experimental models, suggesting the possibility that *TCF7L2* rs7903146 may represent a facet of a common genetic predisposition to both diabetes and CVD (doi: 10.1371/journal.pone.0016341). Unfortunately, as no information about postprandial glycemia was available, we were not able to investigate this correlation.

Interestingly, we found a stronger association of the allele with CAD in KTRs than in the general population, as for PTDM. This increased strength of association could be explained by the fact that genetic predisposition can emerge more clearly in KTx setting due to a higher event rate caused by interaction of polymorphisms with transplant-specific environmental risk factors, such as IS therapy and chronic renal failure.

On this basis, can genetic analysis of rs7903146 be useful in clinical practice?

To test this possibility, we elaborated and compared predictive models for PTDM and CAD based on TCF7L2 genotype and clinical variables. Our results showed that rs7903146 significantly improved predictive ability of risk models based only upon clinical factors, suggesting that information on TCF7L2 genotype could be useful for identification of KTRs at higher risk of PTDM and CAD. For instance, the multivariate model including this SNP and the main relevant clinical variables seems to have a good predictive ability for post-transplant CAD (AUROC=0.790) and the inclusion of this polymorphism significantly improved the predictive ability of the clinical risk model in our cohort (delta AUROC: +0.112; p =0.003). Availability of this genetic information prior to surgery would have important consequences: patients could be preemptively informed about their risk of PTDM and its associated risks; steroid withdrawal could be considered in high-risk patients, trying to balance metabolic benefits with a potentially increased risk of rejection (doi: 10.1007/s00228-012-1292-8); early and intensive insulin therapy to control hyperglycemia could be implemented in the immediate post-operative phase to minimize glucotoxic effect on beta cells; dietary interventions and life-style modifications aimed at reducing overweight could be aggressively carried out long before KTx.

On the other hand, recommendations from a recent international consensus meeting actually do not support adaptation of immunosuppressive therapy with the aim of minimizing PTDM risk due to lack of sufficient evidence (doi: 10.1111/ajt.12850). Although these limits are true considering the general populations of KTx recipients as a whole, a better stratification of PTDM risk through genetic polymorphisms could help identify a subset of high-risk patients in whom preventive measures such as steroid-sparing could instead be justified.

Moreover, the availability of a predictive model for CAD prior to KTx in non-diabetic patients would entail important consequences: high-risk patients could be identified even in the absence of diabetes and other traditional risk factors and could be preemptively informed about their

risk of developing a cardiac ischemic event after KTx. For example, the 5-years risk of developing a cardiac ischemic event in our cohort is as high as 9.2% in carriers of the rs7903146 TT genotype, which indeed cannot be neglected. Many crucial aspects of KTx management could be affected, including early steroid withdrawal, resumption of anti-aggregant therapy immediately after KTx, aggressive use of insulin to control post-KTx hyperglycemic spikes, maintenance of adequate hematocrit, employment of statin and beta-blockers.

Which "phenotype characterization" is helpful?

Apart from genes, environmental factors may be the key determinants of some post-transplant complications. Some "external" factors are common to the general population, like already shown for PTDM and CAD, while other are peculiar of KTRs. However "discovering" a KTR-specific risk factor may be interesting also for the general population: some risk factors which are more evident in the transplant setting may have a relevance also in immunocompetent patients.

To identify immunosuppression-related risk factors we focused on complications that are more common in KTR than in the general population. Vascular events are a major cause of morbidity and mortality; however, there are many confounders, including the already existing vascular damage (related to the previous CKD), the extent of residual renal dysfunction and therapies, both IS and concomitant.

On the other side, both infections and malignancies have a much higher incidence in KTRs: therefore, we focused mainly on virus-associated malignancies, using as "environmental factor" viral replication. Indeed, genetic predisposition could probably play a role, but, as shown by our results, this does not seem the case, apart from PTLDs.

Antiaggregant and vascular events

We investigated the role of antiaggregant (AAT) and anticoagulant (VKA) therapies on early CVE: our results suggest that neither pre-transplant AAT or VKA therapies "*per se*" or their suspension are associated with a higher risk of CVE.

The main risk factor for early post-KTx CVEs are previous CVE, but not the use of AAT and its subsequent withdrawal. Interestingly enough, no associations were observed between CVE and any immunosuppressive drug. Resumption of AAT after KTx occurred in our Center after a longer time than the one reported by other groups [doi: 10.1111/j.1399-0012.2010.01293.x.], mainly because we considered AAT as a relative contraindication to kidney biopsy, a relatively common procedure in the first weeks after transplant. However the rate of major CVE during the first three months after transplant is in keep with data reported from literature [PMID: 23050275] and this temporary therapy withdrawal might be considered safe. However, a cautious assessment of risk-benefit profile should be made in each patient as high-risk patients, such as those with previous CVE, may have an advantage in resuming secondary prophylaxis earlier.

Unexpectedly we found that CVE are associated to cinacalcet use at transplantation, probably because patients on this drug have had a more severe and long-lasting hyperparathyroidism, potentially leading to a more diffuse vascular damage. Moreover cinacalcet discontinuation after KTx has been widely associated with a rebound of PTH and calcium: further studies evaluating the role of cinacalcet on KTx early outcomes would be recommended, for example comparing post-KTx outcomes of patients enrolled in large multicenter studies (ie: EVOLVE, ADVANCE, OPTIMA trials).

Beta Papillomavirus and NMSC

This study extended previous observations from our group on the presence of active β–HPV infection in skin tumors from KTRs through the detection of viral protein expression, including the E4 and L1 proteins (doi: 10.1038/modpathol.2013). In this new round of screening, we analyzed 70 skin tumors derived from 21 patients and found positive staining in 3 tumors that were all removed from the same patient. This female patient in question was exceptional in that she received her kidney transplant 25 years ago, and developed more than 15 skin cancers and multiple pre-malignant skin lesions. Consistent with her clinical picture revealing multiple actinic keratosis on her forehead and dorsum of the hands, β–HPV viral protein expression was observed in 3 skin tumors, corresponding to 2 KA and the pathological hyperplastic border of an SCC. The 2 KA were classified as benign proliferative lesions that, especially in the immunosuppressed setting, are considered to be at high risk of malignant progression. These results are fully consistent with our previous reports and strengthen the notion that β–HPV are actively replicating in the pathological skin of organ transplant recipients and can therefore cooperate with other carcinogenic agents, such as UVB, favoring skin cancer promotion and progression (doi:10.1016/j.virol.2015.02.004, doi:10.1016/j.ctrv.2013.08.005). This hypothesis is compatible with the proposed 'hit-and-run' mechanisms of carcinogenesis, with cutaneous β–HPV possibly being important for tumor initiation and progression, but not necessary for tumor maintenance. These assumptions can explain the low virus detection rates found in this kind of study. The observed stimulation of basal cell proliferation in the β–HPV-infected tissues is similar to the situation seen in α–HPV-associated neoplasia and may contribute to the transformation process without necessarily being maintained in the more advanced disease (doi: 10.1038/modpathol.2015.52).

β−HPV infection can be detected in cutaneous intraepidermal precursor lesions of KTR, although its presence is never maintained in more advanced disease states and thus more difficult to observe.

BK virus and urinary tract malignancies

Another form of cancer with an incidence rate known to be significantly increased in the KTR setting regards kidney and urinary tract tumors; their incidence in KTRs is 4 to 10 times higher than in the general population. Of note, urinary tract cancer was statistically more frequent in patients with a previous documented diagnosis of BKVAN in comparison with those who never experienced this virus-associated complication. Reactivation of polyomavirus is associated with allograft viral nephropathy through ascending infection in the renal tubules. Emerging evidence is also indicating a causal link between viral reactivation and urinary tract carcinogenesis or RCC.

Although BKV in urothelial carcinoma is distinctly rare in the general population, a recent report from a large KTR cohort, together with case reports and small series in the literature, and data from a transgenic mouse model suggest a significant rate of HPyV positivity in this cancer, at least in the kidney transplant population (doi: 10.1111/ajt.13550, doi:10.1038/sj.bjc.6604711). In addition, Kenan et al. recently documented genomic integration of BKPyV in tumor cells associated with robust dysregulated large T antigen expression in a high-grade urothelial carcinoma arising in a renal allograft (doi: 10.1002/path.4584). In our kidney tumor series, we failed to detect any large T antigen expression by immunohistochemistry in the tumor cells, while very strong expression was found in the urothelium lining of the ureter in a patient who had undergone nephrectomy of the native kidney for RCC soon after diagnosis of BKVAN in the allograft kidney. The FISH signal in these cells was very strong with a diffuse pattern and the appearance of large dots, very much resembling sites of intense viral genome replication. In one

bladder carcinoma, very strong and homogeneous large T antigen expression was observed in the tumor mass of a moderately differentiated urothelial carcinoma. This uniform staining has also been reported in other tumor series (doi: 10.1111/ajt.13550) and together with the recent finding of integrated truncated BK virus in a renal tumor displaying uniform large T-antigen expression (doi: 10.1002/path.4584) indicates that deregulated large T antigen expression may be associated with the transformation process. Accordingly, p16INK4 was highly expressed as expected for virus-induced dysplastic cells (doi: 10.1111/ajt.13550). When we looked at the FISH signal with a BKPyV DNA probe, the proportion of positive nuclei was significantly lower compared with the homogeneous distribution of large T antigen staining. Experiments are in progress to demonstrate viral integration; however, the discrepancy between protein expression and the FISH signal may suggest that clonal expansion of the transformed cells has occurred in this case.

Although the sample size in our series is still limited, our findings support the notion that the BK virus, in addition to entering a lytic life cycle, resulting in the loss of renal tubule cells in BKVAN, can enter a non-lytic life cycle accompanied by deregulated expression of the oncogenic large T antigen in transformed cells.

EBV vs. Genes in PTLD

We've put a lot of efforts in determining risk factors for PTLDs: they are a rare and lethal complication of KTx, with a disease-specific mortality rate up to 30%. Moreover its SIR is very high, particularly for large B cell Lymphoma (about 10) and some lesions are peculiar of KTRs, like early lesions, which are composed by oligoclonal EBV-infected malignant lymphocytes. EBV plays a major role in their pathogenesis, particularly in pediatric transplantation, in which a primary EBV infection is relatively common, and in PTLDs occurring within 2 years from transplantation. PTLDs of elderly patients with a long lasting

transplant are usually assumed to occur through other pathways, probably not strictly dependent on EBV replication. Even considering these confounders, PTLDs are a very intriguing complication for research purposes: they are probably the result of a close interaction between host immune system, immunosuppressive drugs, EBV and other oncogenic viruses (HHV8, possibly BKV and HHV6) and mutagens.

To date risk factors on PTLD are not well defined and even recent "wide" registry studies (doi: 10.1111/tri.12744) focus on epidemiological descriptions rather than inferential analyses, while the attention is shifting from PTLD occurrence to PTLD effects on patient and graft survival (doi: 10.1111/tri.12375). Indeed, there is a wide consensus on PTLD incidence being highest in the first year and after the tenth year form transplant with a bimodal distribution, and on EBV seronegativity as the main risk factor, with an HR varying from 5 to 20 (doi: 10.1016/j.trim.2015.04.003). Moreover, different solid organ transplants (heart, lung, pancreas, kidney, liver) have a different risk for PTLDs, but obviously they cannot be considered a "risk factor"; still, it is interesting to note that the highest risk is in observed in transplants requiring the highest IS (thoracic organs) and lower in non-life saving transplants in which IS can be tapered more safely (kidney).

We have analyzed two cohorts, the Novara-gene cohort and the Parma case-cohort study, which included long lasting transplants and both early and late PTLDs. Particularly in the Parma cohort we were able to determine the relationship of the PTLDs with EBV in 20 cases. Interestingly, in neither cohort EBV serostatus at the time of KTx was significantly associated with PTLDs, even if OR overlapped with literature data (Novara: OR=4.70; 95%CI: 0.536-41.3; Parma: OR=1.822; 95%CI: 0.833-3.989).

The interaction between EBV and IS therapies in determining PTLDs is particularly clear in the Parma study, which enrolled 49 cases and 196 matched controls. Indeed, EBV serology was an effect modifier of the relationship between lymphocyte depleting agents (ATG) and PTLD: there was no association with ATG in EBV positive patients, while they seemed to be

protective in EBV negative patients (OR=0.323; 95%CI: 0.034-3.083; p=0.307), but possibly due to patient selection bias. Truly, in the past the use of ATG was relatively uncommon in EBV negative patients due to an expected increase in PTLD risk and EBV negative patients who received ATG have been probably carefully selected.

Moreover, a high daily CNI dose (above the cohort median, being tacrolimus > 0.075 mg/kg or cyclosporine > 3.25 mg/kg) was associated with an increased risk of PTLDs only in EBV negative patients (OR=3.451; 95%CI=0.811-14.67), but not in EBV positive patients (OR=1.818; 95%CI=0.788-4.195). This finding is consistent with the hypothesis by which a sustained viral replication is needed to induce a PTLD: EBV seronegative patients often develop a primary EBV infection, but if the patient is able to rapidly clear the virus (ie: is receiving a low dose IS) the relative risk of a PTLD is relatively low (1.30 in this study). Moreover, in EBV positive patients, "standard" drug monitoring is not an adequate marker of sustained EBV replication and therefore PTLD risk. However some EBV-positive patients may be at a higher risk of PTLD due to an increased IS biological effect due to an altered intracellular CNI clearance (ie: *MDR1* "slow transporters") or by a genetic predisposition that can be "triggered" by a subacute EBV infection (ie: *TCF7L2* rs7903146 TT genotype). These factors were investigated in the Novara-gene cohort, and partially discussed in a previous paragraph (*Which gene variants are useful?*). The effect of *TCF7L2* is possibly a direct effect on lymphocytes. Its product (Transcription Factor 4, TCF4) recognizes an Ephrussi-box ('E-box') binding site -a motif first identified in immunoglobulin enhancers. It is widely expressed and *TCF7L2* has multiple alternatively spliced transcript variants that encode different proteins with still unknown properties. TCF4 in the adult is involved mainly in the pathways of cell terminal differentiation and apoptosis: the rs7903146 T allele (less TCF4 function) has been associated with a "slower" differentiation of fibroblasts, yielding to increased collagen production, and to an increased apoptosis of pancreatic beta-cells after a sovra-maximal glucose stimulus (ie: glucotoxicity). Indeed, given the wide spectrum of TCFs

functions, a reduction in the activity of one of them may indeed produce very different biological effects in different cell population. We can speculate that B cells of patients with a less functional variant (ie: rs7903146 T allele) have a defective differentiation towards B memory and plasmacells, yielding eventually to a relatively less effective specific humoral response, including the "memory response" of EBV positive patients. Moreover, B cells might tend to remain for a longer time in lymphnodes during their centrocyte and centroblast differentiation stage (doi: 10.1073/pnas.1205299110): this is actually the most likely state to gather mutations yielding to malignant lymphoma (diffuse large B cell lymphoma, the most common in KTRs).

Even if the observed genetic association are not completely explained by current knowledge, they may explain the reason why to date no single "therapy variable" has been strongly associated with PTLDs, like we observed in the Parma study. Certainly "more immunosuppressed" patients are at higher risk for virus-related malignancies (like heart transplant recipients), but in the current setting of KTx IS there are very few (if not none) severely IS patients.

Moreover, the proposed risk model for PTLDs is definitely intriguing: it is able to stratify accurately the risk of PTLDs (AUROC=0.891) and is able to determine which patient is at high risk for EBV reactivation (EBV seronegative and *MDR1* slow transporters), those at a high risk for PTLD "*per se*" (*TCF7L2* low expressors) and those with both risk factors.

How to manage these patients will be matter of study: patients with a higher risk of EBV reactivation could benefit from a therapy with mTORi or with a low dose CNI (as shown in the Parma study) or even with a prolonged valganciclovir prophylaxis. Patients at the highest risk of PTLD will probably benefit also from a dedicated screening program, which would be useless if applied to the entire KTR population. As far as we know this is the first reliable model (AUROC>0.80) predicting PTLDs: it may be worth testing a similar model in other IS population, like those with autoimmune diseases or long term cancer survivors.

Potential applications and conclusions

The main results of this PhD program were risk stratification models for common transplant complications, which are an unmet need for the transplant community. The first application of these models could be pre-transplant screening and information: based on the variants of three genes (*CYP3A5* haplotypes, *MDR1* rs1128503 and *TCF7L2* rs7903146) we could be able to reliably predict the individual risk of post-transplant diabetes, ischemic heat events and post-transplant lymphomas. As these complications are potentially lethal or impact severely quality of life, patients at high risk should be correctly informed.

Moreover pre-transplant screening for variations in these gene may enable physician to modulate IS therapy based on their predicted pharmacokinetics and risk of complications. Before advising for specific clinical choices, randomized control trials should test these hypothesis: for example, regarding diabetes, it is well known that tacrolimus acts mainly on beta cells, while it has a relatively low impact on insulin resistance, therefore it may be substituted by cyclosporine only in patients at high risk for diabetes due to beta-cell insufficiency (second transplant, *TCF7L2* rs7903146 TT carriers), while other measures (like the use of euglycemic agents, as glucagon agonists-liraglutide- or glycosuric drugs-empagliflozin) could be used in more "insulin resistant prone" patients (ie: obese, older patients). On vascular events, primary prophylaxis has not been tested in KTRs: however, in high risk patients (ie: *TCF7L2* rs7903146 TT carriers, previous CVE or previous therapy with cinacalcet) it seems a feasible option and even an early start appears to be safe in our cohort (doi: 10.1007/s40620-015-0185-1).

Regarding PTLDs, patients with a higher risk of EBV reactivation could benefit from a therapy with mTORi or with a low dose CNI (as shown in the Parma study) or even with a prolonged valganciclovir prophylaxis. Patients at the highest risk of PTLD will probably benefit also from a dedicated screening program, which would be useless if applied to the entire KTR population.

Moreover after transplantation novel screening methods for virus-induced malignancies could be implemented based on our findings. The case for BKV is “historically” interesting: screening for BKVAN was first performed with urinary decoy cells, then viruria and viremia, and nowadays viremia is considered the standard screening for BKVAN. Indeed patients with a BKVAN were at a high risk for malignancies of the urinary tract: viruria, more than viremia, is an earlier marker of subclinical BKV replication in the bladder and its role as a predictor of urinary tract cancer should be investigated, possibly identifying those patients with a sustained bladder BKV replication regardless of BKVAN. These patients could be given chemoprophylaxis, for example with mTORi, or undergo a more strict screening program. Similarly for NMSC, a sustained beta-HPV replication is a favoring factor to accumulate mutations yielding to NMSC. A primary infection acquired during IS therapy is more likely to be sustained, even if subclinical, also because there are no specific anti-viral agents: a novel infection can be demonstrated by the appearance of a new anti-HPV antibody (ie: against a different serotype than before KTx). These patients could benefit from a maximal NMSC prophylaxis or from more accurate screening. Moreover patients with an active beta-HPV replication before KTx -even if it may be hard to measure- could receive an HPV vaccine before surgery: it has been reported that alpha-HPV vaccine may indeed improve diffuse warts and frequently relapsing NSMC.

In conclusion, viruses, host, immune system and therapies strictly interact and determine transplant complications: in each patient the relative contribution of each factor may be very different. The aim of personalized medicine is to determine how much each risk factor increases the risk of a complication in each individual patient: the developed models have a good AUROC and are able to differentiate between different pathogenetic moments. Based on this knowledge, we will need first to confirm these models in other cohorts and then to test the “biologically plausible” therapy and screening “personalizations” in patients carrying similar characteristics. Indeed the way towards personalized medicine is still long and probably more tortuous than what we believed few years ago, but still worth pursuing.